BIOLOGICAL, BIOCHEMICAL AND BIOMEDICAL ASPECTS OF ACTINOMYCETES

PART B

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32

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Akadémiai Kiadó, Budapest

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OF ACTINOMYCETES

Proceedings of the Sixth International Symposium on Actinomycetes Biology, Debrecen, Hungary, 26—30 August, 1985

Edited by
G. SZABÓ, S. BIRÓ and
M. GOODFELLOW

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The material of the proceedings is coming from the sixth of a series of scientific meetings held in every three years on the biology of Actinomycetes. These conferences are unique opportunities for specialists studying all aspects of Actinomycetes to meet.

This volume of the Proceedings of the 6th International Symposium on Actinomycetes Biology contains the full text of the lectures read at nine plenary sessions, in a somewhat abbreviated form, the papers delivered at eight mini-symposia which followed the plenary sessions with the participation of specialists of a given, narrower field of interest. The abstracts of the seven poster sessions are also included.

The titles of the plenary sessions indicate the fields which were covered on the biology of Actinomycetes: genetics; physiology and biosynthesis of primary metabolites; biosynthesis of secondary metabolites; biochemistry; morphology and ultrastructure; taxonomy; pathogenicity and immunology; ecology and epidemiology; differentiation.

There were about 500 participants from 27 countries. This time the number of scientists taking part at the Actinomycetes Symposium was the highest compared to previous ones.

The Proceedings of the Symposium give an excellent overview of an important group of microorganisms for specialists working with them in antibiotic industry, in human or veterinary medicine, in agriculture or in fundamental research.

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PART B

MORPHOLOGY AND ULTRASTRUCTURE

Plenary Session

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COLONY DEVELOPMENT IN STREPTOMYCES

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Introduction

Actinomycetes constitute a group of microorganisms within the Procaryotic Kingdom which shows many similarities with microscopic fungi, with which they are a classical example of convergent evolution. Specially relevant in this context are the morphology and mycelial organization of their colonies as well as their systems of reproduction. One may speculate that these similarities arise from a common adaptation to fulfill their metabolic functions in the absence of water in their immediate environment.

The life cycle of streptomycetes growing on solid media involves the formation of two different types of mycelia: firstly, a substrate mycelium that grows on and into the culture medium and, as the colony matures, a second type of mycelium which grows into the air and will finally form the spores.

During colony growth, aerial hyphae develop in absence of water and, because of this, must be protected from dehydration. On the other hand, and due to the previous growth of the substrate mycelium, aerial hyphae develop under unfavourable conditions such as the exhaustion of nutrients and accumulation of inhibitory compounds in the culture medium.

Changes in colony organization of Streptomyces during the developmental cycle were studied by Wildermuth (1970). However, very little information is available about the physiological events that occur during aerial mycelium growth and sporogenesis. This may, in part, be due to the technical difficulties imposed by the fact that these differentiation processes take place only on solid media. It must be kept in mind that, as Kalakoutskii and Agre (1976) suggested, the physiological and biochemical data obtained with submerged shaken cultures must be taken with considerable caution when interpreting morphogenetic changes observable in surface cultures.

The cellophane transfer technique is a useful tool to achieve a better understanding of the physiological and biochemical events that take place during the different stages of the streptomycetes life

cycle. By using this technique a reliable correlation may be established between the morphogenetic changes observed throughout the development of the colonies and the physiological and biochemical changes that accompany them.

Colony development and mycelial organization

a) Semi-thin sections of whole colonies

The structural complexity of the colonies of streptomycetes makes TEM studies of their development a laborious and time consuming process. However, formation of aerial mycelium and changes in the mycelial organization of the colonies can be easily and accurately detected by examining semi-thin sections of whole colonies under the light microscope. Semi-thin sections of whole colonies enable rapid identification of the different regions of the colony and any modification of the mycelial distribution within the colonies. Moreover, this technique allows to select given parts of sections of entire colonies for a more detailed study in the electron microscope. Using this technique, the observed development pattern of S.antibioticus colonies (Méndez et al. 1985a), was essentially in agreement with previous results described by Wildermuth (1970). Although in our experiments, substrate mycelium, grew as a thin layer formed by a dense network of hyphae on the surface of the solid medium, and as a large zone occupied by hyphae that grew more sparsely and penetrated deeply into the culture medium. Aerial mycelium was formed by a loose network of hyphae which grew into the air. Only hyphae from the upper region of the colony were surrounded by a thin sheath (a characteristic structure of aerial mycelium).

b) Cellophane transfer technique

Many studies on streptomycetes have been made by using the cellophane transfer techniques. We previously verified the effect of this technique on the pattern of colony development in order to apply it subsequently to studies concerning mycelial organization in the colony. In spite of the fact that this procedure introduced some minor disturbances in the whole organization of the colonies (e.g., substrate mycelium remains located on the cellophane and does not grow into the culture medium), differentiation processes such as aerial growth and sporogenesis followed the same pattern as those incubated directly on the culture medium (Méndez et al. 1985b). Moreover, changes in surface coloration, proposed firstly by Wildermuth (1970) as a useful indicator of colony development in streptomycetes, were identical and occurred at the same times of incubation as those incubated without cellophane.

With this procedure, the mycelium can be separated from the culture medium, allowing the interruption of colony growth at specific time intervals, as well as the study of the physiological changes that take place during the life cycle.

Physiological studies during colony development

a) Changes in dry weight of colonies during the life cycle

The colony growth was followed by measuring variations in dry cell weight. The dry cell weight increased during growth of the substrate mycelium (Fig. 1), slowed down shortly before the aerial hyphae started to form and increased slightly during aerial mycelium development. This slight increase was mainly due to the accumulation of reserve compounds (in the form of glycogen) which occurs in S.antibioticus during colony maturation, as commented below.

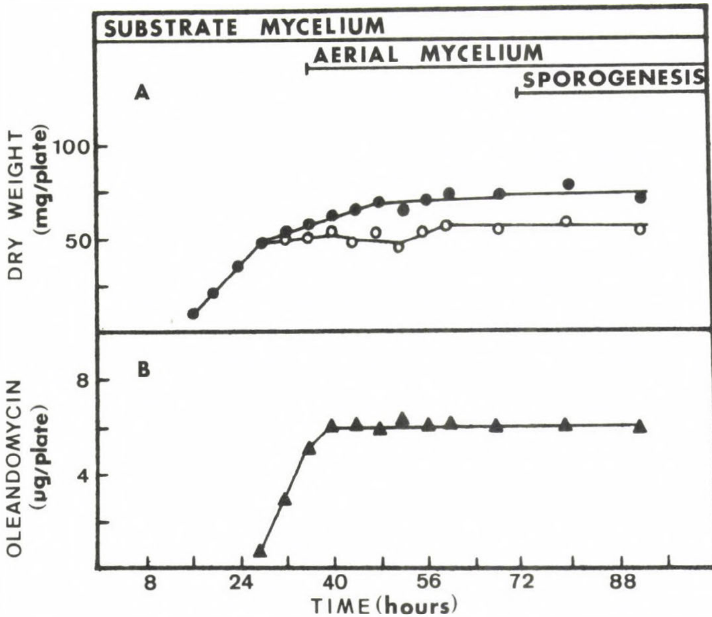

Fig. 1. Changes in dry weight (A) and oleandomycin production (B), during colony development in S.antibioticus. Symbols: ●, total dry weight; ○, dry weight without glycogen; ▲, oleandomycin accumulation in the medium. The different stages of colony development are shown at the top of the figure.

b) Antibiotic synthesis during colony development

The ability to synthesize oleandomycin during colony growth was also investigated. Antibiotic activity started to be detected when the growth rate started to decrease. From this moment onwards, this activity increased continuosly, reaching a maximum value at the onset of the aerial mycelium growth. Thereafter and during the development of the

aerial hyphae, the levels of oleandomycin in the culture medium remained constant (Fig. 1). So, significant amounts of oleandomycin were present in the culture medium during aerial mycelium growth. This fact supports the previous suggestion that the role of antibiotics synthesized by streptomycetes may be to protect the lysing substrate hyphae from other bacteria present in the soil, thus preserving them as a source of nutrients for aerial growth (Chater and Merrick, 1979).

c) Substrate mycelium as a source of nutrients for aerial mycelium growth

Aerial mycelium formation starts once the nutritional conditions of the medium have been considerably modified as a consequence of the previous growth of the substrate mycelium. Furthermore, aerial hyphae develop on the substrate hyphae, many of which have, at this time, suffered extensive lysis (Wildermuth, 1970; Méndez et al., 1985a). These facts have led to the suggestion that the substrate mycelium could be used as a nutritional support for aerial mycelium development. This hypothesis has been tested with autoradiographic techniques and by studying the effect of nutrient starvation on aerial mycelium formation (Méndez et al., 1985b).

Autoradiographs obtained from sections of young colonies with only substrate hyphae showed homogeneous labelling throughout the mycelium (Fig. 2a). However, when these colonies were transferred to a non radioactive medium, aerial growth was clearly observed and different zones of labelling could be seen throughout the colony (Fig. 2b). In the upper zone (which contains the aerial hyphae), the labelling was uniform but more sparse than below. Microscopic examination of thin sections stained with toluidine blue revealed that labelling was clearly located on the aerial hyphae. In the lower zone, occupied by the substrate mycelium, two different regions may be distinguished: a more intensely labelled region located in the uppermost substrate hyphae (which contains entire cells), and a region with low intensity of labelling in the basal zone of the colony, which also contained substrate hyphae but in an advanced stage of disintegration. As the colony matured, and sporulation took place, radioactivity in the substrate region of the colony decreased and both sporulating aerial hyphae and disintegrated substrate hyphae appeared equally labelled (Fig. 2c). So, the radioactivity, initially present in the substrate mycelium, progressively moved towards the upper zone of the colony as the aerial mycelium developed.

On the other hand, aerial mycelium development may also take place in the absence of an exogenous supply of nutrients (Méndez et al., 1985b). Substrate mycelium of a non-agarolytic *Streptomyces* strain, grown on sterile cellophane disks and transferred onto a medium lacking nutrients (2% Noble Agar in distilled water), showed a layer of aerial hyphae that developed on the substrate mycelium (Fig. 3).

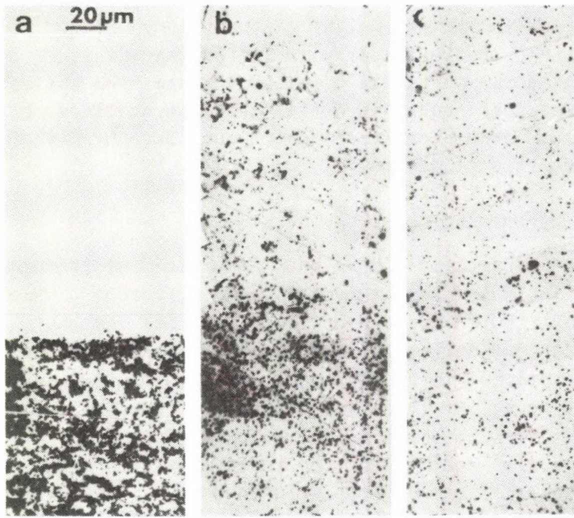

Fig. 2. Distribution of label during colony development in S.antibioticus. Colonies were grown for 24 h on nutrient medium containing ^{3}H-Leucine and then placed on cold medium during aerial mycelium development. Colonies recently transferred to cold medium contained a full-labelled substrate mycelium (a). After 24 h of incubation in cold medium, the intensity of labelling in the substrate mycelium decreased and a uniform labelling appeared throughout the aerial hyphae (b). After 48 h of growth in cold medium, the whole colony was labelled with the same intensity (c).

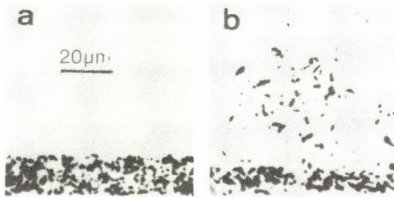

Fig. 3. Thin sections of S.antibioticus colonies during growth on a solid medium lacking nutrients. Colonies with only substrate mycelium (a) were placed onto Agar medium (2% Noble Agar in distilled water). After 48 h of incubation at 28°C, aerial hyphae developed on the surface of the colonies (b).

It therefore seems clear that compounds present in the substrate mycelium may be used as nutrients for serial mycelium growth. Several lines of evidence support this conclusion. Firstly, during colony development, aerial mycelium growth is accompanied by an extensive lysis of the substrate hyphae. Moreover, the important development of aerial

mycelium that takes place during the cell cycle is not accompanied by a significant increase in the dry weight of the colony. Secondly, data from autoradiographic studies clearly indicated that at least certain compounds present in the substrate mycelium migrate from the base of the colony towards the upper region as aerial mycelium develops. Finally, aerial growth occurs under conditions where substrate hyphae are the only available nutritional source.

d) Carbohydrate content during colony development

Glycogen and trehalose have been found in many sporulating fungi and bacteria, including streptomycetes, and different physiological roles have been attributed to each of them. For this reason, the study of carbohydrate content during colony development seemed of interest.

Glycogen started to be measurable at the onset of aerial mycelium formation (Fig. 4). At this time, only 7% of the initial ninhydrin-positive compounds were detected in the culture medium, whereas the glucose concentration was about 50% of the initial value. During growth of the aerial mycelium, glycogen accumulation continued, reaching a maximum (18% of the total dry cell weight) just before the onset of sporulation. From this moment on, the glycogen content of the mycelium decreased gradually.

Accumulation of glycogen in the substrate mycelium may be triggered by the exhaustion of the nitrogen source, as observed in many microorganisms (Dawes and Senior, 1973). Several observations, however, suggest that part, if not all, the glycogen synthesized by the substrate hyphae is not utilized by these cells. Despite the lysis of many substrate hyphae containing glycogen, the total glycogen content of the colony increased steadily during aerial hyphae growth (which at this time of colony development do not accumulate glycogen). In this period more than 25% of the initial glucose is still present in the culture medium, which seems to make glycogen degradation unnecessary as a source of carbon and energy. In addition, many polysaccharide granules were visible among the cell debris in the substrate mycelium. This seems to indicate that substrate hyphae death was not due to a shortage of carbon source, as their reserves remained unused after lysis. A clear decrease in glycogen levels of the colony only occured during the sporulation phase when glucose in the medium approached exhaustion.

The location of the glycogen in the colony during the differentiation process was studied by cytochemical techniques (Thiery, 1967). After 24 h of incubation, the colony contained only substrate mycelium and no polysaccharide granules were present in the hyphae. After about 36 h of incubation, two different regions could be distinguished (Fig. 5a). The lower region contained a large and dense network of substrate hyphae whereas in the upper region a loose network of aerial hyphae started to be formed. In the substrate region many cells appeared completely full of glycogen granules (Fig. 5b). In the basal zone of the

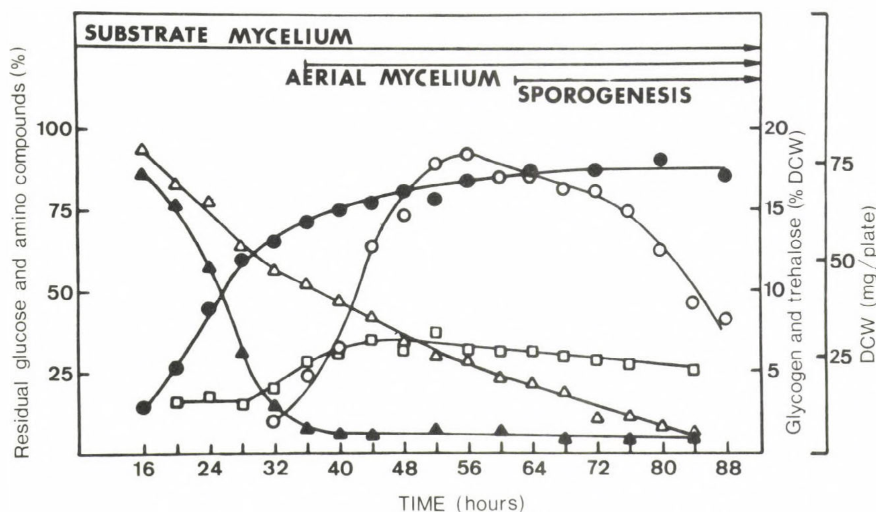

Fig. 4. Growth, sugar accumulation and nutrient depletion during colony development of S.antibioticus. The microorganism was grown on solid medium with 1‰ asparagine, covered with a cellophane membrane. The mycelium was removed from the plates and dry cell weight (●), accumulated glycogen (○) and trehalose (□) were determined. Residual glucose (△) and ninhydrin-positive compounds (▲) were measured in samples of medium previously homogenized in distilled water. The different stages of colony development are indicated at the top of the figure. All determinations were done in triplicate.

colony, substrate hyphae were lysed and devoid of glycogen (Fig. 5c). In a 48 h colony, a well developed aerial mycelium could be observed. At this time of colony development many substrate hyphae were lysed but still retained part of their glycogen content among the cell debris.

In sporulating colonies (after 72 h of incubation) the substrate mycelium was completely lysed, although glycogen granules were still visible. In the aerial region a new round of glycogen synthesis could be observed. It is important to note that in this region and at this time of colony development, glycogen synthesis took place only in sporulating hyphae (Fig. 6), and in no case were glycogen granules seen in the cytoplasm of non-sporulating aerial hyphae. During the sporulation process, the synthesis of glycogen coincided with the formation of the sporulation septum (Fig. 6a) and the maximum polysaccharide accumulation was reached during the first stages of maturation (Fig. 6b). Later, the number of granules progressively decreased and in mature spores no glycogen granules were observed (Fig. 6c). Chemical analysis of isolated spores did not reveal the presence of glycogen.

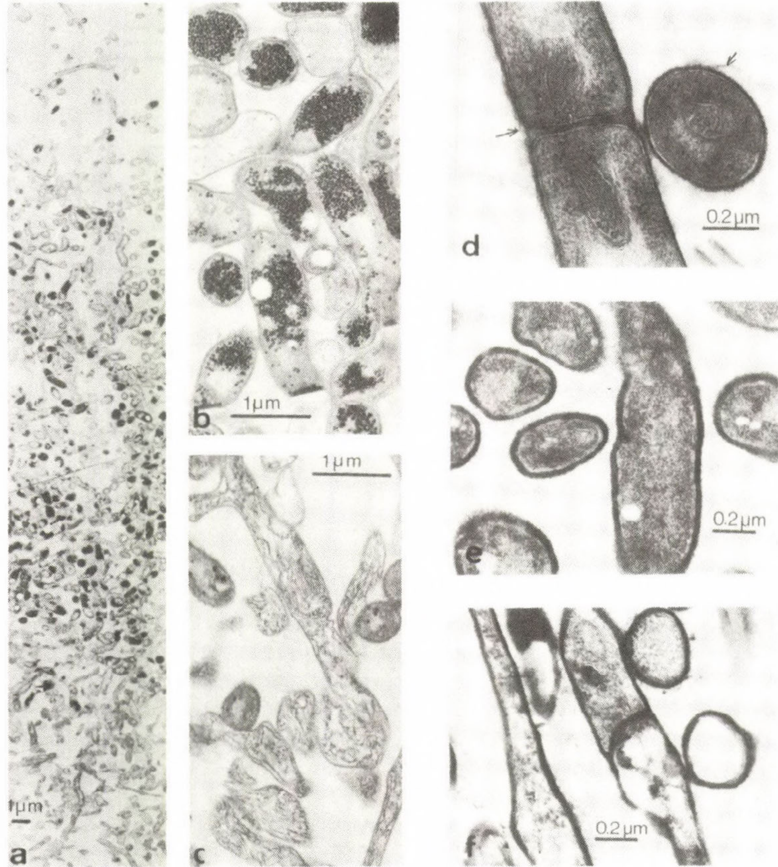

Fig. 5. a, b and c, thin sections of S.antibioticus colonies stained with silver proteinate to demonstrate polysaccharides. Vertical section of a colony grown for 36 h, in which aerial hyphae have started to form (a). In the central region of the colony many substrate hyphae are full of glycogen granules (b), whereas in the basal zone the substrate hyphae are mostly lysed and lack glycogen (c). d, e and f, selected parts of vertical sections of colonies showing ultrastructural characteristics of hyphae from different regions. Sheathed hypha (arrows) from the aerial region of a colony grown for 36 h (d). Substrate hypha from the basal region of the same colony (e). Completely lysed substrate mycelium from the basal region of sporulating colonies after 72 h growth (f).

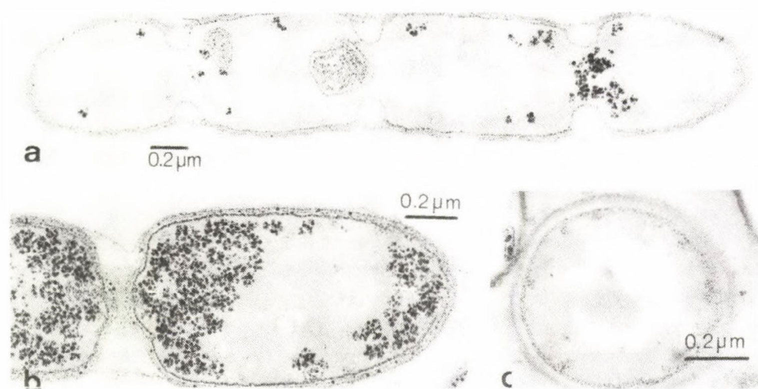

Fig. 6. Sequential development of glycogen granules during sporogenesis. The appearance of glycogen granules coincided with the formation of the sporulation septa (a). Glycogen content increased until maturation began (b). In mature spores no glycogen granules were observed (c).

With respect to the role of the glycogen accumulated during sporogenesis, the absence of this polymer in mature spores clearly excludes any role as a reserve compound for the germination process. The gradual disappearance of glycogen during maturation suggests its involvement in the synthesis of some spore components or as a source of energy to complete the sporulation process as has been proposed for other sporulating microorganisms (Bergere et al., 1980).

The behaviour of trehalose during colony development was quite different to that of glycogen. Trehalose was detected in the substrate hyphae from the initial stages of growth, and its levels remained constant during the first 28 h of substrate growth (Fig. 4). Trehalose synthesis, contrary to glycogen, took place in presence of an available nitrogen source. During growth of the aerial mycelium, total trehalose content of the colony increased from 3% to 7% of the dry cell weight, remaining at this level throughout the entire sporulation process. In the different types of cells, trehalose content (expressed as percentage of dry cell weight) was 2% for substrate hyphae, 5% for aerial hyphae and 12% for spores. In S.antibioticus, trehalose was present in all stages of colony development and did not seem to behave as a typical storage carbohydrate. In addition, its localization differed from that of glycogen, with a greater accumulation in the aerial mycelium and, specially in the spores.

Concluding remarks

During colony development in streptomycetes aerial mycelium formation begins when the nutritional conditions start to be adverse for vegetative growth. At this moment, many hyphae of substrate mycelium are in state of lysis, and compounds from the substrate mycelium may be used as nutrients for the development of the aerial mycelium. In this sense, aerial mycelium behaves as a nutritional parasite on the substrate hyphae.

Because of the growth in absence of water, aerial hyphae must be protected from dehydration. The hydrophobic sheath that surrounds these hyphae must likely help to avoid this problem. We suggest that trehalose may also play a role in protecting aerial hyphae from dehydration. Trehalose is known to protect the structural and functional integrity of isolated membranes under conditions of desiccation (Crowe et al., 1984). Taking into account the preferential accumulation of trehalose in the regions of the colony farthest from the humidity of the culture medium, it is quite tempting to propose an involvement of this compound in protecting the aerial mycelium and/or the spores from desiccation. We have, in fact, found evidence of such an effect with exogenous trehalose added to mycelium and spores (unpublished results). Other possible functions for trehalose must be borne in mind: trehalose may be used for the initial stages of germination (Hey-Ferguson et al., 1973) or as an endogenous source of energy during dormancy (Barton et al., 1982). At the present none of the hypothetical functions can be excluded and further studies are necessary to determine the role of trehalose during the life cycle of Streptomyces.

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SPORE DEVELOPMENT IN SPORANGIA-FORMING ACTINOMYCETES

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INTRODUCTION

Nine genera of the sporangia-forming actinomycetes were traditionally classified into the family Actinoplanaceae (Bland and Couch, 1981). They produce their spores within special receptacles, the sporangia. After spore dispersal, the empty sporangial envelope remains. With the exception of Streptosporangium, all other genera have motile spores with different types of flagellation. The spores can be globose, ovoid, rod-shaped or curved. They are formed in branched or unbranched chains which can be arranged in parallel rows, spirals or irregularly. Depending on the affiliation of the genus, the sporangia contain one, two, few or many spores. The shapes of the sporangia range from spherical, oval, cylindrical, claviform, lobate to irregular. These morphological characters are enough to determine sporangiate isolates to the genus level (Couch and Bland, 1974). The results of chemotaxonomical studies and ribosomal RNA cataloguing lead to the separation of the sporangiate actinomycetes into two groups and to the combination with non-sporangiate genera (Goodfellow and Cross, 1983).

A brief description of the morphology of sporangia of some genera shall be given. The genera Spirillospora and Planomonospora are described in somewhat more detail.

Abbreviations:

LM: light microscope; SEM: scanning electron microscope; TEM: transmission electron microscope; cw: cross wall; di: diaphragm; fe: fibrous elements; hw: hyphal wall; se: sporangial envelope; sh: sporogenous hypha; si: sporangial initial; sp: sporangiophore; so: spore; sw: spore wall.

MATERIAL AND METHODS

The strains investigated are stated in the legends of Figs. 1 to 6. Colonies were grown on artificial soil extract agar in small Petri-plates for several days at temperatures up to 30° C. The culture media and the preparation techniques were described previously (Vobis, 1981; Vobis and Zimmermann,1984; Vobis and Kothe, 1985). For micrographs a Wild M 20 (LM), a Leitz AMR 1200 B (SEM) and a Siemens IA (TEM) were used.

RESULTS AND DISCUSSION

Actinoplanes and Amorphosporangium

The species of both genera have spherical to irregular sporangia containing numerous globose spores. The sporangia can be developed at the tip of palisade hyphae (Couch, 1950), which are anchored in the agar medium (Fig. 1,A). The ultrastructure of spore formation was investigated in various strains of Actinoplanes by Lechevalier and Holbert (1965), Lechevalier et al. (1966), Sharples et al. (1974) and Vobis and Kothe (1985). The morphology of the irregular sporangia of Amorphosporangium species was studied in SEM by Locci (1976).

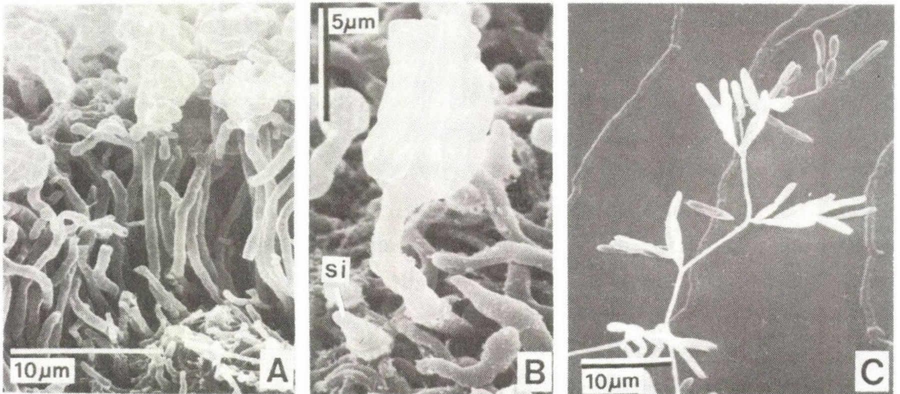

Fig. 1. (A) Sporangia of Actinoplanes sp. (strain MB-VE 1092) developed on palisade hyphae (SEM). (B) Substrate hyphae, sporangial initial and sporangium of Ampullariella regularis (strain MB-SE 1) on the surface of agar (SEM). (C) Bundles of sporangia of Planobispora rosea (strain ATCC 23866) on aerial hypha grown on cover slip (SEM).

Ampullariella and Pilimelia

The species of Ampullariella and Pilimelia have rod-shaped spores, which are arranged generally in parallel rows. The sporangia are produced at the surface of the substrate. The sporangiophore arised out of the aqueous milieu of the agar medium into the air. The point of transition was observable due to the different contrast in SEM (Fig. 1,B). At the surface of sporangia, an interesting hairy structure, not known until now, was described in Ampullariella pekinensis (Seino, 1983). The spore formation in the Pilimelia species was studied previously (Vobis, 1984).

Dactylosporangium

The formation of the zoospores of Dactylosporangium had been claimed to be endogenous (Sharples et al., 1974). A new investigation of the spore development in a well sporulating isolate undoubtedly proved that the mode of sporulation is similar to that found in Actinoplanes or Pilimelia (Vobis and Kothe, 1985), as already presumed by Ensign (1978).

Streptosporangium and Planobispora

The multispored and spherical sporangia of Streptosporangium contain one unbranched chain of spores which are coiled into spirals. The spores are non-motile. The ultrastructure of the spore development was studied by Lechevalier et al. (1966). The sporangia of Planobispora were produced in bundles on short ramifications of aerial hyphae (Fig. 1,C). To clarify the mode of spore formation in this genus, it will be necessary to investigate ultrathin sections in TEM.

Spirillospora

The development and ultrastructure of the sporangia of Spirillospora strains were studied in SEM and TEM. After 15 days of incubation at 30° C, the surface of the colonies of Spirillospora albida was completely covered with sporangia developed on aerial mycelium (Fig. 2,A). The tip of a hypha coiled into some narrow windings and branched already at this early stage of sporangial development (Fig. 2,B; 4,A,B). The narrow branches coiled outside parallel to the inner windings to form a compact small sporangium (Fig. 2,C; 4,C). The sporangiophore remained inserted laterally until maturation of the sporangium (Fig. 2,G; 4,D). The snail-like spirals of the sporogenous hyphae could be seen through the sporangial envelope (Fig. 2,D). If the envelope ruptured i.e. by preparation, the parallel windings

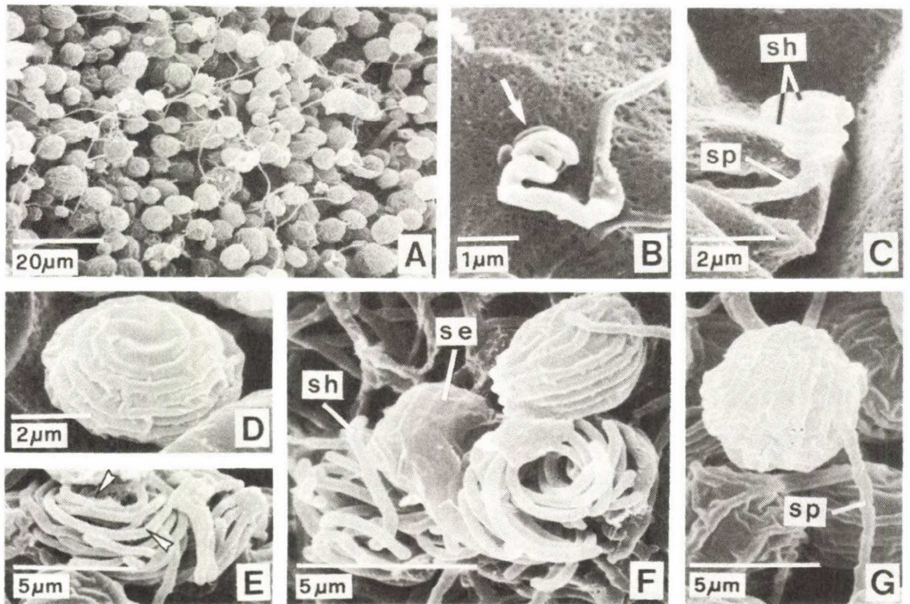

Fig. 2. Sporangial development in Spirillospora albida (strain ATCC 15331) in SEM. (A) Sporangia at the surface of a colony. (B) Coiled hyphal tip with branching (arrow) . (C) Sporangium in an early stage of development. (D) Spirals of sporogenous hyphae covered by the sporangial envelope. (E) Branching of sporogenous hyphae (arrows). (F) Uncovered sporogenous hyphae. (G) Mature spherical sporangium.

could be observed more clearly (Fig. 2,F), even the ramifications could be demonstrated (Fig. 2,E). Normally the branchings were observed only in sections (Fig. 3,B). Small two-layered cross walls divided the sporogenous hyphae into oblong segments (Fig. 3,A,B), each of which became a spore (Fig. 4,D). The mature spores were rod-like, slightly curved and surrounded by a one-layered spore wall (Fig. 3, G). The sporangiophore was covered by a thin sheath (Fig. 3,E). It was composed of small interwoven fibrous elements (unpublished result), which was characteristic also for the aerial hyphae (Fig. 3,D). The sheath of the sporangiophore continoued directly into the sporangial envelope (Fig. 3,F). Ultrathin sections of the envelope demonstrated that it was composed of fibrous elements, embedded in an electron dense material (Fig. 3,C). The results of the studies presented in Figs. 2 and 3 are summarized in a diagram (Fig. 4,A-D).

The earlier descriptions of spore formation of Couch (1963), Lechevalier et al. (1966) and Locci (1976) were also taken in consideration.

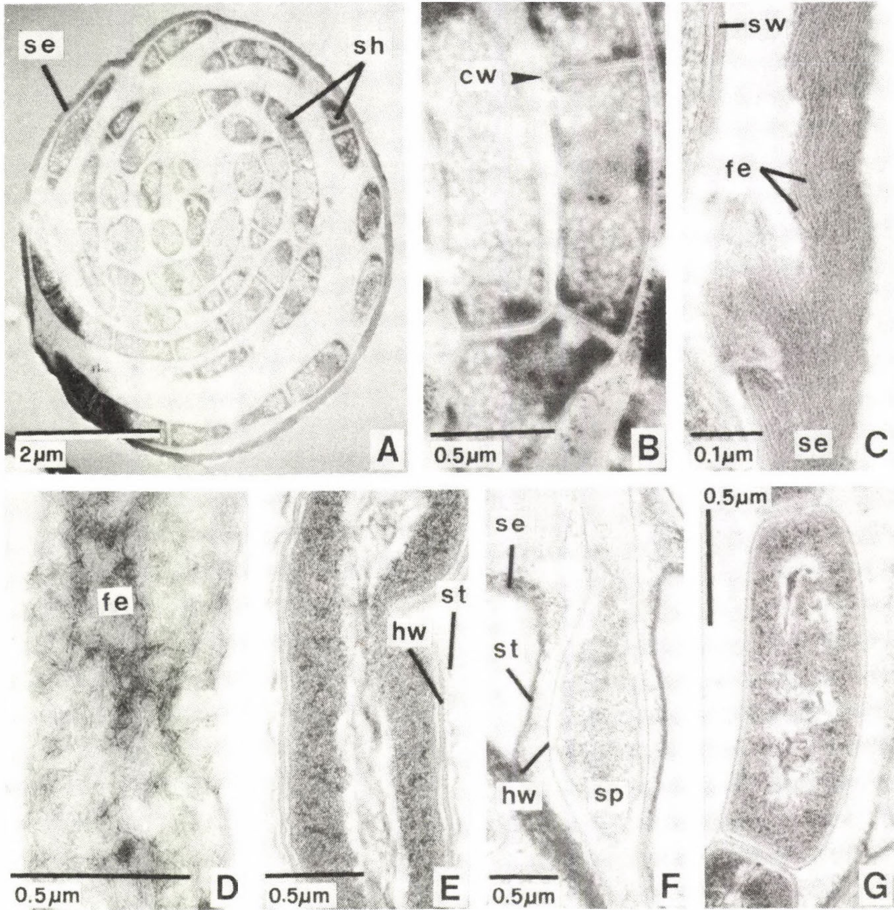

Fig. 3. Fine structure of Spirillospora rubra (strain MB-SK 31) (A,B,C,E) and Spirillospora albida (strain ATCC 15331) (D,F,G). (A) Section through a mature sporangium. (B) Branched and septate sporogenous hypha. (C) Thick sporangial envelope with fibrous elements. (D) Negatively contrasted fibrous elements of an aerial hypha. (E) Longitudinal section through the sporangiophore. (F) Penetration point of the sporangiophore into the sporangium. (G) Mature spore within the sporangium.

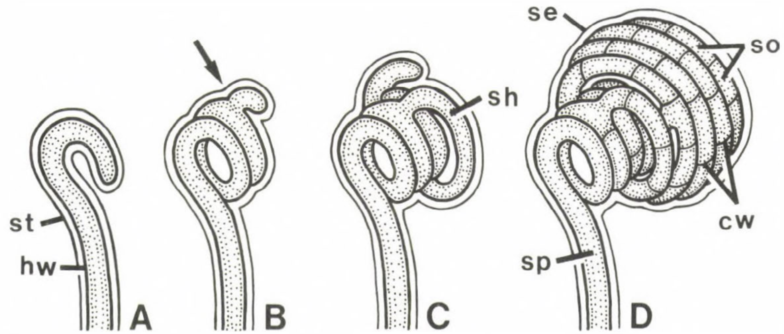

Fig. 4. Diagram of the sporangial development in Spirillospora species. (A) Coiling of a hyphal tip. (B) Narrow windings with branching (arrow). (C) Loose outer windings developed additionally. (D) Sporangium with septate sporogenous hyphae; sporangiophore inserted laterally.

Planomonospora

After 10 days of incubation, numerous sporangia were produced terminally and on side branches of the aerial hyphae of Planomonospora parontospora (Fig. 5,A). They sat vertically on the sporangiophores forming parallel double rows (Fig. 5,B,C). The sporangial initials were developed from the tip of a slightly curved sporangiophore which elongated parallel to the previous sporangium (Fig. 5,F). After reaching the full length, the sporogenous hypha was divided by a double layered cross wall and separated additionally by a diaphragm (Vobis and Kothe, 1985). The material of the diaphragm was derived from the sheath (Fig. 5,E,F). The wall of the sporogenous cell and the sheath increased to form a one-layered spore wall and the sporangial envelope, respectively (Fig. 5,C,D,E). The fibrous elements of the envelope were sometimes detectable in ultrathin sections (Fig. 5,D).

The strains of Planomonospora venezuelensis produced their sporangia singly or in bundles forming a characteristic palm leaf pattern (Fig. 6, A,B,E). The sporangia developed from an apical elongation of the sporangiophore. This apical part of the hypha swelled into a clavate shape and was separated at the base by a double layered cross wall and a diaphragm (unpublished result). The thin wall of the sporogenous cell thickened to form a one-layered spore wall (Fig. 6,C,D). The sporangial envelope originated from the sheath of the apical part of the sporangiophore. The palm

leaf pattern can be explained by the subsequent alternate formation of new sporangia from the tip of the sporangiophore (Fig. 6,A,E).

The morphology and ultrastructure of the sporangia of Planomonospora species were investigated repeatedly by LM, SEM and TEM (Locci and Petrolini Baldan, 1971; Sharples et al., 1974; Thiemann, 1970; Thiemann et al., 1967; Williams, 1970; Williams et al., 1973). A short summary of our studies shall be given. The pattern of spore development is just as in other sporangiate actinomycetes, i.e. in Actinoplanes, Dactylosporangium or Streptosporangium (Vobis and Kothe, 1985). It corresponds to the category

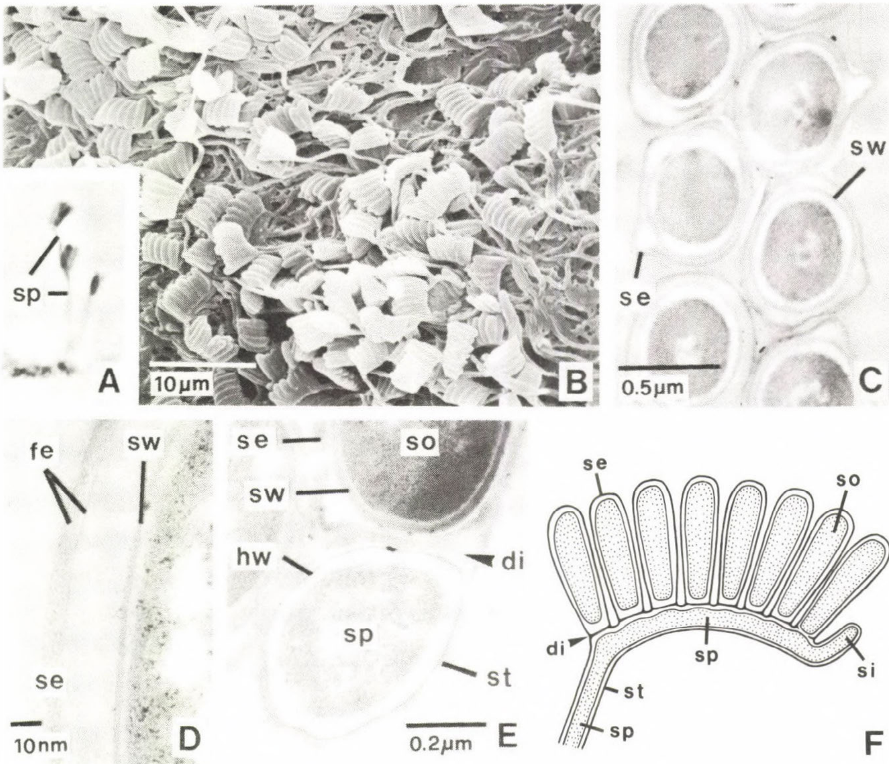

Fig. 5. Sporangia of Planomonospora parontospora (strain ATCC 23863) in LM (A), SEM (B) and TEM (C,D,E). (A) Sporangiophore with few, young sporangia. (B) Aerial mycelium with abundant sporangia arranged in parallel double rows. (C) Cross section through monosporous sporangia. (D) Sporangial envelope with fibrous elements. (E) Base of a mature sporangium. (F) Diagram of the development of sporangiophore and sporangia.

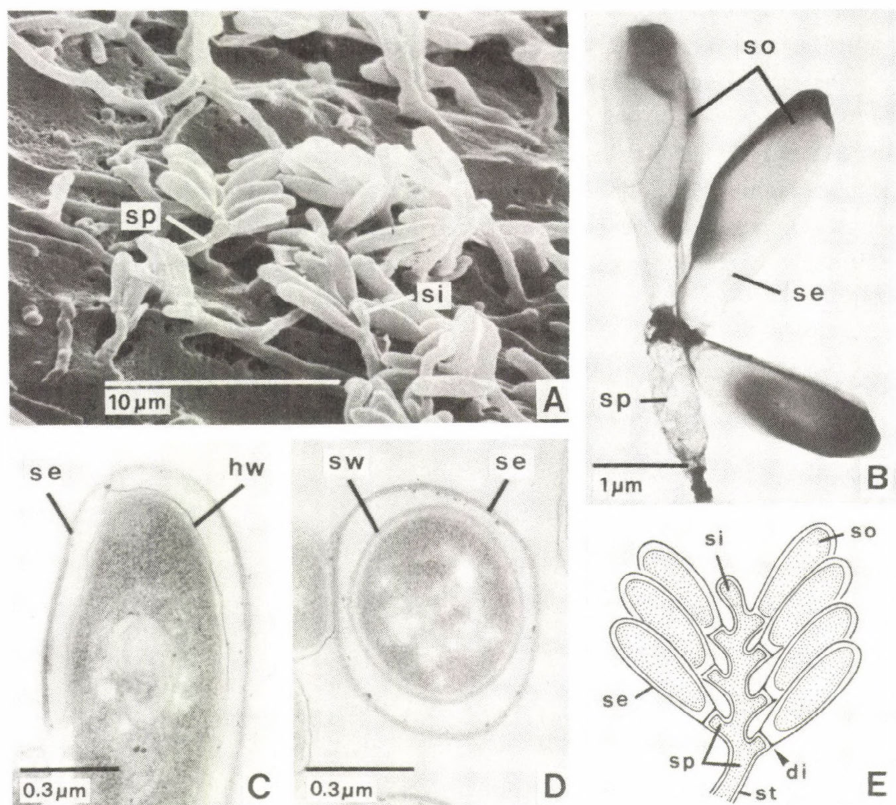

Fig. 6. Sporangia of Planomonospora venezuelensis, strain ATCC 2385 in SEM (A), and strain MB-SE 100 in TEM (B,C,D). (A) Sporangia at various stages of development. (B) Whole mount preparation of a bundle of three monosporous sporangia. (C) Longitudinal section through the tip of a young sporangium. (D) Cross section of a mature sporangium. (E) Development of sporangia in diagram, forming the palm leaf pattern.

"spores of hyphal origin" of Cross and Attwell (1975). The sporangial envelope originates from the fibrous sheath; a special opening mechanism at the tip of the sporangium is not detectable. The base of the sporangium is separated from the sporangiophore by a substance, which is derivated from the envelope. It is called diaphragm and is also observable in the sporangia of Planobispora (Vobis and Kothe, 1985). Prosumably the diaphragm is the intrasporangial substance which swells in water and plays an important role during spore releasing as described by Thiemann (1970).

To describe the pattern of spore development, Locci and Sharples (1983) introduced mycological terms, which are normally used to characterize the various conidiogeneses in deuteromycetes. After critical assessment of the validitation of these terms, this can represent a new starting point of a more precise description of sporogenesis in actinomycetes.

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CHROMOSOME STRUCTURE AND DNA-BINDING PROTEINS IN STREPTOMYCETES

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The structure of the chromosome of streptomycetes is described by the highly ordered packaging of the double-stranded DNA in the nucleoid which involves interactions with RNA and proteins.
The primary and secondary DNA structure of streptomycetes is in principle the same as in other bacteria. Streptomyces DNAs exhibit the most extremes in base composition ranging from 69.8 to 76.4 mole-% in GC content (Gladek and Zakrzewska 1984, Zimmer and Venner 1964). 5 methylcytosine has been frequently detected in *Streptomyces* DNA as minor base.
Due to the high GC content *Streptomyces* DNA exerts a high stability against heat and acid denaturation (Zimmer and Triebel 1969).
The DNA forms a characteristic double-stranded closed ring structure. Different data exist for the length estimation of chromosomal DNA in streptomycetes. Using DNA reassociation kinetics Benigni et al. (1975) estimated a genome size of 6.33-6.77 x 10^9 Da for *S. rimosus* and of 7.09-7.23 x 10^9 Da for *S. coelicolor* corresponding to 3.5 to 3.7 mm in length. Values for the *E. coli* chromosome are 2.7 x 10^9 Da and 1.2 mm. According to these results the *Streptomyces* chromosome would be 3 times longer than that of *E. coli*.
Gladek and Zakrzewska (1984) determined the genome size of 13 *Streptomyces* species and 2 *Streptoverticillium* strains by another reassociation method which yields more realistic values. Their values ranged from 3.69 to 5.15 x 10^9 Da. Thus the size of the chromosome in streptomycetes seems to be only 1.5 times larger than those of *E. coli*.
Very little is known about the tertiary and quaternary DNA structure in streptomycetes which means the folding pattern and packaging of DNA within the nucleoid. The DNA thread of 3.6 mm (Benigni et al. 1975) or 1.8 mm (Gladek and Zakrzewska 1984) respectively must be folded in such a manner, that it occupies a small volume of 0.4-0.7 µm in diameter only and that the package allows replication, segregation, and transcription without tearing or breaking of the DNA strands.

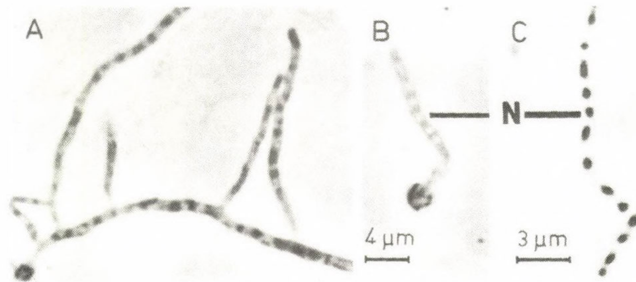

Fig. 1. Shape and distribution of nucleoids in hyphae of Streptomyces hygroscopicus. A and B are viable cells growing on medium supplemented by 20 % gelatine and investigated by phase contrast microscopy; C is a hypha after HCl-Giemsa staining. Nu = nucleoid, Sp = spore.

Nucleoids in situ

Light microscopic investigations demonstrate that the chromosomal DNA is localized in discrete nucleoid regions within the growing hyphae (Fig. 1).
After fixation and staining (e.g. by the HCl-Giemsa method) nucleoids appear as spherical, ellipsoidal or dump-bell shaped heavily stained bodies varying in size between 0.3-0.7 µm and separated by zones of cytoplasm. The average distance of two nucleoids is about 1.9 µm.
Similar forms and similar distribution of the nucleoids can be seen in viable cells by using phase contrast microscopy and 20 % gelatine in the medium.
Electron microscopic investigations of the nucleoids by the ultrathin section technique revealed a fine structure similar to that of nucleoids in other bacteria (Fig. 2) (Hobot et al. 1985). Defined nucleoid regions corresponding to those obtained by phase contrast microscopy can be obtained after osmium tetroxide fixation. In ultrathin sections the nucleoid appears as an electron transparent area more or less clearly demarcated from the cytoplasm but without a surrounding membrane structure.
The nucleoid contains thin electron dense fibres about 2.5 nm in diameter, arranged as a more or less regular network or in parallel bundles. As in other bacteria, the pattern of the fibres does not allow any conclusion concerning the packaging of the DNA threads within the nucleoid.

Isolated nucleoids

Important data about the structural organization of the bacterial chromosome have been obtained when it became possible to isolate nucleoids and to investigate the chromosome in the folded state using biochemical and electron microscopic methods (Charret et al. 1980, DWORSKI 1975, Kavenoff and Bowen 1976, Kleppe et al. 1979, Stonington and Pettijohn 1971, Van Ness and Pettijohn 1979, Worcel and Burgi 1972, 1974).

During last years we have studied nucleoids isolated from S. hygroscopicus (Sarfert et al. 1983). Vegetative hyphae, grown for 18-30 hrs in a complex AL 58 medium supplemented

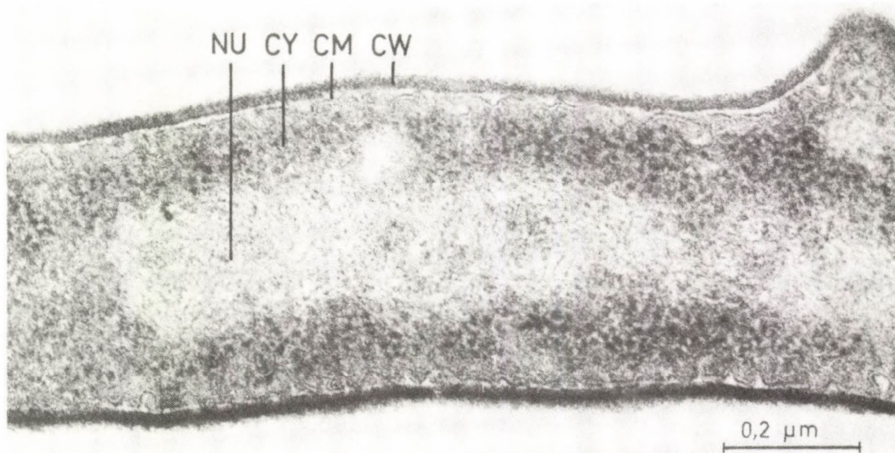

Fig. 2. Nucleoid region (NU) in a hypha of Streptomyces hygroscopicus showing characteristic fibrillar fine structure. CW: cell wall; CM: cytoplasmic membrane; CY: cytoplasm with ribosomes. Ultrathin section of osmium tetroxide fixed specimen.

with ^3H thymidine in a final concentration of 2-6 µCi/ml, were washed in TRIS buffer (pH 8.2) supplemented with 20 % sucrose and digested with lysozyme (500 µg/ml), Na_3EDTA (5 mM) phenylmethylsulfonyl fluoride (0.5 mM), Brij-58 (0.5 %), Na-deoxycholate (0.2 %), spermidine (0.6 mM), and NaCl (0.2-1 M). The nucleoid fraction has been separated from the lysate by sucrose gradient centrifugation (0-30 %) at 8000 g for 1 h.

The isolated nucleoids sediment between 1500 and 1000 S. These sedimentation coefficients agree with those of isolated membrane-free nucleoids of E. coli (Korch et al. 1976, Worcel et al. 1973, Worcel and Burgi 1974).

The isolated nucleoid of S. hygroscopicus consists of 55 % DNA, 20 % RNA, and 25 % protein.

The major portion of the DNA in the isolated nucleoids is in a superhelical state as shown by ethidium bromide intercalation experiments. An increase in the ethidium bromide concentration from 0 to 10 µg/ml caused first a loss of negative superhelical turns, measured as a decrease in the S-values, followed by creation of positive superhelical turns, visible as an increase in the sedimentation rate. The minimum in the sedimentation rate at 2 µg/ml ethidium bromide, representing an equilibrium between the number of titratable superhelical turns removed and those originally present, was the same as in the nucleoids of E. coli as well as for intracellular SV 40 and λ closed-circular DNA (Worcel and Burgi 1972). The intercalation experiments demonstrated that the DNA exists in a superhelical state in nucleoids isolated from S. hygroscopicus. Taken into account the corrected value of unwinding of double-stranded DNA by ethidium bromide (Wang 1974) the superhelical concentration is one negative super-

helical turn per 200 base pairs. This is similar to the superhelix concentration in ccc DNA and in nucleoid DNA of *E. coli* (Worcel and Burgi 1972). The original value of 400 bp representing one superhelical turn given by Worcel and Burgi (1972) has to be corrected by a factor of two according to the finding of Wang (1974). Based on a genome size of 5 x 10^9 Da the total number of negative superhelical turns would be about 40 000 per chromosome in streptomycetes.
RNAse treatment (50 μg/ml, 3 min) lowers the sedimentation coefficients to 800 S. The sedimentation coefficients of these nucleoids show similar changes upon increasing ethidium bromide concentrations as untreated nucleoids. Obviously, the RNAse causes only a partial loosening of the superhelical structure, and the RNA does not play the dominant role in the folding of the DNA.
Proteinase K treatment at 37 ^{o}C likewise caused a decrease of the sedimentation coefficient. This indicates that the protein component in the nucleoids has a significance in the packaging of the DNA (Sarfert et al. 1983).

Electron microscopy of isolated nucleoids

Isolated nucleoids can be investigated in the electron microscope by using the DNA spreading technique according to Kleinschmidt (1968). Most of the nucleoids of *S. hygroscopicus* appeared more or less damaged. This may be attributed to incomplete inactivation of proteases and nucleases during lysis procedure, mechanical shear, too strong or too poor spreading on the hypophase and too weak attachment at the surface of the filmed grid resulting in removal during staining and washing procedure.
A partially unfolded nucleoid is shown in Fig. 3. Large amounts of DNA threads emerge from the elongated central fiber structures. Most DNA strands form loops of different length. Others are more than 20 μm in length and show free ends. It is not clear whether both central fiber structures and the associated DNA strands belong to two chromosomes or whether they are two halves of one chromosome. The second interpretation would be consistent with a consideration that the chromosomal DNA has to be packed up as two separate halves in one nucleoid. This is one assumption of a model concerning the structural organization of the bacterial chromosome based on light and electron microscopic analyses of the nucleoid shape during the cell cycle in *E. coli* (Gumpert 1983).
A better preserved nucleoid is shown in Fig. 4. It is characterized by a dense core structure (0.5 x 0.2 μm) and intact DNA loops. It seems that the high protein content of the *Streptomyces* nucleoid is one reason for the relatively large core structure. Membrane-free nucleoids of *E. coli* showed less dense cores (Kavenoff and Bowen 1976).
More than 60 % of the loops are 2-4 μm in length. The others are shorter or longer. Strands with free ends are rare. In some loops a characteristic superhelical configuration with more or less extended windings is preserved. 94 loops of 2-4 μm in length are countable in the nucleoid (Fig. 4). If one consider that the longer loops corespond to two loops of that typical length, the total number would increase to 116.

Fig. 3. Partially unfolded membrane-free nucleoid isolated from Streptomyces hygroscopicus. Note the two central fiber structures connected by several strands. Many open and supercoiled loops and strands with free ends are extremely long. 30 μl of the nucleoid fraction were mixed with the spreading solution containing 40 μl of a mixture of formamide (60 %) and TRIS (0.5 M, pH 8.5), 10 μl cytochrome C (0.5 %) and 20 μl distilled water and spread on a hypophase (formamide 10 % + TRIS 0.01 M, pH 8.5). Monolayers were sampled by parlodion-coated grids. They were stained with uranyl acetate (1 %), rotary shadowed with platinum-iridium and investigated in a TESLA BS 540 electron microscope.

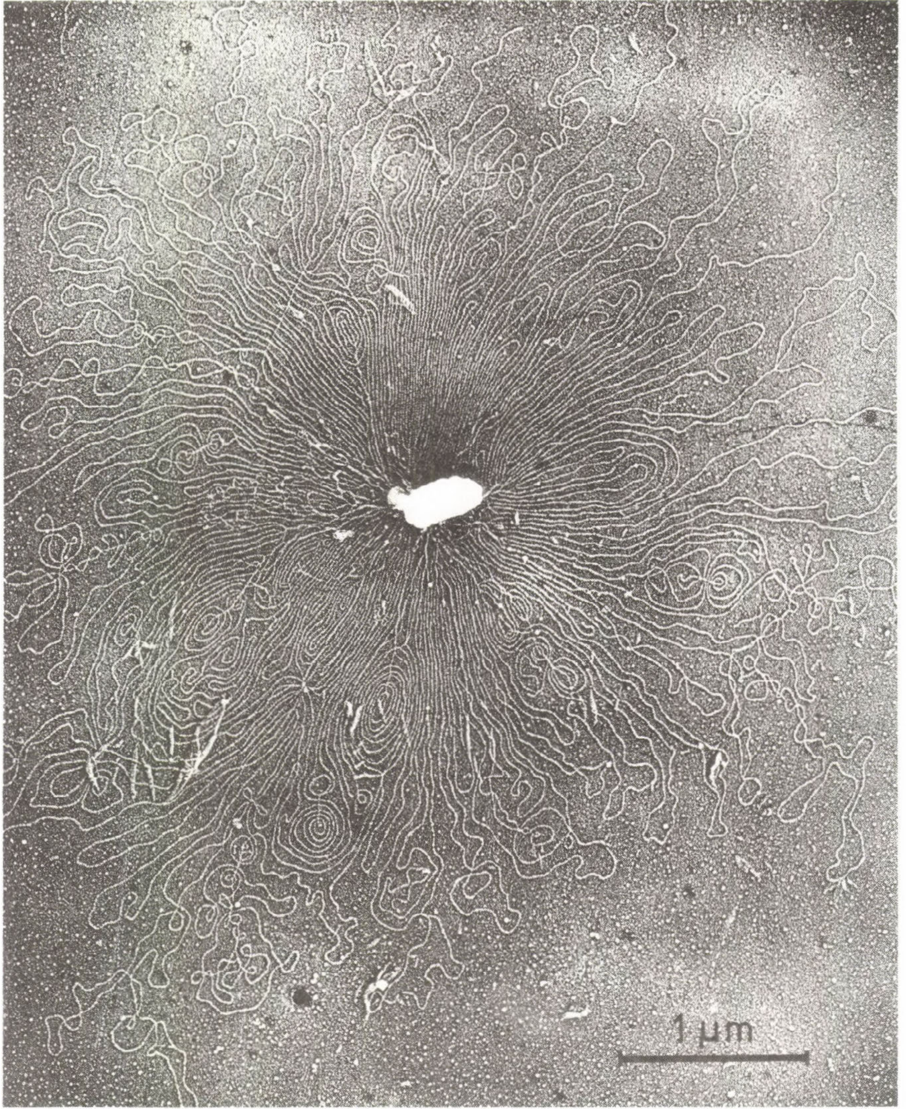

Fig. 4. Isolated nucleoid from Streptomyces hygroscopicus characterized by a dense core structure and intact loops of DNA strands emerge.

Between 65 and 130 loops have been counted by Kavenoff and Ryder (1976) in nucleoids of E. coli.

These loop numbers are in the same order of magnitude as the number of domains of supercoiling present in the bacterial chromosome. Data from relaxation experiments (Pettijohn 1982) lead to the conclusion that the whole bacterial chromosome is subdivided into 100 ± 30 separate topological units representing domains of supercoiling. The ends of these units are fixed sites on the DNA double helix that limit its rotation. Other authors calculated about 50 topologically independent domains (Sinden and Pettijohn 1981, Worcel and Burgi 1972). Obviously, the DNA of such units can be folded and unfolded independently from the folded state of adjacent domains. One might conclude that the DNA loops recognizable in spread nucleoids correspond to the domains of supercoiling.
An exact measurement of the total contour length of the DNA threads in a spread nucleoid is not possible since the high concentration of threads in the narrow zone around the core does not allow a sufficient clear resolution. Free contour lengths of 550-900 μm have been measured very roughly in several nucleoids similar to that in Fig. 4. These values would correspond to 40-75 % of the length of an E. coli chromosome (1.2 mm). Supposing the Streptomyces chromosome is 3 times or 1.5 times longer than that of E. coli, the amount of DNA threads seen in Fig. 4 would represent 25 % or 50 % respectively of the total chromosomal DNA of S. hygroscopicus.

The large amount of DNA strands has to be packed up in a highly ordered manner in the growing cell. In view of the folding and function of the chromosomal domains the presence of specific DNA-binding proteins are of special interest.

DNA-binding proteins

Several single-stranded and double-stranded DNA-binding proteins are known to interact with chromosomal DNA in bacteria (Geider and Hoffmann-Berling 1981). Among them are histone-like proteins which seem to play a role in the folding of DNA threads. Several such "scaffolding proteins" were identified recently in E. coli showing molecular weights between 9 and 28 kDa (Kishi et al. 1982, Lammi et al. 1984, Rouviere-Yaniv and Gros 1975, Yamazaki et al. 1984). Similar proteins have been detected in isolated nucleoid of Bac. subtilis (Nakayama et al. 1981), while, nothing is known about such proteins in streptomycetes.
A characteristic protein called S protein has been detected when isolated nucleoids from S. hygroscopicus were analysed by SDS polyacrylamide gel electrophoresis (Fig. 5). The same was found in lysates of cells treated with lysozyme or by sonication. The protein was also present in acid soluble protein extracts after sonication of S. levoris, S. lividans, and S. coelicolor. It was absent in the protein extract of Thermoactinomyces vulgaris and only traces could be detected in those of Nocardioides albus (Sarfert et al. 1983).
The S protein has nearly the same position in the SDS polyacrylamide gel as lysozyme. The position between histon H2A and H4 may indicate its histone-like character (Fig. 5). In Triton-urea polyacrylamide gel electrophoresis, however, it behaved different from the histones.
DNA affinity chromatography showed that the S protein represents a homogeneous component of about 15 kDa in molecular

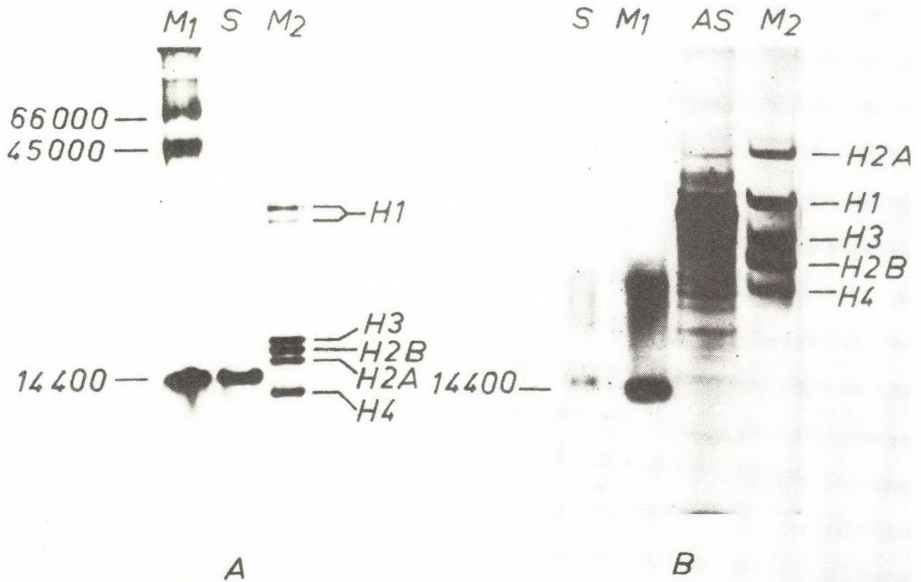

Fig. 5. Polyacrylamide gel electrophoresis of S protein from Streptomyces hygroscopicus in SDS (sodium dodecylsulfate) according to Weber and Osborn (1969) (A) and in Triton-urea according to Zweidler (1978) (B). M1: protein markers for molecular weight determination; bovine serum albumin (66 kDa), ovalbumin (45 kDa), lysozyme (14.4 kDa); M2: total histone from rat liver; S: S protein; AS: acid soluble proteins.

Fig. 6. SDS polyacrylamide gel electrophoresis of S protein from isolated nucleoids of Streptomyces hygroscopicus after digestion by proteinase K.

1: calf thymus chromatin
2: proteinase K
3: untreated nucleoid
4: proteinase K treatment 20 min, 20 °C
5: proteinase K treatment 20 min, 37 °C
6: lysozyme

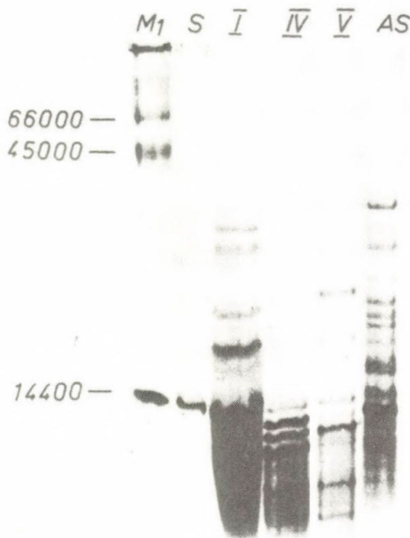

Fig. 7. SDS polyacrylamide gel electrophoresis of ASB proteins of Streptomyces hygroscopicus. Isolation procedure:preparation of cell lysates by sonication, extraction with 0.25 M H_2SO_4, ethanolic precipitation, solubilisation in TRIS EDTA buffer (pH 8.1) by dialysis, DNA cellulose column chromatography according to Sjastad et al. (1983),elution in a KCl gradient (0-1.5 M). M1:marker proteins bovine serum albumin (66 kDa), ovalbumin (45 kDa), lysozyme (14.4 kDa); S: purified S protein; I: proteins in the flow-through fraction; IV: ASB proteins in the highest peak at 0.2 M KCl; V: proteins from a low peak at 0.35 M KCl; AS: proteins in the total acid soluble extract.

weight. After digestion of the nucleoid by proteinase K (20 min at 37 °C) the S protein band disappeared in the SDS polyacrylamide gel electropherogram (Fig. 6).
After isolation and separation of the acid soluble proteins from total cell lysates by DNA cellulose column chromatography and subsequent SDS polyacrylamide gel electrophoresis further DNA-binding proteins could be detected (Fig. 7). These ASB (acid soluble binding) proteins occurred mainly in one peak after elution with 0-0.2 M KCl. Their molecular weights are lower than that of the S protein. In Triton-urea (8 M) gel electrophoresis their bands are located near to H4 histone. Probably they might represent proteins of histone-like character.
Experiments on the interaction of S and ASB proteins with calf thymus DNA showed different effects. The S protein had no stabilizing effect proved by melting temperature measurements. The ASB proteins, however, showed an increase in the melting temperature for calf thymus DNA of about ΔT_m = 15 °C in 2 mM NaCl. This is a relatively large stabilization effect and it demonstrates that ASB proteins are double-stranded DNA-binding proteins. Further experiments are required, however, to show the function of these low molecular weight proteins upon DNA-binding in the nucleoid.
Several possibilities might explain the contradiction between the failure of a stabilizing effect of the S protein on DNA in vitro and its significance for the integrity of nucleoids proved by proteinase K digestion. One explanation could be that the S protein is involved in the binding of single-stranded segments of DNA loops by a limited number of binding sites only. Nothing is known whether nuclesome-like struc-

tures exist in Streptomyces as it is suggested in E. coli (Pettijohn 1982, Rouviere-Yaniv et al. 1979). We hope that further characterization of these DNA-binding proteins will contribute to a better understanding of the structural organization and function of the chromosomal DNA in streptomycetes.

We thank Mrs. Monika Völkel and A. Engelmann for excellent technical assistance and Dr. S. Klaus for the help in applying the monolayer technique.

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STREPTOMYCES sp. CHITIN DEVELOPMENT DURING SPORULATION

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Streptomyces spore ornamentation has been considered a stable and therefore a useful taxonomic characteristic (Tresner, Davies and Backus, 1961). In 1955, Vernon reported the "patterned nature" of what he described as a sheath surrounding Streptomyces sporulating hyphae (Vernon, 1955). Stock cultures and freshly isolated strains all produced a "sheath" which apparently split longitudinally releasing the spores. The sheath did not stain with the lipid stain, Sudan IV although Erikson (1947) had previously indicated Sudan IV staining of spore chains.

Hopwood and Glauert (1961) further developed the sheath concept of a "loose covering" composed of fibrils. Their observations (1961; Glauert & Hopwood, 1961) were based upon electron microscopy of surface images from negatively stained material or carbon replicas of freeze-dried samples. The conclusion was that the rodlet (fiber) structures were the outermost spore component.

Utilizing freeze-etch replication technology, numerous authors have since revealed a more complex picture of the Streptomyces spore sheath. Wildermuth, Wehrli and Horne (1971) demonstrated clear images of the fibrillar mosaic (rodlet mosaic) (which was earlier described by Hopwood and Glauert (1961)) and in addition identified an additional amorphous layer of material outside the fibrous layer. This amorphous layer often masked the clear images of the fiber mosaic.

Williams, Bradshaw, Costerton and Forge (1972) used a combination of sample preparation techniques to present a developmental story of Streptomyces spore sheath. Young vegetative hyphae lack a sheath, but the sheath developed prior to the onset of sporulation. These authors also reported an amorphous component of the spore sheath and hypothesized that the fine fibrillar structures may be a stage in development of spore sheath elements.

In 1978 we presented a spore envelope model for Streptomyces coelicolor A3(2) (Smucker & Pfister 1978). Based on a suite of sample preparation methods, the model presented the fibrillar component as structurally distinct from the more loosely associated sheath. Freeze-etch replication disclosed two distinctly defined components located outside the fibrous layer.

Roles of the fibrillar elements and sheath in spore germination was examined by several authors. Whole mount preparations and carbon replicas of whole cells consistently reveal fibrillar elements present with spores, but not on the emergent germ tube itself (Vernon, 1955; Hopwood and Glauert, 1961; Sharples & Williams, 1976; Williams, Bradshaw, Costerton & Forge, 1972; Strunk, 1978).

Only several accounts have addressed effective isolation of the fibrous spore component and analysis of the fibers. Current evidence based upon rigorous extraction procedures, X-ray diffraction, complete hydrolysis of extracted fibers coupled with TLC and colorimetric chemistry, enzymatic sensitivity, and structural similarities to crab chitin support the hypothesis that the fibrils occurring during sporulation in Streptomyces are chitin (Smucker and Pfister, 1978; Smucker, 1984a).

Despite the numerous reports of Streptomyces sheath and the fibrous layer of sporulating cultures, there has been a general lack of interest in this area of research since the early 1970's. Some of the conceptual difficulty of fibrillar component significance may reside in the implications of the term "sheath". Strictly speaking, a sheath is a temporary structure having no essential integral role in activity of the ensheathed structure. In the present case, the prevailing concept views the fibrous layer covering Streptomyces spores during development as a consequence of being part of the sheath. It follows that the fibrous component must be only temporary and therefore does not participate in spore ontogeny throughout germination.

One objective of this report is to present new evidence for the role of chitin fibers in spore ontogeny throughout germination events. Another objective is to clarify the concept of sheath and to distinguish the fibrous component from the sheath. Present results will be discussed in the context of genetic, biochemical and ultrastructural evidence.

MATERIALS AND METHODS

Cultures and Media

Streptomyces coelicolor A3(2) (ISP#5049) and S. bambergiensis (ISP #5590) were provided by E. B. Shirling from the ISP culture collection. Streptomyces griseus (OSU #500) was obtained from the Ohio State University. A few strains isolated from Lake Erie sediment were also used in the ultrastructural studies. Cultures were stored in liquid nitrogen for long term maintenance and grown on glycerol-asparagine medium or chitin-agar medium for ultrastructural studies, as previously described (Smucker & Simon, 1981; Smucker and Pfister, 1978). Spores used for germination studies were harvested from sucrose-nitrate agar medium using glass-beads to harvest the spores. Unless otherwise described, all experiments were run at 28°C. Submerged cultures were aerated on a reciprocal platform at 150 RPM with 1" oscillations. Media used in the submerged culture experiments were in Tris-buffered salts media (pH 7.61) with or without 95% acetylated regenerated chitin (Smucker and Kim, 1984). For the germination study, 240 ug/ml chloramphenicol (Sigma) was added to the basic salts medium without any carbon or nitrogen sources. The level of chloramphenicol used, completely blocked any protein synthesis during or following the heat shock treatment of spores (7 min at 52°C) (Ensign, 1978).

Colloidal Gold:Chitinase Label

Chitinase was harvested and further purified by size exclusion and ion exchange chromatography, and its mode of action was confirmed by HPLC (Smucker, Warnes, and Haviland, in press). Collolidal gold was made by the tannic acid/citric acid reduction method of Muhlpfordt (1982). Protein-Au conjugates were prepared by the methods of Molano, Bowers, and Cabib (1980). Whole cells were collected on grids and stained for various periods of time with the colloidal gold-chitinase label. Details of these procedures will be published elsewhere.

Chitinase Assay

Chitinase hydrolysis of ^{3}H-chitin was monitored by liquid scintillation counting of released trichloracetic acid-soluble oligomer (Molano et al, 1977; Smucker and Kim, 1984).

Electron Microscopy

Samples of aerial growth were obtained by the grid-impression technique for negative staining and shadow-casting. The grid impression details, embedding and sectioning, and the freeze-fracture replica methods have been reported elsewhere (Smucker and Pfister, 1978).

RESULTS

Figures 1, 2 and 3 document representative examples of freeze-etch images which illustrate the participation of fiber mosaic in spore maturation of several strains. In Fig. 1 the centripetal formation of mosaic fibers demonstrates a developmental role for the fibers in spore maturation. This image of the fibers and those in Fig. 2 and 3 clearly distinguish the fibrous component and the external material which is more clearly visualized in Fig. 4. This layer is not amorphous (Wildermuth, et al. 1971), but has a granular characteristic, therefore the label, "granular matrix".

Sites of chitinase-Au labeling on whole spores (Fig. 5) are on the exposed fibrils. This staining pattern is consistent with freeze-etch images showing placement of chitin fibers (this paper Fig. 1-3, and Smucker & Pfister, 1978).

Streptomyces griseus and S. bambergiensis spores harvested from sucrose-nitrate agar, and inoculated in fresh medium (with or without glucose) both contained chitinase activity. A chitinase would be expected since the chitin fibers surround spores and the hydrolytic enzyme would be required to participate in the process of spore envelope alteration during germination and outgrowth. Glucose is known to repress vegetative Streptomyces carbohydrase synthesis (unpublished results), therefore, this chitinase observed in S. griseus should be spore-specific. S. bambergiensis spores heat-shocked in the presence of 250 ug/ml chloramphenicol also contained chitinase activity. Lysozyme is known to bind to and hydrolyze chitin, but 10^3 times less than for chitinase kinetics (Skujins, et al 1973). Spore lysozyme therefore would not likely contribute significantly to the activity observed since the assay

was terminated after 160 min and there were less than 10^6 spores/ml. Only 200 ul of supernate was analyzed per assay. There was 0.16 $mU \cdot ml^{-1}$ chitinase activity associated with the spores and there was no difference between control and chloramphenicol treatments.

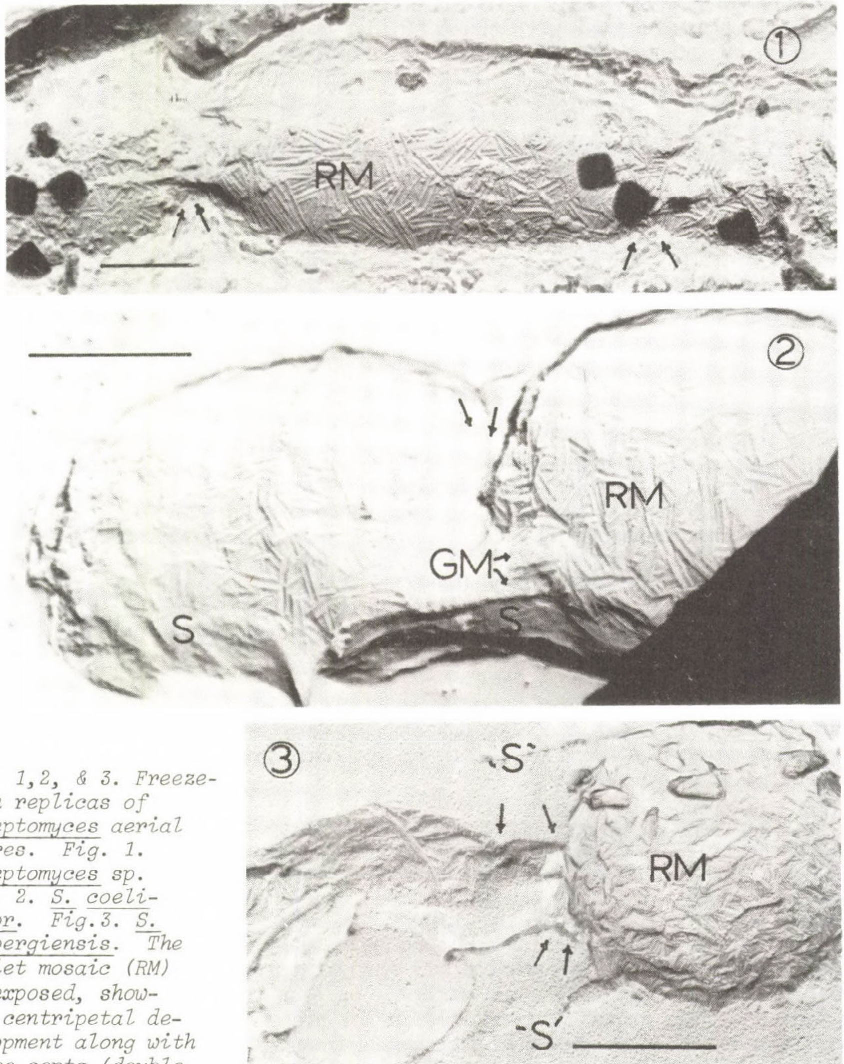


Fig. 1,2, & 3. Freeze-etch replicas of Streptomyces aerial spores. Fig. 1. Streptomyces sp. Fig. 2. S. coelicolor. Fig.3. S. bambergiensis. The rodlet mosaic (RM) is exposed, showing centripetal development along with spore septa (double arrows). The sheath (S) is clearly visible, bridging spores (compare to Fig. 8). A few fibrils of the rodlet mosaic (RM) type are also visible in the granular matrix (GM) between spores in Fig. 2.

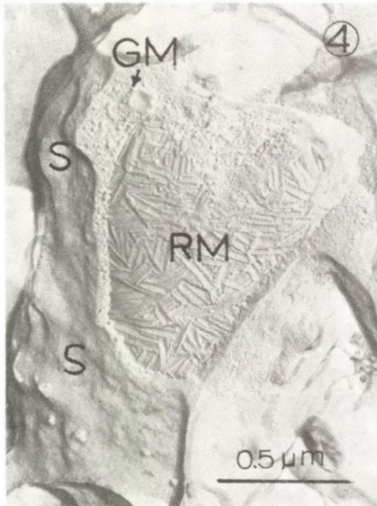


Fig. 4. S. coelicolor aerial spore. The surface sheath is clearly distinguished from the granular matrix and the fibers of the rodlet mosaic.

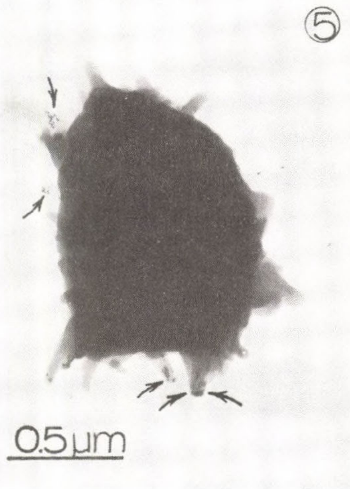
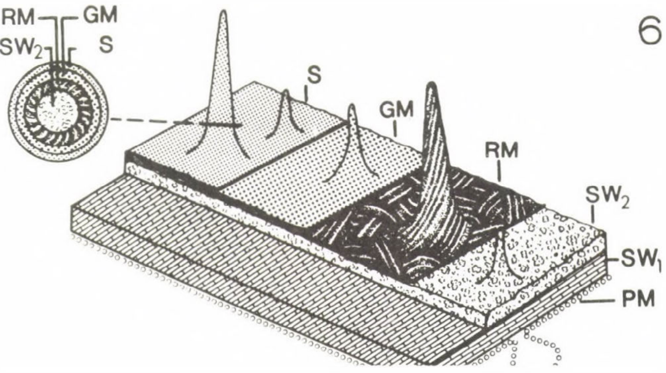


Fig. 5. Whole mount of S. bambergiensis aerial spore harvested from sucrose-nitrate medium. The colloidal-gold chitinase complex only labeled the spore where broken hairs exposed fibril regions (arrows). Surface coat did not stain.

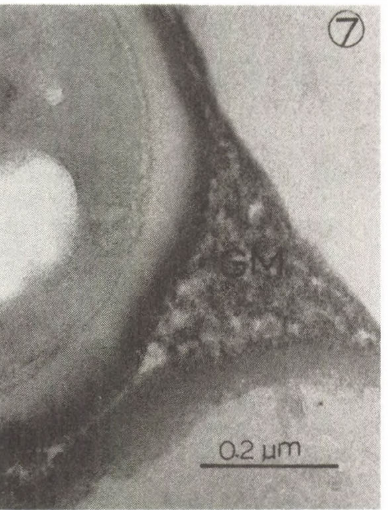
DISCUSSION

Consistent with the reports of Wildermuth, Wehrli, and Horne (1971) and Williams, et al (1972) we have demonstrated an amorphous Streptomyces envelope component, the granular matrix in (Fig. 2, 3, 4). Our previous (Smucker & Pfister, 1978) and current ultrastructural evidence clearly place this matrix outside the fibrous components. Fig. 6 is a model based upon hundreds of ultrastructural observations of stock cultures and fresh isolates using negatively stained, shadowed whole mounts (Smucker & Pfister, 1978), thin sections and thick sections studied by high voltage electron microscopy (Smucker, 1984b), and freeze-etch replicas. The fibrous component is clearly distinguishable from the outer material. Granular mosaic is at least in part analogous to the interspore matrix which is autolyzed during spore maturation. An outermost sheath of material (unknown composition) then remains loosely associated with the spores (Fig. 7 & 8). If the fracture plane in Fig. 9 is compared with the sectioned images of Fig. 7 & 8, one can clearly see the separate ontogeny of fibrous mosaic and the sheath.

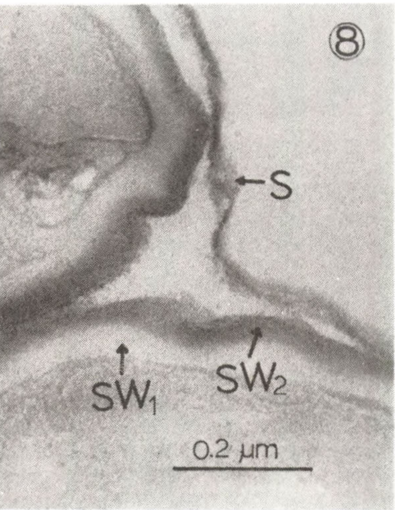
Genetic evidence for the separate fibrous layer development was discussed in the work of Strunk (1984). At the surface of an Amy$^-$ strain of S. griseus there was secondary cell wall thickening and cross wall splitting resulting in aborted spores. There was complete absence of a "distinct" surface sheath but, there was production of the "rodlet" fibers. The ultrastructural information from this Amy$^-$ strain considered together with our ultrastructural studies, appear to provide a basis of



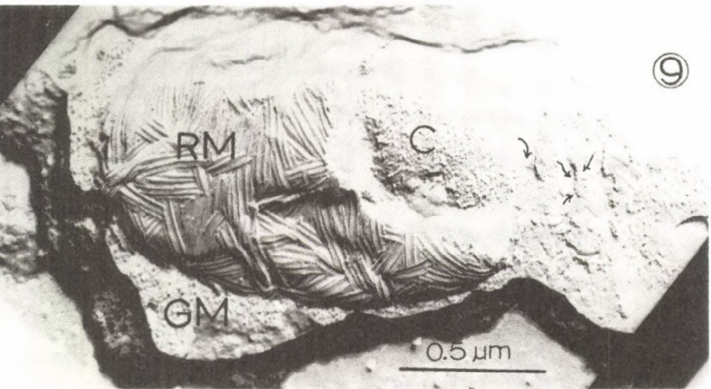
6
RM
GM
SW2
S
S
GM
RM
SW2
SW1
PM



⑦
GM
0.2 µm



⑧
S
SW1
SW2
0.2 µm



⑨
RM
C
GM
0.5 µm

changing the concept of sheath in Streptomyces. The fibrous (rodlet) component is ultrastructurally and developmentally a distinct entity from the outer sheath. The fibrous layer develops centripetally, following the contours of crosswalls, surrounds mature spores, and remains with the spore throughout germination and outgrowth events.

A spore-bound chitinase apparently is associated with the spores since chloramphenicol treatment of spores didn't affect the levels of chitinase detected. This spore bound chitinase may be distinct from chitinase produced during growth on exogenous chitin as a nutritional source. In the structural and chemical work reported here, and previously, only glycerol, glucose, or sucrose were used as carbon sources. The spore-associated chitinase is expected if the chitin fibers must be digested prior to germ tube outgrowth.

When ultrastructural studies of sporogenesis are viewed as a whole, it should be apparent that the fibrous (rodlet) mosaic of both smooth and ornamented strains develops independently of the loosely fitted sporogenic sheath. Observations of evidence which support a separate ontogeny of: 1) Streptomyces fibrous mosaic, which develops between mature spores, and 2) sheath, which is often loosely associated are outlined below:

1. Chater described and mapped morphogenetic mutants in S. coelicolor A3(2) in which a whi-99 mutant formed chains of rod-shaped spores. This block in development is significant from the wild type spore which proceeds to develop ellipsoidal spores, with fully developed transverse envelope invagination (Chater, 1972). For significance, see Fig. 1, 2 and 3 and 6-9 which show centripetal development of the fiber mosaic and the separate granular interspore matrix with sheath region.

2. Centripetal development of spore envelopes occurred with no discernable loose sheath around the mature spores (Hopwood and Chater, 1961). S. lividans (Chater, 1984) is a more recently studied streptomycete which also shows this pattern. The hairy-spored S. bambergiensis (Smucker, 1984a) and S. coeruleorubidus (Blumauerova, et al, 1978) also show this characteristic in mature spores.

3. Strunk (1984) reported that both her work and that of Bradshaw and Williams (1976) observed rodlets on sporulating structures of amy⁻strains of S. griseus when the sheath was completely absent on sporulating hyphae.

←

Fig. 6. Synoptic model of Streptomyces spore envelope. The inner wall (SW_1), outer wall layer (SW_2), rodlet mosaic of fibers (RM), granular matrix (GM), and the outer sheath (S) are presented. Fig. 7 & 8. Thin sections of S. coelicolor aerial spores. The granular matrix in Fig. 7 appears partially degraded and is completely autolyzed in Fig. 8, leaving the outer sheath detached. Fig. 9. Freeze-etch replica of S. coelicolor. Fractures occurred revealing cytoplasm ribosomes, RM, GM, and RM fibers in the interspore region (arrows).

4. If the chitin fibrils of the fiber mosaic are surrounding mature spores and the mosaic is digested during spore germ tube emergence, then a spore-carried chitinase would be expected. Chitinase is indeed part of aerial

   spores of at least two Streptomyces, S. griseus (Smucker & Kim, 1984) and S. bambergiensis (present work).

5. Fibers of the S. coelicolor fiber mosaic have been isolated and separated from any contaminating amorphous or membranous material (Smucker & Pfister, 1978).

CONCLUSIONS

Based on a whole series of evidence, the term "fibrous sheath" should be discarded when Streptomyces aerial hyphae and spores are evaluated. Certainly there is a frequently observed loosely spore-associated material seen in sections, but there is now clear evidence that this "sheath" is a separate entity from the fibrillar sporogenic component. The fibrous material in some strains becomes part of the spore decoration, but develops so as to encompass the mature spore requiring germ tubes to emerge through the fibrous layer. A proper concept of the fibrous spore component would be better developed if the outer sheath were simply called "sheath". The fibrous mosaic, which appears to be under separate gene control and develops centripetally along with the wall septal region, would better be called the fibril mosaic.

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BIOCHEMISTRY OF THE INITIATION OF GERMINATION OF STREPTOMYCES SPORES

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I. INTRODUCTION

Spore-forming organisms are reduced to a narrow range of prokaryotic and eukaryotic organisms and typical representative of both types of cells are bacterial and fungal spores, respectively. Streptomycetes are a group of spore-forming gram-positive bacteria with a life cycle unique among bacteria (Fig.1). When these organisms are grown on an adequate solid medium, the spores germinate and grow as a higly branched substrate (vegetative) mycelium firmly attached to the solid surface. Later, and on the top of this substrate mycelium, will arise specialized aerial hyphae originating the aerial mycelium in which, by multiple septation, will give rise to chains of spores. In an optimal nutritional and physico-chemical environment, spores undergo some morphological and physiological changes given rise to a vegetative cell which will regenerate the cycle. The term "germination" used for describing these changes is usually employed as for fungal spore germination instead as for **Bacillus** spores. So, and following the terminology proposed by Cross & Attwell (1975), it comprises the period leading to the emergence of germ tubes. It can be divided into three sequential morphological and biochemical stages : darkening, swelling and germ tube emergence (Hardisson et al. 1978). The first stage, darkening, is sometimes referred to as initiation of germination and, in this paper, we use both terms (darkening and initiation) as synonymous.

Fig. 1. DEVELOPMENTAL CYCLE OF Streptomyces.

It is not our intention to present here an exhaustive recopilation of the existing information about streptomycete spores, which has been perfectly documented in the excellent comprehensive reviews of Kalakoutskii & Agre (1976) and Ensign (1978). The aim of this paper is just only to summarize the actual knowledge on the biochemistry of some early events of spore germination: the role of divalent cations and energetic metabolism on the initiation of germination and the involvement of the macromolecular biosynthetic machinery on the germination process.

II. DIVALENT CATIONS AND ENERGETIC METABOLISM IN THE INITIATION OF GERMINATION

Unlike **Bacillus** endospores which show a criptobiotic state, **Streptomyces** spores can only be considered as in a resting state. So, although in the dry state **Streptomyces** spores do not show endogenous respiration (Hirsch & Ensign, 1978), when wetted endogenous respiration can be measured (Hardisson et al, 1978 ; Hirsch & Ensign, 1978 ; Garcia Diaz et al, 1983). However, the rate of endogenous and exogenous respiration have been shown to vary with different **Streptomyces** spores (Table 1) showing a clear correlation with the adenylate energy charge status (Garcia Diaz et al, 1983).

Table 1. RESPIRATION AND ADENYLATE ENERGY CHARGE OF SPORES OF DIFFERENT Streptomyces SPECIES

ORGANISM	RESPIRATION		ENERGY CHARGE
	ENDOGENOUS	EXOGENOUS	
S. fradiae	1.11	1.22	0.20
S. antibioticus	10.79	18.50	0.47
S. coelicolor	15.64	23.21	0.49
S. viridochromogenes	31.19	43.54	0.55

Respiration is expressed as QO_2 values : $\mu l\ O_2$ /h/mg dry weight. Energy charge was calculated from the equation : ATP + ½ ADP/AMP + ADP + ATP.

Possibly, the endogenous respiration could be originated through the metabolism of a glucose disaccharide (trehalose) which is present at a high content in spores of different **Streptomyces** species (Hey-Ferguson et al, 1973 ; Hirsch & Ensign, 1978). Additionally, **Streptomyces** spores posseses the uptake systems necessary for the incorporation of several substrates which can be oxidized (Hirsch & Ensign, 1978 ; Salas & Hardisson, 1981). However, it is noteworthy the fact that dormant spores oxidize glucose to CO_2 but do not increase their ATP content (Ensign et al, 1980 ; Garcia Diaz et al, 1983).

In its dormant state, **Streptomyces** spores contain a very high calcium level (1.1% to 2.1% of the dry weight in different species) and normal levels for other mono and divalent cations (Salas et al, 1983). Accumulation of this

calcium occurs preferentially during the sporulation process and spore calcium is mostly located in the integument fraction. Possibly, although it has not been proved, these calcium ions can be neutralizing anionic sites in the spore cell wall and, in some way, contributing to the maintaining of dormancy. Upon incubation of spores in the presence of the adequate divalent cations, which induce darkening of spores and loss of heat resistance, calcium is released from the spores as one of the earliest events in the initiation of germination (Salas et al, 1983). Calcium release from spores is not an energy-requiring process, while those events associated with darkening (loss of heat resistance, decrease in absorbance, excretion of spore carbon, etc) are blocked by metabolic inhibitors . Therefore, two different stages can be distinguish during initiation of germination : a first stage, characterized by the release of calcium and energy-independent, and a second stage energy-requiring in which the loss of heat resistance, the decrease in absorbance and the darkening of spores take place.

What are the initiators of germination?. Initiation of germination has ben shown to be dependent only on the presence of some divalent ions : Ca^{2+} , Sr^{2+} or Ba^{2+} ions for **S.viridochromogenes** (Eaton & Ensign, 1980) and Mg^{2+} , Fe^{2+} , Mn^{2+} or Zn^{2+} for **S. antibioticus** (Salas et al, 1983). The addition to dormant spores of one of these cations induces the triggering of the initiation of germination with the subsequent darkening of spores. In **S. viridochromogenes**, it has been suggested that the triggering site of the calcium-initiated germination is external to the cytoplasmic membrane (Eaton & Ensign, 1980). Whatever it may be located, the exact nature of the interaction between the initiator cation and the dormant spore remains to be investigated.

As mentioned above, with the exception of calcium release, the biochemical events occurring during initiation are blocked by metabolic inhibitors (Hardisson et al, 1978 ; Eaton & Ensign ,1980). However, this process does not require the addition of an exogenous energy source (Hardisson et al, 1978 ; Hirsch & Ensign, 1978). It is therefore assumed that the spore must mobilize endogenous energetics reserves. In this sense and by measuring the release of CO_2 as a consequence of the metabolism of differentially labeled glucose by spores, the participation of both the Embden-Meyerhof-Parnas (EMP) and the pentose phosphate (PP) pathways in the catabolism of glucose has been clearly proved (Salas et al, 1981). Interestingly, the amount of glucose metabolized by each pathway seems to be regulated by the existence or the absence of germinative conditions. Thus, under nongerminative conditions (incubation of dormant spores in distilled water), the PP pathway plays a major role in glucose catabolism. In contrast, when spores are incubated in germinative conditions, the EMP pathway tend to have a more active participation and through the germination process the ratio of the participation of both catabolic routes is more

favorable to the PP pathway. In isolated membrane fractions obtained from S. antibioticus dormant spores, we have detected the existence of a functional electron transport chain able to reoxidize the NADH produced in the glucose catabolism with the subsequent energy production (Table 2). The increasing role of

TABLE 2. ASSAY OF DIFFERENT SEGMENTS OF THE MEMBRANE-BOUND ELECTRON TRANSPORT CHAIN OF S. antibioticus SPORES

STAGE	NADH OXIDASE	NADH DEHYDROG.	NADPH DEHYDROG.	NADH-CYTOCHROME c-REDUCTASE
Dormant spores	0.87	31.42	39.25	69.48
Dark spores	0.33	11.83	80.15	39.06
Swollen spores	3.75	27.25	12.77	21.41
Mycelium	12.28	37.02	19.22	3.62

Enzyme activities are the average of six independent determinations and are expressed as :

nmoles NADH or NADPH oxidized / min / mg protein.

nmoles DCPIP reduced / min / mg protein.

nmoles cytochrome c reduced / min / mg protein.

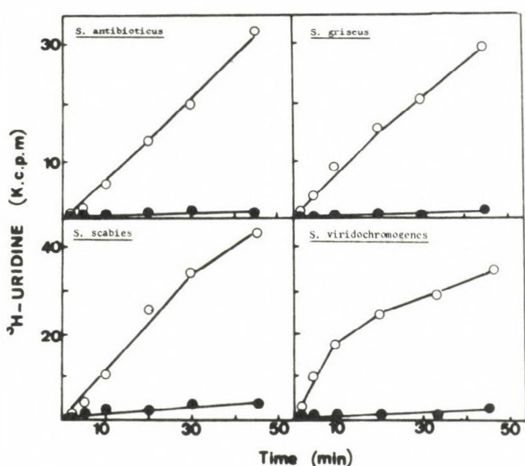
the PP pathway during germination would induce a decrease in the NADH levels and a concomitant increase in NADPH production. Reoxidation of this NADPH through the spore electron transport chain is also possible given that NADPH dehydrogenase is also present in the spore membrane and its activity increases during initiation of germination (Table 2). Regarding the spore electron transport chain it is noteworthy the fact that the NADH-cytochrome c reductase activity continuously decreases during germination, showing the highest level of activity in the dormant spore. In contrast, the ability to oxidize NADH increases increases during germination (Table 2). Both facts suggest that probably during germination there might be changes in the qualitative composition of spore electron transport chain. In this sense, quantitative changes in the b, c and a-type cytochromes of spores have been reported for several Streptomyces species (Taptykova et al. 1969).

III. MACROMOLECULAR BIOSYNTHESIS AND GERMINATION

Dormant spores of Streptomyces do not initiate germination in distilled water. However, protein and RNA synthesis (but no DNA synthesis) take place when spores are incubated in distilled water (Hardisson et al. 1980). The energy for these biosynthetic processes may be obtained from the degradation of trehalose and the further catabolism of the produced glucose. Additionally, the demand for precursors for the biosynthesis of both macromolecules may be fulfilled by the existence of a complete amino acid pool and five nucleosides (Garcia Diaz et al . 1983) and the turnover of some spore proteins with the participation of proteases which have been detected in cell-free extracts of spores (Guijarro et al. 1983).

The possible existence of a stable mRNA fraction in dormant spores have been until recently a controversial

subject. Attempts to detect the presence of polysomes in dormant spores of S. granaticolor (Mikulik et al. 1975) and of S. antibioticus (L.M.Quirós, unpublished results) were unsuccesful, probably due to their high inestability during spore breakage and the fact that spore ribosomes contain associated a ribonuclease activity (Mikulik et al. 1975). Some authors have claimed that spores of S. granaticolor (Mikulik et al. 1977) and of S. viridochromogenes (Hirsch & Ensign , 1975) do not contain any functional mRNA. On the other hand, such a mRNA fraction have been shown to exist in spores of S. antibioticus (Hardisson et al. 1980). All these experiments were made on the basis that rifampicin is an inhibitor of initiation of transcription (Wehrli et al. 1968 ; Sippel & Hartmann, 1979). Therefore, if spores contain stable mRNA and are permeable and sensitive to the drug, the incorporation of a labeled precursor into protein in the presence of rifampicin will represent the translation of preexistent mRNAs. In S. granaticolor spores, rifampicin does not completely inhibit RNA synthesis during the first 10 min of germination (Mikulik et al. 1984) and, therefore, the authors could not distinguish whether the synthesis of proteins in the presence of the drug was due to stable mRNA or to de novo synthesized mRNA species. The apparent contradiction on the existence of this stable mRNA fraction in spores has been recently resolved. Using a simple and unique method for growing and harvesting of spores, we have shown that four different Streptomyces species (S. antibioticus, S. griseus , S.scabies and S. viridochromogenes) were very sensitive to rifampicin (Fig. 2) and, all of them, showed ^{3}H-leucine incorporation into TCA-insoluble material in the presence of rifampicin (Quirós et al. 1985) (Fig. 3).



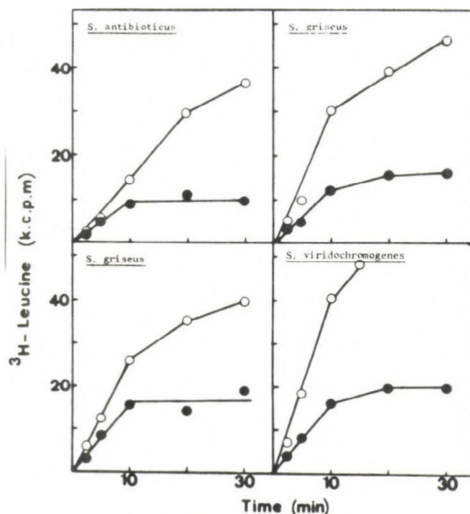


Fig.2. EFFECT OF RIFAMPICIN ON RNA SYNTHESIS BY SPORES

Fig.3. EFFECT OF RIFAMPICIN ON PROTEIN SYNTHESIS BY SPORES

(○) Control, no rifampicin ; (●) Rifampicin , 15 μg/ml

These experiments have been shown in our laboratory to be reproducible using different batches of spores and taking the

additional precaution of including rifampicin in the process of harvesting of spores to avoid the possibility that mRNA synthesis takes place during the preparation of the spore suspensions. The general pattern of labelled proteins after the translation of the stable mRNA fraction was also studied by polyacrylamide gel electrophoresis and fluorography and showed a great similarity in the four species with the predominance of low molecular weight polypeptides ($<$ 18 kDa), a clearly defined protein of 42 kDa and a band of 54 kDa present in three species and undetected in one specie. Subcellular fractionation of these proteins did not show a clear and preferential location in any fraction. Radioactivity incorporated into teguments, membrane, ribosome and soluble fractions were 26% , 19% , 19% and 36% of the total , respectively. Incorporation into the teguments fraction may not be very significative since may represent the labeling of proteins due to contaminant membranes and ribosomes in that fraction. In contrast, the incorporation into the membrane and ribosomal fractions may represent a greater incorpoation since their protein content was very much lower than that of the soluble fraction, i.e. the specific activity for incorporation would be much greater. Once translation of the stable mRNA fraction has occurred, the role of many of the proteins coded must takes place early in germination, since the proteins coded by the stable mRNA of the four Streptomyces species are subjected to a high degradation rate, about 20-30% of the coded proteins are degraded in the first hour of germination (Guijarro et al. 1983 ; Quirós et al. 1985).

Recent studies suggest that dormant spores may have a less eficient protein biosynthesis machinery and, early in germination, its normal activity being restored. This defect seems to be located in the ribosomes and, particularly. in the ribosomal proteins. We have analysed the 70 S ribosomal proteins of spores and mycelium of S.antibioticus by high-pressure liquid chromatography (HPLC) and two-dimensional SDS/polyacrylamide gel electrophoresis (2D-PAGE). The HPLC

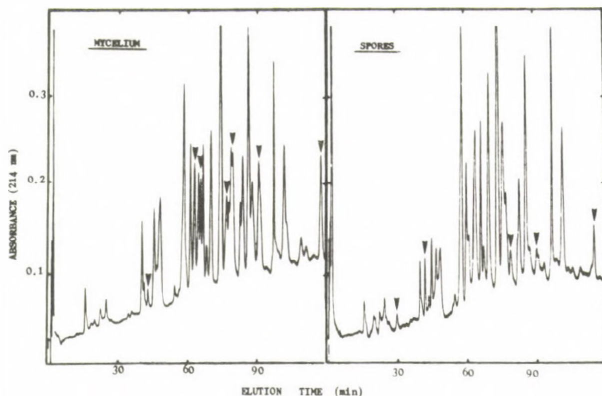


Fig.4. HIGH-PRESSURE LIQUID CHROMATOGRAPHY OF RIBOSOMAL PROTEINS OF MYCELIUM AND SPORES OF S. antibioticus.

chromatograms showed differences in the protein composition of both type of ribosomes (Fig. 4) and by 2D-PAGE several polypeptides were found to be present in mycelial ribosomes and absent in spore ribosomes. The absence of those proteins from spore ribosomes may explain our observation that both types of ribosomes show different sedimentation coefficient when centrifuged through a linear sucrose gradient. Differences in the ribosomal protein pattern of spores and mycelium of S. granaticolor have been also reported (Janda et al. 1981 ; Mikulik et al. 1984) and, in this organism, it has been shown that at least three proteins absent from dormant spores ribosomes were synthesized in the first minutes of initiation of germination (Mikulik et al. 1984). It is possible that these proteins would correspond to the low molecular weight polypeptides coded by the stable mRNA fraction.

Quantitative changes have been also found in the absolute number of ribosomes per cell during germination. After initiation of spore germination (darkening of spores) the number of ribosomes per mg cell protein is double when compared with that of dormant spores (Table 3).

TABLE 3. DETERMINATION OF THE NUMBER OF RIBOSOMES DURING GERMINATION AND VEGETATIVE GROWTH OF Streptomyces antibioticus

STAGE	RNA / PROTEIN	100 x rRNA / TOTAL RNA	rRNA / PROTEIN	RIBOSOMES / mg PROTEIN
DORMANT SPORES	0.57	37	0.21	8.14 x 10
DARK SPORES	0.92	42	0.39	15.12 x 10
SWOLLEN SPORES	1.25	61	0.75	29.09 x 10
MYCELIUM	0.89	65	0.58	22.59 x 10

The number of ribosomes per milligram of protein was calculated by dividing the values of rRNA/mg protein by the weight of a 70 S ribosome. The molecular weight of a 70 S mole divided by the Avogadro's number yields the weight of rRNA per 70 S ribosome, i.e. 2.57×10^{-18} g/ribosome.

Swollen spores, which show the highest metabolic and biosynthetic rates in germination (Hardisson et al. 1978 ; Hardisson et al. 1980 ; Salas & Hardisson, 1981), seems to posses the highest number of ribosomes even greater than that of the vegetative mycelium (Table 2).

RNA synthesis by S. antibioticus spores starts before 5 min of germination. A high proportion of the total RNA synthesized at the initiation of germination corresponds to stable RNA (80% when labeling takes place at 15 min of germination) (Guijarro et al. 1982). Possibly this stable RNA fraction represents the biosynthesis of rRNA and tRNA (L.M.Quirós, unpublished results). Other interesting parameter for the RNA biosynthesis is the long half-life of the mRNAs synthesized at the initiation of germination: 20 and 11 minutes for labeling at 15 and 60 minutes of germination, respectively (Guijarro et al. 1983). These values are quite

high when compared to the 1-3 minutes values normally reported for bacteria. Later on germination and during vegetative growth the half-life values for mRNA decrease to 5-6 minutes, which probably reflects the considerable slower growth rate of Streptomyces in comparison with unicellular bacteria.

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TAXONOMY

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ACTINOMYCETE SYSTEMATICS: PRESENT STATE AND FUTURE PROSPECTS

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INTRODUCTION

The development and application of new and reliable biochemical, chemical and molecular biological techniques are revolutionising actinomycete systematics and challenging the traditional morphological definition of the order Actinomycetales. There has also been a concomitant increase in the application of computer-assisted numerical taxonomic procedures to handle the large amounts of quantitative data generated by many of the new techniques (Goodfellow et al., 1985). As a result of these developments established actinomycete genera and species have been defined more precisely, novel taxa have been proposed for new centres of variation and poorly circumscribed species reduced to synonyms of well defined taxospecies. Nearly sixty actinomycete genera are now recognised and one of the four volumes of the forthcoming edition of Bergey's Manual of Systematic Bacteriology will be devoted to the actinomycetes. The recent improvements in actinomycete classification are now providing a sound basis for the introduction of accurate and standardised methods for the identification of genera and species and for the detection of novel taxa.

Most of the new taxonomic methods are of limited value in detecting relationships among distantly related species, genera and families. However, a number of powerful methods are available for establishing phylogenetic relationships (Table 1). In an impressive series of 16S/ribosomal RNA cataloguing studies it has been shown that Gram-positive bacteria form a distinct phyletic line which can be divided into two branches on the basis of DNA base composition (Stackebrandt & Woese, 1981). The actinomycete line, which includes many bacteria previously assigned to coryneform taxa, accommodates organisms with a guanine (G) plus cytosine (C) content above ca 55 mol.% and can readily be distinguished from the low G plus C ($<$ 50 mol.%) Bacillus - Clostridium - Lactobacillus branch. Interestingly, the genera Micrococcus and Stomatococcus, traditionally classified with other Gram-positive spherical bacteria, notably Staphylococcus, belong to the actinomycete phyletic branch (Stackebrandt & Woese, 1981; Stackebrandt et al., 1983). In marked contrast, the thermophilic genus Thermoactinomyces, long regarded as a bone fide actinomycete because of its ability to form branched hyphae which carry many lateral spores on both substrate and aerial hyphae, is phylogenetically close to other aerobic, endospore-forming bacteria (Goodfellow & Cross, 1984). Such findings bear eloquent testimony to the dangers inherent in constructing taxonomies solely on morphological features. They also raise the important question as to what is, or what is not, an actinomycete.

THE PHYLOGENY OF THE ACTINOMYCETES

It is now possible to classify actinomycete taxa at the suprageneric level on the basis of their evolutionary relationships. Thus, the methods outlined below (Table 1) can be used to measure large phylogenetic distances and the fine structure of the resulting higher taxa

can then be detected in DNA:DNA pairing, phage typing, numerical phenetic and immunological studies. Taxa defined in this way can be circumscribed using a wide array of biochemical, chemical, morphological and physiological markers. Indeed, the delineation of suprageneric groups make it possible to interpret the discontinuous distribution of specific chemical markers within a phylogenetic context, a theme pursued by Erko Stackebrandt elsewhere in this volume.

Table 1 Methods for constructing bacterial phylogenies

Macromolecules	Method	Ranks covered
DNA	DNA:DNA pairing	Closely related species
RNA	DNA:16/23 S RNA pairing	Species - order
	5/16 S rRNA oligonucleotide sequencing	Species - kingdom
	Secondary structures	Species - family
Proteins	Comparative immunology	Species - family
	Electrophoretic patterns	Closely related species
	Sequences of proteins with conserved primary structures	Species - order

The phylogenetic diversity within the actinomycete line has been used to assign genera to a number of aggregate groups. Goodfellow and Cross (1984) recognised eight such groups but considered that it was premature to equate them with higher ranks in the taxonomic hierarchy. The aggregate taxa recognised by these workers have been updated in light of recent studies (Table 2).

Table 2 Suprageneric groups, wall chemotype, peptidoglycan type and DNA base composition of actinomycetes, bifidobacteria and propionibacteria

Group/Genus	Wall Chemotype *	Peptidoglycan Type *	Mole % G + C
ACTINOBACTERIA			
A. Arthrobacter	VI	A3α	59-66
Cellulomonas	VIII	A4β	71-77
Dermatophilus	III	A1γ	57-59
Micrococcus	VI	A3α	66-75
Oerskovia	VI	A4α	70-75
Promicromonospora	VI	A4α	73-74
Renibacterium	VI	ND	53-54
Rothia	VI	A3α	54-57
Stomatococcus	VI	A3α	56-60
B. Agromyces	VII	B2γ	71-76
Aureobacterium	VIII	B2β	65-75
Curtobacterium	VIII	B2β	69-75
Microbacterium	VI	B1α	69-70

con't ...

Table 2 (con't)

Group/Genus	Wall Chemotype *	Peptidoglycan Type *	Wall % G + C
C. Actinomyces	V, VI	A4 α, A4 β, A5 α, A5 β	60-73
Arcanobacterium	VI	A5 α	50-52
D. Arachnia	I	A3 γ	63-65
Pimelobacter	I	A3 γ	69-74
E. Brevibacterium	III	A1 γ, A4 β	60-64
ACTINOPLANETES			
Actinoplanes			72-73
Amorphosporangium			71
Ampullariella	II	A1 γ	72-73
Dactylosporangium			71-73
Micromonospora			71-73
Pilimelia			ND
Glycomyces	II	ND	71-73
BIFIDOBACTERIA			
Bifidobacterium	VI, VIII	A3 α, A3 β A4 α, A4 β	52-65
KITASATOSPORIA			
Kitasatosporia	I, III	ND	66-73
MADUROMYCETES			
Actinomadura A			66-69
Microbispora			70-74
Microtetraspora A			ND
Planobispora	III	A1 γ	70-72
Planomonospora			72
Spirillospora			71-73
Streptosporangium			69-71
MICROPOLYSPORAS			
Actinosporangium	IV	ND	64
Micropolyspora			ND
Pseudonocardia	IV	A1 γ	79
Saccharomonospora			74-75
Saccharopolyspora			77
MULTILOCULAR SPORANGIA			
Frankia	III	ND	68-72
Geodermatophilus	III	A1γ	73-75
NOCARDIOFORMS			
Caseobacter			60-67
Corynebacterium			51-59
Mycobacterium	IV	A1 γ	62-70
Nocardia			64-69
Rhodococcus			59-69
"aurantiaca" taxon			ND

con't ...

Table 2 (con't)

Group/Genus	Wall Chemotype *	Peptidoglycan Type *	Wall % G + C
NOCARDIOIDES			
Nocardioides	I	A3γ	ND
PROPIONIBACTERIA			
Propionibacterium	I, II	A1γ, A3β, A3γ	57-68
STREPTOMYCETES			
Intrasporangium			ND
Sporichthya			ND
Streptomyces	I	A3γ	69-78
Streptoverticillium			69-73
Kineosporia	I		ND
THERMOMONOSPORAS			
Actinomadura B		A1γ	65-70
Actinosynnema			ND
Microtetraspora B			ND
Nocardiopsis	III	ND	65-76
Saccharothrix			73
Streptoalloteichus			ND
Thermomonospora			ND

Data from Collins et al. (1983); Fischer et al. (1983); Goodfellow & Cross (1984); Goodfellow & Minnikin (1985); Gustafson et al. (1985); Labeda et al. (1984, 1985); Minnikin et al. (1978); Omura et al. (1983); Stackebrandt et al. (1983); Suzuki & Komagata (1983) and Takahashi et al. (1984).

* Wall chemotypes and peptidoglycan type after Lechevalier & Lechevalier (1970) and Schleifer & Kandler (1972), respectively. ND, not determined.

The coherence of aggregate groups such as the Actinoplanetes, Maduromycetes, Nocardioforms and Streptomycetes is underpinned by an impressive battery of taxonomic evidence (Goodfellow & Cross, 1984; Stackebrandt, this volume) but it seems possible that other groups such as the Actinobacteria and Thermomonosporas are heterogeneous. Indeed, a number of putative evolutionary lines can be recognised amongst the actinobacteria (Table 2), a group which includes organisms previously classified as coryneform bacteria (Minnikin et al., 1978).

TOWARDS A REVISED DEFINITION OF AN ACTINOMYCETE

The actinomycetes historically have been defined and recognised solely on morphological grounds, that is, as bacteria able to form narrow hyphae or filaments that show some degree of true branching. The application of molecular biological techniques to microbial systematics shows that the possession of branching filaments does not automatically place a strain in the actinomycetes, conversely the absence of branching hyphae does not necessarily exclude an organism from this group of bacteria. There are several examples of mycelial-forming actinomycetes being more closely related to rod-shaped and coccoid bacteria than to other branching actinomycetes, e.g. Agromyces to Curtobacterium, Dermatophilus to Micrococcus, and Oerskovia and Promicromonospora to Cellulomonas. The discovery that the morphologically simple corynebacteria are phylogenetically close to the more highly differentiated

mycobacteria, nocardiae and rhodococci, is less unexpected given the early taxonomic history of these organisms (Goodfellow & Minnikin, 1981). The close phyletic relationship between mycelial and amycelial taxa can lead to difficulties in reconciling molecular biological data with other kinds of taxonomic evidence (Prauser, this volume).

The order Actinomycetales needs to be redefined given the inclusion of taxa containing pleomorphic and coccoid organisms and the exclusion of thermoactinomycetes. It is not too surprising that it is proving difficult to find simple unifying characters for the definition of the order given the wide range of biochemical, chemical, morphological and physiological properties shown by actinomycetes. However, the fact that actinomycetes do not show such deep phyletic divisions as groups such as the spirochaetes (Paster et al., 1984), clostridia and pseudomonads (Stackebrandt & Woese, 1981) is an argument in favour of recognising the reshaped order Actinomycetales.

Actinomycetes are Gram-positive bacteria with a high G plus C content in their DNA (ca. > 55 mole %) which form a natural group on the basis of rRNA oligonucleotide sequencing and nucleic acid pairing data. The order accommodates genera that exhibit a range of morphological features extending from the coccus, e.g. Micrococcus and Stomatococcus, through fragmenting hyphal forms, e.g. Rothia and Rhodococcus, to genera with a permanent and highly differentiated branched mycelium, e.g. in Actinoplanes and Streptoverticillium. Some, but not all genera, form spores which include motile zoospores and specialised structures that resist desiccation and mild heat but do not display the organisation and marked resistance properties of endospores (so excluding the genus Thermoactinomyces). There are no clear-cut encompassing chemotaxonomic characters but the genera can be assigned to two groups on the grounds of wall envelope composition. The larger, and more heterogeneous group, can be compared with classical Gram-positive bacilli and cocci as the envelope consists of a lipid-containing plasma membrane surrounded by a lipid-free wall composed of peptidoglycan and other polymers. In addition to these components, the organisms in the second group have walls containing substantial amounts of covalently bound long-chain hydroxy acids, the mycolic acids.

NUMERICAL CLASSIFICATION

The application of powerful molecular biological techniques in establishing relationships amongst actinomycetes is to be welcomed but it is important not to lose sight of two of the primary objectives in microbial systematics, namely the provision of a stable nomenclature to allow effective communication and the supply of reliable systems of identification for the non-specialist. Sound classification is a prerequisite for accurate identification and this helps to explain why much of the recent thrust in actinomycete systematics has been directed towards unravelling the subgeneric composition of some poorly defined genera.

Chemical markers are being increasingly used to describe and distinguish between actinomycete genera (Goodfellow & Minnikin, 1981; Goodfellow & Cross, 1984) but conventional numerical taxonomy has been shown to be the most effective method of determining relationships at the subgeneric level. In essence, numerical taxonomy (see Goodfellow et al., 1985) involves the construction of a large data base for many organisms which are assigned to clusters on the basis of overall similarity. Initially, all features are given equal weight but, once a numerical classification has been obtained, cluster specific properties can be chosen and, after reproducibility studies, used to design identification schemes. This strategy is in sharp contrast to the traditional practice in actinomycete systematics as species are recognised not by a small number of subjectively chosen morphological and staining features but by overall similarities based on many equally weighted characters. Numerical phenetic surveys have provided updated classifications of several actinomycete genera (see Goodfellow & Cross, 1984) including Actinomadura (Athalye et al., 1985); Streptoverticillium (Locci et al., 1981) and Thermomonospora (McCarthy & Cross, 1984).

Conventional numerical taxonomy with its emphasis on many strains and tests remains the method of choice for circumscribing taxospecies. It is, however, imperative to evaluate

numerical taxonomies in the context of data derived from other independent taxonomic methods, notably DNA pairing and serological studies, as similarities between strains can be distorted by several factors (see Goodfellow et al., 1985). The assumption in such polyphasic studies is that the congruence between classifications derived from the application of different methods is a measure of the reliance that can be placed in a taxonomy. It is surprising that little interest has been shown in the development and application of statistical procedures for comparing different kinds of quantitative data though the lack of activity may reflect the difficulty of measuring similarity values between each and every pair of strains in nucleic acid pairing and serological studies. The usual practice in such work is to determine the similarity values of the entire set of test strains against a restricted number of reference strains for which nucleic acid preparations and antisera are available. A statistical method, based upon principal components analysis, is now available for establishing a complete or derived matrix from a partial matrix by treating entries in the latter as quantitative character states (Sneath, 1983). This new method has been used to compare DNA pairing and numerical phenetic data obtained in a pilot study on streptomycetes (Mordarski et al., this volume).

DETECTION AND DESCRIPTION OF NEW TAXA

A sound classification is needed to prove novelty and minimal guidelines are required for the description of new species. The Approved List of Bacterial Names (Skerman et al., 1980) led to the demise of many actinomycete taxa that had been defined by a few subjectively weighted characters, notably morphological features. Recommendation 30b of the International Code of Nomenclature of Bacteria (Lapage et al., 1975), which calls for the introduction of recommended minimal standards for the definition of new species, was designed to prevent any further proliferation of poorly described species. Minimal standards have been proposed for assigning an organism to the genus Mycobacterium (Wayne et al., 1986) but novelty, like beauty, still tends to remain in the eye of the beholder. In other words, novelty can frequently be seen as a distillate of the aims, interests and judgement of the investigator (Williams et al., 1984). As these vary markedly, so do the criteria invoked to justify novelty. An idea of both the previous and present disparities in the number of species assigned to actinomycete genera can be gleaned from Table 3. The number of species in a genus is an approximate measure of its natural diversity, but is also influenced by the aims of the systematist and the criteria used to define the species. Some current developments can be exemplified by reference to the genus Streptomyces.

Table 3 Numbers of species in selected actinomycete genera

Genus	1974	1986	Genus	1974	1986
Streptomyces	463	142	Micromonospora	16	9
Mycobacterium	29	55	Nocardia	31	9
Actinomadura	-	28	Cellulomonas	1	7
Corynebacterium	39	16	Thermomonospora	2	5
Arthrobacter	7	15	Dactylosporangium	2	4
Actinoplanes	4	14	Pseudonocardia	1	3
Rhodococcus	-	14	Planobispora	2	2
Streptoverticillium	11	13	Planomonospora	2	2
Actinomyces	5	10	Frankia	10	1
Microbispora	9	10	Oerskovia	-	2
Micrococcus	3	9	Rothia	1	1

Compiled from Bergey's Manual of Determinative Bacteriology (Buchanan & Gibbons, 1974) and the forthcoming edition of Bergey's Manual of Systematic Bacteriology.

Until recently, newly isolated streptomycete species were recognised by little other than their ability to produce novel secondary metabolites. In the last edition of Bergey's Manual of Determinative Bacteriology, Pridham and Tresner (1974) recognised 463 validly described species, defined by a limited number of standardised criteria. This number has been sharply reduced to 142 (Williams et al., 1987) using numerical phenetic data. The genus has also been redefined and now encompasses the genera Actinopycnidium, Actinosporangium, Chainia, Elytrosporangium and Kitasatoa, all of which were originally morphological in concept (Goodfellow et al., 1986a-d). Improved classification has also fostered a reduction in the number of species assigned to the genera Corynebacterium, Frankia, Nocardia and Streptoverticillium, and more extensive taxonomic studies have led to the recognition of additional species of Actinomyces, Actinoplanes, Cellulomonas, Mycobacterium and Thermomonospora (Table 3). Similarly, polyphasic taxonomic investigations have led to the establishment or re-introduction of genera such as Arcanobacterium, Aureobacterium, Brevibacterium, Microbacterium, Nocardiopsis, Oerskovia, Rhodococcus and Stomatococcus.

Procedures used to distinguish novel from established taxa should be based on sound taxonomic principles. Numerical phenetic surveys of restricted groups of bacteria can be used to detect new centres of variation (see Goodfellow et al., 1985; Cross & Makkar, this volume) but this approach is time-consuming and can be laborious. However, rapid and reliable chemosystematic and computerised data handling systems are now being developed for characterising novelty (see Goodfellow & Minnikin, 1985), as well as for classification (Embley et al., this volume) and identification (Yano et al., this volume). The examination of fatty acid profiles using a microcomputer-based package called SIMCA may provide an easy and reliable way of detecting novel taxa (see O'Donnell, this volume). It is essential in such quantitative studies that the chemical data are suitable for multivariate analysis (see Saddler, this volume).

IDENTIFICATION

The dearth of objective criteria for the delineation of actinomycete genera and species has hindered many aspects of research and development on actinomycetes. This is especially so in areas such as ecology, laboratory diagnosis, industrial screening programmes and microbial technology. The challenge of identifying unknown actinomycetes has rarely been seen as a distinctive and major task so that current practice mainly consists of a jumbled mixture of more or less useful techniques with little evidence of any rational design. The application of widely differing techniques to diverse actinomycete taxa is often based more on scientific folklore than on established fact.

Identification of unknown actinomycetes is essentially a twofold procedure. Thus, reliable criteria are needed to assign an organism to a genus prior to the selection of the appropriate battery of tests for the identification of individual species. A combination of chemical and morphological characters are recommended for identification to the genus level (Minnikin et al., 1978; Goodfellow & Cross, 1984; Goodfellow & Minnikin, 1985), and a host of biochemical, physiological and serological tests are usually available for the differentiation of species. However, many of the more conventional biochemical tests are based upon methods that were discovered in the early days of microbiology and employed empirically. Even where single enzymatic reactions are involved the detection agents used and the conditions of the test are such that sensitive methods are often lacking. Significant improvements in the biochemical identification of actinomycetes await the development of highly sensitive methods for the detection and possible measurement of specific enzyme activity.

Classifications with high information contents provide sound bases for the construction of accurate identification schemes. One of the advantages of conventional numerical taxonomy is that it provides a pool of quantitative data on the test reactions of the strains within each of the defined clusters. This is usually expressed as the percentage of the organisms within each cluster which show a positive state for each character studied. Once classification is complete, a data base can be trawled and presumptive diagnostic characters

abstracted. Presumptive tests found to be reproducible can then be used (a posteriori weighting) to formulate dichotomous keys, diagnostic tables and computerised identification matrices.

Computer-assisted identification schemes are to be preferred to the more widely used monothetic sequential keys and diagnostic tables which are more prone to test error (Sneath,1974). Numerical taxonomies are now being used to construct identification matrices (see Goodfellow et al.,1985), which contain the minimum number of selected characters required for discrimination between the taxa previously defined by numerical taxonomy. Once formed the matrices can be used for the probabilistic identification of unknown strains. Few numerical classifications of bacteria have been supported by probabilistic identification schemes, possibly because of the extensive work associated with reproducibility studies (Wayne et al., 1976). However, theoretically sound, workable computer-assisted procedures derived from numerical taxonomic data bases are now available for the identification of selected actinobacteria (Seiler,1983), slow-growing mycobacteria (Wayne et al., 1980), streptomycetes (Williams et al.,1983,1987) and streptoverticillia (Williams et al.,1985; Locci et al., this volume). Information from the data base on Streptomyces has not only been used to build a probability matrix for the identification of unknown strains from both aquatic and terrestrial habitats (see Ortiz-Ortiz et al., 1984), but has also been used to formulate and evaluate media designed to isolate members of the streptomycete community other than those usually recovered on conventional isolation media (Williams et al., 1984).

FUTURE DEVELOPMENTS

The impetus given by the recent impressive advances in actinomycete systematics needs to be maintained if many of the remaining taxonomic challenges are to be met in the foreseeable future. Further applications and developments of chemical, numerical and molecular biological methods will undoubtedly enhance our understanding of the biological relationships of actinomycetes, especially at the rank of genus and above. Aggregate groups recognised at the supra-generic level will be formally equated with higher ranks in the taxonomic hierarchy and will be the subject of broadly based comparative studies. The delineation of phylogenetically coherent taxa above the genus level will allow all kinds of taxonomic data to be interpreted within an evolutionary context. Phylogenetic markers will be weighted for the descriptions of families, genera and species. Improvements in actinomyete classification will also provide a much needed spur to the development of recommended minimal standards for the description of new species.

More specifically, modern taxonomic methods will be applied to the revision of poorly defined taxa and to the detection of novel isolates. Attention will be focussed towards devising quick, accurate and standardised methods for the creation of high quality data bases which will be exploited to the benefit of actinomycete biology generally. Such studies will be facilitated by the introduction of miniaturised and automated techniques and by use of statistics needed to marshal the large amounts of data generated. The need for better methods of data handling is a consequence of the changes in microbial systematics that have occurred with the introduction of analytical instruments including gas chromatographs, high performance liquid chromatographs and mass spectrometers. Improved statistics will also be required to interlock data sets derived from the application of independent techniques.

Accurate identification depends critically on good classification and on careful test standardisation. Improvements in classification will provide a sound base for the development of numerical identification. Identification matrices of the type presented by Locci et al. (this volume) will be increasingly used for computer-assisted identification of unknown actinomycetes. Chemical methods will also be used in the numerical identification of taxa and for the detection of novel actinomycetes (O'Donnell, this volume).

In conclusion, it can be said that the application of new theoretical concepts to actinomycete classification and identification has provided insights that have drastically

altered our views of the relationships between actinomycete taxa. The emerging natural taxonomy of the actinomycetes is not only of value in its own right but will also form an essential part of a developing microbial technology.

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THE SIGNIFICANCE OF "WALL TYPES" IN PHYLOGENETICALLY BASED TAXONOMIC STUDIES ON ACTINOMYCETES

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INTRODUCTION

The last 6 years have seen an enormous increase in information about the hierarchic structure of the phylogenetically coherent group of actinomycetes and their non-actinomycete-like relatives. The data have been comprehensively dealt with by Stackebrandt (1982), Stackebrandt and Woese (1981a,b), Stackebrandt et al., (1981, 1983) and Stackebrandt and Schleifer (1984). The most reliable approaches used today are determination of full 16S rRNA sequences, 16S rRNA cataloguing, determination of rRNA cistron similarities and DNA-DNA hybridization. Since the primary structure of the rRNAs is more conserved than the average primary structure of the entire genome, the analyses of 16S/23S rRNAs allow determination of moderate to even most remote relationships while DNA hybridization can only be applied to closely related species (Fig. 1). Results of each of the 4 ap-

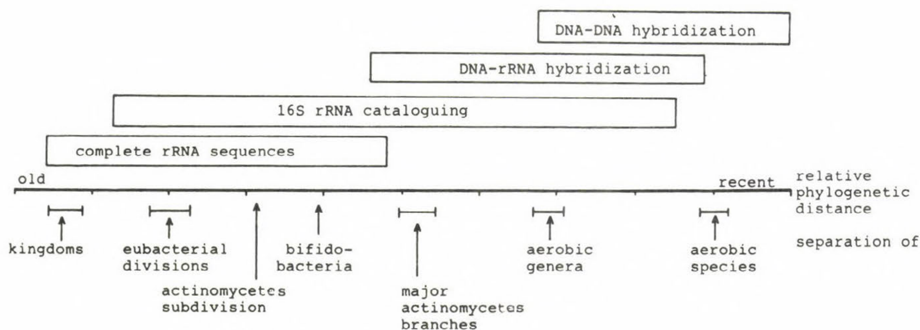

Fig. 1 Schematic illustration of the range of phylogenetic distances which can reliably be covered by the application of varous molecular genetic methods.

proaches lead to the generation of dendrograms, which can be used to depict one composite tree (Fowler et al., 1985). Fig. 2 displays the main lines of the Actinomycetales.

THE PROBLEM

A dendrogram (phylogenetic tree) represents for the first instance the evolution of the investigated molecule only. It may however, represent the phylogeny of the corresponding organisms, provided that the molecule used fulfills the requirements of a reliable phylogenetic marker, e.g. genetic stability, conserved primary structure, constancy in function. (Stackebrandt and Woese 1981a).
Although it is tempting to use the branching pattern of the dendrogram to delineate taxonomic ranks one should avoid to do so. For two reasons this strategy is meaningless for taxonomic purposes. (1) There are several examples showing that the evolution of the genotype does not parallel with the evolution of the phenotype. The genus *Clostridium* and the order Actinomycetales may serve as an example: The genus *Clostridium* is an ancient group of eubacteria while members of the Actinomycetales constitute a much younger group in evolutionary terms (low similarity coefficients for clostridia versus high values for actinomycetes). On the other hand, looking at the phenotype, clostridial species, despite their age, show a remarkable small number of distinguishing features as compared to the actinomycetes with their enormous diversity in morphological, biochemical and physiological properties. Within the order Actinomycetales genera and families are separated from each other by Sab values of about 0.7 and 0.5, respectively. Projecting the situation seen within this order unreflectively on the genus *Clostridium* as defined today, we would end in a high number of genera and families. Many of the genera would comprise a small number of species only. Applying this strategy the other way round, the order Actinomycetales would have to be described as a single genus. The impractability of this approach is obvious. (2) At present, the mere skeleton of a phylogenetic dendrogram is worthless for the identification part of taxonomy. Since hybridization and sequencing methods used today are still slow, expensive, and restricted to a few scientists only, the phenotypic characterization is absolutely necessary for describing taxa. However, the near future may see techniques available that allow a rapid identification and description of isolates at the molecular level. These techniques (e.g. full rRNA sequences) will make identification more reliable than the traditional phenotypic characterization.

PROKARYOTIC TAXONOMY SHOULD BE BASED ON THE GENOTYPE

It could be shown in various examples that most of those markers used so far in an artificial system have little or no significance at all in defining reliable taxonomic ranks. Since phenotypic characters cannot be used to measure phylogenetic distances the backbone of a hierarchic classification scheme has to be provided by the genealogical grouping of organisms. In no case should taxa be established which are inconsistent

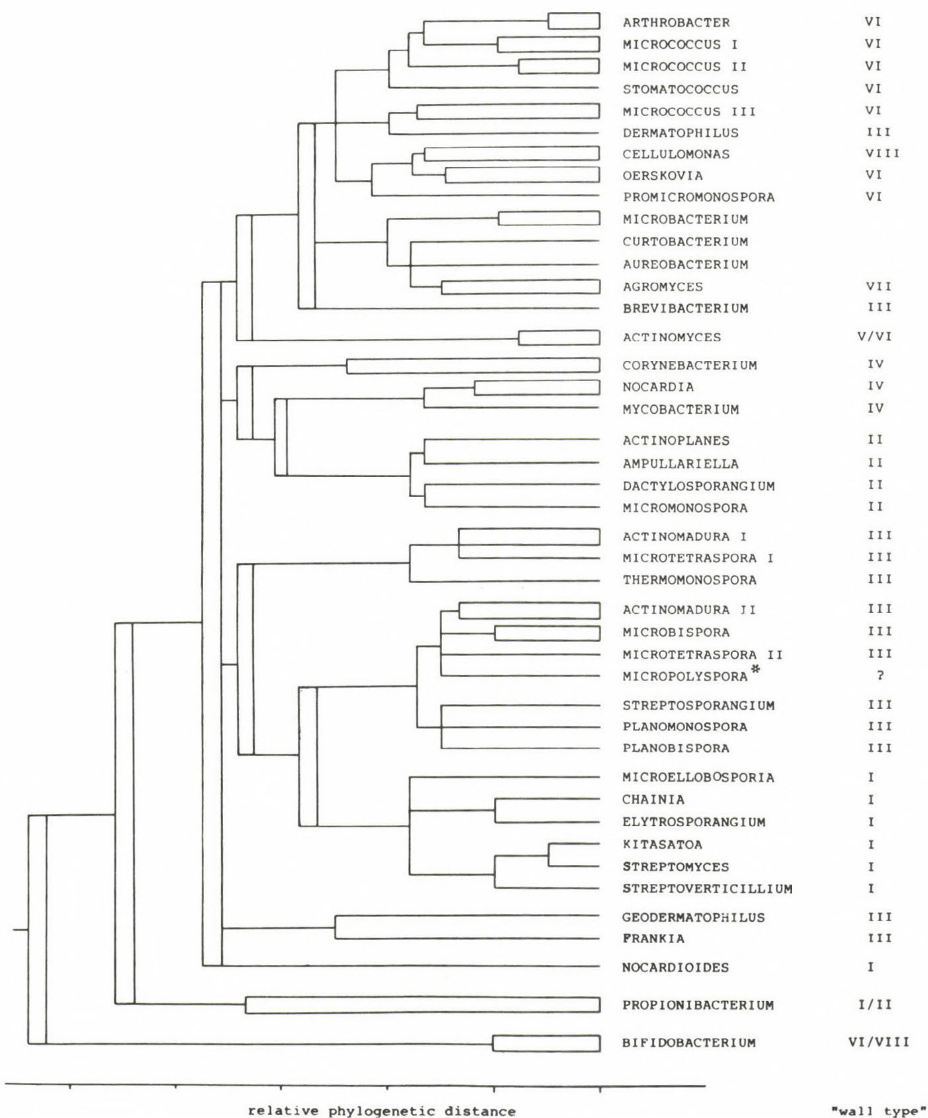

Fig. 2 The main branches of descent of the subdivision of actinomycetes. Boxes indicate the range of relationships of a group of species. ***Micropolyspora angiospora**

with the phylogenetic clustering of their members. With no doubt has the phylogenetic approach already markedly improved the taxonomy of various prokaryotic taxa - especially those taxa whose description and characterization had been based almost exclusively upon simple features. The genus is the only level at which phylogenetic and phenotypic groupings match ratherwell; this can be explained by the proper choice of phenotypic characters, selected by chance without knowing that these features are indeed suitable for describing phylogenetic clusters.

Today the significance of a marker can be determined best by following its distribution along the phylogenetic branching pattern. Since one marker alone, however, will in most cases be unsufficient to define a taxon properly, this strategy has to extended to a set of characters. After all, clusters of organisms will emerge from the phylogenetic tree which are characterized by a unique collection of characters. Members of each cluster are defined by the same set of characters while different clusters (phylogenetic neighbours) are distinguished from each other by different combinations of features.

Applying this strategy to actinomycetes a number of markers were shown to be of taxonomic value. These markers exhibit the same advantages: they are widely, if not universally distributed, they show a high phenotypic variability, they do not seem to be influenced by changes in growth conditions and, since they are coded for by several genes, they are genetically stable; peptidoglycan, fatty acids, lipids, mycolic acids, DNA G+C content, and, although of less significance, whole cell sugars, as well as fermentation and products and, in certain taxa, morphology, are markers that can be relied upon.

"WALL TYPES" VERSUS "PEPTIDOGLYCAN TYPES"

Actinomycete taxonomists are accustomed to "wall types", introduced by Lechevalier and Lechevalier (1970). The types have played a dominant role in the establishment of actinomycete taxa, since, for the first time in systematic studies on actinomycetes, a chemotaxonomic marker had demonstrated its power. Certain "wall types" and diagnostic whole cell sugars were shown to correlate rather well which was used to split the large group of genera showing type III into two subgroups. While the "wall types" restrict the information of the complete amino acid structure of the peptidoglycan to one or two acids only, Schleifer and Kandler (1972), by proposing "peptidoglycan groups" and "types", demonstrated the significance of the high chemical variability of the peptidoglycan as a chemotaxonomic marker. Following the distribution of "wall types" along the phylogenetic structure of a representative number of actinomycete genera, one is able to determine the taxonomic significance of this marker (Fig. 2).

THE DISTRIBUTION OF "WALL TYPES"

"Wall type" I (LL-A_2pm, glycine)

Members of 3 taxa exhibit "wall type" I, i.e. *Streptomyces* and related genera, *Nocardioides*, and *Propionibacterium*. While

Streptomycetes form a coherent cluster with regard to the peptidoglycan, Propionibacterium does not: P. agnes, P. avidium and P. thoenii have LL-A_2pm, while other species (see below) have m-A_2pm. Differences in morphology and spore formation, major end products of fermentation, relationship to oxygen, major isoprenoid quinones (Collins and Jones, 1981) and DNA G+C content facilitate the classification of "wall type" I actinomycetes into one of the three taxa.
"Wall type" I corresponds to "peptidoglycan type" LL-A_2pm, directly cross linked (variation A3γ); glycine forms the interpeptide bridge.

"Wall type" II (meso-A_2pm, glycine)

This type has been found so far exclusively in members of a phylogenetically coherent group defined by Actinoplanes and relatives. The "wall type" is actually of more significance than originally anticipated. The traditional classification has placed Micromonospora away from the genera Actinoplanes, Amorphosporangium, Ampullariella and Dactylosporangium because of differences in morphology.
Phenotypic characters supporting the close phylogenetic relationship of these genera are whole cell sugar pattern, presence of glycine in position 1 of the peptide subunit, and glycolated muramic acid. Members of Micromonospora show some heterogeneity in the chemistry of the peptidoglycan: some species have traces of LL-A_2pm, while others have 3-hydroxy-A_2pm instead of m-A_2pm (Kawamoto et al., 1981). A closer look for these exceptions may be helpful for taxonomic studies at the strain level.
"Wall type" II corresponds to "peptidoglycan type" m-A_2pm, directly crosslinked (variation A3γ'). Propionibacterium freudenreichii also contains m-A_2pm and glycine. In this species, however, glycine forms the interpeptide bridge of peptidoglycan.

"Wall type" III (meso-A_2pm)

This type is mosts widely distributed among eubacteria, defining almost all Gram-negatives, cyanobacteria and many genera of the Gram-positives with a low DNA G+C content (Schleifer and Kandler, 1972). It is therefore not surprising to find this type also to be present in various taxa of the Actinomycetales. Phylogenetically they do not group together and members of the different taxa are distinguished from each other by a broad spectrum of phenotypic characters. While Brevibacterium, Dermatophilus, Geodermatophilus and Frankia can be classified rather easily, the situation is more complicated within the taxa of sporeforming actinomycetes. Mono-, bi-, and polysporate organisms are clearly separated from the sporangiate organisms of the genera Streptosporangium, Planobispora and Planomonospora.
Each of the genera Actinomadura, Microbispora, Microtetraspora, Micropolyspora and Nocardiopsis is poorly described. Fig. 3 shows the results of RNA cataloguing and DNA hybridization experiments (Fowler et al., 1985; Fischer et al., 1983; Poschner et al., 1985). Actinomadura embraces two subgroups each being worthy of genus status and each containing members from diffe-

rent genera. Nocardiopsis constitutes a heterogenous collection of organisms. Characterization by whole cell sugars are of restricted value. The most valuable support for the phylogenetic data came from studies on lipid- and isoprenoid composition (Tab. 1) (Fischer et al., 1983; Poschner et al., 1985), and from numerical analyses (Athalye et al., 1985).

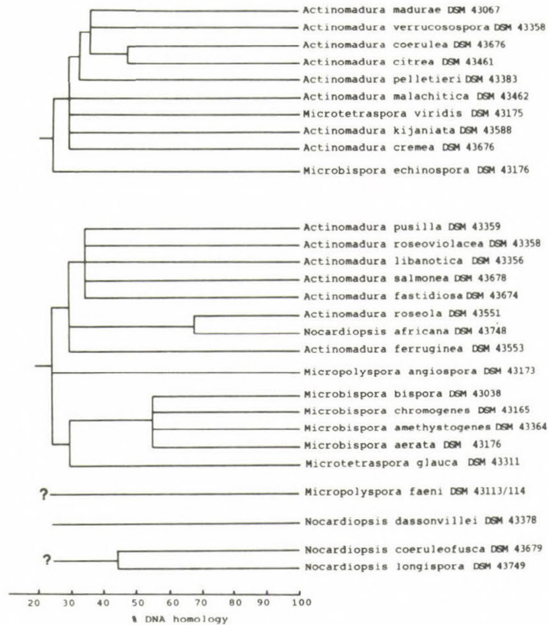

Fig. 3 Relationships of polysporate actinomycetes as determined by DNA-DNA hybridization. Questionmarks indicate that the position of the respective organisms within the phylogenetic tree (Fig. 2) have not yet been determined

"Wall type" IV (meso-A_2pm, arabinose, galactose)

Actinomycetes exhibiting "wall type" IV are easily recognized by the presence of an arabinogalactane and mycolic acids. Despite these uniting features, the genus Corynebacterium has traditionally not been classified with Mycobacterium, Nocardia, Rhodococcus and relatives but was placed into the "coryneform group of organisms" (Rogosa et al., 1974). However, there is no doubt that Corynebacterium, together with other members of the CMN group, forms a second major subbranch within the order Actinomycetales, whose members are defined by coryneform morphology. Phylogenetically the CMN group forms a cluster which in addition contains Actinoplanes and related taxa. Unfortunately a few representatives only of the CMN group have been investi-

Tab. 1 Distribution of major isoprenoids and diagnostic fatty acids in members of the two groups of Actinomadura (Fig. 3) and other polysporate actinomycetes exhibiting "wall type" III

Phylogenetic taxon	major isoprenoids	diagnostic fatty acids
A. madurae, A. pelletieri, A. coerulea, A. malachitica, A. verrucosospora, A. citrea, A. kijaniati, A. cremea, Microtetraspora viridis, Microbispora echinospora	MK9-(H_4),(H_6)	16:0, 18:1, 18-me
A. pusilla, A. roseoviolaceae, A. libanotica, A. salmonea, A. fastidiosa, A. roseola, Nocardiopsis africana, A. ferruginea	MK9-(H_2),(H_4)	
Micropolyspora angiospora		17-me
Microbispora bispora, M. chromogenes, M.amethystogenes, M. aerata, Microtetraspora glauca	MK9(H_0), MK9-(H_2),(H_4)	
Micropolyspora faeni	MK9(H_4),MK10(H_4)	i/ai-15/17
Nocardiopsis dassonvillei	MK10-(H_2),(H_4),(H_6)	i-16, ai-17, 18-me
Nocardiopsis coeruleofusca[1] Nocardiopsis longispora[1]	MK9-(H_4)	i-16, i/ai-15/17, 17-me

i-iso, ai-anteiso, 17(18)-me-10-methylhepta(octa)decanoic acid
1-hydroxy fatty acids are present.

gated so far by RNA characterization, making the branching pattern precarious. The relative phylogenetic distance separating members of the MN group from Actinoplanes and relatives are small, allowing changes of the pattern with more organisms investigated. The placement of Actinoplanes and relatives within the radiation of the CMN group is not supported by chemotaxonomic properties.

"Wall type" V (lysine, ornithine)

Actinomycetes exhibiting "wall type" V are members of the genus Actinomyces, e.g. A. israelii, A. naeslundii, A. viscosus and A. odontolyticus. As for A. israelii, analysis of the peptidoglycan showed ornithine on position 3 of the peptide subunit while lysine and D-glutamic acid form the interpeptide bridge (Weiss et al., 1981). Although lysine and ornithine may occur in other actinomycetes (in other molar ratios than 1:1, however), "wall type" V, like types II and IV, is a valuable mar-

ker; no other actinomycetes have been found so far to possess this peptidoglycan composition.

"Wall type" VI (lysine, aspartic acid, galactose)

This type is found in members of several phylogenetically unrelated genera, i.e. Oerskovia, Actinomyces and Bifidobacterium. Actually, "wall type" VI is ill defined since analysis of the peptidoglycan (Schleifer and Kandler, 1972; Lauer and Kandler, 1983) has revealed a high degree of chemical variation: Lys-Ser-Asp in Cellulomonas (Oerskovia) cartae, Lys-Thr-Asp and Lys-Thr-Glu in Oerskovia turbata, Lys-Asp in several species of Bifidobacterium and Lys-Ser-Asp in Bifidobacterium bifidum. Moreover, lysine and ornithine can replace each other in many bifidobacteria; in these cases both amino acids are present in organisms of one population, simulating the presence of "wall type" V.

"Wall type" VII (2,4-diaminobutyric acid, glycine, (lysine))

The presence of 2,4-diaminobutyric acid (DAB) is a valuable indication that the respective strain belongs into the phylogenetically coherent cluster defined by Agromyces, Corynebacterium michiganense, C. mediolanum, Brevibacterium helvolum and others (Schleifer and Kandler, 1972; Döpfer et al., 1982). The "wall type", however, suppresses information about the rich variation that is actually present in this and related peptidoglycan types (group B, according to Schleifer and Kandler, 1972). Other taxa which show a close relationship to Agromyces and relatives are Microbacterium, Brevibacterium imperiale, Curtobacterium and Aureobacterium. Each of the latter taxa are non-actinomycetes-like in morphology but are characterized by the rare group B peptidoglycan.

"Wall type" VIII (ornithine)

As mentioned under "wall type" VI lysine and ornithine can replace each other in bifidobacteria. Orn(Lys)-Asp- and Orn(Lys)-Ala_{2-3}-peptidoglycan types occur in different species. The Orn-Asp type is also present in Cellulomonas flavigena, and ornithine, together with homoserine is present in certain coryneform bacteria with group B peptidoglycan (Schleifer and Kandler, 1972).

"Wall type" IX (meso-A_2pm, various amino acids)

This type has only been found in Mycoplana species so far. Nothing is known about its phylogenetic position. Since Mycoplana strains are described to be Gram-negative they may not be members of the actinomycetes at all.

CONCLUSION

Except for the DAB-containing peptidoglycan ("wall type" VII), occuring in Agromyces and related taxa only, all other "wall types" occur in more than one phylogenetic (and phenetic) cluster. This finding points towards a convergent evolution of the amino acid composition of the primary structure of peptidoglycan. Thus, the cell wall chemistry alone cannot be used as the

sole chemotaxonomic marker to define genera and higher taxa. Nevertheless, for aerobic actinomycetes the establishment of the principal amino acid together with additional chemotaxonomic characters is sufficient to allocate an isolate to one of the phylogenetic groupings. Restricting the analysis to "wall types" only, taxonomically important variations of the peptidoglycan (types V-VIII), present in facultative anaerob and anaerobic actinomycetes, however, may be overlooked. Peptidoglycan types are more difficult to determine but they include the complete information about the amino acid composition, making the description of a taxon more reliable.

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A PROBABILISTIC APPROACH TO THE IDENTIFICATION OF STREPTOVERTICILLIUM SPECIES

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Baldacci (1958) proposed the genus Streptoverticillium to accomodate sporoactinomycetes producing sporophores arranged in verticils. An emendation of the genus was later made and a type species designated (Baldacci et al., 1966; Farina and Locci, 1966; Locci and Petrolini, 1970).

The genera Streptomyces and Streptoverticillium are closely related. However the latter has been clearly distinguished in several numerical phenetic studies (Kurylowicz et al., 1975; Locci et al., 1981; Williams et al., 1983a) and the morphological distinction between the two genera was stressed by studies of the fine structure of Streptoverticillium spores and sporophores (Locci and Petrolini, 1970; Cross et al., 1973). There are therefore sound practical grounds for maintaining the separation of Streptoverticillium and Streptomyces.

Classification and identification of Streptoverticillium species were initially based on spore and mycelium pigmentation. This resulted in the allocation of 40 species to 12 series (Locci et al., 1969; Baldacci and Locci, 1974). More recently, attempts have been made to classify Streptoverticillium species on the basis of overall similarity (Locci et al., 1981; Williams et al., 1983a). Assessment of overall similarity by the simple matching coefficient (S_{SM}; Sokal and Michener, 1958) at the 84% similarity level and clustering by unweighted average linkage (UPGMA) resulted in the clear separation of streptoverticillia from streptomycetes. The streptoverticillia were distributed between 10 multi-membered and 14 single-membered clusters (Locci et al., 1981).

These data provide a sound basis for the development of a probabilistic identification matrix. Such a system has many advantages over the widely used monothetic sequential keys which are very susceptible to test error (Sneath, 1974). The probabilistic matrix developed by Williams et al. (1983b), and based on computer programs devised for the purpose (Sneath, 1979a, 1979b, 1980a, 1980b, 1980c), was applied to the classification data of Locci et al. (1981) to produce a probabilistic identification scheme for Streptoverticillium species.

METHODS

Strains and clusters. The 77 *Streptoverticillium* named species classified by Locci et al. (1981) and their clustering are summarised in Table 1. The clusters were named, where possible, after the earliest validly described species which they contained. Validation of nomenclature not appearing in the Approved List (Skerman et al., 1980) has been recently proposed (Locci, 1985). The type species of the genus (*Streptoverticillium baldaccii*) is maintained.

Selection of most diagnostic characters. The diagnostic value of all characters was determined and ranked using the CHARSEP program (Sneath, 1979b), which provides several separation indices for each test. The most diagnostic tests were progressively reduced to the minimum number which provided good identification scores for the Hypothetical Median Organism of each cluster (Sneath, 1980b). The character selection was checked by applying the DIACHAR program (Sneath, 1980a) which ranks the diagnostic scores of each character for each group in an identification matrix and also gives the sum of scores of all characters for each taxon.

Characters selected for the identification matrix. The characters selected are those used in the identification matrix (Williams et al., 1985). In all cases, the inoculum consisted of dense spore and/or mycelial suspensions in sterile water. Negative results were re-tested to reduce the possibility of false readings due to poor viability of the inoculum. Incubation was at 27°C unless stated. All media were adjusted to pH 7.0. Details of the tests are given in Williams et al. (1985).

Evaluation of the identification matrix. Cluster overlap was assessed using the OVERMAT program (Sneath, 1980c) which determines overlap between groups in a matrix containing percentage positive values for character states. The significance of any overlap is assessed against selected critical values (v_0), which in this case were 1% and 10%.

Identification scores were obtained by using the MATIDEN program (Sneath, 1979a) which provides the best scores for a known or unknown strain against the matrix. The identification coefficients determined were the Willcox probability (Willcox et al., 1973), taxonomic distance and standard error of the taxonomic distance. With the former a score approaching 1.0 indicates a high probability of identification, low scores for taxonomic distance indicate relatedness and acceptable values for the standard error of taxonomic distance are less than about 2.0 - 3.0, with negative values indicating that the unknown is closer to the centroid than average. Fuller details of these coefficients can be obtained from Sneath (1979a) and Williams et al. (1983b). In addition to providing the scores

Table 1. Designation and source of cultures assigned to clusters

CLUSTER 1 (Streptoverticillium baldaccii): Stv.baldaccii, ATCC 23564, Stv.biverticillatum, CBS 211.62, Stv.distallicum, NRRL2886, Stv.fervens, ATCC 27429, Stv.flavopersicum, ATCC 19756, Stv.griseoverticillatum, ATCC 27236, Stv.hiroshimense, ATCC 19772, Stv.kentuckense, ATCC ' 12691, Stv.netropsis, ATCC 23940, Stv.roseoverticillatum, ATCC 19807, Stv.roseoverticillatum subsp.albosporum, ATCC 25189, Stv.rubrochlorinum, LIA 0084, Stv.rubroverticillatum, LIA 0251, and Stv.salmonis, NRRL 1472.

CLUSTER 2 (Stv.cinnamoneum): Stv.blastmyceticum, ATCC 19731, Stv.cinnamoneum, ATCC 11874, Stv.cinnamoneum subsp.albosporum, ATCC 25186, Stv.cinnamoneum subsp.lanosum, ATCC 25187, Stv.cinnamoneum subsp.sparsum, ATCC 25185, Stv.eurocidicum, ATCC 27428, Stv.lavenduligriseum, ATCC 13306, Stv.mediocidicum subsp.multiverticillatum, KCC S-0285, Stv.olivoreticuli, ATCC 23943, Stv.parvisporogenes, ATCC 12568, Stv.paucisporogenes, ATCC 12596, Stv.septatum, ATCC 27464, and Stv.sporiferum, ATCC 25188.

CLUSTER 3 (Stv.griseocarneum): Stv.abikoense, ATCC 12766(=IPV 2027), Stv.ardum, ATCC 27417, Stv.cinnamoneum subsp.azocolutum, ATCC 12686, Stv.griseocarneum, ATCC 12628, Stv.luteoverticillatum, ATCC 23933, Stv.mashuense, ATCC 23934, Stv.olivoverticillatum, ATCC 25480, Stv.pentaticum subsp.jenense, KCC S-0211, Stv.triculaminicum, KCC S-0242, and Stv.tropicalense, ATCC 17963.

CLUSTER 4 (Stv.hachijoense): Stv.hachijoense, ATCC 19679 (=IPV 2014 and 2057).

CLUSTER 5 (Stv.salmonis): Stv.aureoversales, ATCC 15853 and LIA 0882, and Stv.salmonis, NRRL 1472.

CLUSTER 6 (Stv.ladakanum): Stv.ladakanum subsp.ladakanum, ATCC27441, and Stv.verticillum, ATCC 15003.

CLUSTER 7 (Stv.mobaraense): Stv.luteoreticuli, ATCC 27446, Stv.mobaraense, ATCC 29032, and Stv.thioluteum, ATCC 12310(=IPV 2050).

CLUSTER 8 (Stv.morookaense): Stv.aspergilloides, ATCC 14804, and Stv.morookaense, ATCC 19166.

CLUSTER 9 (Stv.abikoense): Stv.abikoense, ATCC 12766 (=IPV 2225), Stv.ehimense, ATCC 23903, Stv.rimofaciens, ATCC 22166, Stv.takataense, ATCC 27469, and Stv.waksmanii, ATCC 12629.

CLUSTER 10 (Stv.olivoreticuli): Stv.hachijoense subsp.takahagiense, IPV 2255, and Stv.olivoreticuli subsp.cellulophilum, ATCC 21632.

SINGLE MEMBER CLUSTERS: Stv.albireticuli, ISP 5051, Stv.alboverticillatum, IPV 2254, Stv.album, NRRL 2401, Stv.kashmirense, ATCC 27439, Stv.kishiwadense, ATCC 25464, Stv.lilacinum, ATCC 23930, Stv.orinoci, ATCC 23202, Stv.rectiverticillatum, ATCC 19845, Stv.reticuli subsp.protomycicum, KCC S-0180, Stv.sapporonense, ATCC 21532, Stv.thioluteum, ATCC 12310(=IPV 2262), Stv.verticillium subsp.quintum, IPV 2253, Stv.verticillum subsp.tsukushiense, ATCC 21633, and Stv.viridoflavum, IPV 2258.

for the best fit to the matrix, MATIDEN also lists scores for the two next best alternatives, properties of the unknown which are atypical of the best fit taxon and characters which distinguish the unknown from the two nearest taxa.

Identification coefficients for the Hypothetical Median Organism of each cluster were determined by the MOSTTYP program (Sneath, 1980b), which provides the best scores available by an entirely typical example of each group in a matrix.

Finally the matrix was evaluated by determining the identification scores for strains from each cluster. Strains were selected randomly and the results of the appropriate tests obtained in the classification study (Locci et al., 1981) were input.

RESULTS

Selection of characters most diagnostic of the clusters

The diagnostic scores provided by the CHARSEP program (Sneath, 1979b) and the evaluation of decreasing numbers of the best 50 tests by MOSTTYP (Sneath, 1980b) indicated that a minimum of 41 characters were required. Therefore these were used to construct an identification matrix consisting of 24 clusters x 41 characters (Williams et al., 1985).

The diagnostic value of these characters,as indicated for example by their VSP indices (Sneath and Johnson, 1972), ranged from 84.23% (utilization of methionine) and 81.16% (degradation of DNA) to 46.56% (cottony aerial mycelium) and 45.54% (yellow spores). Values overall were higher than those obtained for the streptomycete matrix (Williams et al., 1983b), due to the inclusion of the single member clusters, but adherence to the strict sequential selection procedure proved to be necessary to obtain a workable matrix.

Final assessment of the test selection using the DIARCHAR program (Sneath, 1980a) produced sums of scores ranging from 11.54 to 20.34, with those for single member clusters inevitably being very high (Table 2). Values for multi-membered clusters were higher than those of Williams et al. (1983b) which ranged from 9.36 to 18.56. The comparatively low scores for clusters 2 and 3 indicated that they were less sharply defined than the others.

Evaluation of the identification matrix

Assessment of the overlap between clusters in the matrix, provided by the OVERMAT program (Sneath, 1980c) indicated that it was insignificant at a critical overlap value (v_o) of 1% in most cases. The exceptions were clusters 1 and 5, 2 and 4, and 3 and 4 which showed some overlap at a v_o value of 10%.

Table 2. Sum of scores of the most diagnostic character-states for each cluster provided by the DIACHAR program

Cluster	No. of strains	Sum of scores
1. Stv.baldaccii	17	13.35
2. Stv.cinnamoneum	14	11.54
3. Stv.griseocarneum	13	11.56
4. Stv.hachijoense	2	18.10
5. Stv.salmonis	3	19.01
6. Stv.ladakanum	2	18.81
7. Stv.mobaraense	3	14.19
8. Stv.morookaense	2	20.04
9. Stv.abikoense	5	16.64
10. Stv.olivoreticuli	2	20.34

Scores for single member clusters ranged from 23.87 (Stv.alboverticillatum) to 26.55 (Stv.orinoci).

Identification scores for the Hypothetical Median Organism (MOSTTYP, Sneath, 1980b) can be regarded as the best attainable for each cluster. Application of MOSTTYP to the final matrix produced good scores for clusters 1 to 10 (Table 3), with Willcox probabilities of 0.999 or 1.000 (except for cluster 2), low taxonomic distances (0.171 - 0.260) and highly negative standard errors of taxonomic distance. Clearly this program is irrelevant to single member clusters.

Table 3. Identification scores for the Hypothetical Median Organism of each cluster provided by the MOSTTYP program

Cluster	Identification scores		
	Willcox probability	Taxonomic distance	Standard error of tax.distance
1. Stv.baldaccii	0.999	0.220	-3.560
2. Stv.cinnamoneum	0.996	0.249	-3.200
3. Stv.griseocarneum	0.999	0.260	-3.189
4. Stv.hachijoense	1.000	0.247	-2.953
5. Stv.salmonis	0.999	0.171	-4.095
6. Stv.ladakanum	1.000	0.259	-2.914
7. Stv.mobaraense	1.000	0.256	-3.099
8. Stv.morookaense	1.000	0.221	-3.059
9. Stv.abikoense	1.000	0.219	-3.609
10. Stv.olivoreticuli	1.000	0.221	-3.059

Identification scores for cluster representatives using data from the classification study are given in Table 4. All strains identified to their parent clusters, the vast majority doing so with clearly acceptable scores for each coefficient. The one notable exception was Stv.mashuense, ATCC 23934, a representative of cluster 3, which had a low Willcox probability. As anticipated, all single member cluster strains identified correctly with highly significant scores.

Table 4. Identification scores for cluster representatives using classification data (Locci et al., 1981)

Cluster No.	Cluster representative	IDENTIFICATION SCORES Willcox probability	Taxonomic distance	Standard error of tax.distance
1.	Stv.baldaccii, ATCC 23654	1.000	0.331	-0.620
	Stv.kentuckense, ATCC 12691	0.978	0.349	-0.166
	Stv.hiroshimense, ATCC 19772	1.000	0.261	-2.393
2.	Stv.cinnamoneum, ATCC 11874	0.996	0.285	-2.177
	Stv.paucisporogenes, ATCC 12596	1.000	0.270	-2.536
	Stv.sporiferum, ATCC 25188	1.000	0.375	-0.014
3.	Stv.griseocarneum, ATCC 12628	1.000	0.375	-0.342
	Stv.mashuense, ATCC 23934	0.828	0.395	0.127
	Stv.tropicalense, ATCC 17963	0.997	0.370	-0.448
4.	Stv.hachijoense, ATCC 19679	1.000	0.247	-0.449
5.	Stv.aureoversales, ATCC 15853	1.000	0.233	-0.831
	Stv.salmonis, NRRL 1472	1.000	0.266	0.336
6.	Stv.ladakanum subsp. ladakanum, ATCC 27441	1.000	0.260	-0.393
	Stv.verticillum, ATCC 15003	1.000	0.260	-0.393
7.	Stv.mobaraense, ATCC 29032	1.000	0.300	-1.020
	Stv.thioluteum, ATCC 12310	1.000	0.320	-0.496
8.	Stv.aspergilloides, ATCC 14804	1.000	0.221	-0.597
9.	Stv.rimofaciens, ATCC 22166	1.000	0.303	-0.654
	Stv.abikoense, ATCC 12766	1.000	0.362	0.965
10.	Stv.hachijoense subsp. takahagiense, IPV 2255	1.000	0.221	-0.597

DISCUSSION

The results of this study, together with those of Williams et al. (1983b) on streptomycetes underline the potential value of numerical

classification data for the construction of a probabilistic identification system. This with the subsequent evaluation and application of the identification matrix, are facilitated by the various computer programs designed specifically for these tasks.

Unlike the streptomycete matrix, which was restricted in size by practicality to the major clusters defined in the classification study (Williams et al., 1983a), all the streptoverticillia classified by Locci et al.(1981) were included. Therefore the identification matrix contained data for most of the type species in the genus. Although some of the computer programs employed in the construction and testing of the matrix were somewhat irrelevant for the single member clusters, we were able to produce a workable matrix which encompassed all the taxa defined by Locci et al.(1981). It should therefore serve as a useful reference system for future identification of unknown strains.

The minimum number (41) of tests required for construction of a reliable matrix was coincidentally the same as that used to differentiate between the 23 major streptomycete clusters (Williams et al., 1983b). As most of the 24 streptoverticillia clusters were smaller than those of the streptomycetes, this was surprising. This was probably due to the greater degree of overlap which occurred between some of the larger streptoverticillia clusters; the number of tests required is clearly dependant on the variation between clusters. Although the phenetic classification studies on streptomycetes and streptoverticillia had many tests in common, it is interesting to note that the resulting identification matrices shared only 8 characters.

The tests selected for the streptoverticillia matrix covered a wide range of properties, including carbon source utilization, degradation, growth inhibition and antibiosis. However, morphological and pigmentation characteristics, which have been given great emphasis in previous attempts to group Streptoverticillium species (Locci et al., 1969; Baldacci and Locci, 1974) were not well represented. Only white spores, yellow spores and cottony aerial growth were included, and theis VSP indices (determined by the CHARSEP program, Sneath, 1979b) were relatively low at 51.14%, 45.29% and 46.56% respectively. The best VSP score for pigmentation of the substrate mycelium (yellow) was 45.29%, just excluding it from the matrix, while other pigments were of little or no diagnostic value. Likewise, soluble pigments were of little value, the best VSP score being 24.58% for yellow/brown pigmentation. Thus the use of these "traditional" characters for streptoverticillia results in highly artificial groupings.

In the evaluation of the streptomycete system, unknown isolates were tested and identified against the matrix (Williams et al., 1983b). This was not feasible with streptoverticillia as few are available and they appear to be difficult to isolate from soil and other habitats. Most of the type cultures have originated from workers screening isolates for antibiotics; 30 of the 40 species listed by Baldacci and Locci (1974) produced named antibiotics and another 7 showed anti-microbial activity.

Information on the sources and methods used to detect these strains is generally unavailable. Therefore, the ecology of streptoverticillia is still somewhat enigmatic. The results presented here should facilitate identification of any future isolates obtained and might also serve as a basis for the development of more objective isolation procedures, as demonstrated for streptomycetes (Vickers et al., 1985). Provision of a range of unknown isolates would allow evaluation of the matrix using strains not involved in its construction, which is the ultimate test of any identification system (Sneath and Sokal, 1973). Isolation of more strains should also eventually lead to a more accurate assessment of overall variation and speciation within the genus. The disproportionate number of single member clusters defined by Locci et al., (1981) may well be a reflection of the inadequacies of current sampling and isolation procedures.

Despite these problems, this study has provided a theoretically sound, workable identification system for known *Streptoverticillium* species, which is comparable with that previously devised for the closely related *Streptomyces* genus.

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EVALUATION OF SPECIES GROUPS IN THE GENUS STREPTOMYCES

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INTRODUCTION

The application of numerical taxonomy, molecular genetic and other powerful taxonomic methods has led to significant improvements in bacterial systematics (Goodfellow & Board, 1980). in general, good congruence has been found between DNA nucleotide sequence homology and numerical phenetic data (Staley & Colwell, 1973), a trend that extends to the actinomycete genera Nocardia (Mordarski et al., 1977a) and Rhodococcus (Mordarski et al. 1977b, 1980). Taxospecies have been found to show at least 70% DNA homology with lower binding values taken to reflect significant genetic divergence (Brenner, 1973). The genus Streptomyces has not featured in such comparative studies though both numerical phenetic (Silvestri et al., 1962) and DNA pairing data (Tewfik & Bradley, 1967; Okanishi et al., 1972) have shown the taxon to be overspeciated. in the most comprehensive numerical phenetic survey to date (Williams et al., 1983) the type strains of over 300 species of Streptomyces were assigned to 23 major clusters or subclusters (6 to 38 strains), 37 minor clusters ($<$ 5 strains) and 13 single member clusters. The minor and single member clusters were considered to form species and the major clusters equated with species groups. The largest cluster, the Streptomyces albidoflavus species group (cluster 1), was divided into three subgroups.

It can be important to evaluate numerical classifications using other taxonomic methods as similarity values between strains may be distorted by factors such as growth rate differences and sampling and test error (Goodfellow & Wayne, 1982). Indeed, the agreement found between classifications derived from the application of several independent taxonomic methods is a measure of the reliance that can be placed in a taxonomy (Jones & Sackin, 1980). In the present study, the extent of binding between DNA preparations from S. albidoflavus and related strains and reference DNA from single representatives of the subclusters of phenon 1 was determined. A comparison was then made between the numerical similarities and the DNA homology values of the test strains against the three reference systems.

MATERIALS AND METHODS

Test strains and growth conditions

Details on the source of the test strains (Table 2) can be found in Williams et al. (1983). Binomials in inverted commas are not on the Approved Lists of Bacterial Names (Skerman et al., 1980) and have not been validly published since 1 January 1980.

To obtain biomass, the organisms were grown in shake flasks (160 strokes per minute) containing Sauton's broth (Mordarska et al., 1972) at 30°C for 18 hours. Cultures were then checked for purity, harvested by centrifugation, washed in 0.15M saline - 0.1M ethylenetetraacetate (pH 8.0), and stored at -20°C.

Extraction of DNA

Purified DNA, isolated using the modification of the method of Saito and Miura (1963) described by Mordarski et al. (1976), was stored at 5°C in 0.1 x SSC (SSC = 0.15M NaCl + 0.015M trisodium citrate) containing a few drops of chloroform.

Analysis of DNA base compositions

The moles percent guanine (G) plus cytosine (C) of the DNA preparations were estimated using the thermal denaturation method of Marmur and Doty (1962).

Preparation of radioactive DNA for homology studies

Thymine-labelled DNA from uracil-labelled cells was prepared, extracted and purified as before (Mordarski et al., 1976, 1977b). Labelled reference DNA was prepared from Streptomyces alboviridis ISP5326, Streptomyces coelicolor ISP5233 and Streptomyces olivaceus ISP5072; the specific activities of the samples were 5064, 3533 and 2937 counts per minute per microgram, respectively.

Fixation of denatured/high molecular weight DNA on membrane filters and DNA:DNA pairing

Homology values between the test strains were estimated by determining the extent to which immobilised single-stranded DNA bound labelled reference DNA in solution. The reassociation was performed on nitrocellulose membranes (Sartorius SM-1140) for 20 hours at 48°C in 0.5 ml 23 x normal strength SSC, 0.5 ml formamide and 0.25 ml DNA (120 µg/ml) in 1 x SSC (Okanishi & Gregory, 1970; Toyama et al., 1974). Additional details of the methods used have been described by Mordarski et al. (1976). Experiments were repeated four or five times and the results expressed as mean average values.

Thermal stability of DNA:DNA duplexes

The thermal stability of duplexes formed between filter bound DNA and reference DNA preparations was determined from Tm(e) values. [TM(e) is the temperature at which half of the reassociated reference DNA becomes dissociated and eluted from the test DNA bound to the filter. The midpoint of the thermal elution, ΔTm(e), was found by subtracting the Tm(e) of the molecular hybrids of the heterologous system from the Tm(e) of the homologous hybrids (Okanishi & Gregory, 1970)].

When pairing was complete, the filters were dried and eluted with 0.1 x SSC solution at temperature increments of 5°C over the range 70 to 100°C. The radioactive samples were assayed in the scintillation fluid of Bray (1960).

Computation

Numerical analyses were carried out using the DNA homology data and the similarity values obtained with the corresponding strains in the numerical phenetic survey of Williams et al. (1983); the latter were based upon the simple matching coefficient (S_{SM}; Sokal & Michener, 1958). The taxonomic structure found in the data sets was expressed in dendrograms derived from the analysis of Euclidean distances with the unweighted pair group method with the arithmetic averages (UPGMA) algorithm (Sneath, 1983). The DNA pairing and numerical phenetic data were compared using the product moment correlation coefficient (Sneath & Sokal, 1973).

RESULTS

DNA base compositions

The DNA from the test strains fell within the range 71.0 to 76.4 mole % G plus C (Table 1).

Table 1. DNA base composition of clusters and subclusters

Subcluster	Range	Cluster	Range
1A	73.7 - 75.6	16	73.8 - 75.9
1B	71.7 - 76.4	55	71.7 - 74.4
1C	71.0 - 75.2	61	74.1 - 72.5

DNA:DNA PAIRING

The DNA preparations from most of the subcluster 1A strains showed extensive duplex formation with reference DNA from S. coelicolor ISP5233 but relatively little homology with that from the other two reference systems (Table 2). S. albidus ISP5320 and S. alboniger ISP5043 clearly belong to the S. coelicolor homology group. It is also interesting that all of the strains showing homology values of over 70% with this reference system formed notably stable duplexes. In contrast, DNA from strains assigned to subclusters 1B and 1C tended to show relatively low degrees of homology with reference DNA from S. alboviridis ISP5326 and S. olivaceus ISP5077, respectively. However, "S. oligocarbophilus" ISP5589 belongs to the S. alboviridis homology group as does "S. craterifer" ISP5296 from subcluster 1A. The DNA from S. erythraeus ISP5517 showed a relatively high similarity with reference DNA from the S. olivaceus system but in this instance there was evidence of mismatching.

Taxonomic structure derived from the incomplete data sets

The UPGMA dendrograms based on the Euclidean distances derived from the S_{SM} and DNA homology values are shown in Figures 1 and 2. With only two exceptions the subcluster 1A, 1B and 1C strains were recovered in corresponding phena in the analysis based upon S_{SM} values (Fig. 1). The remaining strains were assigned to a second phenon that was heterogeneous but well separated from the rest. In the case of the DNA pairing values the results were less clear cut though a main cluster containing subcluster 1A strains plus two subcluster 1B strains was evident (Fig. 2). A less well circumscribed phenon contained some subcluster 1B strains with two subcluster 1A strains and single strains from subcluster 1C and cluster 16. The remaining phenon encompassed a miscellany of strains, most from subclusters 1B and 1C with the rest from subcluster 1A and clusters 16, 55 and 61.

Comparison of numerical phenetic and DNA pairing data

The mean DNA pairing and S_{SM} values of the reference strains with the organisms from the numerically defined taxa are shown in Table 3. Good congruence was found between the two sets of data when the mean S_{SM} values were over 80% and the corresponding DNA homology values around 75%. However, it is evident that DNA pairing falls away dramatically even for S_{SM} values of just under 80%. The highest correlation between the DNA pairing and numerical phenetic data was found between the reference strains and the subcluster to which they belong (Table 4).

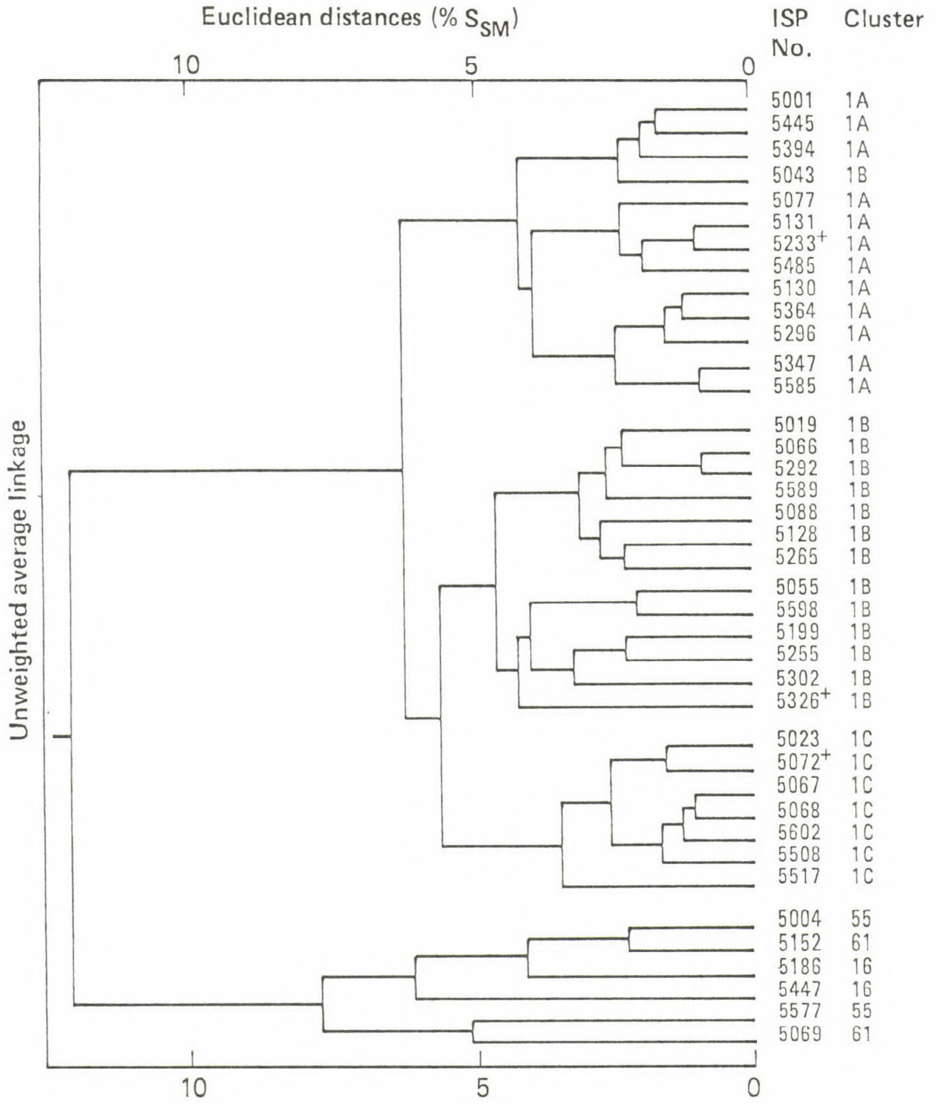

Fig. 1 UPGMA dendrogram of similarity values showing the relationships between the test strains

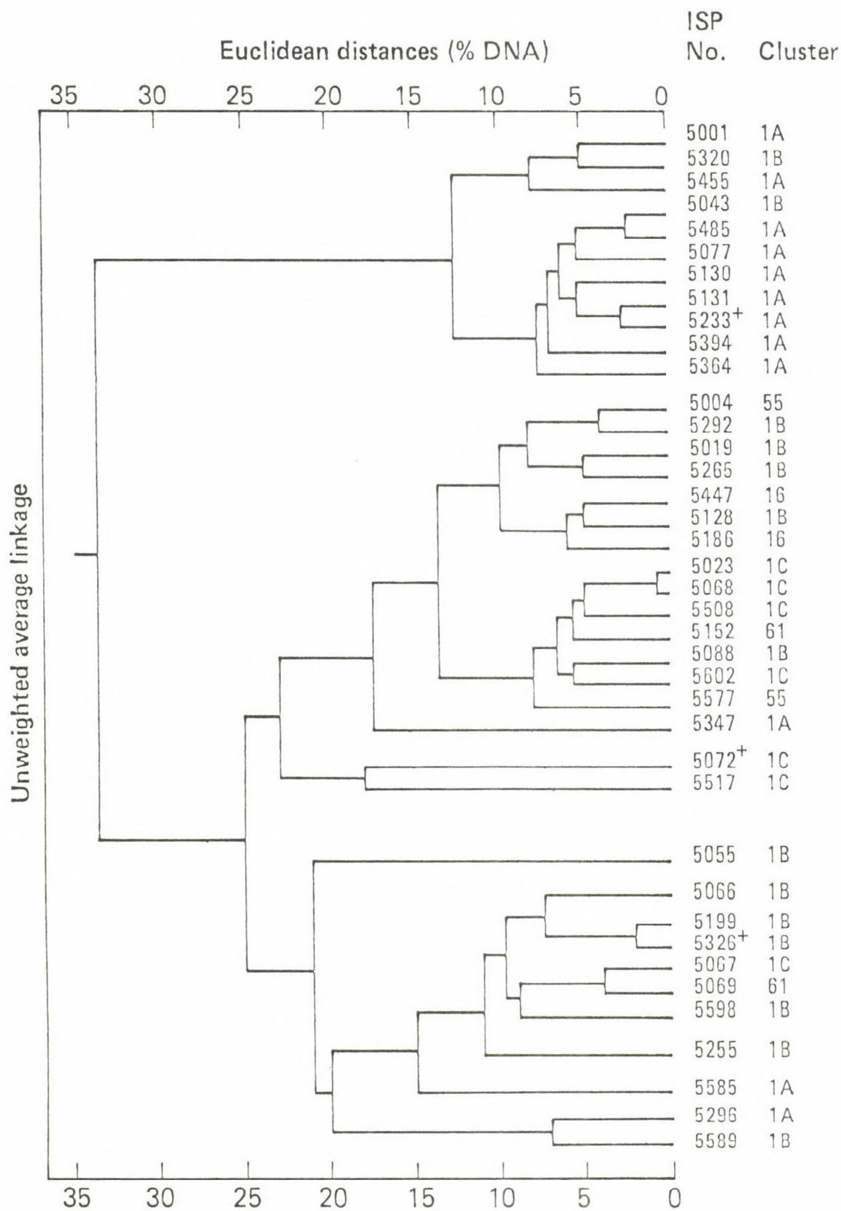

Fig. 2 UPGMA dendrogram of DNA homology values showing the relationships between the test strains

Table 2. Reassociation of DNA samples from test strains with DNA from reference strains *Streptomyces coelicolor* ISP5233, *Streptomyces alboviridis* ISP5326 and *Streptomyces olivaceus* ISP5072*

DNA source	ISP Number	Labelled DNA source: *S. coelicolor* ISP5233 Relative amount of DNA bound (Subcluster 1A)	ΔTm(e)	*S. alboviridis* ISP5326 Relative amount of DNA bound Subcluster 1B	ΔTm(e)	*S. olivaceus* ISP5072 Relative amount of DNA bound (Subcluster 1C)	ΔTm(e)
Subcluster 1A**							
S. albidoflavus	5455	84 ± 1+	1.5	15 ± 2		20 ± 0	
S. canescens	5001	71 ± 1	0.5	19 ± 0.5		22 ± 0	
"*S. citreus*"	5364	98 ± 1	0	14 ± 1		10 ± 0.5	
S. coelicolor	5233++	100		19 ± 0.5		30 ± 2	11.5
"*S. coriofaciens*"	5485	92 ± 1.5	1.5	23 ± 0.5		26 ± 0.5	
"*S. craterifer*"	5296	29 ± 0		86 ± 2.5	0	20 ± 0	
S. felleus	5130	104 ± 4	0.5	16 ± 0.5		31 ± 0	11.5
S. limosus	5131	92 ± 1.5	1.5	18 ± 0.5		32 ± 0.5	11
S. odorifer	5347	42 ± 1	1	33 ± 2	4	11 ± 0.5	
S. rutgersensis	5077	86 ± 0	1.5	26 ± 0.5		34 ± 1	12.5
"*S. sampsonii*"	5394	84 ± 1	1.5	17 ± 0.5		24 ± 1	
"*S. tetanusemus*"	5585	20 ± 0.5		62 ± 0	0	13 ± 0.5	
Subcluster 1B							
"*S. albidus*"	5320	76 ± 3	2	13 ± 1		17 ± 0.5	
S. alboniger	5043	82 ± 1	1.5	28 ± 2		24 ± 1	
S. alboviridis	5326+	32 ± 2	5.5	100		23 ± 0	
"*S. aureus*"	5055	32 ± 2	9	57 ± 0.5	9	25 ± 0.5	
S. bacilleris	5598	28 ± 0.5		42 ± 1	4.5	18 ± 0	
S. chrysomallus	5128	23 ± 0.5		17 ± 0.5	1.5	25 ± 1.5	
S. citreofluorescens	5265	11 ± 1		15 ± 0		9 ± 0	
S. globisporus	5199	29 ± 0		41 ± 0.5	8	24 ± 0	
S. griseobrunneus	5066	34 ± 0	2.5	55 ± 0.5	3	30 ± 1	12.5
S. niveus	5088	27 ± 0		25 ± 0.5		32 ± 0	10.5
"*S. oligocarbophilus*"	5589	30 ± 1	6	79 ± 0.5	0.5	31 ± 0	12.5
S. pluricolorescens	5019	13 ± 0		20 ± 0.5		11 ± 0.5	
S. sindenensis	5255	23 ± 1		34 ± 1.5	5.5	16 ± 0	
S. spheroides	5292	13 ± 0		10 ± 0		9 ± 0.5	
Subcluster 1C							
S. erythraeus	5517	31 ± 0.5	6.5	22 ± 1		64 ± 1	4
S. griseolus	5067	33 ± 0.5	3.5	39 ± 2	2.5	30 ± 0.5	12.5
S. halstedii	5068	25 ± 0		28 ± 2		23 ± 1.5	
"*S. humifer*"	5602	31 ± 1	3.5	30 ± 0	9	31 ± 0	11.5
"*S. naraensis*"	5508	32 ± 2	4.5	30 ± 2	10	21 ± 0	
S. nitrosporeus	5023	25 ± 1		28 ± 0.5		24 ± 0.5	
S. olivaceus	5072+	42 ± 0	1	26 ± 1		100	
Cluster 16							
S. almquistii	5447	28 ± 0		13 ± 0.5		18 ± 0.5	
S. aminophilus	5186	23 ± 0		7 ± 0.5		14 ± 1	
Cluster 55							
Streptoverticillium griseocarneum	5004	15 ± 0.5		12 ± 1		15 ± 0.5	
Stv. septatum	5577	27 ± 0		18 ± 0		22 ± 0	
Cluster 61							
S. flavotricini	5152	25 ± 0		24 ± 1		21 ± 1	
S. lavendulae	5069	34 ± 1.5	4	38 ± 2	1	38 ± 0.5	12.5
Control							
Micrococcus lysodeikticus	ATCC 4696	7 ± 0		5 ± 0		6 ± 0	

* Details of methods used and explanation of ΔTm(e) can be found in the text.

** Taxa defined in the numerical phenetic survey of Williams *et al*. (1983).

\+ Standard error.

++ Reference strains.

Table 3. Means of reassociation of DNA samples and S_{SM} coefficients of reference strains with organisms in each of the numerically circumscribed taxa

Taxon	S. coelicolor ISP5233 (Subcluster 1A)		S. alboviridis ISP5326 (Subcluster (1B)		S. olivaceus ISP5072 (Subcluster 1C)	
	% DNA	% S_{SM}	% DNA	% S_{SM}	% DNA	% S_{SM}
Subcluster						
1A	75.2	84.8	29.0	79.3	23.6	78.6
1B	33.1	77.1	38.3	85.4	20.8	78.6
1C	31.3	76.3	29.0	84.6	43.1	87.1
Cluster						
16	25.5	71.0	10.0	74.5	16.0	77.0
55	21.0	70.5	15.0	70.0	18.5	64.0
61	29.5	66.5	31.0	68.0	29.5	66.5

Table 4. Comparison of the DNA reassociation (% relative DNA bound) and the phenetic similarity (% S_{SM}) between reference strains and the phenetic groups expressed as correlation coefficients

Phenetic groups	Reference strains		
	S. coelicolor ISP5233	S. alboviridis ISP5326	S. olivaceus ISP5072
All clusters	0.53	0.49	0.31
Cluster 1	0.58	0.91	0.65
Subcluster of reference strain	0.77	1.00	0.91

DISCUSSION

The analysis of the similarity values on the test strains led to the construction of a dendrogram very similar to that obtained by Williams et al. (1983). As expected, clusters formed around the three reference strains. The recovery of S. albidus ISP5320 and S. alboniger ISP5043 in subcluster 1A was especially interesting as these strains fell into the S. coelicolor DNA homology group. The separation of strains from clusters 16, 55 and 61 from those in cluster 1 is also consistent with the results of the earlier study. It is possible that the grouping of these strains in a single phenon is due to the compression effect on satellites given that all of the cluster 1 reference strains shared high overall values.

The dendrogram based on the DNA homology values proved more difficult to interpret. However, most of the subcluster 1A strains formed a distinct DNA homology group which also contained the two subcluster 1B strains mentioned above. It is also noteworthy that the subcluster 1A strains have DNA that falls within the narrow range 73.7 to 75.6 mole % G plus C. A second cluster encompassed some subcluster 1B strains together with two subcluster 1A strains, one subcluster 1C strain and a representative of cluster 61. It was also encouraging that both clusters 1 and 2 contained a reference strain. The remaining reference strain,

S. olivaceus ISP5072, fell into the final cluster composed of the balance of the subcluster 1B and 1C strains and representatives of clusters 16, 55 and 61. It is difficult to explain this taxon though it is evident that the DNA pairing data were not following the S_{SM} values. Possibly the least expected finding is that the DNA values do not show the clusters 16, 55 and 61 pushed together into a single phenon as noted earlier. It is possible, however, that some of the higher DNA values between cluster 1 and the other clusters have produced this mixed cluster.

The DNA pairing data are in line with those from earlier studies in showing that the genus Streptomyces is overspeciated (Tewfik & Bradley, 1967; Okanishi et al., 1972). In the present study, strains sharing DNA homology values of over 70% with a reference system were considered as genomic species provided that the duplexes were stable. Streptomyces coelicolor, S. albidoflavus, S. albidus, S. alboniger, S. canescens, "S. citreus", "S. coriofaciens", S. fellus, S. limosus, S. rutgerensis and S. sampsonii formed one such taxon and S. alboviridis, "S. craterifer" and "S. oligocarbophilus" the nucleus of a second group. None of the test strains showed extensive stable duplexes with reference DNA from S. olivaceus ISP5072.

The partial congruence found between the similarity and DNA homology values helps to highlight an important problem in bacterial systematics that is likely to occur increasingly as more analytical and statistical procedures are applied in bacterial classification and identification. Thus, given two incompatible or partially compatible classifications, which criteria can be employed to decide which, if any, of the taxonomic structures is most fitting? In such instances the search for other taxonomic markers may not resolve the situation fully. Indeed, it seems more likely that more will be gained if greater attention were given to detailed work on strain selection, to reproducibility testing, to the taxonomic significance of particular tests or characters, and to the development of appropriate statistical procedures capable of interlocking classifications from different sources (Sneath, 1985). It is also possible that lack of congruence may have a biological significance of some importance but unambiguously good quality data are needed before such new vistas can be explored with vigour.

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THE CELLULOMONAS, OERSKOVIA, PROMICROMONOSPORA COMPLEX

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INTRODUCTION

The three genera Cellulomonas, Oerskovia and Promicromonospora were frequently discussed jointly during the last years. Cellulomonas was placed among the coryneform genera, whereas Oerskovia and Promicromonospora display a nocardioform life cycle (Prauser 1970). Colonies of all these organisms are mainly smooth and yellow to whitish.

The differentiation of the genera, which seemed to be clear (Tab. 1), is now debatable. Additional biochemical evidence was elaborated (Tab. 2), molecular biological data were supplied (summarized in Stackebrandt and Schleifer 1984), new organisms were introduced in this taxonomic area (Stackebrandt and Kandler 1980, Jáger et al. 1983), and new combinations were proposed (Stackebrandt et al. 1982).

Tab. 1: Differentiating characters (around 1975)

Character	Cellulomonas	Oerskovia	NMOs	Promicromonospora
CW diamino acid	ornithine	lysine	lysine	lysine
Mycelia	-	+	+	+
Motility	+ or -	+	-	-
Aerial hyphae	-	-	-	+
Cellulase	+	-	+	-

Cellulomonas, once a genus of the family Corynebacteriaceae, lost its placement in this family (Rogosa et al. 1974). Oerskovia and Promicromonospora were also discussed as "in search of a family" (Lechevalier and Lechevalier 1981). Stackebrandt and Schleifer (1984) on molecular biological ground offer the view of harbouring all these organisms together with a redefined genus Arthrobacter in the family Arthrobacteraceae.

In this article some new information will be discussed in the context of present knowledge. Taxonomic conclusions will be offered.

Tab. 2: Biochemical and molecular characters common to *Cellulomonas, Oerskovia* and *Promicromonospora*

Mol% G + C of DNA	70 to 75 (Tm) (a, b, c, d, e)
Prevailing menaquinones	MK-9 (H_4) (f, g, h)
Diagnostic phospholipids	phosphatidyl glycerol, glucosamine-containing unknowns (i, j, k)
Diagnostic fatty acids	anteiso/iso (l)
Intergeneric level of S_{AB} values of 16S rRNA	0.71 to 0.63 (m)
Intergeneric DNA homologies	around 20 % (e, n, Tab. 6)

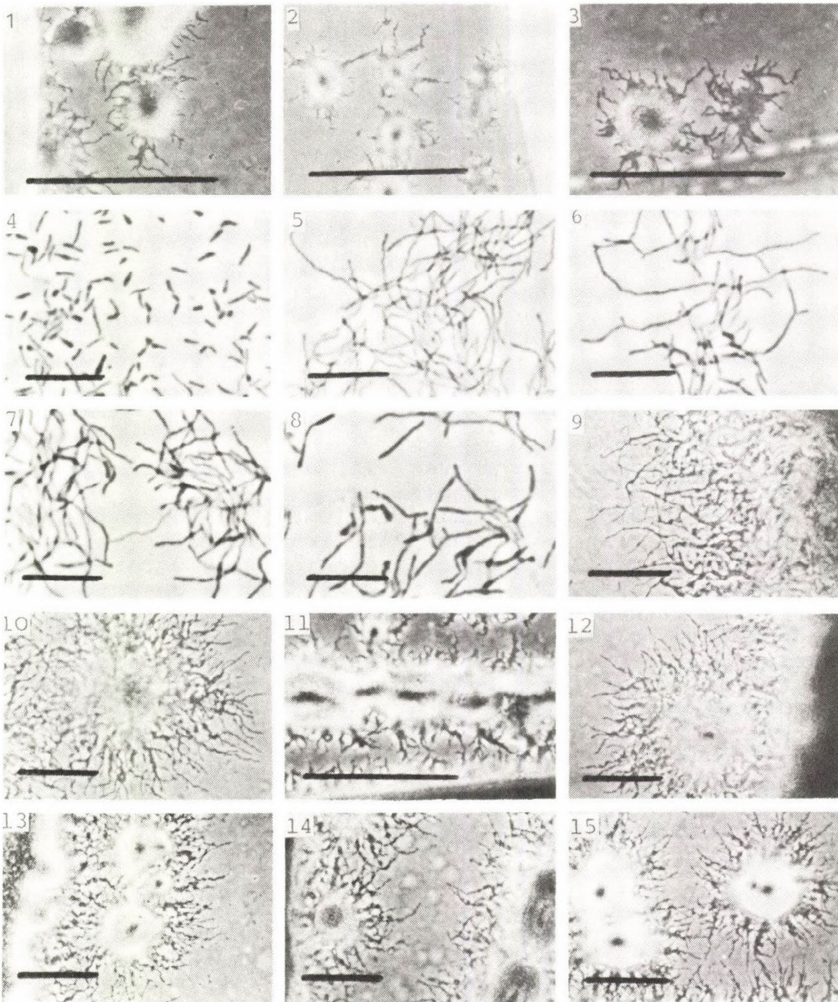
(a) Tsyganov et al. 1966, (b) Yamaguchi 1967, (c) Prauser 1966, (d) Sukapure et al. 1970, (e) Stackebrandt and Kandler 1979, (f) Yamada et al. 1976, (g) Collins et al. 1979, (h) Collins and Jones 1981, (i) Lechevalier et al. 1977 (*Promicromonospora*: additionally acyl phosphatidyl glycerol), (j) Lechevalier et al. 1981, (k) Minnikin et al. 1979 (*Cellulomonas* partly: ai-15/15:0), (l) Andreyev et al. 1983 (reports ai-15/16:0/i-15:1 for *O. turbata* and ai-15/16:0 for *O. xanthineolytica*), (m) Stackebrandt et al. 1983, (n) Stackebrandt et al. 1980

MORPHOLOGY

Among the numerous contributions to morphology it may be referred to Stackebrandt and Kandler (1979) concerning *Cellulomonas*, to Kretschmer (1981) with regard to *Oerskovia* and to Yevtushenko et al. (1982) and Kuimova et al. (1983) for *Promicromonospora*.

Mycelial development, fragmentation of hyphae and cell shape depend primarily on the composition of the medium and on the age and kind of culture. In submerged shaken culture cellulomonads - with the exception of *Cellulomonas cartae* - produce mainly rodlike to coryneform elements (Fig. 4, see also figures of Stackebrandt and Kandler 1979). Oerskoviae including the "nonmotile organisms" (NMOs) of Lechevalier (1972), *Cellulomonas cartae* and promicromonosporae show irregular, occasionally branched, subsequently fragmenting hyphae of differring length at early stages of the logarithmic phase (Fig. 5, 6, 7, 8).

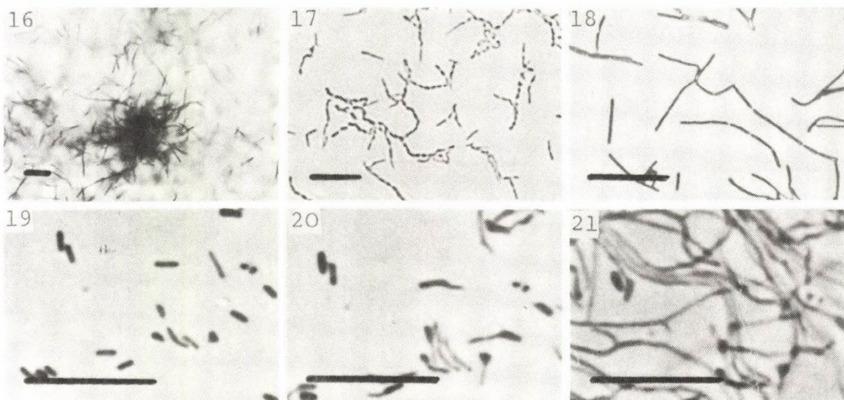
In contrast to the usual view the colony fringes of all *Cellulomonas* species studied (see Tab. 4) show at least in limited regions on poor media and at early stages a more or less hyphal or even mycelial appearance (Fig. 1, 2, 3). Oerskoviae and related organisms develop in agar cultures mycelia the hyphae of which break up into rodlike to coccoid elements (Fig. 10, 11, 12, 13, 14, 15). I. e., there are



Fragmenting mycelia on agar media, 26 h, 28°C. Bar=100 µm. (1) Cellulomonas flavigena NCIB 8037, a; (2) C. biazotea NCIB 8077, c; (3) C. fimi NCIB 8980, a

Submerged shaken culture, 26 h, 28°C, b. Bar= 10 µm. (4) C. biazotea; (5) Oerskovia turbata Ørskov 27; (6) Cellulomonas cartae DSM 20106; (7) Brevibacterium fermentans NCIB 9949; (8) Promicromonospora citrea ATCC 15908

Mycelia, partly fragmenting, on agar media, 28 h, 28°C, a (9) Promicromonospora citrea; (10) Oerskovia turbata; (11) O. xanthineolytica IMRU 3959; (12) Cellulomonas cartae; (13) NMO Type A, Ballou; (14) Nocardia cellulans NCIB 8868; (15) Promicromonospora enterophila DFA-19 T. Bar=100 µm



Promicromonospora citrea VKM 791, aerial mycelium and development of its fragments. Bar = 10 µm. (16) 28 h, 28°C, a: aerial mycelium; (17) coverslip gently pressed on aerial mycelium, slip transfered on slide coated with a film of agar: beginning fragmentation of hyphae; (18) 5 d, 28°C, d, slip handled as described, however transfered on slide without agar or water: hyphae completely fragmented; (19) removal of aerial hyphae as described before, adhering material transfered on slide with agar film b: fragments of different size and shape; (20) same place after incubation for 9 h at 28°C: beginning growth; (21) same place after 18 h: formation of mycelia

Media applied: a) Bennett-saccharose agar, b) complex organic medium 79 (Prauser and Falta 1968), c) Mannose glycerol agar, d) Oatmeal agar (Shirling and Gottlieb 1966)

transitions concerning the microscopical appearance of agar-grown colonies between cellulomonads and oerskoviae. As a rule, the mycelial development is most distinct with promicromonosporae (Fig. 9).

The aerial mycelium of Promicromonospora citrea was reported to be poor and sterile (Krassilnikov et al. 1961). All strains studied (see Tab. 5) produce aerial hyphae which may be seen at least microscopically (especially in old laboratory strains) or by the naked eye as forming a scanty aerial mycelium. Occasionally, the colonies are covered by a consistent and dense mat of aerial mycelium. The aerial hyphae are straight or slightly bent, are unbranched or rarely and sparsely branched (Fig. 16). The aerial hyphae break up completely into rodlike elements (Fig. 17, 18, 19), which give rise to new mycelia (Fig. 19, 20, 21). Thus, the life cycle of Promicromonospora citrea resembles the cycles of Nocardia and Nocardioides.

CELL WALL COMPOSITION

For Promicromonospora citrea lysine was reported as diamino acid of the cell wall peptidoglycan (Yamaguchi 1965). Cellulomonas biazotea could be differentiated by the occurrence of ornithine from the lysine-containing strain Ørskov 27 (Prauser 1966), which was subsequently attributed to Oerskovia turbata. Tab. 3 includes more detailed and extensive information.

Tab. 3: Peptidoglycan types

Taxon	Peptidoglycan type
Cellulomonas flavigena	L-Orn-D-Asp (a)
" biazotea	L-Orn-D-Glu (a)
" cellasea	L-Orn-D-Glu (b)
" gelida	L-Orn-D-Glu (b)
Oerskovia turbata	L-Lys-L-Thr-D-Asp (c)
	L-Lys-D-Glu (D-Asp) (d)
Oerskovia xanthineolytica	L-Lys-D-Ser-D-Asp (c)
Brevibacterium fermentans	L-Lys-D-Ser-D-Asp (b)
" lyticum	L-Lys-D-Ser-D-Asp (e)
Cellulomonas cartae	L-Lys-D-Ser-D-Asp (e)
"Corynebacterium manihot"	L-Lys-D-Ser-D-Asp (e)
Nocardia cellulans	L-Lys-D-Ser-D-Asp (b)
"Arthrobacter luteus"	L-Lys-D-Ser-D-Asp (e)
Promicromonospora citrea	L-Lys-Ala_2 (f)

(a) Schleifer and Kandler 1972, (b) Stackebrandt et al. 1980, (c) Seidl et al. 1980, (d) Stackebrandt and Schleifer 1984, (e) Stackebrandt et al. 1978, (f) Stackebrandt et al. 1983

PHAGE HOST RANGE PATTERNS

Susceptibility to the attack of phages was found to correspond closely to classifications which were established by DNA:DNA hybridization and by comparative analysis of oligonucleotides of 16S ribosomal RNA (Prauser 1984). For example, strains of all genera attributed to the family Streptomycetaceae by use of the latter methods (Stackebrandt and Schleifer 1984), strains of the genera Actinoplanes, Micromonospora and others, which were placed in the family Actinoplanaceae (ibid.), and strains of the Nocardia-Rhodococcus complex are susceptible to phages of each's own specific set (Prauser 1984, Prauser and Falta 1968). Also clearing effects, i. e. phage-dependent effects without phage propagation, were shown to be taxon-specific (Prauser 1981).

Among the large number of phages which we isolated from various types of soil, and which were tested against many strains representing the majority of the coryneform, nocardioform and sporoactinomycete genera, 13 of these phages were found active against strains of the genus Oerskovia (Tab. 4).

Tab. 4: Selected host range patterns of Oerskovia phages

Taxon	O-phages 2	3	12	13	8	11
Oerskovia turbata T	+	+	+	-	+	+
" "	+	+	+	-	-	-
Promicromonospora enterophila T	+	+	-	-	-	o
" "	o	o	-	-	-	o
NMO Type B	+	+	+	-	+	o
Oerskovia xanthineolytica T	-	-	-	-	o	-
" "	-	-	-	+	+	+
Cellulomonas cartae T	-	-	-	+	+	+
NMO Type A	-	-	-	o	o	o
Nocardia cellulans T	-	-	-	+	+	+
Brevibacterium fermentans T	-	-	-	+	o	o
"Corynebacterium manihot"	-	-	-	-	o	-
Cellulomonas spp. (5 species) T	-	-	-	-	-	-
Promicromonospora citrea (26 strains including T)	-	-	-	-	-	-

+: lysis, o: clearing effect, -: no response, T: type strains, see legends to Fig. 1 - 21

At least one of six further phages was effective against individual strains among the total of 26 strains studied of the hitherto monotypic genus Promicromonospora citrea (Tab. 5).

Tab. 5: Host range patterns of Promicromonospora phages

Strain No.	P-phages 1	2	3	4	5	6	Strain No.	P-phages 1	2	3	4	5	6
ATCC 15908	+	+	+	+	+	+	LL G-165	+	+	+	+	+	+
393-9	o	-	o	+	o	o	LL G-173	o	o	+	-	+	-
604-56	-	+	+	+	+	-	LL G-201	-	-	+	+	-	-
623-3	-	+	o	-	+	-	VKM 791	-	-	+	+	-	+
480	-	+	+	+	+	-	VKM 796	o	+	+	+	+	o
1196-130	+	+	+	+	+	-	VKM 784	-	o	+	+	o	-
1196-112	+	+	+	+	+	+	1084-2908	-	+	+	-	+	-
1196-17	-	-	+	+	-	-	1084-3280	-	-	+	-	-	-
1196-63	o	+	+	+	+	-	1084-3525	-	-	+	-	-	-
1196-45	o	+	o	o	-	-	1136-47	-	+	+	+	+	-
1196-282	o	o	+	+	+	-	VKM 788	-	-	o	+	-	-
VKM 783	o	o	+	-	-	o	VKM 789	-	+	+	+	+	-
VKM 787	o	-	+	-	+	-	VKM 790	-	-	+	+	-	o

+: lysis, o: clearing effect, -: no response

Four phages were specific for the type strain of Cellulomonas fimi (Tab. 4). There were no cross reactions of phages of the three sets among these three genera (Tab. 4) or with strains of other coryneform, nocardioform and sporoactinomycete genera.

The selected six phages which are active against Oerskovia and related organisms may be arranged in three subgroups as shown in Tab. 4. Two of these subgroups are specific for each of two particular subunits of the genus Oerskovia, whereas the third one covers the whole range of the oerskoviae.

MOLECULAR BIOLOGICAL EVIDENCE

The Mol% G + C of the DNAs is similar for the three genera (Tab. 2).

From the studies of Stackebrandt et al. (1980) three groups of DNA homology emerge:

- Oerskovia turbata, showing DNA homologies with i) Oerskovia xanthineolytica, Nocardia cellulans, "Corynebacterium manihot", Cellulomonas cartae and Brevibacterium fermentans in the range of 49 to 37 %; and with ii) Cellulomonas flavigena, C. cellasea and C. gelida of 24 to 22 %;
- Oerskovia xanthineolytica, Nocardia cellulans, "Corynebacterium manihot", Cellulomonas cartae and Brevibacterium fermentans - with group internal homologies between 91 and 52 % - displaying homologies to i) Oerskovia turbata between 43 and 39 %, and to ii) the cellulomonads given above between 23 and 17 %;
- Cellulomonas spp. as resulting from the low homologies between 24 and 17 % with organisms of the two former groups.

In a preceding study including all valid Cellulomonas species it was always Cellulomonas cartae that hybridized at the lowest level (23 to 12 %) (Stackebrandt and Kandler 1979).

In our own studies, which were mainly performed by J. Felsberg (now Institute of Molecular Genetics, Prague), we subjected type and reference strains of species of the genera Cellulomonas, Oerskovia and Promicromonospora to DNA:DNA hybridization. As far as identical taxa are involved our results (Tab. 6) correspond closely to those of Stackebrandt and Kandler (1979) and Stackebrandt et al. (1980). Oerskovia turbata appears well separated from Oerskovia xanthineolytica and its relatives as well as the whole genus from the cellulomonads and from Promicromonospora. Cellulomonas cartae clearly joins the oerskoviae. Using labelled DNA of Cellulomonas flavigena the oerskoviae were not separated too distinctly. Remarkable to some degree are the homologies in the range of 55 to 43 % among the promicromonosporae. In spite of these low degrees of homology the respective strains could not yet be attributed to different species of this genus by the study of conventional characters.

As a result of comparative analyses of oligonucleotides of the 16S ribosomal RNA the species Oerskovia turbata and Cellulomonas flavigena turned out to be related at the S_{AB} level of 0.71 (Stackebrandt et al. 1983). The respective values for Oerskovia turbata and Promicromonospora citrea on the one side and for the latter species and Cellulomonas

flavigena on the other are 0.66 and 0.63. Arthrobacter globiformis joins the three species under discussion at S_{AB} levels in the range of 0.63 and 0.67.

Tab. 6: DNA homologies among strains of Cellulomonas, Oerskovia and Promicromonospora

Filter DNA from		^{14}C-DNA from Oer. tur. T	Cel. car. T	Cel. fla. T	Pm. cit. T
Oerskovia turbata	Ørskov 27 T	100	36	26	23
" "	IMET 7006	75	38	24	22
Oerskovia xanthineolytica	IMRU 3959 T	30	66	23	18
" "	LL Y 13-4	28	60	21	19
Cellulomonas cartae	DSM 20106 T	30	100	24	20
"Corynebacterium manihot"	NCIB 9097	33	68	26	22
Cellulomonas flavigena	NCIB 8037 T	21	21	100	20
" biazotea	NCIB 8077 T	21	19	28	23
" subalbus	NCIB 8075 T	22	22	33	21
" fimi	NCIB 8980 T	19	25	30	18
Promicromonospora citrea	RIA 562 T	18	24	19	100
" "	604-56	19	25	22	53
" "	623-3	17	23	21	52
" "	393-1	18	22	19	55
" "	LL G-165	17	22	18	45
" "	LL G-173	15	16	18	43
" "	1084-2908	17	20	19	44
Micrococcus luteus	ATCC 9341	9	12	13	11
Pseudomonas fluorescens	ATCC 13525	4	4	8	3

T: Type strain, |: intragenus DNA homology,
||: intraspecies DNA homology

TAXONOMIC CONCLUSIONS

We follow the proposal of Stackebrandt and Schleifer (1984) to understand the genus Arthrobacter, including Micrococcus, and the organisms discussed in this article as nucleus of a newly to be established family. However, according to the priority of Cellulomonas over Arthrobacter the proposed name Arthrobacteriaceae (sic) should be avoided in favour of the name Cellulomonadaceae. Apart from the viewpoint of priority the genus Cellulomonas would be more suitable as nomenclatural type, since Arthrobacter was always a heterogenous and frequently confused taxon.

Cellulomonas cartae, in the present author's opinion, was misclassified from the early beginning. The reasons for its transfer to the genus Oerskovia are summarized in Tab. 7, as well as reasons against the proposed union of Oerskovia and Cellulomonas. As shown in Tabs. 3 and 4 different cell wall

composition and phage susceptibility clearly - and simply in practice - differentiate these organisms. The levels of DNA: DNA homology and the S_{AB} values may be subject to discussion. In both cases there are values which are not too distant from each other. However, the areas never overlap. We should be aware that - as well as in numerical taxonomy - no definite values will be available to define the borderlines of taxonomic ranks.

Tab. 7: Characters in favour of placing Cellulomonas cartae to the genus Oerskovia and reasons against the union of Oerskovia and Cellulomonas

Peptidoglycan type	L-Lys-D-Ser-D-Asp
Phage susceptibility	to Oerskovia phages, being ineffective against cellulomonads
Appearance in shaken culture (exponential phase)	irregular, partly branching hyphae of different length
DNA homology	within the range of oerskoviae (49 to 37 % with O. turbata; low with cellulomonads (23 to 17 %)
Comparative analysis of oligonucleotides of 16S ribosomal RNA	no imperative reason to unite Oerskovia with Cellulomonas on the S_{AB} level of 0.71
Numerical taxonomy	not localized in the area of cellulomonads; localized in the area of organisms which are similar or synonymous to Oerskovia xanthineolytica (a, b)

(a) Seiler et al. 1977, (b) Bousfield et al. 1983

Whichever place the "cartae" taxon will find, the rules of the International Code of Nomenclature of Bacteria (1976) should be observed. If the synonymies stated by Stackebrandt et al. (1982) are real, the correct name would be Cellulomonas cellulans (Metcalf and Brown 1957) followed by the names of the authors of the combination. Under the same premise and according to the present author's proposal the name would be Oerskovia cellulans (Metcalf and Brown 1957) N. N. against the interest of Oerskovia xanthineolytica.

Promicromonospora enterophila Jáger et al. 1983 should be transfered to the genus Oerskovia: i) The organism is susceptible to Oerskovia phages (O-phages), particularly to those specific for Oerskovia turbata (Tab. 5), ii) aerial mycelium is lacking, iii) motility was observed for two strains in shaken culture, iv) on agar media the microscopical appearance is indistinguishable from that of oerskoviae.

On the base of the preceding discussions three proposals are offered concerning the redefinition of the genera Cellulo-

monas, Oerskovia and Promicromonospora (Tab. 8). The respective biochemical data are given in Tab. 2.

Tab. 8: Redefinition of the genera Cellulomonas, Oerskovia and Promicromonospora

Genus Cellulomonas	(Bergey et al. 1923; Stackebrandt, Seiler and Schleifer 1982) N.N.
Colony fringe:	More or less filamentous or mycelial in young cultures on poor media. Mostly smooth in older cultures
Cell shape:	Rods straight, angular, slightly curved or irregular. Occasionally branched. Cocci may occur in older cultures
Motility:	Motile or nonmotile
Spores:	Lacking
Aerial mycelium:	Lacking
Phage susceptibility:	Not susceptible to Oerskovia and Promicromonospora phages (O- and P-phages)
Aerobic or facultatively anaerobic	

Genus Oerskovia	(Prauser, Lechevalier and Lechevalier 1970) N.N.
Mycelium:	More or less extended on and in agar media
Hyphae:	Break up into motile or nonmotile elements
Spores:	Observed in some strains, non-heat-resistant
Aerial mycelium:	Lacking
Phage susceptibility:	To Oerskovia phages (O-phages)
Facultative anaerobiosis may occur on special media	

Genus Promicromonospora	(Krassilnikov, Kalakoutskii and Kirillova 1961) N.N.
Substrate mycelium:	Extensively branching on and into agar media. More or less extended in shaken cultures
Substrate hyphae:	Break up into nonmotile elements of various size and shape which produce new mycelia
Aerial hyphae:	At least microscopically visible, not or sparsely branching. Break up into rodlike elements, which produce new mycelia
Spores:	Lacking
Polymorphism:	Different from strain to strain. May be substantial in submerged culture

Table 8 continued

Growth:	Pasty to leathery in agar cultures
Phage susceptibility:	To Promicromonospora phages (P-phages)
Aerobic	

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CHEMICAL AND NUMERICAL METHODS IN THE CHARACTERISATION OF NOVEL ISOLATES

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INTRODUCTION

The selective isolation and characterisation of novel microorganisms from natural environments is an important aspect of most industrial screening programs in which the objective is the discovery and evaluation of new metabolites or alternative biocatalytic processes. For maximum efficiency, it is essential that the procedures used to differentiate novel isolates from established taxa are based on sound taxonomic principles which themselves rely on the objective interpretation of information held in high quality data bases. Data on the chemical composition of actinomycetes and in particular their lipid and peptidoglycan composition (Minnikin & O'Donnell, 1984) have made a significant contribution to the current state of actinomycete systematics. Indeed, for some taxa, details of their lipid and wall diamino acid composition are an essential part of the species description. Despite the high diagnostic value of such characters, much of the data is interpreted qualitatively for the presence or absence of particular components. This has limited the interpretation of chemotaxonomic profiles to definitions of taxa at the generic or suprageneric levels. The object of this paper is to demonstrate the increased taxonomic information obtained by analysing the relative proportions of fatty acids in Streptomyces quantitatively and to show how such an approach can be incorporated into a screening program designed to select novel actinomycetes.

Statistical Methods

Statistically, the characterisation and detection of novel isolates is essentially a problem of pattern recognition in which the unknown fatty acid profile is compared with a library of known profiles and its similarity or dissimilarity determined. This

comparison can be either _qualitative_, in which similarity is dependent on the presence or absence of particular peaks or variables, or it can be _quantitative_. In a quantitative analysis, the relative proportions of the variables, as well as their presence or absence, are used to compare or group the profiles. The majority of chemotaxonomic studies employ the latter procedure subjectively with the criterion for group or taxon membership determined by the operator and dependent on the possession of 'large amounts' or 'small amounts' of a particular component. However, the application of multivariate statistical procedures to continuous data such as fatty acid profiles (Drucker, 1981; Bousfield _et al_. 1983; Jantzen _et al_. 1975; O'Donnell 1985; O'Donnell _et al_. 1985) provides a reliable and objective alternative to such an approach. The requirements for improved methods of data interpretation reflect the changes in bacterial systematics that have occurred in recent years with the introduction of analytical instruments such as gas chromatographs, high performance liquid chromatographs, mass spectrometers and electrophoresis apparatus (Goodfellow & Minnikin, 1985). The data generated by these instruments is composed of signals, curve forms or numbers and the need to convert these data into information on the original sample has led to the development of a new field in analytical chemistry, _chemometrics_. It is important to remember, however, that although the statistical methods have been devised by statisticians and applied mathematicians, the problems of experimental design and interpretation remain those of the microbiologist.

The data analysis system employed in the present study, SIMCA (Soft Independent Modelling of Class Analogy) is a commercially available package (SEPANOVA AB, Enskede, Sweden) running on a variety of microcomputers and mainframes. SIMCA pattern recognition uses the well established statistical technique of principal components analysis (Gower, 1966) to identify unknown samples. The basis of the SIMCA method of identification is to describe each taxon or group of strains by a separate principal components model. This procedure of disjoint principal components analysis (Wold, 1976; Wold & Sjostrom, 1977) means that the final data matrix can include information on a variety of taxa in which the within group variation is modelled independently and therefore not assumed to be equal. This offers considerable advantages in bacterial systematics since bacterial taxa rarely show equal intraspecies homogeneity. The allocation of unknown chromatograms to each class model is determined by linear multiple regression (Blomquist _et al_. 1979a,b). Using this procedure, successful identification relies on the

residual standard deviation, obtained when the unknown is tested against a given principal components model, being within the known standard deviation of the test class or taxon. Geometrically, a principal components model can be regarded as representing a region in multidimensional space in which the standard deviation is used to define a confidence interval for the taxon. Unknown isolates can be considered as belonging to the class if they fall within the corresponding confidence interval. Samples which do not allocate to any of the class models used to describe a particular taxon may belong to hitherto unrepresented classes and therefore constitute 'novel' isolates. This definition of 'novelty' has been adopted with respect to the following example on the characterisation of streptomycete and acidophilic actinomycete fatty acids.

The application of SIMCA pattern recognition to actinomycete fatty acids

Organisms

The test strains (Table1) were grown in shake flasks for 7-10 days at 30°C in modified Sauton's medium (Mordarska *et al*. 1972). Previous studies on the effect of growth on fatty acid profiles (Saddler *et al*. in press) had shown that incubation for 7-10 days in a standardised liquid culture resulted in reproducible fatty acid profiles essential for quantitative statistical analysis. Cultures were checked for purity, killed by shaking with formalin (1% v/v), separated by centrifugation, washed with distilled water and freeze dried.

Extraction and analysis of fatty acid methyl esters

Biomass (50 mg) was examined using a recently developed whole organism alkaline methanolysis procedure (Minnikin *et al*. unpublished). Analytical and preparative thin-layer chromatography (TLC) was done as previously described (O'Donnell *et al*., 1982). Gas chromatography of petroleum ether extracts of the purified fatty acid methyl esters (FAMES) was carried out using a Shimadzu GC Mini-2 fitted with a 25 m bonded OV-1 fused silica capillary column (Alltech Associates) and flame ionisation detectors. The gas chromatograph was temperature programmed from 100°C to 200°C at 4°C min^{-1}. The retention time and relative proportions of each FAME, expressed as a percentage of the total area to remove the effects of sample size, were measured using a Shimadzu CE1B computing integrator. The identity of individual esters was established by comparison with the retention times of standard FAMES mixtures.

TABLE 1. DESIGNATION AND SOURCE OF STRAINS

Test strains	Designation and source of strains[1]
1-26	John Lonsdale: JL6, 161, 163, 165, 289, 271, 273, 286, 290, 333, 334, 336, 337, 338, 176, 240, 58, 104
Cluster 1B[2]	S. bacillans, ISP5598, ATCC 15855 S. cavourensis, ISP5300, ATCC 14889 S. fluorescens, ISP5203, ATCC 15860 S. griseobrunneus, ISP5066, ATCC 19762
S. cyaneus[2]	S. cyaneus, ISP5108, ATCC 14923 S. collinus, ISP5129, ATCC 19743 S. resistomycificus, ISP5133, ATCC 19804 S. coralus[3], ISP5256, ATCC 23901 S. arenae, ISP5293, ATCC 25428

1. ISP, International Streptomyces Project; ATCC, American Type Culture Collection.
2. Strains used to define class models
3. Included in cluster 19 (S. diastaticus) using the S_{sm} co-efficient and cluster 18 (S. cyaneus) using the S_j co-efficient (Williams et al. 1983)

Results and Discussion

All data analyses were carried out using the SIMCA statistical package running under CP/M on an APPLE II+ microcomputer. The result of applying principal components analysis to the fatty acids of the acidophilic isolates (Table 1) is shown in Figure 1. Strains which clustered together in a numerical phenetic study (Lonsdale & Goodfellow, unpublished data) have been linked together. Since this analysis requires no 'a priori' information on the clustering of strains (classification), this output (Figure 1) demonstrates a close correlation between the fatty acid and numerical phenetic classfications. With the exception of strains 15 and 16 (176,240, Table 1) which contained hydroxy-DAP (diaminopimellic acid), analysis of the cell wall amino acid composition (Lonsdale & Goodfellow, unpublished data) showed that LL-DAP was the major diamino acid. The presence of a cell wall type I suggested a relationship with the genus Streptomyces (Lechevalier & Lechevalier, 1970; Minnikin & O'Donnell, 1984). On the basis of an extensive numerical phenetic study (Williams et al. 1983), the genus Streptomyces is thought to comprise 19 major and 40 minor clusters. The object of the

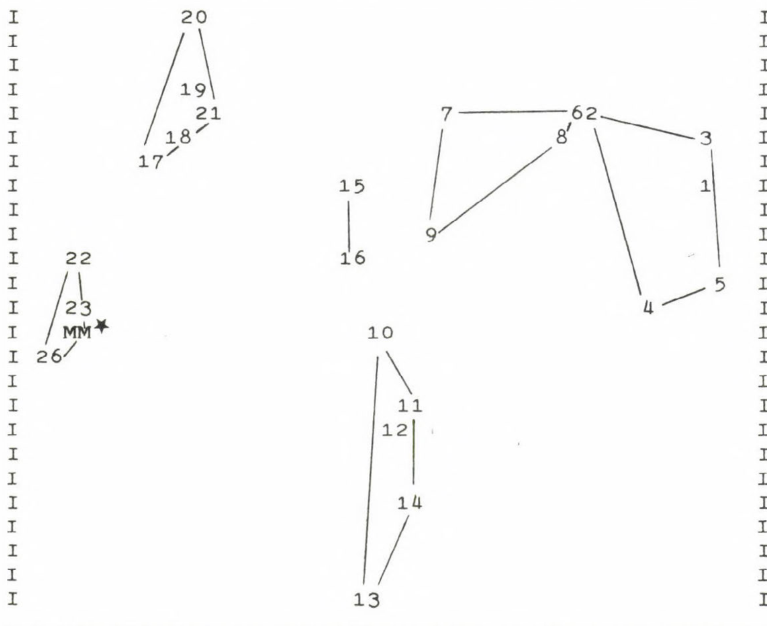

Figure 1. Principal components analysis of the fatty acid data on the acidophilic isolates. Plot shows the first (x-axis) and second (y-axis) principal components. Strains clustering together in a numerical phenetic study (Lonsdale & Goodfellow, unpublished) have been linked together. For strain identities see Table 1.

present investigation was to determine whether or not the acidophilic isolates represented new centres of taxonomic variation ('novel isolates') or could be assigned to one of the existing streptomycete taxa. This involves the construction of principal component models for each of the Streptomyces clusters and calculating the similarity between these models and the acidophilic isolates using linear regression (Blomquist et al. 1979a,b). Although work on the construction of the models is still in progress, thereby delaying a decision on the 'novelty' of the acidophilic isolates, the principals involved can be demonstrated with reference to two classes, Streptomyces cyaneus and Cluster 1B (Williams et al. 1983). Fatty acid methyl esters from representatives

of each class were extracted and analysed as described above. For the SIMCA analyses separate principal component models were constructed for S. cyaneus and Cluster 1B. Classes were not separately scaled (Blomquist et al. 1979a,b). The number of statistically significant components necessary to describe each class model was determined by cross-validation (Wold, 1978). The cross-validation procedure provides a useful estimation of how much the variation within a class data matrix accurately describes the class (systematic variation, Blomquist et al. 1979a) and how much is 'random noise'. In this way the statistical stability and therefore the predictive value of each principal component model can be evaluated (Blomquist et al. 1979a,b). For S. cyaneus and Cluster 1B the cross-validation procedure recovered two significant components from each data matrix. The result of plotting the distance (object standard deviation) of each of the acidophilic isolates from S.cyaneus (y-axis) and Cluster 1B (x-axis) is shown in Figure 2. Imposing 95% class confidence intervals divides the plot into four distinct regions. Isolates found in region A can be identified as belonging to Cluster 1B and those in region D to S.cyaneus. All of the acidophilic strains are found in region B. Since this area lies outside the class confidence intervals of both taxa, this result indicates that none of our acidophilic isolates can be allocated to either S. cyaneus or Cluster 1B. Organisms allocating to region C are not uniquely assigned to either class and may represent intermediates.

Conclusions

Although the work outlined above is preliminary it does demonstrate the potential of SIMCA pattern recognition in improving the interpretation of bacterial fatty acid data. As an identification system the success of such procedures depends on the construction and testing of statistically as well as microbiologically significant principal component models. Two major obstacles to the use of fatty acid data in this way can be recognised. The first of these is that the variation in fatty acid profiles is not taxon specific. However, this lack of congruence between classifications derived using different analytical procedures depends on the criteria used for the circumscription of taxa and highlights an important and as yet untackled problem in bacterial systematics (Sneath, 1985; O'Donnell et al. 1985). The second obstacle to such procedures is that many established bacterial taxa are very heterogeneous with respect to the strains they encompass. The within group variance in fatty acid composition wil reflect

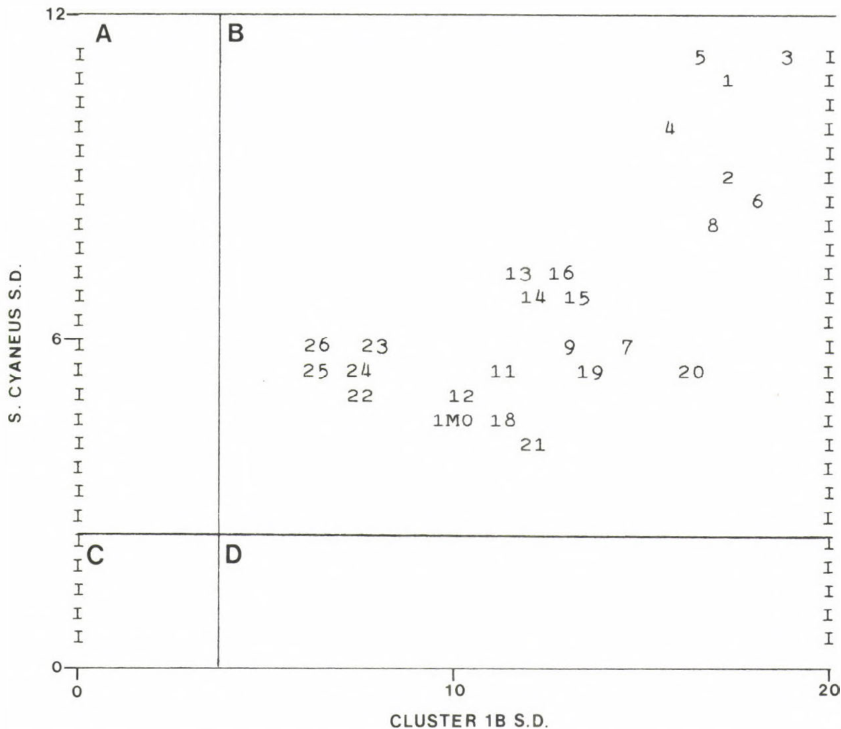

Figure 2. Class distance plot for S. cyaneus and Cluster 1B. Each of the acidophilic isolates was fitted to both class models. Distances (standard deviations) from S. cyaneus are represented along the y-axis and distances from Cluster 1B along the x-axis. The plot is divided into four regions (A,B,C,D) using the 95% confidence limits of each class. For the identities of strains see Table 1.

this diversity making it difficult to construct statistically stable and reliable principal component models. It is not surprising, therefore, to conclude that the successful identification and characterisation of novel isolates depends on the existence of a sound classification system.

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TAXONOMY

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CHEMICAL CRITERIA IN THE CLASSIFICATION OF SOME MYCOLATELESS WALL CHEMOTYPE IV ACTINOMYCETES

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INTRODUCTION

Actinomycetes that lack mycolic acids but have a peptidoglycan containing meso-diaminopimelic acid and wall-associated arabinogalactan polymers (wall chemotype IV sensu Lechevalier & Lechevalier, 1970) are currently assigned to the genera Actinopolyspora, Micropolyspora (Faenia), Pseudonocardia, Saccharomonospora and Saccharopolyspora or remain mis-classified in the genus Nocardia. It is not clear whether all five of the taxa merit generic status or if they form a distinct evolutionary group but they do have many features in common (Goodfellow & Cross, 1984; Goodfellow & Minnikin, 1984). The present investigation is part of a polyphasic study undertaken to unravel the taxonomy of mycolateless actinomycetes with a wall chemotype IV. The primary aim of this study was to determine the value of fatty acid, isoprenoid quinone and polar lipid composition in representative strains of the five genera mentioned above.

MATERIALS AND METHODS

Test strains

All but one of the organisms (Table 1) were grown in shake flasks of nutrient broth around their optimum temperature (30 or 45°C) and harvested in early stationary phase as described earlier (Embley et al., 1984). The type strain of Actinopolyspora halophila was cultivated in a medium with a high salt concentration (Gochnauer et al., 1975).

Lipid analyses

Fatty acid methyl esters were prepared and analysed by capillary gas chromatography (GC) using published procedures (Embley et al., 1984). Techniques developed by Minnikin et al. (1984) were employed for the extraction and analysis of isoprenoid quinones and polar lipids. Quantification of menaquinone isoprenologues was by reverse phase high performance liquid chromatography (Collins, 1985). The fatty acid data were examined using Gower's coefficient (S_G) and the complete linkage (furthest neighbour) algorithm (O'Donnell et al., 1985) and the results presented in a dendrogram.

RESULTS AND DISCUSSION

In line with earlier studies all of the test strains lacked mycolic acids but contained major amounts of iso- and anteiso-methyl branched fatty acids with 15, 16 and 17 carbons and minor amounts of 10-methyl branched components (Goodfellow & Minnikin, 1984; Kroppenstedt, 1985). The Micropolyspora faeni, Pseudonocardia thermophila and Saccharopolyspora

hirsuta strains possessed compounds which gave similar mass spectra to 10-methyl hexadecanoic, heptadecanoic and octadecanoic acids, respectively, but had much shorter chromatographic retention times. The organisms fell into two broad groups on the basis of fatty acid composition (Fig. 1).

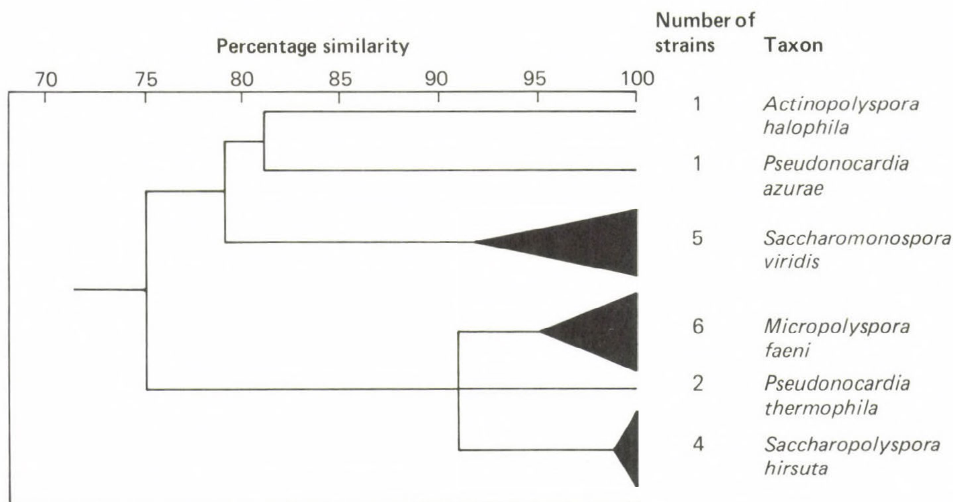

Figure 1. An abbreviated dendrogram showing the relationships between the test strains based on the analysis of fatty acid methyl ester composition using Gower's coefficient and the complete linkage clustering algorithm.

Results of the isoprenoid quinone and polar lipid analyses are in good agreement with those obtained in earlier studies (Lechevalier et al., 1977; Kroppenstedt, 1985). Apart from the type strain of Pseudonocardia thermophila all of the organisms including Pseudonocardia azurae contained major amounts of tetrahydrogenated menaquinones with nine isoprene units (Table 1). Further studies are required to determine whether the distribution of the minor components is taxonomically significant. It was possible to distinguish all of the taxa on the basis of polar lipid composition (Table 1). Thus, all strains contained diphosphatidylglycerol, phosphatidylglycerol and phosphatidylinositol but the distribution of phosphatidylcholine and nitrogenous lipids was discontinuous. Several partially characterised glycolipids and phospholipids were also detected in the test strains.

These results together with those of other preliminary studies suggest that lipid analyses may help to clarify the taxonomy of mycolateless actinomycetes with a wall chemotype IV.

Table 1. Menaquinone and polar lipid composition of test strains.

Taxon[1]	Menaquinone isoprenologue (%)[2]					Diagnostic phospholipid[3]
	$MK-8(H_4)$	$MK-9(H_4)$	$MK-9(H_6)$	$MK-9(H_8)$	$MK-10(H_5)$	
Actinopolyspora halophila (1)	2	65	-	-	22	PC,P(1),G(1)
Micropolyspora faeni (6)	-	48-58	8-17	5-30	0-10	PC,PN(2), P(4),G(9)
Pseudonocardia azurae (1)	5.2	90.6	4.2	-	-	PN(2),P(2),G(2)
Pseudonocardia thermophila (1)	96	-	-	-	-	PC(2),PN(2),PGL, P(1),G(6)
Saccharomonospora viridis (6)	18-24	60-70	1-10	-	-	PN(3),P(3), G(3)
Saccharopolyspora hirsuta (4)	0-4	82-98	8-11	-	-	PC(2),PN(2), P(4),G(2)

[1]Numbers in brackets indicate number of strains. Type strains of all taxa were studied.

[2]Abbreviations exemplified by $MK-9(H_4)$, menaquinone having nine isoprene units two of which are hydrogenated.

[3]All of the strains contained diphosphatidylglycerol, phosphatidylglycerol and phosphatidylinositol. Abbreviations: PC, phosphatidylcholine; PN, ninhydrin positive phospholipid; P, unidentified phospholipid; PGL, phosphoglycolipid; G, unidentified glycolipid. Figures in brackets indicate number of spots.

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CONTINUOUS PROBLEM WITH THE NOCARDIA MEDITERRANEI TAXON

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INTRODUCTION

There has been a continuing search for the correct taxonomic position of rifamycin producing actinomycetes /"mediterranei" cluster/. Hitherto, three names have existed in the literature - Streptomyces mediterranei, Nocardia mediterranea and Nocardia mediterranei - which suggests that the problem remains to be solved. The morphological, numerical phonetic and chemotaxonomic data /Alderson et al., 1981/ as well as phage host range studies /Prauser, 1984/ showed that the members of the taxon belong neither to the genus Streptomyces nor to Nocardia.

Nevertheless, the name Nocardia mediterranei is used in the title of this paper as it seems at present to be the most correct Latin nomenclature.

The aim of our studies was to determine the relationship of a rifamycin producing actinomycete mutant to Nocardia mediterranei type strain from the American Type Culture Collection /ATCC/ using chemotaxonomic and genotaxonomic methods.

MATERIALS AND METHODS

The main strains analysed in the studies were: a rifamycin producing actinomycete mutant CMC L-1-5 and Nocardia mediterranei ATCC13685. For deoxyribonucleic acid /DNA/ analysis the following reference strains were also used: Micrococcus lysodeikticus /c/, Nocardia asteroides ATCC19247, Streptomyces felleus ISP130 and Streptomyces griseus ISP236.

The cell mass was obtained from shake cultures grown on pepton yeast extract dextrose broth at 35° for 48 h.

Cell wall preparations were obtained by crushing wet biomass in an X-press /LKB, Sweden/ and sonic oscillator /MSE, England/ followed by treatment of pellet with pepsin and trypsin /Lechevalier and Lechevalier, 1980/ or entirely pronase /Alderson et al., 1981/.

The cell wall and whole cells analyses were made by paper chromatography /Lechevalier and Gerber, 1970/ and gas liquid chromatography /GLC/ /Sawardeker et al., 1965/.

The fatty acid spectrum was determined by gas chromatography /Lechevalier and Lechevalier, 1980/.

Polar lipids were identified by thin layer chromatography /TLC/ /Minnikin and Abdolrahinazadeh, 1971/.

The mole % quanine plus cytosine /%GC/ and nucleotide sequence homology was determined according to Mordarski et al., /1976/.

RESULTS AND DISCUSSION

Good agreement between the results of chemotaxonomic and genotaxonomic studies of Nocardia mediterranei ATCC13685 and the rifamycin producer CMC L-1-5 was obtained.

The following amino acids were found in the cell wall hydrolysates of the actinomycetes: alanine, meso-diaminopimelic acid /meso-DAP/, some trace of L-DAP, glucosamine, glutamic acid and muramic acid.

Galactose was the main carbohydrate /50-60%/ found in the cell wall as well as whole cell hydrolysates of the strains. Arabinose /1-19%/, glucose /0-12%/, rhamnose /4-7%/ and an unidentified compound /3-12%/ were also detected. The preliminary analysis of the latter by paper chromatography and colour reaction with acid aniline phthalate allowed its tentative identification as madurose. Further studies are required because it is the first time that madurose has been found in the actinomycetes with wall chemotype IV. It is worth stressing that the sugar was only discovered in the hydrolysates of cell wall digested with trypsin and pepsin but not in that treated entirely by pronase. Moreover, it was easier to detect in the cell wall hydrolysates obtained by milder conditions /Lechevalier and Gerber, 1970/.
The same observation concerning arabinose has also been made. These findings could explain why arabinose has not always been detected in hydrolysates from Nocardia mediterranei strains.

The fatty acids of the microorganisms were branched iso- and anteiso- series and it is consistent with earlier studies /Alderson et al., 1981/.

Diphosphatidyl glycerol /DPG/, phosphatidyl ethanolamine /PE1/ and hydroxy fatty acid PE2 were the major components of the polar lipids of the strains studied. In addition phosphatidyl inositol /PI/ and phosphatidyl inositol mannosides /PIM/ also occured in the actinomycetes. Thus, the phospholipid pattern could be compared to type II /Lechevalier and Lechevalier, 1980/.

The nucleotide composition of the DNA preparations from Nocardia mediterranei ATCC13685 and CMC L-1-5 showed a high GC content of 74.1% and 73.9%, respectively.

The DNA preparations from CMC L-1-5 bound significant amounts of reference DNA from Nocardia mediterranei ATCC 13685 /98%/ but showed little affinity with that from Nocardia and Streptomyces reference strains /8-14%/.

The results presented in this paper along with unpublished morphological and physiological observations allowed the

patent rifamycin producer - labelled CMC L-1-5 to be identified as Nocardia mediterranei.

Thus, a proper generic attachment of the "mediterranei" cluster remains to be solved. Further studies on madurose occurrence and its taxonomic value along with hydroxy fatty acid-containing phosphatidyl ethanolamine in the taxon and other actinomycetes with chemotype IV lacking mycolic acids seems to be interesting.

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CELL WALL POLYOL PHOSPHATE POLYMERS OF BACTERIA BELONGING TO THE GENUS ACTINOMADURA

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The teichoic acids, the phosphorus containing anionic polymers, have been found in different genera of the order Actinomycetales /1/. These polymers have several functions which are of value to the cell. It has been shown that important function of teichoic acids is the binding of divalent cations. They may also regulate the activity of the endogenous autolysins /2/.

In an attempt to obtain further information on the occurrence of these polymers in bacteria of the order Actinomycetales we have now examined genus Actinomadura.

We report here evidence that teichoic acids are widely distributed in bacteria of this genus. All 30 strains examined in this survey contained teichoic acids (Table 1). Most of the investigated cultures had poly(glycerol phosphate) teichoic acids. Some of the chains are substituted a little or unsubstituted at all, the others have large amount of glycosyl residues (A.libanotica IMET 9616, A.spadix ATCC 27298, A.vinacea INA 1682). Among them we have found glucosyl, galactosyl, N-acetylglucosaminyl, N-acetylgalactosaminyl and 3-O-methylgalactosyl (madurosyl) units.

Table 1. The teichoic acid type of actinomadurae strains

Species	Strain	Type teichoic acid	Predominant phosphoric esters of acid hydrolysis
1	2	3	4
A.carminata	INA 4281^{T}	glycerol	Gro-P,Gro-2P
A.citrea	INA 1849^{T}	ribitol	Rib-P,AnRib-P
A.citrea	INA 18045	ribitol	Rib-P,AnRib-P
A.citrea	INA 18703	ribitol	Rib-P,AnRib-P
A.coerulea	INA 765^{T}	ribitol	Rib-P,AnRib-P
A.coeruleoviolacea	INA 3564^{T}	glycerol	Gro-P,Gro-2P
A.cremea	INA 292^{T}	glycerol	Gro-P
A.cremea	INA 17721	glycerol	Gro-P
A.cremea	INA 17863	glycerol	Gro-P
A.cremea subsp. rifamycini	INA 1349^{T}	glycerol	Gro-P
A.ferruginea	IMET 9567^{T}	glycerol	Gro-P,Gro-2P
A.fulvescens	INA 3321^{T}	glycerol	Gro-P
A.libanotica	IMET 9616^{T}	glycerol	Gro-P,Gro-2P
A.livida	INA 1678^{T}	glycerol	Gro-P
A.luteofluorescens	IFO 13057^{T}	ribitol	Rib-P,AnRib-P
A.macra	ATCC 31286^{T}	glycerol	Gro-P
A.malachitica	INA 1920^{T}	glycerol	Gro-P,Gro-2P
A.pelletieri	IMET 7141^{T}	ribitol	Rib-P,AnRib-P
A.polychroma	INA 2755^{T}	glycerol	Gro-P,Gro-2P
A.pusilla	ATCC 27296^{T}	glycerol	Gro-P,Gro-2P
A.recticatena	INA 308^{T}	glycerol	Gro-P,Gro-2P
A.roseola	INA 1671^{T}	glycerol	Gro-P,Gro-2P
A.rubra	INA 325^{T}	glycerol	Gro-P,Gro-2P

Table 1 continued

1	2	3	4
A.roseoviolacea	ATCC 27297^T	glycerol	Gro-P,Gro-2P
A.salmonea	INA 2488^T	glycerol	Gro-P,Gro-2P
A.spadix	ATCC 27298^T	glycerol	Gro-P,Gro-2P
A.spiralis	IMET 9621^T	glycerol	Gro-P,Gro-2P
A.turcmeniaca	INA 3344^T	glycerol	Gro-P,Gro-2P
A.vinacea	INA 1662^T	glycerol	Gro-P,Gro-2P
A.umbrina	INA 2309^T	glycerol	Gro-P,Gro-2P
A.fastidiosa*	VKM A-804	glycerol	Gro-P,Gro-2P
A.madurae*	VKM A-809	glycerol	Gro-P
A.verrucosospora*	VKM A-668	ribitol	Rib-P,AnRib-P

*Data of the article (6); T - typical strain; Gro-P - monophosphate glycerol; Gro-2P - diphosphate glycerol; Rib-P - monophosphate ribitol; AnRib-P - anhydroribitol-5-phosphate; INA - Cultures of Institute of New Antibiotics, USSR Academy of Medical Sciences (Moscow, USSR); IMET - Zentralinstitut für Mikrobiologie und Experimentelle Therapie (Jena, GDR); ATCC - American Type Culture Collection (Rockville, USA); VKM - All-Union Collection of Microorganisms, USSR Academy of Sciences (Pustchino, USSR); IFO - Institute of Fermentation (Osaka, Japan).

7 strains contained glycerol teichoic acids of unusual structure. Both acid and alkaline hydrolysis of these polymers did not give diphosphate glycerol. Acid hydrolysis yielded a monophosphate glycerol and different monosaccharides. The nature of the products of an alkaline and acid hydrolysis is consistent with a structure in which neighbouring polyol and sugar units are joined by phosphodiester linkages /poly(glycosylglycerol phosphate) chains/.

Ribitol teichoic acids were found in 6 strains. Besides these cultures contain minute amounts of the poly(glycerol phosphate) chains that will sooner indicate the presence of the lipoteichoic acid than the wall glycerol teichoic acid. All ribitol teichoic acids have glycosyl residues. Some cultures contain heterogeneous poly(ribitol phosphate) chains with various degrees of substitution by a sugar component. This fact was established by investigation of individual polymers which had been separated by electrophoresis.

We did not study in detail acyl groups of teichoic acids. However, aminoacid lysine was revealed among the products of acid and alkaline hydrolysis of the ribitol teichoic acid of A.luteofluorescens IFO 13057 and glycerol teichoic acid of A.spadix ATCC 27298. The detection of lysine and its amide in the ammonolysis products of these polymers provided evidence in favor of the presence of an esteric bond between lysine and poly(polyol phosphate) chain. O-Lysyl substituents were found earlier in the cell wall teichoic acids of some streptomycetes /3/.

The presence of madurose in the structure of teichoic acid is an interesting and new fact. We have established in detail the structure of the cell wall teichoic acid of A.carminata INA 4281. The polymer belongs to 1,3-type and consists of about 8 glycerol phosphate units, two of them have 2-acetamido-2-deoxy-α-D-galactopyranosyl substituent and one - 3-O-methyl-β-D-galactopyranosyl-(1-3)-2-acetamide-2-deoxy-α-D-galactopyranosyl residue at C2 of glycerol (Fig. 1) /4/.

The cell wall of this culture was found to contain besides teichoic acid ($\sim$10%) peptidoglycan ($\sim$20%) which belongs to

Fig. 1. Cell wall teichoic acid of Actinomadura carminata.

type A1γ, protein ($\sim$30%) and one or two polysaccharides, their main compounds being glucose and mannose.

As shown in Table 1, all 4 strains of A. cremea examined contain glycerol teichoic acids which lack poly(glycerol phosphate) chains and 3 strains of A. citrea have ribitol teichoic acid. Analogous results were obtained earlier for the cultures of the genus Streptomyces. All the strains of S.griseus and S.chrysomallus tested contain ribitol teichoic acid and all the strains of S.levoris have glycerol teichoic acid /5/.

Occurrence of certain type of teichoic acid seems to be the species specific characteristic and in some cases it may serve as a helpful additional criterion for taxonomical purpose.

MATERIALS AND METHODS

The cultures of actinomadurae were grown for 72 h at 28°C with shaking in a medium described in /4/. The organisms were harvested by the centrifugation and washed with cold 0.95% NaCl. The cell wall and teichoic acid preparations were obtained as in /3/. The structures of teichoic acids were determined by chemical methods and in the case of A.carminata teichoic acid by NMR ^{13}C spectroscopy too /4/. Acidic, alkaline and enzymatic hydrolysis, ammonolysis as well as analytical procedures, paper chromatography and electrophoresis were carried out as described in /3/.

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GC/MS ANALYSIS OF MYCOLIC ACID MOLECULAR SPECIES AND CONTRIBUTION TO THE CHEMOTAXONOMY OF NEW RHODOCOCCUS SPECIES

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Mycolic acids are the most characteristic components in the cell walls of bacteria belonging to Actinomycetes genera such as Mycobacterium, Nocardia and Rhodococcus. The structure varies greatly given the genus and species, and covers a wide range from C_{20} to C_{88}, consequently mycolic acids have been recognized to be important in the chemotaxonomy of these taxa. However, previously, the structural analysis of mycolic acids has been carried out by direct mass spectrometry which gave the information on the lytic products; meromycolal and fatty acid methyl esters. Recently we have developed an analytical method for examining the intact mycolic acid molecular species composition (Yano et al. 1972, 1978) using a gas chromatography-mass spectrometry system. In this report, we have examined the mycolic acid molecular species composition of new species of Rhodococcus (and related taxa) using a GC/MS system.

MATERIALS AND METHODS

Cells of Nocardia and Rhodococcus were grown aerobically in a medium containing 1% glucose, 0.5% peptone and 0.5% yeast extract at pH 7.0. Lipids were extracted with chloroform-methanol (2:1, v/v) and the residues were hydrolyzed with 10% KOH. After acidification, fatty acids were extracted with hexane and methylated with benzene-methanol-H_2SO_4 (10: 20:1, v/v). Mycolic acid methyl esters were separated on thin-layer chromatography of silica gel with a solvent of hexane-ether (4:1, v/v) and recovered from the gel. After trimethylsilylation with BSTFA-pyridine (2:1, v/v), TMS methyl mycolate was subjected to the gas chromatography-mass spectrometry system with a column of 1% OV-1 (50cm x 0.3cm) at the temperature of 250 to 300°C. The mass spectra were recorded with a Hitachi M-80B GC/MS system with ionization energy 20eV and the acceleration voltage 3KV.

RESULTS AND DISCUSSION

Thin-layer chromatograms of fatty acid methyl esters gave clear spot(s) corresponding to mycolic acid methyl esters (Fig. 1). The spots were recovered and subjected to the

GC/MS system after trimethylsilylation. The results revealed that each species possessed a characteristic profile; R. rhodochrous (C36-48), R. rubropertinctus (C50-62), R. equi (C28-38, C40-48), R. chubuensis (C56-66), R. obuensis (C56-66), R. aichiensis (C56-66), R. roseus (C36-46), R. terrae (C52-62), R. aurantiacus (C64-76)(Fig. 2, 3 and 4).

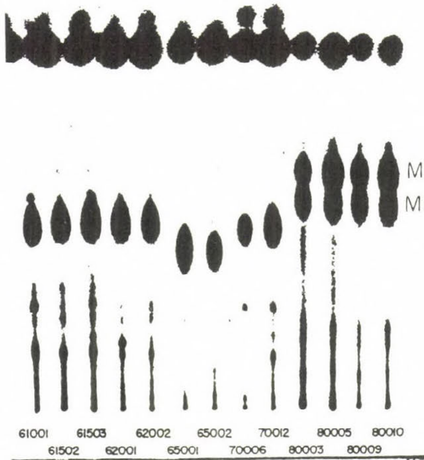

Fig.1. TLC of methyl mycolate from various Rhodococcus species.

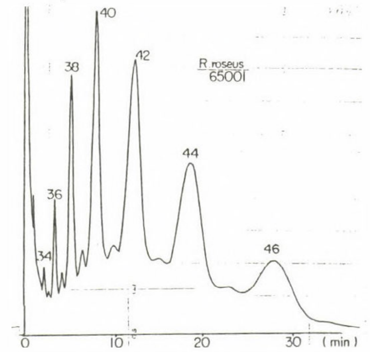

Fig.2. GLC of TMS methyl mycolate from R. roseus 65001.

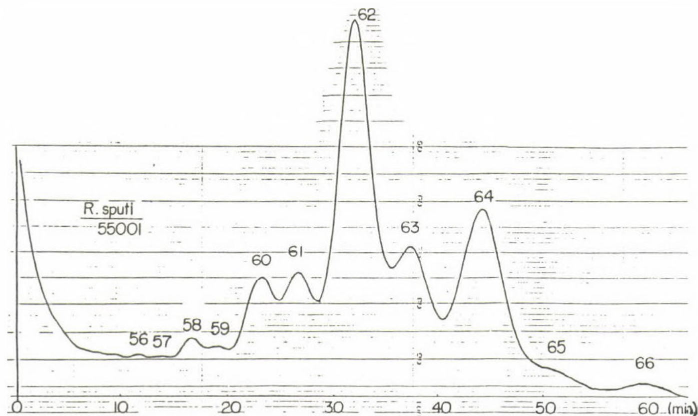

Fig.3. GLC of TMS methyl mycolate from R. sputi 55001

Table 1. Distinguishing characters between the genera *Rhodococcus* and *Nocardia*

Character	Number of strains showing positive reaction (%)																
	R. bronchialis n=13	R. sputi n= 3	R. rubropertinctus n= 5	R. terrae n= 3	R. rhodochrous n= 7	R. chubuensis n=3	R. aichiensis n= 5	R. obuensis n= 7	R. ruber n=11	R. equi n= 6	R. coprophilus n= 4	'Gordona aurantiaca' n= 9	N. asteroides (A) n= 9	N. asteroides (B) n=14	N. brasiliensis n= 7	N. otitidis-caviarum n=10	N. farcinica n=27
1. Temporary mycelium	0	0	0	0	0	0	0	0	91	0	75	0	100	100	100	100	100
2. Long rods (> 7 µm in length)	38	0	0	0	0	0	0	0	100	0	100	0	100	100	100	100	100
3. Growth at 42 C	8	0	0	0	0	0	100	0	0	0	0	11	56	93	0	100	100
4. ß-Galactosidase	0	0	0	0	0	0	0	0	9	67	0	100	100	100	100	100	100
5. Sucrose as C source	100	100	100	100	100	100	100	100	91	0	0	100	0	0	14	10	26
6. Ethanol as C source	77	100	100	100	100	100	100	29	100	83	0	78	0	29	0	0	89
7. Acid from mannose	69	100	100	100	100	100	100	57	18	0	0	89	11	7	0	0	30
8. Sorbitol as C source	0	100	100	100	0	100	0	57	100	0	0	89	0	0	0	0	0
9. Nitrite as N source	15	0	0	0	0	0	0	0	9	0	0	44	67	50	29	70	30
10. Resistance to 5-fluorouracil (20 µg/ml)	0	0	0	0	0	0	0	0	0	0	0	78	89	7	86	100	96
11. Resistance to mitomycin C (5 µg/ml)	0	0	0	0	0	0	0	0	0	0	0	78	100	100	100	100	100
12. Nitrate reduction (24 hours)*	100	67	100	100	100	67	60	57	91	50	100	11	100	93	0	60	30
Mycolic acids: Average carbon numbers	64	61-62	58-59	58	44	61-62	62	61-62	44	42	?	71-73	44-52	54-56	52	?	?
Mycolic acids: α-unit	18	16,18	16,18	16,18	14,16	16(18)	16	16	12,14,16	12,14,16	?	20-22	14,16	16	16	?	?
Mycolic acids: β-unit	40-48	40-48	34-44	22-32	40-48	40-48	40-48	40-48	22-32	18-34	?	46-54	22-34	36-44	32-40	?	?
Mycolic acids: Double bond numbers	2-6	2-6	1-5	1-5	0,1(2)	2-6	2-6	2-6	0,1,2	0,1	?	2-7	0,1,3	0-4	0-3	?	?

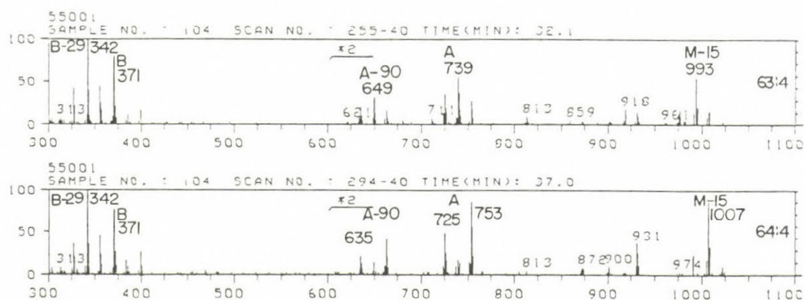

Fig.4. Mass spectra of TMS methyl mycolate from R. sputi 55001

These results indicate the GC/MS profiles of mycolic acids give the most precise information on the chemotaxonomy of this group of bacteria (Table 1).

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DIFFERENTIATION OF NOCARDIAE AND MYCOBACTERIA BY THIN-LAYER AND GAS CHROMATOGRAPHIC ANALYSES OF MYCOLIC AND FATTY ACID METHYL ESTERS

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INTRODUCTION

Identification of clinically significant actinomycetes and mycobacteria in the routine laboratory is often difficult and time-consuming. In this communication we therefore wish to report the results of our biochemical studies which were aimed at facilitating the routine identification of selected species of aerobic actinomycetes and mycobacteria. Main emphasis was placed upon the fatty acid spectrum and the mycolic acid pyrolytic products.

MATERIALS AND METHODS

Cultures: Stock cultures and clinical isolates were taken from the culture collection of the Institute of Hygiene of the University of Cologne (HIK) and included: Nocardia farcinica - 3 strains; N. asteroides, N. orientalis, N. brasiliensis, and N. otitidis-caviarum - 2 strains of each species; N. phenotolerans, N. transvalensis, N. vaccinii, N. carnea, N. aerocolonigenes, Rhodococcus ruber, R. rhodnii, R. equi, R. spec., Actinomadura pelletieri, Nocardiopsis dassonvillei, Streptomyces gibsonii, S. somaliensis, Mycobacterium farcinogenes var. tchadense, M. farcinogenes var. senegalense, M. fortuitum, M. smegmatis, M. xenopi, M. gordonae, M. bovis, M. kansasii, M. avium, and M. tuberculosis - 1 strain of each species. The nocardiae, streptomycetes and actinomadurae were grown in shake culture in brain heart infusion broth (BHI), whereas the mycobacteria were grown in TB broth.

Acid Methanolysis: The lyophilized cells were transesterified by the method of Minnikin et al. (1980).
Thin-Layer Chromatography: Thin-layer chromatography (TLC) was carried out using 20 x 20 Merck silica gel 60 F_{254} aluminium sheets (Merck no. 5554) and a twice development in a solvent of light petroleum-acetone (95:5 v/v). The mycolic and fatty acid methyl esters were detected with UV light (366 nm) after spraying with 2,7-dichlorofluorescein (Merck no. 9677).
Gas-Liquid-Chromatography: Gas-chromatographic analysis was carried out on a model 7400 Hewlett-Packard gas chromatograph, equipped with a dual flame ionization detector and a glass column of 3 m by 0,2 mm inside diameter. Samples were analyzed with two phases: (1) polar phase, 10% Silar 10 C on gas chrom 100/120 mesh; (2) non-polar phase, 3% OV-1 on gas chrom 100/120. Carrier gas: nitrogen 50 ml/min; temperature programme: 160 to 280° C at 6° C/min for the OV-1 column and 160 to 240° C at 4° C/min for the silar 10 C column; detector temperatures: 285° C and 260° C respectively; injector temperatures: 250° C for fatty acids and 310° C for mycolic acids.

RESULTS AND DISCUSSION

Distribution of Simple Fatty Acids

The analysis of the fatty acid methyl esters prepared from Streptomyces and Actinomadura gave the following results: a) Occurrence of the homologous series of straight-chain saturated acids ranging from C_{12} to C_{17} in the case of Streptomyces and from C_{14} to C_{19} in the case of Actinomadura. b) Occurrence of the homologous series of saturated branched-chain acids from C_{12} to C_{17}, with C_{14}, C_{15}, C_{16} and C_{17} as the main components in Streptomyces. In the case of actinomadurae, the most prominent branched-chain fatty acids were the C_{14} to C_{19} acids with C_{16}, C_{17} and C_{18} branched-chain acids as the major components. c) In addition to the iso- and anteiso branched-chain fatty acids, the actinomadurae contained tuberculostearic acid (10 methyl-octadecanoic acid). The analysis of the fatty acids of nocardiae chiefly revealed: a) Straight-chain fatty acids ranging from C_{14} to C_{19} with few strains having straight-chain fatty acids up to C_{24}. b) The following unsaturated straight-chain fatty acids were identified: hexadec-9-enoic and octadec-9-enoic acids in the case of nocardiae having up to C_{19} straight-chain fatty acids and cis-11-eicosenoic, cis-13-docosenoic and cis-tetracosenoic acids in the case of nocardiae which contained fatty acids ranging up to C_{24}. c) With regard to the branched-chain fatty acids it was found that 10-methyl-hexadecanoic, 10-methyl-heptadecanoic and 10-methyl-octadecanoic acids were the main fatty acids in those nocardial species which possess mycolic acids. Tuberculostearic acid was the main component among these three branched acids while the remaining two were only present in small or trace amounts. d) The nocardial species which do not possess mycolic acids showed characteristic fatty acid patterns which differed from those of the nocardial species with nocardomycolic acids but were related to the fatty acid patterns of the streptomycetes. These species had straight-chain fatty acids ranging from C_{14} to C_{18} and branched-chain fatty acids of the iso-anteiso-type ranging from C_{14} to C_{18}. They did not contain any 10-methyl branched-chain acids.

Chromatography of the mycobacterial fatty acid methyl esters established the presence of straight-chain saturated acids and straight-chain monoenoic acids ranging from C_{14} to C_{26}. Almost all of the mycobacterial species did not contain any branched-chain fatty acids of the iso-anteiso-type but contained the 10-methylated branched-chain acids of C_{16}, C_{17} and C_{18} with 10-methyl C_{18} and 10-methyl C_{16} as the main components. Oleic acid and its derivatives as well as palmitic acid approximately constituted about 75% of the total fatty acids of mycobacterial species.

Mycolic Acids of Nocardia and Mycobacterium

Mycolic acid composition as determined by TLC: Typical thin-layer chromatograms of the mycolic acids of different taxa are shown in Fig. 1. The methanolysates from the mycobacteria gave three distinct TLC patterns. a) The first one examplified by M. tuberculosis, M. bovis, M. kansasii and M. gordonae exhibited three spots which correspond, in the order of increasing mobility, to i) ketomycolates, ii) methoxymycolates and iii) methyl mycolates devoid of oxygen functions other than the 3-hydroxy ester system. b) The second pattern which was produced by methanolysates of M. avium and M. xenopi consisted, in the order of increasing mobility, of i) a homologue of 2-eicosanol, ii) ω-carboxymycolate methyl esters, iii) ketomycolates, iv) an unknown component and v) methyl mycolates with the 3-hydroxy esters as the only oxygen functions. c) The third pattern which is examplified by M. fortuitum, M. smegmatis and M. senegalense showed the simplest structure with two spots corresponding to 3-hydroxy ester mycolates, the lower spot being more

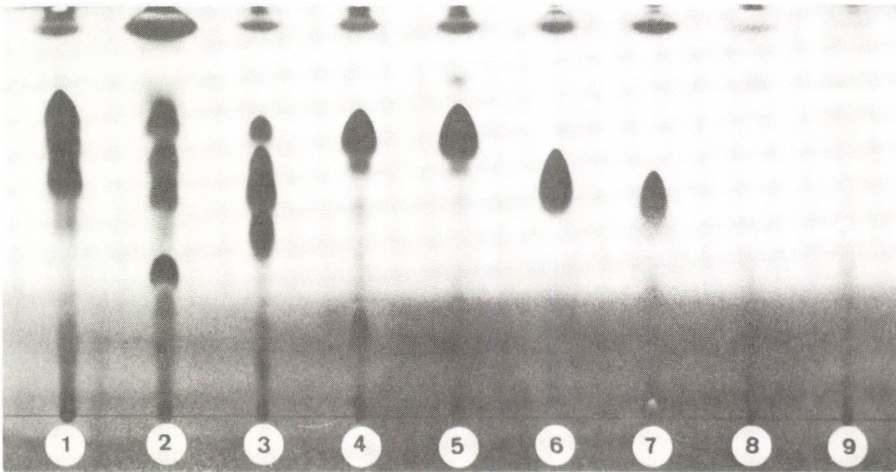

Fig. 1: TLC of whole-organism methanolysates of 1: M. tuberculosis; 2: M. avium; 3: M. bovis; 4: M. fortuitum; 5: M. farcinogenes var. senegalense; 6: N. asteroides, Biovar A_1; 7: R. equi; 8: S. gibsonii; 9: A. pelletieri. Developing system: light petroleum (b.p.60-80°C)-acetone (95:5) to 8.5 cm for three times.

polar and having lower molecular weight than the upper one. d) The methanolysates from Nocardia and Rhodococcus contained only a single spot which corresponds to 3-hydroxy mycolate. The lower mobility of the mycolate from Nocardia and Rhodococcus in comparison to those of mycobacteria is due to the smaller molecular size of these mycolates and constitutes the basis for differentiation of these taxa (Goodfellow et al. 1976, Schaal, 1985).
Mycolic acid pyrolytic products: The fatty acids produced by the thermal breakdown of mycolic acids from Nocardia and Rhodococcus at high temperature split to release unbranched saturated fatty esters having 12 to 18 carbon atoms, whereas those from mycobacteria released C_{22} to C_{26} fatty esters. The distinction between mycolic acids from nocardiae and rhodococci was based on the aldehyde moiety formed during pyrolysis. In the case of Rhodococcus, this aldehyde moiety was small and eluted from the column, whereas the aldehydes of Nocardia were of large size and were retained in the column.

N. farcinica NCTC 4524 contained mycobacterial mycolic acids whereas N. farcinica ATCC 3318 contained nocardomycolic acids. This confirmed the results of other investigators (Asselineau et al., 1969). The fatty acid profiles and the mycolic pyrolytic products support the view that strains formerly labelled N. farcinica belong to two different taxa that are currently designated N. farcinica and M. farcinogenes.The mycolic acid containing taxa are all very similar in their pattern of simple long-chain fatty acids. All contain mixtures of straight-chain unsaturated and 10-methyl branched acids especially tuberculostearic acid. Iso- and anteiso-methyl branched acids are characteristic of actinomycete taxa such as Streptomyces (Ballio et al., 1965), Actinomadura (Bowie et al., 1972) and the so-called nocardiae that do not contain mycolic acids.

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FATTY ACID AND MENAQUINONE COMPOSITION OF STREPTOMYCES CYANEUS NCIB 9616 AT DIFFERENT STAGES IN THE GROWTH CYCLE

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INTRODUCTION

Chemical characters have been used primarily in the classification of bacterial taxa at generic and supra-generic levels. To date most chemosystematic studies have been limited to a visual comparison of results generated by analytical techniques such as gas-chromatography and high performance liquid chromatography. However, examination of quantitative data, using appropriate cluster analysis techniques has given valuable information for characterisation at species level and below (see O'Donnell, 1985).

The present study was designed to monitor changes in quantitative fatty acid and isoprenoid quinone composition associated with the growth cycle of Streptomyces cyaneus (neé coerulescens) NCIB9616 and to establish the suitability of the resultant lipid profiles for multivariate statistical analyses.

MATERIALS AND METHODS

Biomass was prepared in 15 x 1L flasks containing 300ml of modified Sauton's medium (Mordarska et al., 1972). Two liquid cultures were harvested at 1,3,7,10 and 14 days and the biomass freeze-dried. The remaining five flasks were used to monitor growth, by sampling at 4 hourly intervals and determining the increase in dried biomass per ml, with time.

Biomass was examined for fatty acids and isoprenoid quinones. The former were extracted using a whole-organism alkaline methanolysis procedure (D.E. Minnikin et al., unpublished data) and the fatty acid methyl esters so formed were analysed by gas chromatography (O'Donnell et al., 1982). Isoprenoid quinones were extracted by shaking with a biphasic mixture of petroleum ether (b.p. 60-80°C) and methanolic saline (Minnikin et al., 1984). Quantitative information of the isoprenoid quinone composition was obtained by high performance liquid chromatography.

RESULTS AND DISCUSSION

Growth was monitored in separate though identical flasks. Cultures harvested at 1d were in lag phase, those at 3d in early logarithmic phase, those at day 7 had reached stationary phase and by day 14 death phase had started. The results of the gas chromatographic analyses of the fatty acids are shown in Figures 1a to 1c: it is evident that the major components were straight chain saturated and branched chain iso and anteiso fatty acids. The results indicate that the onset of logarithmic growth was accompanied by a small shift in quantitative fatty acid composition.

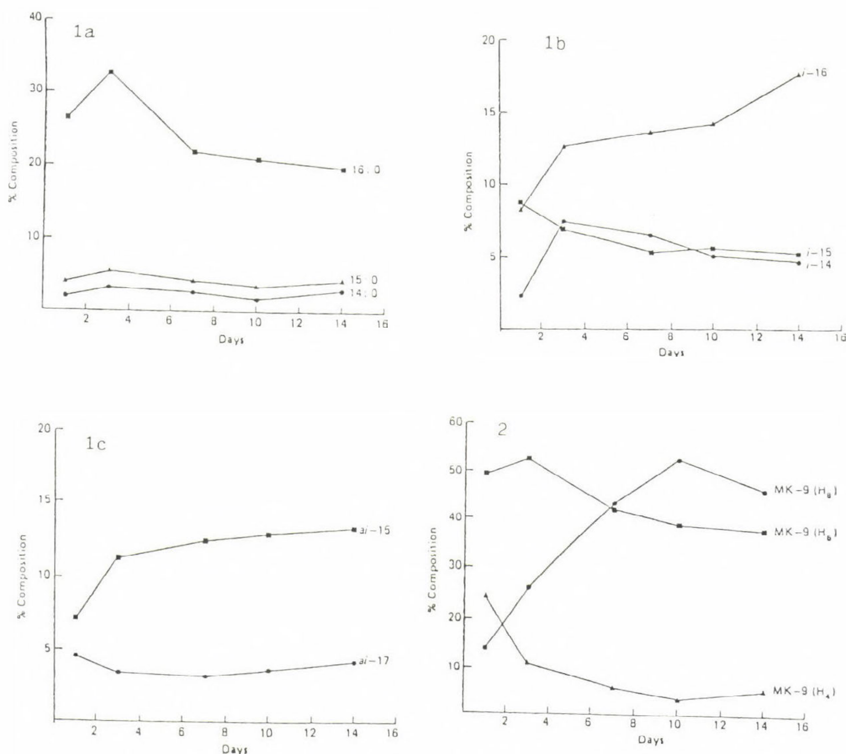

Fig. 1 Percentage fatty acid composition of Streptomyces cyaneus NCIB 9616 at different stages of the growth cycle. (a) ■ , hexadecanoic acid (16:0), ▲ , pentadecanoic acid (15:0) and ● , tetradecanoic acid (14:0); (b) ▲ , 14-methylpentadecanoic acid (i-16); ■ , 13-methyltetradecanoic acid (i-15) and ● , 12-methyltridecanoic acid (i-14); (c) ■ , 12-methyltetradecanoic acid (ai-15) and ● , 14-methylhexadecanoic acid (ai-17).

Fig. 2 Percentage menaquinone composition of Streptomyces cyaneus NCIB 9616 at different stages in the growth cycle. ▲ , MK-9(H_4); ■ , MK-9(H_6) and ● , MK-9(H_8).

However, the fatty acid profile remained relatively constant throughout both the logarithmic and stationary phases of growth. A number of studies have been performed on the effects of culture age on fatty acid composition and although it is difficult to generalise the present findings are comparable with such studies as they show that stability of the profile during logarithmic and stationary phases of growth is preceded by a shift in the relative concentrations of fatty acids (see O'Donnell, 1985).

Menaquinones (MK) with 9 isoprene units and varying degrees of saturation (MK-9[H_4]; MK-9[H_6]; MK-9[H_8]) were the only isoprenoid quinones present in Streptomyces cyaneus NCIB9616. It is evident from the results (Fig. 2) that the menaquinone profile was influenced markedly by culture

age. Thus, over the 14 day period the relative concentration of MK-9[H_8] increased four-fold whilst that of MK-9[H_4] decreased by a similar factor. The importance of harvesting at specific stages of the growth cycle may be especially important in the case of organisms such as streptomycetes, which contain complex mixtures of partially saturated menaquinones. This latter point is particularly valid if data are to be analysed by statistical methods. However, the finding that culture age had only a limited effect on the fatty acid composition of Streptomyces cyaneus NCIB9616 suggests that numerical analysis of the fatty acid profiles of actinomycetes will yield data of taxonomic value without the necessity of monitoring the growth cycle.

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SPORE DOME ACTINOMYCETES

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"Spore dome" actinomycetes, first described by Willoughby (1969), were isolated from leaf litter(eg leaves of Quercus, Acer, Fagus, Salix spp., and various Gramineae) collected from the shores of freshwater lakes, streams and rivers. The litter was air dried (7d at 28°C), suspended in sterile tap water (15-30 min at 20°) and samples of the supernate spread on the surface of colloidal chitin agar containing cycloheximide and nystatin (each at 50 µg/ml). Plates incubated for 14d at 28°C carried a mixture of bacterial and actinomycete colonies. The latter included 'spore domes' (10^4-10^7 cfu g^{-1}dry leaf), recognisable with a low power stereomicroscope and distinguished as white to cream yellow colonies with a shining central dome and lacking aerial mycelium (AM). Leaves rehydrated for longer periods (>55 min) released higher numbers of Actinoplanes spp. (Makkar & Cross, 1982). When transferred and grown on oatmeal agar + 0.1% (w/v) yeast extract 'spore domes' were usually coloured yellow orange with a central dome and rings of satellite domes; colony colour and production of soluble pigments varied from strain to strain. Sections of colonies showed that the surfaces of the domes consisted of a pallisade of hyphae which fragmented into chains of spores. Colonies transferred to tap water rapidly released motile spores (oval to egg-shaped, 1.0 x 1.5-2.5 um) with a polar tuft of flagella.

40 strains were isolated in pure culture from a variety of leaves collected from several freshwater habitats. None formed AM on the many agar media tested. All contained menaquinones MK-9 (H_4) and so differed from Streptomyces [MK-9 (H_6) + MK-9 (H_8)] and Actinosynnema mirum H1011 [MK-9 (H_6) + MK-9 (H_4)]. Cell wall analyses on all strains revealed that the peptidoglycan of 35 contained LL-diaminopimelic acid (DAP) + glycine (9 with traces of meso-DAP) and 5 contained meso-DAP with only traces of LL-DAP and no glycine. The walls did not contain madurose, arabinose, rhamnose or xylose. Phage isolated against wall chemotype I actinomycetes (Streptomyces, Chainia, Streptoverticillium) did not lyse

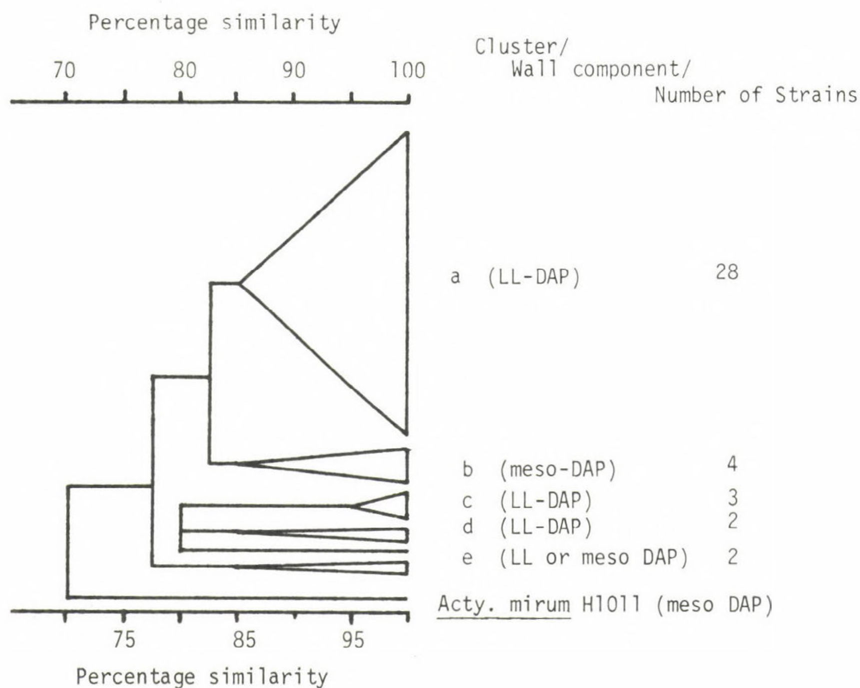

Fig. 1 Simplified dendrogram to display the results of a single linkage cluster analysis employing the S_{SM} coefficient on spore dome actinomycetes and Actinosynnema mirum H1011.

'spore dome' actinomycetes. Phage isolated against 'spore domes' (with LL-DAP walls) lysed both LL-DAP and meso-DAP 'spore dome' strains but were inactive against wall chemotype I streptomycetes, Acty. mirum, and selected species belonging to wall chemotypes II (including Actinoplanes spp.), III and IV.

The 40 'spore dome' strains and Acty. mirum H1011 were subjected to 133 morphological and biochemical tests and the character states analysed by single linkage clustering using S_{SM} and S_J matching coefficients. Test error was estimated at 1.7%. Results expressed in the form of dendrograms (eg S_{SM} analysis Fig. 1) showed that the 'spore dome' actinomycetes were heterogeneous but could be distinguished from Actinosynnema.

Current taxonomic criteria, relying heavily on wall composition, would preclude the 'spore dome' actinomycetes from being accommodated in a single genus, a simple solution that appeared plausible when only morphological characters were considered. Further strains from other habitats and geographical regions must be studied before a final classification can be proposed.

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GENUS KITASATOSPORIA AND ITS PHYLOGENIC POSITION

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The genus Kitasatosporia Omura et al. (1982) was proposed for strains that exhibited new cell wall type, i.e. the organisms had walls containing similar amounts of LL and meso diaminopimelic acid (A_2pm), as well as glycine and galactose. Subsequently, a new species, Kitasatosporia melanogena, was described for strain K55-G-32 (Shimazu et al. 1984).

Kitasatosporia setae (K. setalba) IFO 14216, K. melanogena K55-G-32, and Nocardiopsis dassonvillei IFO 13908, the type strains of the three taxa were used in the present study.

When cultured on agar media, K. setae, K. melanogena and N. dassonvillei formed aerial spore chains consisting of 10 or more spores and mature spores arranged in zig-zag shaped chains. In all three cases the substrate mycelia formed zig-zag shaped hyphae which sometimes fragmented into rods or coccoid elements. When cultured in liquid media, K. setae formed polymorphic submerged spores borne singly, in pairs or as short twisted chains.

Comparison of the chemical properties of the three strains can be summarized as follows. The whole cell hydrolysates or the vegetative mycelia contained m-A_2pm, with minor amounts of L-A_2pm, and galactose in the case of K. setae; m-A_2pm and galactose in K. melanogena, and m-A_2pm in the N. dassonvillei strains. In contrast, the spores of the two Kitasatosporia strains contained L-A_2pm with traces of m-A_2pm. In previous studies the cell walls of the vegetative mycelium of K. setae were found to contain m-A_2pm, minor amount of glycine, and galactose (Takahashi et al. 1984), those of K. melanogena m-A_2pm, glycine and galactose, and those of N. dassonvillei m-A_2pm.

Similarly, the cell walls of aerial and submerged spores of K. setae contained L-A_2pm and glycine (Takahashi et al. 1984). The predominant menaquinone in both species of Kitasatosporia was found to be MK9(H_8) as approved to MK10(H_6) in the N. dassonvillei strain.

These results show that all three species studied share a number of morphological characteristics, but that the Kitasatosporia strains can be distinguished from N. dassonvillei, on the basis of chemical properties.

The genus Kitasatosporia is characterized by the fact that the walls of the vegetative mycelia contain m-A_2pm and those of the spores L-A_2pm. An analogous situation has been reported by Work (1970) in Bacillus sphaericus. Changes in the nature of A_2pm have been reported in the cell walls of mutant and parental strains of actinomycetes (Zakharova et al. 1980).

Cell wall types (CWTs) are stable and useful criteria for the classification of actinomycetes. The presence of many CWTs suggests mutational alterations during the course of evolution. Similarly, differences in diamino acid composition are indicators of phylogenic relationships (see Stackebrandt, this symposium). The vegetative cell wall composition of K. melanogena and K. setae means that they can be assigned to CWT III + galactose but the submerged and aerial spores of these organisms fall into CWT I. These data suggest that the genus Kitasatosporia is a phylogenetic intermediate between CWT I and CWT III taxa or between the acetyl type of CWT IV which contains N-acetylmuramic acid in the peptidoglycan. Likewise, this genus can be seen as an intermediate between CWT I and CWT II taxa or between the glycolyl type of CWT IV through the ancestors of the glycolyl type which contain N-glycolylmuramic acid.

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TAXONOMIC STUDIES OF FRANKIA spp.

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Since 1978 (Callaham et al.) over 50 strains of the endobiotic actinomycete genus, Frankia, have been isolated in pure culture (Baker 1982). Recently we isolated pure cultures of Frankia from nodules of Alnus ferdinandi-coburghii and Alnus tinctoria; the latter was successfully inoculated into its host plant.

In this paper we present the results of morphological, chemical and physiological studies, including guanine-plus-cytosine content of DNA, of strain At2.

MATERIAL AND METHODS

Strains: Af1 was isolated from nodules of Alnus ferdinandi-coburghii collected in Kumming, Yunnan Province, and At2 from nodules of Alnus tinctoria collected in Shenyang, Liaoning Province.

Reference strains: Frankia spp. (Ai rI2) and Frankia spp. (CPI1) were kindly provided by Ms. M. P. Lechevalier.

Isolation: Single root nodules were washed with water, sterilized in 0.1% $HgCl_2$ for 10-20 min and then rinsed five times with sterile distilled water. The sterilized nodules were then cut into thin slices. Each slice was put into a test tube containing liquid medium "S", S+Tw80 or QMod and incubated at 28°C for 1-3 months, i.e. until colonies appeared.

Whole cell wall composition: Whole cell analysis was performed after Hasegawa et al. (1983), and by the methods of Becker (1964) and Lechevalier et al. (1980).

Physiological and chemical properties: The procedures of Lechevalier et al. (1983) were used for physiological and carbohydrate utilization tests.

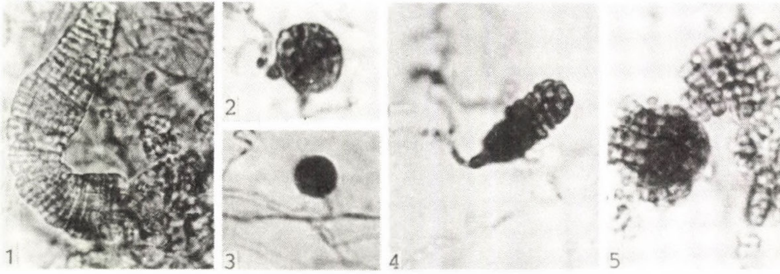
Guanine-plus-Cytosine content: Deoxyribonucleic acid (DNA) extraction and purification were carried out according to the method of Marmur (1961). DNA extracted from E. coli (AS 1.365) was used as a reference.

RESULTS

Morphology: Strains Af1 and At2 produced long branching mycelium (about 1 µm in diameter) bearing terminal, lateral and intercalary sporangia, which were variable in size and shape: typically spherical, club-shaped or irregular-shaped, small - 5 µm in diameter, moderate - 10-15 µm in diameter, or large - about 18x28 µm in diameter (Figs 1, 2, 4). The sporangiospores were produced by division of hyphae in several planes in the sporangia; they were spherical, stereoscopic shaped, 1-1.2 µm in diameter, without flagella and non-motile (Fig. 5). Vesicles, 3-5 µm in diameter (Fig. 3) were found in recently isolated cultures but were difficult to find in older cultures.

Whole cell wall composition: All strains of Af1 and At2 had a wall chemotype III, i.e. they contained meso-DAP galactose and xylose (sugar pattern D).

Physiological type: The influence of Tween 80 and glucose on the growth of the test strains is given in Fig. 6. The marked synergistic effect on growth of the addition of 0.2% Tween 80 to glucose-containing "S" media is apparent. The yield of dry mycelium in Tween 80-containing media is higher than the different concentrations of glucose in "S" media lacking 0.2% Tween 80. In this medium the mycelium grows very poorly, sometimes not at all. These results show that strains Af1 and At2 fall into the physiological type B group since a considerable



Figs 1, 2, Sporangia of Af1 (x 1000); 3, vesicle of Af1 (x 1000); 4, sporangia of At2 (x 1000); 5, sporangiospores of At2 (x 1000).

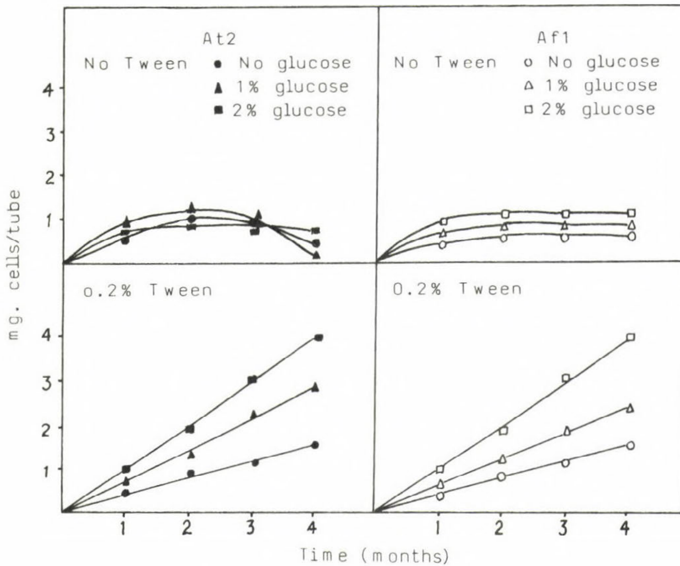


Fig. 6. Influence of Tween 80 and glucose on the growth of Af1 and At2 in "S" medium.

synergistic effect on growth was found in media containing both Tween 80 and glucose (Lechevalier et al. 1983).

Carbohydrate utilization: Strains Af1, At2 and the typical culture of type "CPI1" did not utilize any of carbohydrates

tested at 0.5% concentration, namely L(+)-arabinose, D(+)-glucose, glyceral, maltose, sucrose, D-sorbitol and D(+)-xylose. Typical cultures of type A "AirI2" utilized D(+)-glucose, maltose, sucrose but not D(+)-xylose though it can produce acid from the latter. According to Lechevalier, all cultures belonging to type B do not use any of the carbohydrates without the synergistic action of Tween 80. This fact proved that cultures Af1 and At2 belong to physiological type B.

Guanine-plus-Cytosine content of DNA: The DNA base composition of strain At2 was 69.54 mol% G plus.

DISCUSSION

According to morphology, cell wall composition and infectivity, we consider strains Af1, At2 to be members of the genus *Frankia*. The species of other actinomycetes genera are identified by morphological and physiological properties and other criteria. *Frankia* also differs from other actinomycetes since it is an endobiotic microorganism. Reliable properties are still not available for the circumscription of species of *Frankia* though strains assigned to this taxon do exhibit some differences in physiological properties. We agree with Lechevalier's suggestion (1984) that using the term *Frankia* spp. is better at the moment than introducing new specific names for organisms in the genus. The abbreviation of host plant and the number of the strain should be given after the term. Thus strain Af1, which was isolated from *Alnus ferdinandi-coburghii* becomes *Frankia* spp. Af1 and strain At2, from *Alnus tinctoria* becomes *Frankia* spp. At2.

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GENUS MICROMONOSPORA: TAXONOMY, ANTIBIOTIC SENSITIVITY AND ANTIBIOTIC PRODUCTION

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More than 500 actinomycetes belonging to the genus Micromonospora (Ørskov, 1923) were isolated from natural substrates. On the basis of cultural and morphological features the isolates were divided into 9 subgroups: Aurantiaca (orange), Cinnamomea (brown), Cinnamomea-vinacea (brown-crimson), Cinnamomea-olivacea (brown-olive), Nigra (black), Nigra-violacea (black-violet), Lilacinescens (lilac tinged), Coerulea (dark-blue) and Brunnea (grey-brown). One hundred and five cultures included in these subgroups were tested for their sensitivity to 18 antibiotics. A correlation was observed between the antibiotic sensitivity spectra of the Micromonospora cultures and the subgroup to which they belonged. The antibiograms were indicative of a definite heterogeneity within the genus and justified dividing the cultures into separate subgroups. The Aurantiaca subgroup was recognized by its selective sensitivity to rifampicin, the Coerulea subgroup by its resistance to erythromycin, benzylpenicillin and other β-lactam antibiotics, and the Lilacinescens subgroup by an extremely high sensitivity to the majority of the antibiotics tested and to nalidixic acid. The four subgroups with moderate antibiotic sensitivity, i.e. Cinnamomea, Cinnamomea-vinacea, Nigra and Nigra-violacea gave similar antibiograms, but differed markedly in their capacity to produce aminoglycoside and macrolide antibiotics (see below). The Brunnea and Cinnamomea-olivacea subgroups were not examined for antibiotic sensitivity.

Micromonospora strains produce many antibiotics, aminoglycosides such as gentamicin, sisomicin and fortimicin, and

macrolides such as rosamicin, juvenimicin and megalomicin. The majority of the antibiotic-producing organisms belonged to four subgroups: Cinnamomea, Cinnamomea-vinacea, Nigra and Nigra-violacea, the cultures of which possessed moderate and related antibiotic sensitivity. Antibiotics of the gentamicin type, i.e. gentamicin, sisomicin, verdamicin, sagamicin, antibiotic G-52, antibiotic G-418 and others were produced by cultures of the Nigra and Nigra-violacea subgroups. The Cinnamomea and Cinnamomea-vinacea subgroups included organisms producing macrolide antibiotics such as rosamicin, juvenimicin and megalomicin and macrolactone antibiotics of the isumenolide type. Moreover, by production of the gentamicin type aminoglycosides the Nigra and Nigra-violacea subgroups were related, while the Cinnamomea and Cinnamomea-vinacea subgroups were related by the ability to produce macrolide antibiotics. The Cinnamomea-olivacea subgroup was characterized by an ability to produce aminoglycoside antibiotics with a new structure, i.e. the fortimicins. It should be noted that there were practically no antibiotic-producing organisms in the Brunnea, Coerulea and Lilacinescens subgroups, these subgroups possessed increased antibiotic sensitivity. Probably, the cultures of these subgroups, which are rarely isolated, require special attention. The Aurantiaca subgroup as a whole is moderately sensitive to antibiotics, but antibiotic-producing organisms were not found in the subgroup. However, we isolated organisms producing isumenolides and rifamycins using a new test system for screening β-lactamase inhibitors and selective media with high concentrations of rifampicin. While the cultures of the Aurantiaca subgroup are characterized by high selective sensitivity to rifampicins, the rifamycin-producing cultures were practically insensitive to this antibiotic.

It can be concluded that on the analysis of the cultural and morphological features, antibiotic sensitivity and on the capacity for production of definite antibiotics, Micromonospora strains can be classified into 9 subgroups. Moreover, a correlation is observed between the taxonomic features and sensitivity to antibiotics or the capacity for their production. This correlation is significant when screening for new anti-

biotics, since it provides information on the potentialities of a taxon with respect to antibiotic biosynthesis. Analysis of the capacity for antibiotic production and antibiotic sensitivity is also important in the systematics of the genus *Micromonospora*.

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TAXONOMY

Poster Abstracts

STABILITY OF NUMERICAL CLASSIFICATIONS

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Classical morphological and pigmentation properties represented more than 25% of the unit characters included in a recent numerical phenetic survey of Streptomyces and related taxa (Williams et al., 1981, J. gen. Microbiol. 129, 1743-1813). Several of the numerically defined clusters were heterogeneous with respect to the morphological criteria.

In the present study, the morphological suite of characters was masked and the numerical analyses were repeated as before. Cluster composition was not markedly affected by the omission of the morphological properties though some changes were apparent in the arrangement of the clusters. The three subclusters of the S. albidoflavus taxon achieved cluster status at 77.5% S-level in the second analysis. Clusters 18 (S. cyaneus) and 19 (S. diastaticus) proved to be especially unstable.

NUMERICAL TAXONOMIC STUDY OF CELLULOMONAS AND POSSIBLY RELATED GENERA

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Chemotaxonomic, phenetic and phylogenetic evidence indicate a relationship amongst the genera Cellulomonas, Oerskovia and Promicromonospora (Goodfellow and Cross, 1984. In: The Biology of the Actinomycetes, p. 7, Academic Press). Stackebrandt et al. (Arch. Microbiol. 127, 179, 1980) proposed that the genus Oerskovia be reduced to a synonym of Cellulomonas.

In 1983 Jáger et al. (Int. J. Syst. Bact. 33, 525) proposed a new species in the genus Promicromonospora, P. enterophila, which resembled Oerkovia in physiological properties.

Representatives of the taxa were examined for 123 biochemical, morphological and physiological characters. The data were analysed using the simple matching and pattern coefficients and clustering achieved by the UPGMA algorithm (computations: Sneath and Sokal, 1973. Numerical Taxonomy: The Principles and Practice of Numerical Classification: Freeman). Our results support the proposal to reduce Oerskovia to a synonym of Cellulomonas. Strains of P. enterophila clustered with the cellulomonads and may be genuine members of that genus. Other promicromonosporae bore no specific relationship to the Cellulomonas complex and seemed a well defined taxon.

LYSOZYME RESISTANCE IN STREPTOVERTICILLIUM

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Thirteen *Streptoverticillium* strains, representing the major taxonomic clusters within the genus, were able to grow in the presence of lysozyme (468 units/ml). *Streptomyces* were sensitive to the enzyme except for strains from the *Stym. lavendulae* and *Stym. rimosus* clusters. Resistance to lysozyme could not be attributed to glycolyl muramic acid because all *Streptoverticillium* and *Streptomyces* species analysed contained acetyl muramic acid in their peptidoglycan. Lysozyme became associated with the hyphae of the resistant strains and was not inactivated by soluble inhibitors or enzymes in the growth medium.
A selective medium containing lysozyme has been devised for the isolation of streptoverticillia from soil samples.

SELECTIVE ISOLATION OF BIOACTIVE STREPTOMYCETES

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A number of *Streptomyces* species groups defined in the numerical taxonomic study of Williams et al. (1983) were studied for physiological characters useful for design of selective isolation media. Several media were tested and the most successful was the medium designed for isolation of *S. violaceoniger* (cluster 32) species group. This medium allowed selective growth of members of cluster 32 but inhibited other streptomycetes. The medium was tested with soil and proved to be a good medium for isolation of streptomycetes.

SELECTIVE ISOLATION AND CHEMICAL CHARACTERISATION OF AUTOTROPHIC ACTINOMYCETES

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Eighty autotrophic actinomycetes were isolated by liquid enrichment. Strains were examined using an integrated chemotaxonomic procedure (O'Donnell et al. 1985. In: Chemical Methods in Bacterial Systematics, pp. 131-143, eds. M. Goodfellow and D.E. Minnikin. Academic Press, Orlando, USA) in order to establish the value of fatty acids, isoprenoid quinones, polar lipids and wall amino acids in the classification of autotrophic actinomycetes. The fatty acid data were examined quantitatively using multivariate statistics.

TAXONOMIC AND GENETICAL STUDIES OF STREPTOMYCES HYGROSCOPICUS

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Strains of *Streptomyces hygroscopicus* produce a number of important antibiotics including, for example, compounds with anti-fungal, anti-bacterial, anti-viral or herbicidal activities. The selection of related strains for genetical analysis may increase the chances of identifying a natural, plasmid-mediated system for gene transfer in addition to identifying isolates producing related antibiotics. A computer-assisted identification scheme based upon 41 morphological and physiological tests confirmed that strains previously designated as *S. endus, S. sparsogenes, S. violaceoniger* and *S. hygroscopicus* were all closely related.

The appearance of a clear lethal zygosis effect in interactions between some members of this group suggests the occurrence of a plasmid-mediated conjugation system which may form the basis for production of hybrids via a classical recombination system.

The use of ultra-violet irradiation to produce marked strains revealed an inverse relationship between spore survival and the apparent frequency of mutation to sporulation defective and certain antibiotic resistant phenotypes.

Protoplast technology offers the prospect for producing hybrid strains via protoplast fusion or recombinant DNA techniques. Techniques for the production of protoplasts appeared strain-specific, with variations in growth medium composition and carbon sources in protoplasting medium extending the range of strains for which protoplasts could be produced (10^6 - 10^8 per ml). Regeneration of protoplasts to the mycelial form at frequencies of up to 28% was achieved by allowing reversion in agar overlays upon dried regeneration media.

STREPTOMYCES AURANTIOCREMEUS sp. nov. A CAROTENOGENIC ACTINOMYCETE

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S. aurantiocremeus sp. nov. possesses the first type cell wall, forms straight sporofores, oval spores with smooth surface; aerial mycelium is scant, whitish; substrate mycelium is orange. No soluble pigments and melanoids are formed. Glucose, fructose, rhamnose, xylose, arabinose and mannitol are utilized for growth. The culture synthesizes a water-soluble antibiotic of the first group (by Blinov and Khokhlov classification) and a complex of carotenoids which includes at least five components.

EVALUATION OF AN IDENTIFICATION MATRIX FOR STREPTOMYCETES

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As part of the theoretical evaluation of an identification matrix for Streptomyces spp. constructed in this laboratory, the identification scores for randomly selected cluster representatives were determined. To ascertain the range of identification scores possible within a cluster, the identification scores for all strains within selected clusters were determined [using the appropriate test results obtained during the construction of the numerical classification (Williams, Goodfellow, Alderson et al. 1983) upon which this matrix is based] and as a further comparison, the same exercise was performed using an earlier published identification matrix (Williams, Goodfellow, Wellington et al. 1983).

SELECTIVE ISOLATION, NUMERICAL CLASSIFICATION AND CHEMOTAXONOMY OF NOVEL ACIDOPHILIC AND NEUTROTOLERANT ACTINOMYCETES

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Acidophilic and neutrotolerant actinomycetes were isolated from a coniferous woodland soil using starch casein agar supplemented with actidione and nystatin (Williams et al., 1971. Soil Biol. Biochem. 3, 187-195) and various antibacterial antibiotics. Fifty presumptively novel isolates were examined for 98 unit characters and the data analysed using conventional statistics widely applied in numerical phenetic surveys of bacteria from natural habitats (Goodfellow and Dickinson, 1985. In: Computer Assisted Bacterial Systematics, pp. 131-143, eds M. Goodfellow, D.E. Minnikin, Academic Press, Orlando, USA). Good congruence was found between the numerical phenetic and chemical data.

NUCLEIC ACID HOMOLOGY STUDIES APPLIED TO RHODOCOCCUS TAXONOMY

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We determined the degree of binding DNA preparations isolated from 36 strains of rhodococci and labelled DNA from four reference strains selected on the basis of phenetic-numerical studies (Goodfellow and Alderson, Jour. Gen. Microb., 1977, 100, 99-122). Two methods have been used for DNA homology determination: (i) on nitrocellulose membranes, and (ii) in free solution using S_1 nuclease. Both methods were compared and correlated. Optimal conditions for DNA-DNA pairing in free solution in the presence of formamide were established. Data obtained using these two methods were in good agreement.

STUDIES ON THE VARIABILITY OF ACTINOPLANES

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Among the cultural-morphological and physiological properties of about 30 strains of an *Actinoplanes* sp. isolated from soil the shape and size of spore vesicles (sporangia) showed the greatest variability. Strains which proved to be identical according to their physiological features produced globose, club-, umbrella-shaped, lobed, or digitate spore vesicles. The morphology of sporangia can be considered as a good diagnostic feature at the generic as opposed to the specific level.

NUMERICAL ANALYSIS OF NOCARDIA VACCINII

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Twenty-seven actinomycetes isolated from actionorhisal nodules (Pariyskaja et al., 1982; Malishkayte et al., 1984) were assigned to the species *N. vaccinii* using existing identification keys. These strains together with appropriate reference strains of the genus *Nocardia* were analysed using numerical taxonomic methods. Most of the 27 isolates were recovered in a distinct cluster which included the type strain of *N. vaccinii* (VKM Ac 856). The strains isolated from actinorhisal nodules clearly belong to a distinct species of the genus *Nocardia*.

THE SYSTEMATIC POSITION OF TWO SPECIES FORMERLY INCLUDED IN THE GENUS PSEUDONOCARDIA

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The two not validly published species *Pseudonocardia azurea* and *Pseudonocardia fastidiosa* were included in a study designed to clarify the taxonomy of the genus *Pseudonocardia*. The two species differed from the type species of the genus, and from other true *Pseudonocardia* species, in morphology, ultra-structure of septa and in biochemical characteristics. The systematic position of *P. azurea* and *P. fastidiosa* is discussed.

TAXONOMY OF MICROMONOSPORA ISOLATES PRODUCING ANTIBIOTICS OR ENZYME-INHIBITOR

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During screening for microbial metabolites showing biological activity, the following isolates of *Micromonospora* were found to produce either antibiotics or an enzyme-inhibitor: Strain MK-99, antibiotic XK-99; strain MK-101, tubercidin and 7-deazainosine; strain MK-206, antibiotic XK-206; strain MK-230, psicofuranine and strain K-24, K-26 phosphorus-containing tripeptide (inhibitor of angiotensin I converting enzyme). These strains exhibited characteristics that differed from those of established species of the genus *Micromonospora*. Their differentiation from established taxa and the problem of defining *Micromonospora* species are discussed mainly with respect to the following points: (1) Mycelial and diffusible pigments; (2) spore ornamentation; (3) 3-OH-A_2pm and reducing sugars in cell wall; (4) predominant menaquinone; (5) sugar and amino acid utilization and (6) glycosidase activities.

TAXONOMIC STUDIES OF THREE ACARBOSE PRODUCING STRAINS OF ACTINOPLANETES

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Acarbose is a name for α-glucosidase-inhibitors of clinical importance. These inhibitors were first isolated from culture media of three strains of the family Actinoplanaceae. The latter have been examined for morphological, physiological, chemotaxonomical and molecular biological properties. All three strains were found to belong to the genus Actinoplanes and their relationship to established species is discussed.

THE FINE STRUCTURE OF ACTINOMADURA spp.

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The fine structure of the vegetative cells and spores of the type strains of A. carminata (Gause et al., 1973), A. polychroma (Galatenko et al., 1981) and A. citrea (Lavrova et al., 1972) were examined. The first two strains formed "pseudosporangia" (Nomura and Ohara, 1971) consisting of tightly closed spirals of spores with smooth surfaces. The cells' inner structure was typical of actinomycetes but a large variety in septum structure and in cell division in different directions was noted. The spore surface of the last strain was warty or knobby.

A COMPARATIVE POPULATION ANALYSIS OF THE SPECIES - THE BASIS FOR THE IMPROVEMENT OF ACTINOMYCETE SYSTEMATICS

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At present the species systematics of actinomycetes is far from satisfactory since it contains many nominal species that have still to be reduced to synonyms of established species. Theoretically grounded and experimentally confirmed, the perspective of a new approach to species systematics, based on a comparative taxonomical study of spontaneous variants that form the species population, is possible. The analysis of populations became feasible given the discovery of the phenomenon of parallelism (homologous series) in the hereditary variability of actinomycetes (Kuznetsov, V.D., Antibiotiki, 1973, p. 579, No. 7).

NUMERICAL TAXONOMY OF RAPIDLY GROWING MYCOBACTERIA

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Thirty strains of rapidly growing mycobacteria, isolated from soil samples, and ten strains representing nine established species of *Mycobacterium* were studied by numerical taxonomy. The matching coefficient between the test strains based on 63 unit characters was estimated using two different formulae, i.e. the Ssm coefficient was used to examine the biochemical characters and the SJ coefficient for the cultural and morphological characters. The two coefficients were used in the hope that weighing of some characters might be avoided. Clustering was achieved using the unweighed single linkage method. At the similarity level of 85%, forty of the mycobacteria formed five distinct clusters corresponding to *M. chelonei*, *M. fortuitum* (including *M. peregrinum*) and three unnamed clusters. *M. smegmatis*, *M. thamnopheos*, *M. flavescens*, *M. phlei*, *M. vacci* and 13 unnamed cultures were also recognized.

MYCOLIC ACIDS AND PHOSPHOLIPID ANALYSIS OF NOCARDIOFORM ACTINOMYCETES AND RELATED ORGANISMS

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Mycolic acid and phospholipid analyses were carried out on 14 strains of nocardioform Actinomycetes and related organisms. A strain originally named *Nocardia coeliaca* (Gray) Waksman & Henrici and known to have a cell wall type IV lacked mycolic acids and its phospholipid composition did not belong to any known type as both phosphatidylethanolamine and phosphatidylcholine were presented. These findings indicate that the strain should be moved from genus *Nocardia* to a more suitable generic location. It was also found that the type of phospholipids is a more important criterion than fragmentation of mycelia for differentiation between *Streptomyces* and *Nocardioides*.

A NEW SPECIES OF THE GENUS ACTINOMADURA ISOLATED FROM SOIL

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A new species of the genus *Actinomadura* was isolated from soils in the Kinki-district, Japan. The strain produces several new pepsin inhibitors as well as pepstatin. On the basis of chemotaxonomical analysis the strain has been assigned to the genus *Actinomadura* although it is very similar to *Streptomyces* spp. in physiological and morphological features and it does not contain the diagnostic sugar madurose. The organism does, however, contain the uncommon sugar 6-deoxy talose which has been found in strains of the genera *Actinomyces* and *Mycobacterium*. Neither toxicity nor infectious ability were shown when mycelium of the novel isolate was intraperitoneally inoculated into experimental animals.

PLASMID CHARACTERISTICS OF THERMOPHILIC STREPTOMYCES

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Out of 28 thermophilic streptomycetes examined, *S. thermogriseoviolaceus* T272 contained 3 plasmids (pST1, pST2, pST3) and *S. thermoluteus* T422 a single plasmid (pST4). The molecular weights of the plasmids were calculated by experimental formula (contour length 1 $\mu = 2.07 \times 10^6$ daltons). In the case of strain T272 it was shown that genes related to rifampin resistance might be located on pST1 while there was evidence that pST4 might harbour a gene coding for rRNA methylase which resulted in ELS (erythromycin-lincomycin-spiramycin) resistance of T422. It was also shown that the plasmids had no relation with the thermophilic growth of their host strains. Another four of the 28 strains were found to produce substances which inhibited *B. subtilis* ATCC 6633, one of the substances was found to be similar to restocytin. The relationship between the plasmids and the classification of thermophilic streptomycetes is discussed.

NEW GLYCOLIPIDS CONTAINING NOVEL POLYUNSATURATED MYCOLIC ACIDS IN A PSYCHROPHILIC, ACID-FAST BACTERIUM, GORDONA AURANTIACA

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Gordona aurantiaca (Rhodococcus aurantiacus), a unique psychrophilic species isolated from human sputum (Tsukamura et al.), shows a degree of strong acidfastness comparable to mycobacteria. Taxonomically, the species occupies an essentially intermediate position between mycobacteria and nocardiae. We have previously reported the structure of fully separated mycolic acids from this species and revealed that the mycolic acid composition is extremely complicated, consisting mainly of highly unsaturated molecular species, which are useful for cell wall or membrane function in the low temperature environment. In the present study examination of the cell wall lipid composition revealed that G. aurantiaca possessed a large amount of glycolipid containing mycolic acids and trehalose. At least four glycolipids, two of which have not been reported previously, were isolated and identified tentatively as trehalose monomycolate (TMM), -dimycolate (TDM), -polymycolate-1 (GM) and -polymycolate-2 (up. GM). The mycolic acid composition differed significantly with the glycolipid species and the amount of glycolipids (mycolic acids) varied adaptatively. The role of polyunsaturated glycolipids will be discussed.

PATHOGENICITY AND IMMUNOLOGY

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ACTINOBACILLUS ACTINOMYCETEM-COMITANS AND HUMAN ACTINOMYCOSIS

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Recently we have already discussed epidemiological, etiological, microbiological, medical and therapeutic aspects in human actinomycosis on the basis of over 30 years experience as reference laboratory for the diagnosis of this still problematic disease (11, 14, 15, 16, 17, 18). Two preconditions have been postulated which are essential for the development of human actinomycosis:

1) A pathogenic member of the family Actinomycetaceae (mostly A. israelii) must gain access to the interstitial tissue (specific etiologic agent)
2) The actinomycetes need support from a varying collection of concomitant aerobic and/or anaerobic bacteria (unspecific etiologic agents).

Among these concomitant bacteria a special position was attributed to Actinobacillus actinomycetem-comitans, a slender gram-negative, non-motile, non-sporulating and microaerophilic bacterium. In the following paper the presentday knowledge about this fascinating microorganism is summarized and the significance of A. actinomycetem-comitans (shortly "comitans") in human infections is discussed.

In our institute we have diagnosed since 1952 altogether 3334 cases of human actinomycosis. Actinomycetes could be constantly demonstrated in all these patients (in over 80 % the actinomycetes belonged to A. israelii) and in 796 of these cases (= 24 %) we also cultivated comitans. A careful analysis of our material exhibited no correlation between the presence of comitans and the age and sex distribution of the patients. As seen in non-comitans actinomycosis, threequarters of the patients with comitans-supported actinomycotic lesions are males and the age-peak is situated between 20 and 30 years. Interesting is the observation that a similar sex-ratio (ratio males/females 3:1) was found in comitans-caused endocarditis (22).

As shown in table 1, comitans is only seldom present (6 %) in fresh actinomycotic lesions (persistence of symptoms for one week or less) in comparison to a frequency of 30 % in longer

Table 1: Progress of human actinomycosis and presence of *A. actinomycetem-comitans* (data of 1396 patients)

Persistence of actinomycotic symptoms	<1 week	1-2 weeks	2-3 weeks	>3 weeks
Number of cases	97 = 100 %	79 = 100 %	44 = 100 %	1176 = 100 %
thereof with *A.actinomycetem-comitans*	6 = 6 %	15 = 19 %	13 = 30 %	357 = 30 %

lasting processes (at least two weeks). Similarly, in acute inflammations comitans was not so often found as in typical chronic actinomycotic infections (table 2). Therefore we can conclude that comitans is participitating especially in severe, chronic and long lasting actinomycotic processes which rather often raise therapeutical problems, too. We have no explanation for this observation.

Table 2: State of human actinomycosis and presence of *A.actinomycetem-comitans* (data of 1630 patients)

Course of actinomycosis	Number of cases	thereof with *A. actinomycetem-comitans*
chronic	1176 = 100 %	357 = 30 %
acute	130 = 100 %	130 = 7 %
not known	324 = 100 %	66 = 20 %

Some other interesting facts were discovered when we analysed our material as concerns the presence of comitans together with other concomitant bacteria. During last 6 1/2 years we diagnosed 685 cases of human actinomycosis, in 169 of them comitans was cultivable, too. As seen from table 3, actinomycotic lesions with the participition of comitans contained less often aerobic bacteria and streptococci as well as Bacteroides, fusobacteria and *Leptotrichia buccalis* than non-comitans infections. Such disproportions in their frequency of occurence we could not observe with staphylococci (*S. aureus* as well as *S.epidermidis*), propionibacteria, *Eikenella corrodens* and other more rare companions of actinomycetes. Could be, that there exist some growth inhibition phenomena between comitans and other bacteria as it was demonstrated in vitro for streptococci (23). Because all these bacteria including comitans and actinomycetes are normal inhabitants of human mucous membranes such inhibition or potentiation mechanisms may also exist in vivo influencing the composition of our physiological microflora.

What do we know about comitans? This bacterium was first described 1912 by Klinger (8). The following decades only a very few papers dealt with comitans, mostly mentioning its occurence in actinomycotic lesions. Therefore, in 1959 we published

Table 3: Result of aerobic/anaerobic cultures of actinomycotic material and presence of A.actinomycetem-comitans

A. actinomycetem-comitans	+	-
Actinomyces	+	+
Number of cases	169 = 100 %	516 = 100 %
Aerobic sterile	92 = 54 %	197 = 38 %
α-hemolytic streptococci	3 = 2 %	73 = 14 %
ß -hemolytic streptococci	2 = 1 %	32 = 6 %
Microaerophilic streptococci/ Peptococcaceae	39 = 23 %	240 = 47 %
Bacteroides melaninogenicus-group	6 = 4 %	163 = 32 %
Fusobacteria	38 = 22 %	165 = 32 %
Leptotrichia buccalis	7 = 4 %	97 = 19 %
other Bacteroides species	4 = 2 %	75 = 15 %

results of own investigations concerning the differentiation and pathogenic significance of comitans (2,3). Some years later King and Tatum (7) and Page and King (10) presented excellent studies on the biochemical/serological grouping of comitans and on the clinical importance of comitans as the causing agent of non-actinomycotic infections like endocarditis. In 1970 we discussed the fermentative capabilities of 140 comitans-strains (12), in 1972 we reported about serological studies on 100 comitans-strains (13). Comitans can be cultivated without great difficulties, special media are not needed. CO_2 is an important growth-factor for this microaerophilic bacterium, 37°C is the optimal temperature for cultivation. After 2 - 5 days liquid media remain mostly clear, only small granules can be seen which adhere to the wall. On solid media comitans grows in very characteristic colonies which belong to the most beautiful architectonic pictures in bacteriology. The small, hard and adherent colonies show slightly irregular edges and a starlike inner structure (2). Because of this very typical colonyform together with gram-staining comitans can easily be diagnosed, the biochemical differentiation is usually not problematic. Comitans is able to ferment glucose, maltose, mannose and laevulose, nitrate is reduced to nitrite, the catalase-reaction is positiv. H_2S is not produced, oxidase, esculin, indole,urea and gelatin reactions are negative as well as the following tests: arabinose, dulcitol, glycerol,inositol, inulin, lactose, raffinose, rhamnose, salicin, sorbitol, sorbose and sucrose (2, 7, 12). Using differing reactions to mannitol, xylose and galactose comitans-strains can be grouped into 8 biotypes (12). Six antigens could be demonstrated (13) and the agglutination reaction was shown to be useful for epidemiological and ecological studies on comitans. Zambon et al. (27) found three serotypes a, b and c performing precipitation reactions with immunabsorbed antisera.

Already in 1959 we demonstrated (3) that comitans causes some biologically important phemomena like hemolysis, fibrinolysis, coagulation of plasma and the splitting of polymers. In the meantime more virulence-factors could be found: comitans produces a heat-labile leukotoxin, capable of killing human polymorphonuclear leukocytes and monocytes (25, 26), a collagenase (27) and an endotoxin (6) that promotes bone resorption and is active in the Shwartzman-reaction. Furthermore, comitans was shown to inhibit the growth of murine and human fibroblasts and this ability was associated with certain forms of periodontal disease (20). The presence of an immunsuppressive factor in all comitans strains examined was also shown (21): this heat labile factor inhibits the responsiveness of human peripheral blood lymphocytes to different mitogens and antigens. These data clearly demonstrate that comitans is an organism with a broad spectrum of biological activities which are generally accepted to be important bacterial virulence-factors.

Because of known difficulties with actinomycetes in animal experiments only a few studies have been published dealing with the problem of in vivo-symbiosis between comitans and A.israelii. Jordan and Kelly (5) established a mixed actinomycotic infection in a susceptible weanling mose model by using combinations of A. israelii and comitans. Comitans alone was able to produce only one single lesion in one of six mice which was resolved within a few weeks. A. israelii on the other hand caused lesions in all animals but all of them survived over 12 weeks. Interesting is their statement that chronic infections with the highest mortality occured in the animal groups injected with comitans and A. israelii compared with monoinfections by A. israelii alone and comitans.

Comitans is a physiological member of the microflora of the mucous membranes of the upper respiratory tract of healthy humans (3, 24). However, the posssibility of existing nonoral reservoirs of comitans cannot be eliminated (27). The comitans carrier rate is certainly much lower than the carrier frequency of actinomycetes which normally are present in the oral microflora of practically all adults. This obvious difference in the carrier rate may be the explanation for the fact that actinomycetes are not always accompanied by comitans in human actinomycotic lesions. Unfortunately nothing is really known about the physiological role of comitans within the oral microflora.

It is our experience that in infectious processes comitans is nearly always found as a companion of an actinomycete. Since 1952 we cultivated comitans from the material of altogether 844 patients, in 649 of these patients we could analyse anamnestic data concerning localisation, symptomatology and etiology of the disease. As shown in table 4, in 601 of these 649 cases comitans was found together with actinomycetes in clinically typical actinomycotic infections. In only 48 patients we didn't succeed to isolate an actinomycete or another microorganism besides comitans. In 17 of these 48 cases of comitans-monoinfections we received informations that the patient was treated with antibiotics before the material could be collected to perform

bacteriological cultures, mostly a penicillin-therapy was reported. As discussed earlier (3) and as shown in table 5, an intensive penicillin- and/or vaccine-therapy (containing A. israelii) can be the cause of the elimination of the penicillin-sensitive actinomycetes. The penicillin-resistant comitans is able to survive and to support the actinomycotic process also in the absence of actinomycetes. Therefore we believe that in all 17 cases an actinomycete was also present before the therapy was initiated. Concerning the remaining 31 patients we were unable to collect proper informations concerning the therapy or we found no evidence for the initial presence of actinomycetes. Several of these cases could be examined repeatedly and, even though optimal diagnostic procedures were employed, actinomycetes or other bacteria could never be demonstrated. Therefore we may postulate that comitans is mostly a strict companion of actinomycetes in causing severe human actinomycosis. But occasionally comitans can be the etiologic agent of monoinfections without the participipation of actinomycetes. So far documented such infections in our material resembled true actinomycotic processes.

Table 4: A.actinomycetem-comitans in human infectious processes (649 cases)

	Result of culture	
A.actinomycetem-comitans	+	+
Actinomyces	+	-
Number of cases	601	48

Table 5: The effect of penicillin- and/or vaccine-therapy on the presence of A.israelii and/or A.actinomycetem-comitans in actinomycotic processes

Patient	Data of culture	Result of culture A.israelii	A.actinomycetem-comitans
S.P.	Jan. 1956	+	+
	Penicillin →		
	20. II. 1956	-	+
Sch.	11. IX. 1957	+	+
	Sulfaoratren Vaccine →		
	12. IV. 1958	-	+
L.O.	08. IX. 1967	+	+
	Vaccine →		
	03. II. 1968	-	+
F.O.	08. III: 1968	+	+
	Vaccine →		
	20. VII. 1968	-	+

During last years several reports have been published on comitans-monoinfections. Although such a disease is rare, comitans-monoinfections do occur and have been diagnosed in the case of endocarditis, thyroid gland abscess, urinary tract

infection, brain abscess and vertebral osteomyelitis (see 24, 27). Since Mitchell and Gillespie (9) presented their first patient with comitans-endocarditis in 1964 several statements from different countries have been given. Until the end of 1984 we were able to find reports on 60 patients suffering of comitans-endocarditis (1, 19, 22), 15 of them died in spite of chemotherapy. The clinical symptoms and the course of comitans-endocarditis are similar to other subacute bacterial endocarditis infections. Furthermore it seems that comitans may be involved in the pathogenesis of certain periodontal diseases like juvenile periodontitis (24).

As already mentioned comitans strains can have a rather unfavorable antibiotic resistance spectrum. In determining the MICs of 45 antimicrobial agents we could show (4) that all comitans strains tested have been susceptible for the tetracyclines, chloramphenicol, rifampin and cotrimoxazole, but not for the lincomycins.Some of the comitans strains had to be classified as resistant against the penicillins and cephalosporins, some other strains demonstrated resistance towards the nitroimidazoles. It is therefore understandable when the chemotherapy of infections with comitans very often exhibits problems. Because of differences in antibiotic-susceptibility from strain to strain, MIC-testing has to be recommended in severe comitans-infections.

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IN VITRO AND IN VIVO STUDIES ON THE EFFICACY OF VARIOUS ANTIMICROBIAL AGENTS IN THE TREATMENT OF HUMAN NOCARDIOSIS

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INTRODUCTION

In contrast to human actinomycoses and many other bacterial infections nocardiosis and actinomycetoma have retained an unusually high degree of therapy resistance despite the availability of numerous potent antibacterial drugs. This somewhat surprising fact may be attributed to three major reasons: 1. Clinical and microbiological diagnosis of both forms of disease has remained difficult to establish so that suitable therapeutic measures are often delayed. 2. Many patients, at least among those suffering from nocardiosis, are immunologically compromised so that natural defence mechanisms which are usually necessary to assist the action of antibiotics are not fully effective. 3. Most of the aerobic actinomycetes in question exhibit a broad and pronounced natural resistance to many of the antimicrobial drugs currently in use. Methodological problems in susceptibility testing of nocardiae further contribute to increased uncertainty so that the treatment is often empirical rather than based upon proven facts (27 - 30).

Difficulties in assessing nocardial antibiotic resistance patterns *in vitro* may also be responsible for the lack of correlation reported between the results of *in vitro* studies and the activity of the respective drugs in animal models or human infections (17, 21, 24, 25, 26, 31). We, therefore, decided to conduct a comparative study on the *in vitro* and *in vivo* activities of certain antimicrobial agents against pathogenic *Nocardia* species. The results obtained so far shall briefly be discussed in this contribution.

MATERIALS AND METHODS

Organisms: The *Nocardia* strains used in this study were taken from the culture collection of the Cologne Institute of Hygiene (HIK) and included stock cultures as well as recent clinical and environmental isolates. Their species affiliations were as follows: *Nocardia asteroides*, 30 strains; *N. farcinica*, 40 strains; *N. brasiliensis*, 14 strains; *N. otitidis-caviarum*, 10 strains. The cultures were maintained in lyophilized state and subcultured three times on brain heart infusion agar and/or in brain heart infusion broth prior to sensitivity testing.

In vitro susceptibility tests: For assessing the *in vitro* susceptibility of the test organisms, the standardized agar dilution technique described by Schaal and Leischik in 1969 (30) was employed. DST-agar (Oxoid, CM 261) was used for all of the *in vitro* test and presence or absence of growth were

determined microscopically (magnification 100 x) after 28 to 36 hours of incubation at 36 °C. Minimal inhibitory concentrations (MICs) of the 85 substances included in the study were defined as the lowest concentrations of the respective drugs at which no nocardial microcolonies could be detected under the microscope.

Animal models: The in vivo efficacy of selected antimicrobial agents was assessed using the mouse models for acute and chronic nocardiosis described by Schofield and Schaal in 1983 (32). In the animal studies, one strain of N. asteroides (HIK N14) and one strain of N. farcinica (HIK N301) were used as test organisms. Their in vitro antibiotic sensitivity patterns were typical of the respective species with susceptibility of both strains to netilmicin and amikacin, moderate susceptibility of both strains to sulfadiazine, amoxicillin plus clavulanic acid, fusidic acid, and minocycline, and high to moderate susceptibility of N. asteroides to gentamicin and sulfamethoxazole plus trimethoprim, while N. farcinica N301 was resistant to the latter drugs.

In the acute infection model, the mice were challenged by intravenous injections of doses of live nocardial cells which were high enough to cause death in 100% of the control animals within 5 to 7 days. The antibiotics were administered for the first time immediately after infection and the treatment was continued with injections given twice daily for seven days or until the mice died.

Subacute or chronic infections were induced by intravenous injections of smaller doses of live nocardial cells which allowed the test animals to survive for two weeks or longer. In this case, treatment was started 3 to 5 days post infectionem and continued for two weeks or until the mice died.

Apart from sulfadiazine which was applied intraperitoneally all of the antimicrobial agents used were injected intravenously in doses per kg body weight which corresponded to a high dosage treatment in humans. The therapeutic response was assessed by comparing the death rates in the treated group of animals with that in the control group or by comparing the viable counts of nocardial cells obtained from selected internal organs (lungs, liver, spleen, kidneys). For each test, the treated and control groups usually consisted of 50 and 10 animals, respectively.

RESULTS AND DISCUSSION

In Vitro Studies

The results of the in vitro tests are summarized in Tables 1 to 4. Because of the large number of antimicrobials tested, the tables only give the minimal concentrations of the respective antibiotics that inhibited 50% (MIC_{50}) and 90% (MIC_{90}) of the test strains, respectively.

The vast majority of Nocardia strains included in the study were moderately to highly resistant to most of the ß-lactam antibiotics currently in use (Table 1). This appears to be predominantly due to the production of potent ß-lactamases which have been identified in pathogenic nocardiae (20, 34, 39) and which appear to be especially active in members of the species N. brasiliensis and N. otitidis-caviarum. Only a few representatives of the still heterogeneous taxon N. asteroides exhibited moderate in vitro sensitivity to certain penicillins and some of cephalosporins. These findings are in good agreement with most of the reports in the literature although some authors have claimed susceptibility of the N. asteroides complex to certain penicillins and cephalosporins, especially in earlier reports (2,6,9,14,15, 17,21,23,25,29,30,31,38,41). The new ß-lactam compound imipenem (N-formimi-

doyl thienamycin)also showed no therapeutically significant *in vitro* activity against *N. brasiliensis* and *N. otitidis-caviarum*. However, this substance was able to inhibit growth of many of the *N. asteroides* and *N. farcinica* strains at *in vivo* achievable levels. Thus, *N. asteroides* isolates were inhibited at concentrations between 0.39 and 12.5 mg/l. The MIC-values for *N. farcinica* were found to range from 0.78 to 50 mg/l. 90% of the test strains of both species did not grow at 6.25 mg/l (Table 1) so that the majority of *N. asteroides* and *N. farcinica* strains can be considered susceptible or at least moderately susceptible to imipenem.

Table 1

IN VITRO SUSCEPTIBILITY OF PATHOGENIC NOCARDIAE TO 38 ß - LACTAM ANTIBIOTICS

	MIC_{50}* and MIC_{90}* (mg/l) for							
	N. asteroides		*N. farcinica*		*N. brasiliensis*		*N. otitidis-caviarum*	
	MIC		MIC		MIC		MIC	
Antibiotics	50	90	50	90	50	90	50	90
Penicillins :								
Benzyl penicillin epicillin ampicillin amoxicillin mezlocillin azlocillin	6.25 to 50	>100	≥100	>100	> 100	> 100	>100	>100
Ciclacillin carbenicillin ticarcillin piperacillin apalcillin oxacillin flucloxacillin	50 to >100	>100	>100	>100	> 100	> 100	>100	>100
Amoxicillin + 10 mg/l clavulanic acid	6.25	100	12.5	25	12.5	25	>100	>100
Cephalosporins :								
Cephapirine cefamandole cefuroxime cefoxitine cefotaxime cefotiame cefsulodine cefoperazone cefmenoxime ceftizoxime ceforanide	6.25 to 50	50 to >100	25 to >100	100 to >100	>100	>100	> 100	>100
Cephalothin cephacetrile cephradine cefazoline cefazedone cephalexine cefaclor cefadroxile cefatrizine ceftazidime latamoxef	50 to >100	100 to >100	50 to >100	>100	> 100	> 100	>100	>100
N - Formimidoyl thienamycin	3.13	6.25	3.13	6.25	> 100	> 100	>100	>100

* MIC_{50} / MIC_{90} = Minimal inhibitory concentrations (mg/l) of 50 and 90% of test strains, respectively

Schaal, 1985

These in vitro data concur with the results of other investigators (7,10,14, 15,16,17,20) and indicate that thienamycin might be of value for treating human infections due to N. asteroides or N. farcinica.

Another interesting result of our in vitro tests is the effect which was exerted by clavulanic acid when added to amoxicillin (Table 1). In accord with other investigators (20) we found that clavulanic acid at various concentrations was able to inhibit nocardial ß-lactamases so that the MICs of amoxicillin were significantly reduced. The action of clavulanic acid was most striking in N. farcinica, less pronounced in N. asteroides and N. brasiliensis, and nearly lacking in N. otitidis-caviarum. The detailed MIC-values of amoxicillin plus 10 mg/l clavulanic acid ranged from 0.78 to >100 mg/l in N. asteroides, from 3.13 to 25 mg/l in N. farcinica, from 6.25 to 100 mg/l in N. brasiliensis, and from 25 to > 100 mg/l in N. otitidis-caviarum. These in vitro results suggest that amoxicillin combined with clavulanic acid might be of use in the treatment of certain N. farcinica and N. asteroides infections, but would probably be less valuable in managing diseases caused by N. brasiliensis or N. otitidis-caviarum.

The activity of aminoglycoside antibiotics against pathogenic nocardiae was by far more heterogeneous than that of the ß-lactams (Table 2). Only streptomycin and spectinomycin were ineffective against all of the test organisms at therapeutically achievable concentrations. Kanamycin was completely inactive against N. asteroides, N. farcinica and N. brasiliensis, but inhibited growth of N. otitidis-caviarum at surprisingly low levels ranging from 0.095 to 1.56 mg/l. In contrast, most strains of N. asteroides, N. brasiliensis, and N. otitidis-caviarum were moderately to highly sensitive to gentamicin while N. farcinica proved to be completely resistant. Similar results at slightly lower MIC-values were obtained for sisomicin and dibekacin (Table 2). Tobramycin showed a very good inhibitory effect on N. brasiliensis and was moderately active against N. asteroides, but was not able to inhibit N. farcinica and N. otitidis-caviarum at therapeutically achievable concentrations. The only aminoglycosides to which members of all of the Nocardia species tested were susceptible were netilmicin and amikacin (Table 2). In accord with other investigators (2,8,13,15,16,17,22,38) the latter substances would therefore appear to be good candidates for being entered in the list of chemotherapeutics recommendable for the treatment of all forms of nocardial infections. All of the organisms tested were completely resistant to pristinamycin and rifampicin (Table 2). Novobiocin was only active against N. otitidis-caviarum. A good to moderate in vitro sensitivity of N. asteroides and N. farcinica to fusidic acid has already been reported previously (4,21,29,30). The MIC-values for N. brasiliensis were similar to those of N. asteroides and N. farcinica (Table 2). However, N. otitidis-caviarum appeared to be considerably more resistant.

In general, tetracyclines, chloramphenicols, macrolides, and lincomycins were not very active against pathogenic nocardiae (Table 3). In several independent studies with different strains which were performed between 1969 and 1984, we have always obtained similar results. Nevertheless, the international literature is full of controversial opinions concerning the in vitro and in vivo effectivity of these drugs (see 1,2,29,30). In our hands, the only tetracyclines which showed a reasonable degree of in vitro activity against the pathogenic nocardiae were doxycycline and minocycline (Table 3). N. brasiliensis and N. otitidis-caviarum appeared to be slightly more susceptible to tetracyclines in general than were members of the species N. asteroides and N. farcinica. These differences were found to be especially pronounced for demethyl chlortetracycline.

Table 2

IN VITRO SUSCEPTIBILITY OF PATHOGENIC NOCARDIAE TO AMINOGLYCOSIDES, NOVOBIOCIN, PRISTINAMYCIN, FUSIDIC ACID, AND RIFAMPICIN

	MIC $_{50}$* and MIC $_{90}$* (mg/l) for							
	N. asteroides		N. farcinica		N. brasiliensis		N. otitidis - caviarum	
	MIC		MIC		MIC		MIC	
Antibiotics	50	90	50	90	50	90	50	90
Streptomycin	25	> 100	100	>100	50	50	100	> 100
Kanamycin	50	> 100	>100	>100	> 100	> 100	0.78	1.56
Gentamicin	0.78	12.5	100	>100	0.19	0.39	0.19	0.39
Sisomicin	0.19	3.13	25	25	0.19	0.19	0.19	0.19
Tobramycin	0.19	25	50	100	0.048	0.19	25	100
Dibekacin	0.095	6.25	50	100	0.048	0.095	0.095	0.39
Netilmicin	0.095	0.78	0.39	0.78	0.048	0.095	0.095	0.19
Amikacin	0.78	1.56	1.56	3.13	0.78	1.56	1.56	3.13
Spectinomycin	25	> 100	> 100	>100	> 100	> 100	50	100
Novobiocin	100	> 100	>100	>100	> 100	> 100	0.78	3.13
Pristinamycin	50	100	100	>100	50	100	100	> 100
Fusidic acid	1.56	25	3.13	3.13	3.13	6.25	25	50
Rifampicin	> 100	> 100	>100	>100	> 100	> 100	> 100	> 100

* MIC_{50} / MIC_{90} : Minimal inhibitory concentrations (mg/l) of 50 and 90% of test strains, respectively

Schaal, 1985

Apart from a weak in vitro activity of clindamycin against N. otitidis-caviarum, none of the other substances listed in Table 3 produced MICs that might be of therapeutic interest. Thus, doxycycline and minocycline appear to be the only candidates among the substances listed in Table 3 which could have some value in in vivo experiments or for clinical application.

Sulfonamides although commonly considered the drugs of choice for treating nocardial infections generally showed a comparatively weak in vitro activity against pathogenic nocardiae (Table 4). This is also true for sulfadiazine which has been most widely used in the management of human infections due to N. asteroides. Nevertheless, the MIC_{50} and MIC_{90} values for sulfadiazine given in Table 4 do not look so favourable. A more detailed analysis of the data shows that at least certain strains of the pathogenic Nocardia species exhibited a higher in vitro susceptibility. Thus, 8 strains of N. asteroides were inhibited by sulfadiazine concentrations ranging from 0.78 to 25 mg/l. Nine strains of N. farcinica and 10 out of 14 strains of

Table 3

IN VITRO SUSCEPTIBILITY OF PATHOGENIC NOCARDIAE TO TETRACYCLINES, CHLORAMPHENICOLS, MACROLIDES, AND LINCOMYCINS

	MIC^{*}_{50} and MIC^{*}_{90} (mg/l) for							
	N. asteroides		N. farcinica		N. brasiliensis		N. otitidis-caviarum	
	MIC		MIC		MIC		MIC	
Antibiotics	50	90	50	90	50	90	50	90
Chlortetracycline	50	>100	>100	>100	25	50	50	100
Oxytetracycline	50	100	100	>100	50	100	>100	>100
Tetracycline - HCl	25	50	50	100	25	50	25	50
Rolitetracycline	50	>100	100	100	25	50	50	100
Demethylchlor-tetracycline	25	100	100	100	12.5	12.5	12.5	25
Doxycycline	6.25	25	25	50	6.25	6.25	1.56	3.13
Minocycline	6.25	12.5	6.25	12.5	6.25	12.5	3.13	6.25
Methacycline	50	100	100	>100	50	100	50	100
Chloramphenicol	50	100	25	25	100	100	50	100
Thiamphenicol	>100	>100	>100	>100	>100	>100	>100	>100
Erythromycin	>100	>100	>100	>100	50	>100	>100	>100
Oleandomycin	>100	>100	>100	>100	>100	>100	>100	>100
Spiramycin	>100	>100	>100	>100	>100	>100	>100	>100
Lincomycin	>100	>100	>100	>100	>100	>100	>100	>100
Clindamycin	>100	>100	>100	>100	>100	>100	6.25	25

$^{*}MIC_{50}$ / MIC_{90} = Minimal inhibitory concentrations (mg/l) of 50 and 90% of test strains, respectively

Schaal, 1985

N. brasiliensis fell in the same range of MIC-values. However, all of the 10 N. otitidis-caviarum isolates tested grew well at sulfadiazine concentrations up to 100 mg/l so that this species appears to be completely resistant to this sulpha drug.

The other sulpha compounds included in the study gave similar or even slightly more unfavourable results with one exception: Sulfamethoxazole appeared to be distinctly more active against N. asteroides and N. brasiliensis strains (Table 4). The susceptibility of N. brasiliensis was especially pronounced with MIC-values ranging from 0.78 to 6.25 mg/l. The favourable response of certain Nocardia strains to sulfamethoxazole basically explains the promising results reported for the combination of this sulpha compound

Table 4

IN VITRO SUSCEPTIBILITY OF PATHOGENIC NOCARDIAE TO SULPHA DRUGS, IMIDAZOLES, FOSFOMYCIN, VANCOMYCIN, AND PIPEMIDIC ACID

	MIC_{50}* and MIC_{90}* (mg/l) for							
	N. asteroides		N. farcinica		N. brasiliensis		N. otitidis - caviarum	
	MIC		MIC		MIC		MIC	
Antibiotics	50	90	50	90	50	90	50	90
Sulfadiazine	>100	>100	>100	>100	6.25	>100	>100	>100
Sulfamethoxydiazine	>100	>100	>100	>100	>100	>100	>100	>100
Sulfadimethoxine	>100	>100	>100	>100	3.13	>100	>100	>100
Sulfametrole	>100	>100	>100	>100	>100	>100	>100	>100
Sulfamethoxazole	6.25	>100	>100	>100	1.56	3.13	>100	>100
Tetroxoprime	100	>100	>100	>100	>100	>100	>100	>100
Clotrimazole	1.56	3.13	3.13	3.13	3.13	3.13	6.25	6.25
Miconazole	3.13	6.25	12.5	12.5	6.25	6.25	6.25	12.5
Fosmomycin	>100	>100	>100	>100	>100	>100	>100	>100
Vancomycin	>100	>100	>100	>100	>100	>100	>100	>100
Pipemidic acid	100	100	>100	>100	>100	>100	>100	>100

* MIC_{50} / MIC_{90} Minimal inhibitory concentrations (mg/l) of 50 and 90% of test strains, respectively

Schaal, 1985

with trimethoprim in the literature (4,15,16,38) because at least at ratios of 1:10 to 1:20 trimethoprim to sulfamethoxazole, a significant synergistic effect cannot be demonstrated (3,5,19,29,35). Only at a ratio of 9:1 which is probably without value for therapeutic purposes synergism was generally recorded (3). The variable and comparatively weak inhibitory effect of sulfonamides on pathogenic nocardiae is apparantly the reason for treatment failures with these drugs and for the recommendations of a long-term, high-dosage therapy.

It is still uncertain as to whether or not the rather low MIC-values found for clotrimazole and miconazole (Table 4) are of any therapeutic value because these imidazole derivatives do not produce high plasma levels in vivo so that their action on nocardial infections of deep tissues should be limited. Fosfomycin, vancomycin, and pipemidic acid showed no therapeutically utilizable in vitro activity at all (Table 4). The same is true for a variety of antituberculotic drugs that were tested but not included in the tables because they did not show any in vitro effect at the concentrations used.

Animal Studies

Based upon the in vitro results discussed above and upon reports from the literature the following antimicrobials were chosen for application in the mouse model: sulfadiazine, sulfamethoxazole plus trimethoprim, gentamicin,

netilmicin, amikacin, amoxicillin plus clavulanic acid, ticarcillin plus clavulanic acid, minocycline, and fusidic acid.

As could nearly be expected from the _in vitro_ data and from the mode of action of sulpha drugs, the response of acute _N. asteroides_ (N14) and _N. farcinica_ (N301) infections in mice to treatment with sulfadiazine was comparatively weak. The colony counts obtained from the kidneys of the treated animals were not significantly different from those of the control animals over a period of 8 or 9 days (Fig. 1). Surprisingly, the same was true for those animals treated with fusidic acid.

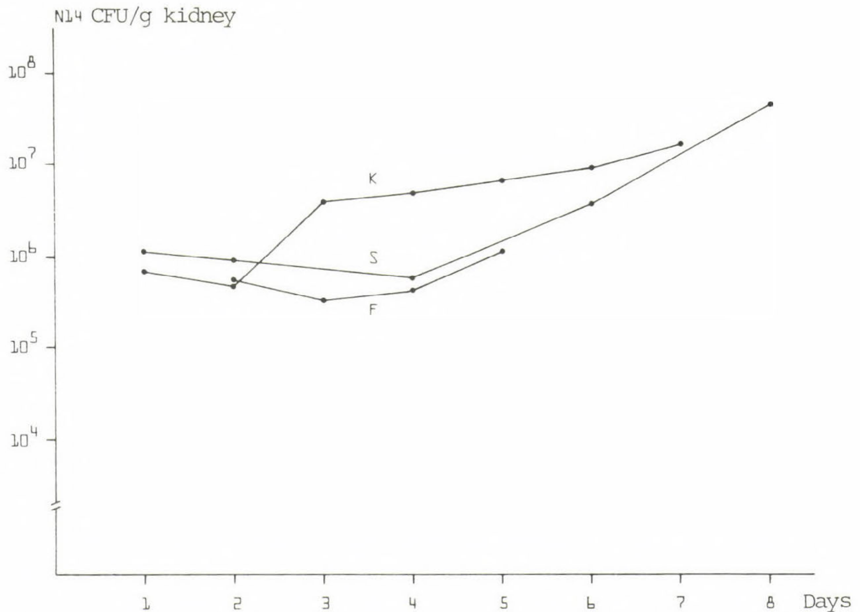

Fig. 1: Colony counts determined at 1 to 8 day time intervals from the kidneys of mice infected with high doses of _N. asteroides_ N14 and treated with sulfadiazine (S) or fusidic acid (F).
K = control group.

The corresponding results for the other drugs included in the study are shown in Figures 2 and 3. The graphs shown in these figures are based upon the viable counts obtained 42 days after infection from the kidneys. Thus, only those of the test substances are mentioned in the figures that allowed survival of the animals for 42 days.

The acute experimental infection due to _N. asteroides_ (N14) was best controlled by amoxicillin plus clavulanic acid (AUG) and amikacin (A), respectively (Fig. 2). Under treatment with these drugs, no viable nocardiae could be recovered from the kidneys when the test was terminated. Application of sulfamethoxazole plus trimethoprim (co-trimoxazole=C), sulfadiazine (S), and minocycline (M) prolonged the survival of the test animals but did not eradicate the infecting microorganisms from the kindeys, at least as far as high doses infections are concerned. Under gentamicin and netilmicin treat-

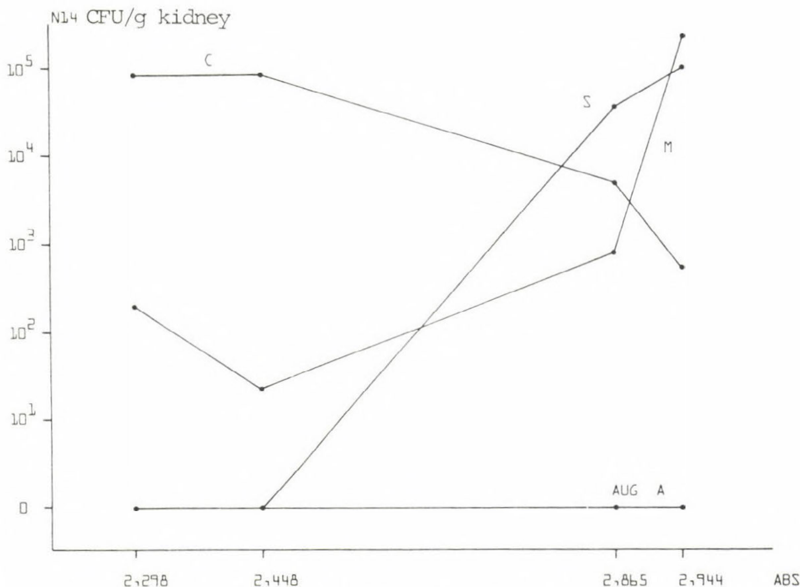

Fig. 2: Colony counts obtained from the kidneys of mice infected with various doses of N. farcinica N301 and treated with amoxicillin plus clavulanic acid (AUG), amoxicillin plus clavulanic acid plus amikacin (AUG + A), minocycline (M), amikacin alone (A), and netilmicin (N).

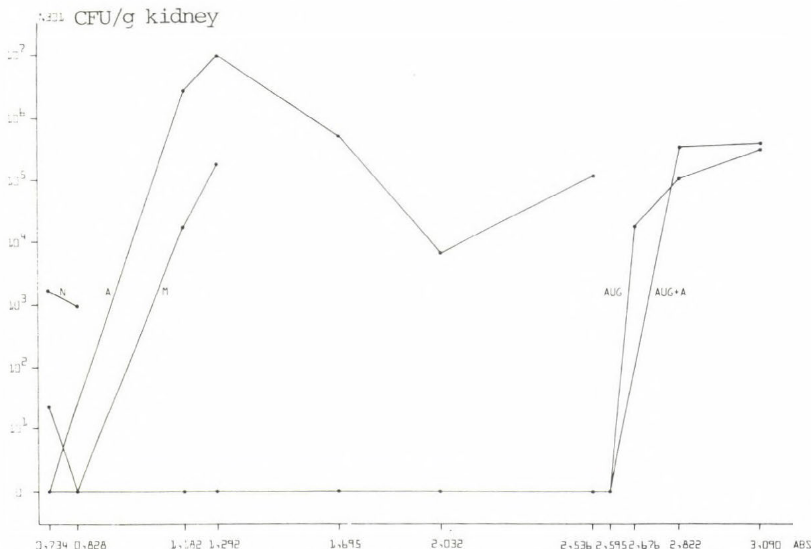

Fig. 3: Colony counts obtained from the kidneys of mice infected with various doses of N. asteroides N14 and treated with amoxicillin plus clavulanic acid (AUG), amikacin (A), minocycline (M), sulfadiazine (S), and co-trimoxazole (C).
ABS = Optical absorption of N. suspensious.

ment, those animals infected with low numbers of viable nocardiae survived and no nocardiae were recovered from their kidneys. However, from the animals which had received higher challenge doses no one was alive 42 days after infection. Treatment with fusidic acid and with ticarcillin plus clavulanic acid led to early death even of those animals which were infected with small numbers of nocardiae. In the case of ticarcillin plus clavulanic acid this might be due to both the lower activity of this combination against N. asteroides and the comparatively low doses at which the combination was used.

The results obtained from the animals infected with N. farcinica (N301) differed considerably from those of the N. asteroides infection (Fig. 3). Only amoxicillin plus clavulanic acid and a triple combination of amoxicillin, clavulanic acid, and amikacin were able to eliminate the infecting organisms from the kidneys over a wide range of infecting dosages. Neither amikacin alone nor netilmicin or minocycline were able to produce this favourable effect (Fig. 3). Fusidic acid, sulfadiazine, sulfamethoxazole plus trimethoprim, gentamicin, and ticarcillin plus clavulanic acid were completely or largely ineffective.

A very similar picture was obtained when the afficacy of the various chemotherapeutics was measured by the percentage of test animals that died under treatment (Fig. 4). Best survival rates of mice infected with N. asteroides were obtained with amoxicillin plus clavulanic acid (AUG) and, surprisingly, with minocycline. Co-trimoxazole, sulfadiazine, and amikacin were less efficient but still showed a marked therapeutic effect. Gentamicin and netilmicin only had a small positive influence on the survival of the test animals. Ticarcillin plus clavulanic acid and fusidic acid again appeared to be completely inactive.

In the acute N. farcinica infection model, amoxicillin plus clavulanic acid and amoxicillin plus clavulanic acid plus amikacin exhibited by far the best therapeutic efficacy (Fig. 4). The response to amikacin and minocycline was less favourable but still marked. Sulfadiazine, ticarcillin plus clavulanic acid, co-trimoxazole, and netilmicin were only able to lower the death rate in those animals which had received small numbers of infecting nocardiae. Gentamicin and fusidic acid showed no effect at all.

The response of the chronic N. asteroides and N. farcinica infections was similar to that of the acute model. Nevertheless, amoxicillin combined with clavulanic acid gave very promising results in both species which were by far more favourable than those obtained with any other antimicrobial agent included in the study.

The survival rates of mice chronically infected with N. asteroides and treated with amoxicillin plus clavulanic acid, minocycline or sulfadiazine are shown in Fig. 5. Under our test conditions, amoxicillin plus clavulanic acid and minocycline were nearly equally effective, whereas sulfadiazine showed results which were only slightly better than that of the untreated control mice. Chronic N. farcinica infections were also well controlled by amoxicillin plus clavulanic acid. The death rates under amikacin and sulfadiazine treatment were considerably higher but still clearly distinct from that of the control group.

The results of the animal experiments were all evaluated statistically. This evaluation showed that the differences noted between the therapeutic efficacy of amoxicillin plus clavulanic acid and the triple combination amoxicillin plus clavulanic acid plus amikacin on the one hand and of all of the other drugs used on the other hand were statistically significant. In addition, the survival rates under amikacin and minocycline treatment were signifi-

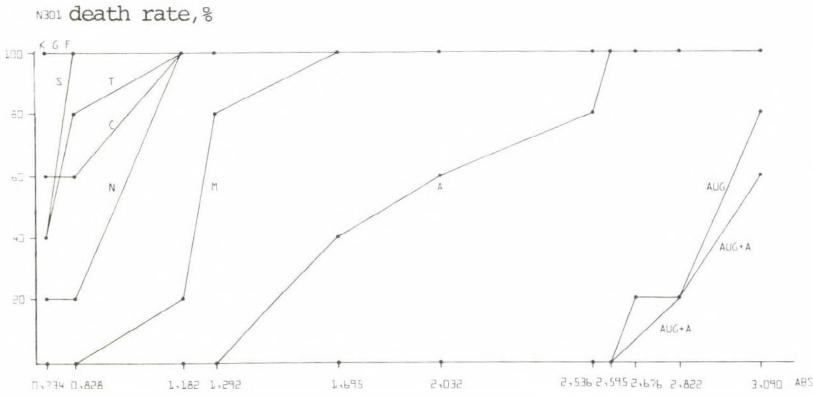

Fig. 4: Death rates observed in mice infected with various doses of N. farcinica N301 and treated with amoxicillin plus clavulanic acid (AUG), amoxicillin plus clavulanic acid plus amikacin (AUG + A), amikacin alone (A), minocycline (M), netilmicin (N), co-trimoxazole (C), ticarcillin plus clavulanic acid (T), sulfadiazine (S), fusidic acid (F), and gentamicin (G).
K = control group.

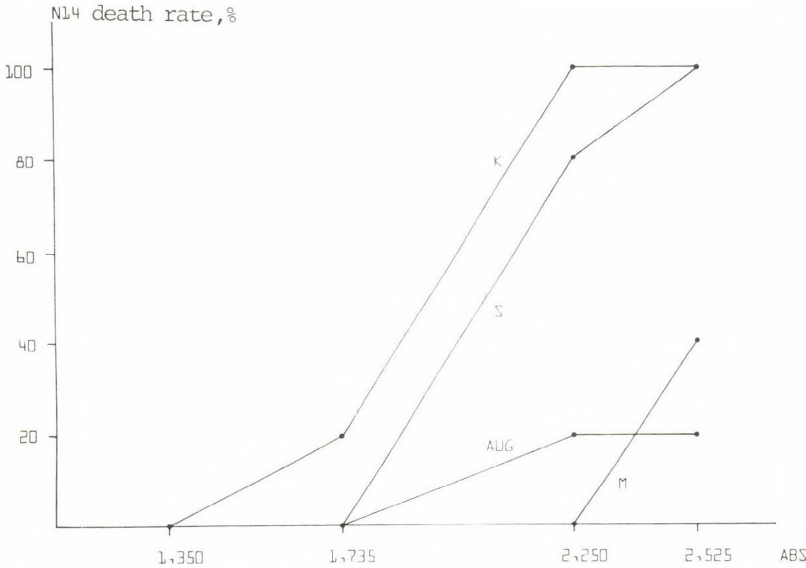

Fig. 5: Death rates observed in mice chronically infected with various doses of N. asteroides N14 and treated with amoxicillin plus clavulanic acid (AUG), minocycline (M), and sulfadiazine (S).
K = control group.

cantly better than that of the untreated control group. However, only in some of the experiments the therapeutic effect of sulfadiazine and co-trimoxazole could be confirmed statistically.

It is difficult to compare our *in vivo* results with relevant data from the literature because animal models as well as dosages and types of application of the antibiotics used were usually different. Nevertheless, it appears that sulfadiazine was not as effective in our hands as it was in most of the other studies (1,12,18,25,26,33,37,40) while the results obtained for minocycline and amikacin correspond well with the data of other investigators (1,40).

In general, our *in vivo* experiments showed that there is a considerable degree of correlation between the activity of certain antimicrobial agents against pathogenic nocardiae determined *in vitro* and the efficacy of these drugs in a mouse model. The only one striking exception which was noted was the lack of *in vivo* efficiency of fusidic acid although this substance had a pronounced inhibitor effect on *N. asteroides* and *N. farcinica* *in vitro*. However, a detailed analysis of the mouse experiments showed that fusidic acid in the dosages used apparently exerted a toxic effect on Swiss Webster mice because the death rates in the fusidic acid treated groups were generally higher than those in the untreated control groups. Therefore, it remains to be clarified as to whether or not fusidic acid is definitely inactive *in vivo*.

Although the results derived from animal studies are usually thought to be more relevant for the application to human infections than are mere *in vitro* data, it remains difficult to predict how human infections would behave under analogous therapeutic procedures. We are, therefore, glad that we have had the opportunity to get some experience with amoxicillin plus clavulanic acid plus amikacin and with imipenem plus amikacin in the treatment of human nocardiosis.

The triple combination amoxicillin plus clavulanic acid plus amikacin was used in a 49 year-old male patient who was suffering from chronic lymphatic leukaemia and had developed severe progressive pneumonia and lung abscess due to *Nocardia farcinica* (36). A few days after a high dosage treatment with amoxicillin, clavulanic acid, and amikacin had been started his condition slowly improved and the lung infiltration as well as the abscess began to resolve. The antibiotic therapy was continued for 3 weeks after which the patient was dismissed from the hospital free of fever and in good condition.

The second case history is that of a 61 year-old male patient who underwent prosthetic heart valve replacement (11). Although the operation had been tolerated well, the patient did not recover properly and developed intermittent fever which did not respond to treatment with cephalosporins and mezlocillin plus tobramycin. Blood cultures showed growth of *N. farcinica* so that the patient was put on oral treatment with 12 g sulfadiazine per day which soon led to intolerability reactions. Amoxicillin plus clavulanic acid plus amikacin in high doses only temporarily reduced fever and leucocyte counts. The antibiotic therapy was therefore changed to imipenem and amikacin in single doses of 1.5 g and 500 mg, respectively, under which the serum of the patient was bactericidal for *N. farcinica* at a dilution of 1:4. Under this treatment, the patient recovered quickly and was dismissed home without further antibiotic treatment.

3 months later the patient was admitted to the hospital again because of septic temperatures. Blood cultures were again positive for *N. farcinica* which was still susceptible to imipenem and amikacin. These drugs were therefore

given again and a replacement of the aortic valve prosthesis was performed. Postoperatively, the patient recovered surprisingly quickly and developed neigher fever nor an acceleration on the erythrocytes sedimentation rate. Three weeks later, the patient was dismissed from the hospital in good condition and has remained well until today.

The results derived from our in vitro and in vivo experiments as well as the two case reports briefly presented here seem to indicate that the treatment of human nocardiosis can be improved by the application of amoxicillin plus clavulanic acid, thienamycin, and amikacin. Possibly, a combination of one of these ß-lactam compunds with amikacin will have the greatest potential for becoming the drugs of choice in the management of human nocardiosis.

SUMMARY

40 strains of Nocardia farcinica (N. asteroides, Biovar B), 30 N. asteroides sensu stricto (N. asteroides, Biovar A), 14 N. brasiliensis, and 10 N. otitidis-caviarum isolates were tested for their in vitro susceptibility to 85 antimicrobial agents using a modified agar dilusion technique. Among the drugs included in the study only a few exhibited in vitro activities which appeared to be of clinical and/or taxonomic interest. As far as N. asteroides and N. farcinica are concerned, favourable results were particularly obtained with the combination of amoxicillin and clavulanic acid, and with N-formimidoyl thienamycin, amikacin, and minocycline. The latter antibiotics were also tested for their protective and curative efficacy in vivo using a mouse model with sulfadiazine as reference substance. In these animal experiments, amikacin, amoxicillin plus clavulanic acid, and minocycline proved to be therapeutically most active being definitely more effective than sulfadiazine. The clinical usefulness of amoxicillin plus clavulanic acid plus amikacin and thienamycin plus amikacin could be confirmed in two patients suffering from serious pulmonary nocardiosis and prosthetic aortic valve endocarditis, respectively.

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DETERMINANTS OF INFECTION IN NOCARDIOSIS

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INTRODUCTION

It is now well established that Nocardia asteroides, N. brasiliensis and N. otitidiscaviarum may be primary pathogens in several animal species including humans (Beaman et al., 1976). Furthermore, progressive disease caused by N. asteroides is frequently recognized within mammalian hosts that have a variety of immunocompromising conditions. Thus, N. asteroides is emerging as an important opportunistic pathogen in both human and veterinary medicine (Beaman and Sugar, 1983). The actual incidence of disease caused by these organisms worldwide is not known and previous attempts to determine their prevalence in humans and other animals have probably grossly underestimated their numbers. Thus, infections in dogs, cattle and humans are not rare, and indeed, may be common (Beaman and Sugar, 1983).

The mechanisms of host resistance to nocardiae as well as the determinants of nocardial pathogenesis have been a focus of investigation for the past several years. In order to dissect the host response to Nocardia and to study the biochemical, and structural basis for nocardial pathogenesis, animal models were developed that would permit simultaneous in vivo and in vitro investigations. Histological and ultrastructural analysis demonstrated that several properties of Nocardia grown in vitro changed during growth within the host. Most of these modifications were visualized in the cell envelope. Furthermore, the less virulent strains of Nocardia exhibited the greatest alterations in structure while the more virulent organisms were affected less (Beaman, 1973). One of the properties that appeared to be readily altered within the host was the degree of acid-fastness. Generally, the nocardiae grown in vivo appeared to be more strongly acid-alcohol-fast than the same organism grown in Brain Heart Infusion or Trypticase soy agar. These acid-fast nocardiae could still be distinguished from acid-fast mycobacteria by pyridine extraction. Nocardial acid-fastness was lost during pyridine extraction whereas mycobacteria remained acid fast after such extraction procedures (Beaman and Burnside, 1973).

Cells walls of N. asteroides have been analyzed chemically and ultrastructurally. It was demonstrated that the cell envelope was chemically and ultrastructurally complex. Significant chemical and structural

modifications occurred within the wall during the growth cycle (Beaman, 1975; Beaman *et al.*, 1981). Furthermore, the specific alterations in composition of the cell wall were dependent upon the strain of *N. asteroides* and appeared to correlate with the relative virulence of that particular strain. Also, the growth stage dependent changes in the envelope appeared to reflect additional and corresponding changes in virulence (Beaman and Maslan, 1978). During the past few years there have been several reviews concerning the pathogenesis of *Nocardia* in humans and other animals. Therefore the discussion presented below will emphasize the more recent observations regarding determinants of infection in nocardiosis (Beaman, 1981a,b; Beaman, 1982; Beaman and Sugar, 1983; Beaman, 1984a,b,c; Filice *et al.*, 1984; Schaal and Beaman, 1984).

MACROPHAGE-NOCARDIA INTERACTIONS

Three strains of *N. asteroides* were selected to serve as models for studying the mechanisms of nocardial pathogenesis because they were significantly different in their virulence for mice. It was found that following intravenous injection, *N. asteroides* GUH-2 was most virulent, *N. asteroides* 14759 was intermediate in virulence and *N. asteroides* 10905 was least virulent when all other experimental conditions were the same (Beaman and Maslan, 1977). These nocardiae exhibited specific organ predilections when injected intravenously into mice, and these "tropisms" were generally the same as those seen in naturally acquired infections (Beaman and Maslan, 1977).

Cells of *N. asteroides* GUH-2 (virulent), *N. asteroides* 14759 (intermediate virulence) and *N. asteroides* 10905 (low virulence) have been incubated *in vitro* with a variety of phagocytic cells obtained from humans, rabbits and mice (Bourgeois and Beaman, 1974; Beaman and Smathers, 1976; Beaman, 1977; Beaman, 1979; Filice *et al.*, 1980a; Davis-Scibienski and Beaman, 1980 a,b,c; Filice *et al.*, 1980b; Black *et al.* 1983; Filice, 1983; Beaman *et al.*, 1985; Black *et al.*, 1985a,b,c). In alveolar macrophages obtained from non-immune animals, the more virulent strains of *Nocardia* were not killed whereas the less virulent organisms (*N. asteroides* 10905) were effectively eliminated. However, since macrophages that reside in different anatomical regions may differ in their phagocytic and bactericidal capacities, experiments were performed to compare nocardial interactions with alveolar, peritoneal, splenic and hepatic (Kupffer cells) macrophages obtained from normal and immunized mice (Black *et al.*, 1985a).

There was good correlation between the *in vitro* functional activities of macrophages obtained from the lungs, liver, peritoneum and spleen of mice compared to the *in vivo* clearance of *N. asteroides* from these sites following either intravenous (IV), intranasal (IN) or intraperitoneal inoculation (Beaman *et al.* 1982; Black *et al.*, 1985a). Each population of macrophage differed significantly in their phagocytic capacities (Table 1). In general, macrophages obtained from immune animals were more bactericidal for *N. asteroides* than macrophages from non-immune animals (Tables 1 and 2). The relative virulence of different strains of *Nocardia* also had an influence on the effectiveness of these macrophages (Tables 1 and 2).

TABLE 1

Comparison of In Vitro functional Activities of Different Populations of Macrophages from Non-Immunized Mice for N. asteroides GUH-2 (virulent) and N. asteroides 10905 (less virulent).

Organism	Macrophage	Phagocytic Index[a]	P-L-fusion[b]	%Killed[c]
N. asteroides GUH-2	Kuppfer	15.4	0	0
	Peritoneal	22.6	25.3	21.7
	Splenic	9.7	38.3	26.4
	Alveolar	16.2	0	0
N. asteroides 10905	Kuppfer	15.5	0	0
	Peritoneal	28.8	75.0	9.1
	Splenic	35.0	39.0	34.6
	Alveolar	28.0	74.2	15.4

a Represents percent of Nocardia phagocytized in 1 hour (MOI = 5:1)
b Phagosome-lysosome fusion based on Acridine orange staining at 3 hrs after infection (Davis-Scibienski and Beaman, 1980a).
c Percent of phagocytized Nocardia killed after 8 hrs incubation within macrophages.

TABLE 2

Comparison of In vitro Functional Activities of Different Populations of Macrophages from Immunized Mice for N. asteroides GUH-2 (Virulent) and N. asteroides 10905 (less virulent).[a]

Organism	Macrophage	Phagocytic Index[b]	% Killed[c]
N. asteroides GUH-2	Kuppfer	17.9	71.7
	Peritoneal	16.1	55.9
	Splenic	11.7	35.0
	Alveolar	21.4	0
N. asteroides 10905	Kuppfer	28.4	52.5
	Peritoneal	32.2	81.5
	Splenic	35.2	68.3
	Alveolar	36.8	58.1

a Mice were immunized with formalin killed cells of N. asteroides GUH-2 (Black et al., 1985).
b Represents percent of Nocardia phagocytized in 1 hour (MOI = 5:1)
c Percent of phagocytized Nocardia killed after 8 hrs incubation within macrophages.

Hepatic Macrophages

Kuppfer cells (hepatic macrophages) from normal mice phagocytized both the virulent and less virulent strain of N. asteroides at the same rate. These macrophages were not able to kill either strain of Nocardia within 8 hours; and, indeed both strains of bacteria grew rapidly within these phagocytes (Table 1). Phagosome-lysosome (P-L) fusion was not seen in Kuppfer cells 3 hours after phagocytizing either strain of Nocardia, but P-L fusion did occur in hepatic macrophages inoculated with Candida used as a control. Kuppfer cells from immunized mice were very different from those obtained from normal mice (Tables 1 and 2). The phagocytic index was increased for both GUH-2 and 10905, however, the degree of enhanced uptake was greatest for 10905. In contrast, the Kuppfer cells killed GUH-2 more effectively than 10905 once these bacteria were phagocytized (Table 2). This may be due, in part, to the immunization of the mice with antigens from N. asteroides GUH-2.

Peritoneal Macrophages

Peritoneal macrophages from normal mice also phagocytized both strains of Nocardia at a similar rate, but the intracellular events that occurred were different for each strain of bacterium (Table 1). N. asteroides GUH-2 was more effective at inhibiting P-L fusion (75% inhibition) than strain 10905 (25% inhibition). Nevertheless, the peritoneal macrophages reduced the colony forming units (CFU) of strain GUH-2 about 20 percent within 8 hours while there was only a 9 percent reduction in CFU of strain 10905 during this same period of time. Even though there appeared to be a difference between the ability of peritoneal macrophages to kill GUH-2 and 10905 (about 2 fold) these differences were probably minor. More importantly, strain 10905 appeared to be relatively more resistant to the lethal effects of the lysosomal contents of normal peritoneal macrophages than GUH-2 (Table 1). The phagocytic ability of peritoneal macrophages obtained from immunized mice was not significantly different from those obtained from normal animals. However, macrophages from immune mice were very effective at killing cells of strain 10905 (Table 2). These phagocytes were not as effective for strain GUH-2 (Table 2).

Alveolar Macrophages

There were clearly defined differences in the interactions between N. asteroides GUH-2 and strain 10905 with alveolar macrophages. For example, alveolar macrophages were more capable of phagocytizing the less virulent 10905. Strain GUH-2 effectively inhibited P-L fusion whereas 10905 did not (Table 1). Furthermore, strain GUH-2 grew within these macrophages while the CFU of strain 10905 were decreased after 8 hours incubation. The immunization of mice with formalin killed cells of N. asteroides GUH-2 did not alter significantly the interaction of alveolar macrophages with GUH-2. In contrast, alveolar macrophages from immunized mice had increased phagocytic uptake and enhanced bactericidal activity against strain 10905 (Table 2).

Splenic Macrophages

Splenic macrophages from normal mice were not effective in phagocytizing cells of N. asteroides GUH-2 while they were more able

to phagocytize cells of strain 10905 (approximately 4 fold increase: Table 1). There was an equal, but modicum, level of P-L fusion for cells of both strains GUH-2 and 10905 following uptake. At 8 hours after uptake the CFU of both strains of Nocardia were reduced similarly. Splenic macrophages from immunized mice demonstrated only a slightly increased killing capacity for GUH-2 whereas they killed 10905 significantly better than macrophages from normal animals. The rate of phagocytosis was not changed in these macrophages (Tables 1 and 2).

As shown above, the effectiveness of host macrophages to phagocytize and inhibit or kill N. asteroides depended upon the anatomical source of the phagocyte, the specific strain of Nocardia, and the immune status of the host. Thus, in normal mice, the order of phagocytic capacity of macrophage populations against strain GUH-2 was peritoneal >Alveolar >Kuppfer >splenic, whereas, for strain 10905 the order was Splenic >Peritoneal >Alveolar >Kuppfer (Table 1). The order of effectiveness of macrophages from immunized mice to kill Nocardia was quite different since Kuppfer cells became most effective at killing strain GUH-2 while peritoneal macrophages killed strain 10905 the best (Table 2).

DETERMINANTS OF NOCARDIAL PATHOGENESIS

In order for Nocardia to be a successful pathogen, it must have the capacity to establish itself within the host. Since the phagocytic cells of the reticuloendothelial system form the major cellular defenses of the host, Nocardia should have defined mechanisms for evading these defenses. As described above, certain populations of macrophages are ineffective at killing N. asteroides. As a consequence, these bacteria are facultatively intracellular pathogens capable of growing within macrophages (Bourgeois and and Beaman, 1974; Beaman and Smathers, 1976; Beaman, 1976; Beaman, 1977; Beaman, 1979; Filice et al. 1980a). Furthermore, it was shown that human polymorphonuclear (PMN) leukocytes and monocytes are also unable to kill virulent strains of N. asteroides (Filice et al., 1980b; Beaman et al., 1985).

Polymorphonuclear phagocytes and macrophages have at least two basic mechanisms for killing microorganisms. One mechanism, involves the generation of toxic oxygen products and represents an oxygen dependent process, while the other is oxygen independent and probably utilizes the toxic and degradative components of lysosomes (Beaman and Beaman, 1984). Virulent microorganisms must be able to either evade, overcome, neutralize or resist these microbicidal activities in order to grow within the host.

Resistance of Nocardia to Oxidative Killing Mechanisms of PMN Leukocytes

Since N. asteroides GUH-2 induces an oxidative metabolic burst upon contact with PMN leukocytes, this organism must be relatively resistant to toxic oxygen metabolites because it is not killed by these phagocytes (Filice et al., 1980b). It was found that the virulent strain GUH-2 produced an unique superoxide dismutase (SOD) that was secreted into the growth environment and became surface associated. The less virulent strain 10905, which was killed by PMN leukocytes, did not secrete this SOD (Beaman et al., 1983). In addition to SOD, N.

asteroides produced large quantities of catalase. Since both of these compounds are important in destroying toxic oxygen metabolites, their role in nocardial pathogenesis was studied (Beaman *et al.*, 1985; Filice, 1983).

It was found that human neutrophils killed approximately 80 percent of the cells of the less virulent strain 10905, but they were unable to kill any of the cells of the virulent GUH-2 at early-stationary-phase of growth. It is important to note, however, that PMN leukocytes were able to kill approximately 50 percent of the cells of strain GUH-2 at log phase of growth after 3 hours incubation. The content of both SOD and catalase within cells of strain GUH-2 is growth stage dependent. Log phase cells secrete more SOD than stationary phase cells however, in contrast, stationary phase cells have approximately 10 times greater amounts of catalase than cells in log phase of growth (Beaman *et al.*, 1985).

Antibody prepared against SOD purified from strain GUH-2 neutralized its activity. When early stationary phase cells of GUH-2 were pretreated with this antibody prior to incubation with PMN leukocytes, it was found that these phagocytes were then able to kill about 50 percent of the bacterial cells within 3 hours. PMN neutrophils did not kill cells of *N. asteroides* GUH-2 that had been incubated with antibody which had little or no anti-SOD activity. Furthermore, pre-treatment of the PMN's with chlorpromazine abbrogated their ability to kill cells of GUH-2 coated with anti-SOD IgG suggesting that the lethal effects of the PMNs for these nocardial cells were due to oxidative metabolites since chlorpromazine inhibited the oxidative metabolic burst of the PMNs without inhibiting their phagocytic capabilities. Thus, SOD on the surface of the cells of GUH-2 helped protect them from the toxic superoxide radicals produced by host phagocytes (Beaman *et al.*, 1985).

SOD dismutates superoxide to produce H O which is also very toxic to bacterial cells. Hydrogen peroxide is broken down by catalase. Therefore catalase was purified from the cytoplasm of strain GUH-2 and combined either alone or in combination with SOD to log phase cells of strains GUH-2 and 10905 prior to and during incubation with PMN leukocytes. Catalase added alone to strain 10905 only partially protected it from being killed, however SOD plus catalase completely protected these cells from oxidative killing by PMNs. Catalase added to log phase cells of GUH-2 protected them from the microbicidal activities of PMN neutrophils while adding SOD had no effect. This was probably due to the fact that log phase cells of GUH-2 already had maximal amounts of surface associated SOD. A mutant of *N. asteroides* GUH-2 which was more virulent during log phase of growth than the parental strain, was isolated (Vistica and Beaman, 1983). This mutant (SCII-C) was shown to possess more than 7 times greater amounts of catalase during log phase than the parental GUH-2. PMN leukocytes did not kill log phase cells of this high catalase mutant. These observations indicate that both catalase and SOD are important for the resistance of *N. asteroides* to the oxygen-dependent microbicidal activities of human phagocytes. Therefore, catalase as well as surface associated and secreted superoxide dismutase are associated with nocardial virulence and appear to represent at least two virulence factors (Beaman, *et al.* 1985).

Even though catalase and SOD appear to represent two important virulence factors, it is clear that there are other attributes that define the mechanisms of nocardial pathogenesis. The bases of nocardial virulence are complex and multifaceted. For example virulent cells of Nocardia have components that are toxic to host cells, modulate phagocyte function, and permit growth within the host. Avirulent cells do not have these capabilities.

As shown in Table 1, virulent N. asteroides GUH-2 inhibits phagosome-lysosome fusion in most populations of macrophages obtained from normal mice. In contrast, the less virulent strain 10905 does not significantly inhibit fusion in either alveolar or peritoneal macrophages. N. asteroides 14759 is of intermediate virulence for mice and it has an intermediate ability to inhibit P-L fusion (Beaman and Maslan, 1977; Davis-Scibienski and Beaman, 1980a,b,c). In Mycobacterium, data support the role of a surface associated sulfolipid as the biochemical agent responsible for inhibition of phagosome-lysosome fusion (Goren et al., 1976; Goren and Brennan, 1979). Prabhudesi et al., (1981) reported that they were able to detect sulfolipids extracted from N. asteroides; however, efforts to detect sulfolipids extracted from N. asteroides GUH-2 were unsuccessful (Feistner and Beaman, unpublished data). Instead, significant amounts of trehalose-dimycolate (cord factor) were found in strain GUH-2. Some of this cord factor material had Rf values on TLC similar to sulfolipid obtained from M. tuberculosis (kindly supplied by M. Goren). Further chemical characterization revealed that this material did not contain detectable sulfolipid (Feistner and Beaman, unpublished data). These results suggested that sulfolipids were not responsible for the inhibition of phagosome-lysosome fusion caused by N. asteroides GUH-2. Therefore, the biochemical basis for the inhibition of P-L fusion by virulent strains of N. asteroides remains unknown.

Modulation of Lysosomal Enzymes by Nocardia

Virulent and less virulent strains of N. asteroides were incubated with murine macrophages obtained from different anatomical regions (Tables 1 and 2). The lysosomal enzymes acid phosphatase, lysozyme, non-specific, esterase and neutral protease were measured within individual macrophages using a computerized cytospectrophotometer (Black et al., 1983; 1985a,b). The cells of virulent strain GUH-2 induced a significant decrease in lysosomal acid-phosphatase activity during phagocytosis by Kuppfer cells, alveolar and peritoneal macrophages from normal mice. This decrease in acid phosphatase activity did not occur following phagocytosis of the less virulent strain 10905 (Black et al., 1983; 1985a). In addition, there was a correlation between the nocardial induced modulation of acid-phosphatase and the ability of various populations of macrophages to either inhibit or kill N. asteroides (Black et al. 1985a). Those macrophages that could kill either virulent or less virulent strains of N. asteroides (primarily those macrophages from immunized mice) had increased lysosomal acid-phosphatase activity following ingestion of increasing numbers of bacteria. In contrast, those macrophages that were unable to either kill or inhibit the growth of Nocardia (especially Kuppfer cells and alveolar macrophages from normal mice) lost significant amounts of acid-phosphatase activity following uptake of increasing numbers of organisms. Acid phosphatase levels remained relatively constant in those macrophages that inhibited the

growth of, but did not kill, cells of Nocardia (Black et al., 1985a). Since it is unlikely that acid-phosphatase was involved in the microbicidal activities of these macrophages, this enzyme might serve as a useful marker for nocardial modulation of overall macrophage function. The specific mechanisms of nocardial induced modulation of acid-phosphatase activity within macrophages is not known. However, lysosomal degranulation appeared not to be the mechanism since the amounts of other lysosomal enzyme markers (non-specific esterase-neutral protease and lysozyme) did not decrease following uptake of Nocardia. Indeed, some of these enzymes increased in activity at the same time acid-phosphatase activity decreased. Furthermore, the rate of decrease in acid phosphatase following phagocytosis of N. asteroides GUH-2 was nearly linear as a function of time. If degranulation had occurred, the loss of enzyme activity would not be linear over a 3 hour incubation period (Black et al. 1985b). Therefore, the differential modulation of lysosomal enzyme activities by virulent and less virulent strains of N. asteroides within different macrophages populations result from generalized mechanims that affect the enzyme activities differently. This reflects the organism's ability to evade the bactericidal mechanisms of macrophages (Black et al., 1983; 1985a,b).

Modulation of Phagosomal pH by Nocardia

The lysosomes within macrophages are acidic with an approximate pH value of five. These lysosomes normally fuse with phagocytic vacuoles, phagosomes, to produce phagolysosomes. Shortly after P-L fusion, the pH of the phagosome drops from near neutrality to pH 5. In addition, the phagosomes may become acidified by mechanisms independent of P-L fusion (Geisow et al., 1981). These two mechanisms of acidification of the phagocytic vacuole probably play an essential role in the bactericidal properties of macrophages. Many bacteria are either inhibited or killed at this low pH, and many of the degradative lysosomal enzymes have their optimal activities in an acidic environment. Microorganisms that have the capacity to neutralize the acidification of the phagosome should be more successful in evading the killing mechanisms of the macrophage.

The phagosomal pH of murine peritoneal macrophages was determined following phagocytosis of virulent and less virulent strains of N. asteroides (Black et al., 1985c). It was found that the less virulent strain 10905 did not effectively prevent phagosomal acidification and the pH of the phagosome containing the Nocardia dropped from about pH 7.5 to approximately pH 6 in 80 minutes (Black et al., 1985c). In sharp contrast, the virulent N. asteroides GUH-2 completely blocked acidification so that the phagosomal pH never went below pH 7 even after 2 hours incubation (Black et al., 1985c). Since the pH of the phagosome remained above pH 7 during prolonged incubation, the virulent strain GUH-2 prevented both early acidification which was independent of P-L fusion and late acidification which occured as the result of P-L fusion. The mechanisms whereby virulent N. asteroides prevent phagosomal acidification and less virulent strains do not are not known; however, it is probable that this capability is important for both the intracellular survival and virulence of this pathogen (Black et al., 1985c).

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HISTOLOGICAL AND ULTRASTRUCTURAL STUDIES OF HUMAN ACTINOMYCETOMAS

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ABSTRACT

Twenty six clinically, histologically and microbiologicaly defined cases of Actinomycetoma (25 from Lara and Falcon States, Venezuela, and 1 from California, USA) were studied. The following etiologic agents were found: Actinomadura madurae, Streptomyces somaliensis, Nocardia brasiliensis, N. asteroides and N. otitidiscaviarum. In addition, 5 of these cases were studied microbiologically by using hypertonic culture media for the isolation of L forms. Cutaneous punch biopsy was used to obtain granules and tissue samples. The ultrastructural study of this material revealed the presence of a cellular infiltrate consisting of PMN leucocytes, activated macrophages, lymphocytes and monocytes. Microorganisms were both extracellular in the granules and intracellular in PMN phagocytes and macrophages.

The granule contained coccoidal, bacillary and filamentous cells. In addition, abnormal pleomorphic organisms were observed. In three cases of Actinomadura, granules inoculated into hypertonic culture media resulted in the growth of spherules that appeared to be like L forms.

INTRODUCTION

Actinomycetomas are localized, chronic, destructive and progressive infections of skin and subcutaneous tissues caused by aerobic actinomycetes. The infections are characterized by subcutaneous granulomas and abscesses, as well as by induration, sinus tract formation and the production of granules. These granules (grains) are colonies of the etiologic agent present within the infected tissue, and they are discharged through single or multiple sinus tracts present in the lesion (1). These infections are generally associated with the tropical and semi-tropical regions of the world (1-9); but, these fistulous tumors also occur in temperate zones (10-13).

Venezuela is a Republic, located at the northern extremity of the South American Continent, with a location between the Equator and the

Tropic of Cancer. Venezuela has a tropical to subtropical climate. Most of the mycetomas that have been reported in the Venezuelan literature (6, 14-20) came from the State of Lara, with additional cases reported from the States of Falcon, Yaracuy and Portuguesa. Lara State has valleys and hills with altitudes that range from 300 to 2500 meters, and this region forms a transitional zone between the coastal mountain range and the Andes. It comprises about 3% of the total area of Venezuela and this state is located in the central western region of the country. Lara State has two major types of forest; (1) a semi-arid (xerophytic) forest with a rain index of 200-500 mm/yr at altitudes up to 400 meters and (2) a deciduous forest with a rain index of 1000-2000 mm/yr at altitudes of up to 2500 meters.

Most cases of Actinomycetoma have been reported from regions with the xerophytic type of forest. This type of forest is dry, with trees and bushes such as *Acacia tortuosa*, *Acacia flexuosa*, *Cereus sp*, *Prosepis fuliflora*, *Amarantus espinosus* and different cactus, such as, *Opuntia caribea* and *Opuntia wentiana* predominating. Since these trees and cactus plants have spines and thorns, it is very common for individuals to puncture their skin while working in this area.

According to Risquez (21), the first Venezuelan case of Actinomycetoma was observed and studied by Rafael Rangel in 1909, and most of these early reports on Actinomycetoma in Venezuela involved lesions located on the feet. The first Venezuelan case of Actinomycetoma not involving the foot was reported by O'Daly in 1938 (23). There have been no detailed studies that have attempted to determine the extent or epidemiology of Actinomycetomas in Venezuela, and most of the publications are reports of clinical cases (14, 16-18, 20). Only Campins, (15, 19) Convit *et al*. (20) and de Albornoz (6) have reviewed cases of Actinomycetoma infections throughout Venezuela.

So far, *Nocardia brasiliensis* has been the most frequently encountered etiologic agent of Actinomycetoma followed, in order of frequency, by *Actinomadura madurae*, *Streptomyces somaliensis*, *Nocardia asteroides*, *Nocardia otitidiscavarum* and *Actinomadura pelletieri* (Serrano, Unpublished Data). In the present report, 25 cases of Actinomycetoma were reviewed clinically, histologically and microbiologically. Of these cases, 23 were from Lara State, and two were from Falcon State (Fig. 1). Four of these 25 cases were studied microbiologically for L-forms by using hypertonic culture media and electron microscopy.

CLINICAL ASPECTS OF PATIENTS

The clinical features of 25 cases of Actinomycetoma are summarized in Table 1. The data were obtained by personal interviews with the patients; by visiting the patient's home and by records kept at the Central Hospital. The information given by the patient was reconfirmed by reviewing his or her previous clinical records in the Dermatology Unit of the Central Hospital. Most cases of Actinomycetoma were from Jimenez County in Lara State (9 cases), followed by Iribarren County (4 cases), Palavecino and Torres Counties (3 cases in each county), Crespo county (2 cases) and from Federation County in Falcon State (2 cases). (Fig. 1).

FIGURE 1. Geographic distribution of the 25 cases studied by County (Distrito) and etiologic agent in Lara and Falcon States, Venezuela.

The average age of the patient was 47 years (range, 17 to 80 years) and 80% of patients were male while 20% were female. This sex distribution is similar to that previously reported by Convit et al. (20). Actinomycetoma involved the foot in 72% of cases, and it involved the body in 16% of the cases. Twelve percent of the cases were localized to the upper extremities. Osteolytic bone lesions was reported in 82% of the cases. Eighty five of the patients were farmers or goat keepers while the remaining 15% had other occupations.

ETIOLOGIC AGENTS AND PATHOLOGY

The culture-proven etiology of Actinomycetoma in Lara and Falcon States are shown in table 1 (12 cases). N. brasiliensis was isolated in 5 cases, N. asteroides in 2 cases, A. madurae in 2 cases, S. somaliensis in 2 cases and N. otitidiscaviarum in 1 case. In each of the 25 cases, the diagnosis was also based on the granule morphology and tissue reaction pattern (1, 24-27).

The presence of a small grain (80 to 100 m in diameter), with a round or oval morphology was observed in hematoxylin and eosin stained sections of mycetomas produced by Nocardia spp (13 cases). In these cases the tissue reaction pattern was that of a microabscess surrounded by granulation tissue. In mycetomas caused by Actinomadura madurae (8 cases) the tentative species diagnosis was based upon the grain morphology. Act. madurae typically forms a multilobed or vermiform shaped grain with a peripheral band that stains deep blue, while the centre is either devoid of colored material or it is a faint blue (1, 24, 26). The granule is surrounded by an eosinophilic zone and the tissue pattern is that of a nonspecific inflammatory reaction with microabscesses, encircled by macrophages with a foamy cytoplasm, resulting in a lesion that has an appearance of a histiocytoma (Fig. 2b). Lesions produced by S. somaliensis (4 cases) have granules that have a compact matrix of amorphous material, and they are brittle upon sectioning. Using Hand E staining, the center of the granule is stained weakly (1, 24, 26) (Fig. 3b-c). The tissue reaction is that of a granuloma with the grain in the center of the lesion, surrounded by PMN leucocytes, lymphocytes, plasma cells and epithelioid cells mixed with multinucleated giant cells, and surrounded peripherally by fibrosis (Fig. 3b-c).

TREATMENT

Therapy with antimicrobial agents appeared to cure 18 of 25 patients (see Table 1). The best results were obtained with Sulfonamides; in particular the combination of trimethroprim (TP)-Sulphamethoxazole (SMT) either alone or in combination with dapsone or streptomycin. Rifampin, ampicillin, erythromycin, tetracycline and ethambutol, were less effective. Surgical amputation was performed in 3 cases (table 1); however, two of these relapsed with infection reappearing at the stump, approximately one year after the surgical treatment. In both cases the infection was treated with TP-SMT, with a good response to the treatment. Out of the 25 cases, 3 individuals died from their infection. One of these patients was a male (case No. 11) whereas two were female (cases No. 9 and 18). In 3 of the five female cases (cases No. 1, 9 and 18) the disease got much worse during pregnancy of these patients.

TABLE 1

EPIDEMIOLOGICAL, CLINICAL, MICROBIOLOGICAL, PATHOLOGICAL AND THERAPEUTICAL FEATURES OF 25 CASES OF ACTINOMYCETOMA IN LARA AND FALCON STATES, VENEZUELA

Case No.	Year first examined	Age & Sex	County; Town	Part of the body affected	Diagnosis made by: Histology	Diagnosis made by: Culture	Bone lesion X-ray	Therapy: Anti-microbial	Therapy: Surgery	Comments
1	1975	17,F	Crespo; Agua Fria	Foot	+	N.brasiliensis +	none	PN:20 days	none	year 1966 injured with stone lesion reappear during pregnancy CURE
2	1984	49,M	Crespo; Paso de Tacarigua	Chest	+	A.madurae	Infiltrative lesion right lung, lytic lesion costal arc	AMP: NR	none	Good response to TP. Improved, under observation
3	1978	21,M	Iribarren; Barquisimeto	Foot	+	A.madurae -	none	TP+SMT: 6 months	none	Initial trauma with spike of baseball shoe CURE
4	1975	40,M	Iribarren; Barquisimeto	Foot	+	N.brasiliensis +	none	SZ:33 days LAS: 4 months	none	Patient originally came from Yaracuy State CURE

TABLE 1 continued

5	1982	54,M	Iribarren; El Garabatal	Foot	+	A.madurae - "L form"like bodies in Hypertonic media	Osteolytic lesion in tarsus pedis and toes	TP+SMT:1year DDS: 1year	none	Initial trauma with a thorn 25 years ago; under treatment not cured
6	1983	46,M	Iribarren; Buena Vista	Shoulder	+	N.brasiliensis +	none	TM+SMT: 16 months PN:NR	none	CURE
7	1965	42,M	Jimenez; Quibor	Foot	+	A.madurae +	Osteolytic lesion 3 and 4th metatarsus and 5th toe	DDS:18 months PN: 18 months TC: 9 months TP+SMT:2years	partially removed	Good response to TM+SMT. CURE
8	1967	49,M	Jimenez; Quibor	Foot	+	A.madurae +	lost definition of cortical margin endosteal bone cavitation, geode	DDS:18 months PN: 4 months SZ: 1 month TR: 8 days ANFB:3 months TP+SMT:6 year	AMP-BK 1973	no good response to drug therapy. Relapse infection in stump a year after amp. CURE
9	1969	36,F	Jimenez; Quibor	Head & Neck	+	S.somaliensis +	Diffuse thickening of skull bones and osteosclerosis frontal parietal right side bone	TR+S:NR PN:NR OTR:9 months DDS:14 months Mi: 80 days	none	Initial trauma thorns from a YACURE tree. Pregnancy increase disease not resolved Death, after cerebral and eye invasion

10	1974	44,M	Jimenez; Quibor	Arm	+	N.brasiliensis	+	Loss of bone density periosteal rising	PN:70 days SZ:15 days TR:20 days	partially removed	Disease started 11 years ago. Good response. CURE
11	1975	75,M	Jimenez; Cuara	Foot	+	Nocardia spp.	-	osteolytic lesions	SZ: 50 days AMP: 20 days TR: 20 days	none	Disease started 15 years ago. Initial trauma with a thorn. Death after acute renal insufficiency
12	1977	38,M	Jimenez; Campo Lindo	Foot	+	A.madurae	-	osteolytic lesions ostersclerosis	TP+SMT: 17 days	none	Initial trauma with truck tire. Evolution disease 6 years. CURE
13	1979	58,M	Jimenez; El Tunal	Leg	+	S. somaliensis	-	none	TP+SMT: 17 months STR:NR	none	Initial trauma with a thorn from Yacure tree. 15 yrs evolution CURE
14	1980	52,M	Jimenez; Maguaje	Foot	+	N. asteroides	+	none	TP+SMT: 6 months	none	Initial trauma thorn from a Cuji tree. 9 months evolution. CURE

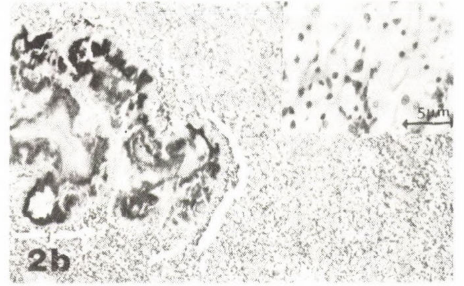
TABLE 1 continued

15	1984	17,F	Jimenez; Quibor	Foot	+	Nocardia spp. -	none	TP+SMT:NR	none	Initial trauma with a cactus thorn. 18 months evolution. CURE
16	1955	66,M	Moran; El Tocuyo	Leg	+	A. madurae -	none	TB+SMT: 1 year DDS:NR STR: 1 month	AMP-AK	31 years evolu- of the disease. CURE
17	1969	42,M	Moran; El Tocuyo	Foot	+	N. caviae +	none	PN:NR	none	8 months evolu- tion CURE
18	1978	35,F	Palave- cino; Guamacire	Back	+	N. brasiliensis +	osteolytic lesion cer- vical and dorsal vertebrate and first ribs.	TP+SMT:NR AMK: 8 days RIF: 23 days DDS:NR KT: 38 days Eth: 6 months	none	Initial trauma with bamboo splints. All drug therapy un- successful. Disease increas- ed with preg- nancy. Death due to septicemia after neurogenic bladder and para- plegic syndrome.
19	1977	57,M	Palave- cino; La Miel	Chest arm	+	N. brasiliensis -	none	PN: 7 days TM+SMT: 2 months	none	CURE
20	1980	33,F	Palave- cino; La Miel	Leg	+	Nocardia spp. -	none	ER:20 days TP+SMT:NR	none	under treatment

21	1976	56,M	Torres; Atarigua	Leg	+	S. somaliensis	+	diffuse osteoporosis	TP+MT:1year DDS:NR AMP:NR RIF:NR	AMP-BK	Infection relapse in stump resolved and CURE
22	1982	60,M	Torres; El paso de Carora	Leg Knee	+	Nocardia spp.	-	osteolytic lesions	TP+SMT: 2 years PN:NR	lesion partially removed	14 years ago, lesion in the knee
23	1978	39,M	Torres; Los Arangues	Foot	+	A. madurae "L form: like structures isolated in hypertonic media	-	osteolytic lesion toes	TP+SMT: 10 months PN:NR DDS:3 years	none	11 years evolution, initial trauma with a cactus thorn. Cure, after 2 years relapse of infection
24	1978	22,M	Urdaneta; Siquisiqui	Foot	+	S. somaliensis	-	none	TP+SMT:NR RIF:NR	none	CURE
25	1972	33,M	Falcon State Federacion; Churuguara	Chest	+	N. brasiliensis	+	none	PN:5 mos.	partially removed	CURE

PN=Penicillin, SZ=Sulfadiazine, AMP=Ampicillin, ER=Erythromycin, TP=Trimethoprim, SMT=Sulphamethoxazole, LAS=Los action Sulfa, DDS=Dapsone, TC=Tetracycline, ANFB=Anfotericin B, TR=Terramycin, S=unspecified sulfonamide, OTR=Oxytetraciclyn, STR=streptomycin, Mi=Miconazole, AMK=Amykacin, NR=Not reported, ETH=ethambutol, RIF=rifampin, AMP=amputation, AMP-BK=amputation below knee, AMP=AK=above knee, + means, diagnosis made based on biopsy or in culture results.

2a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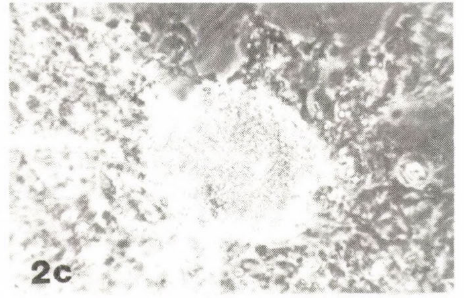
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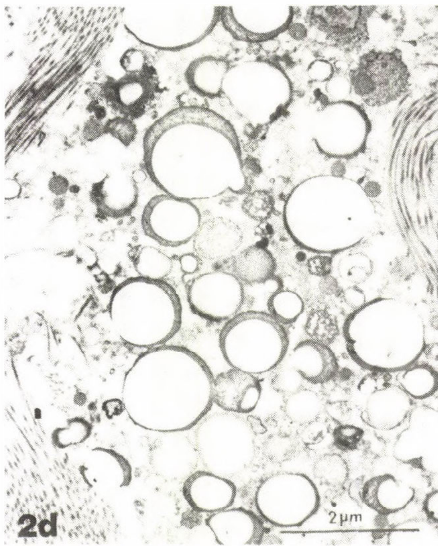
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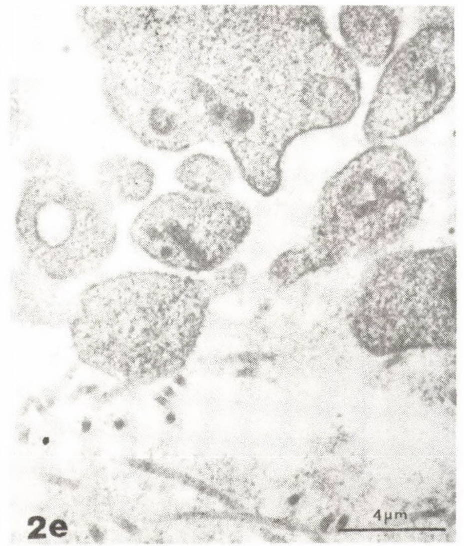
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Cultivation of L-Forms

From the 25 cases presented in table 1, four active cases (cases No. 5, 13, 18 and 23) were studied using "L Form" culture media; and, tissue samples and granules obtained from these patients were observed by electron microscopy. Tissue samples or grains were obtained asceptically by cutaneous punch biopsy from the patients and immediately transferred to a transport liquid medium with the following composition: Glycerol (0.5); polyethylene glycol 4000, (4%); and sucrose (20%). The samples were kept at room temperature and maintained in this medium for up to 15 days without losing viability. The samples were homogenized gently by using a Tekmar tissue homogenizer, and then asceptically transferred to the following media: a) BYE Broth consisting of Brain Heart Infusion (Difco, Detroit, MI); fresh yeast extract (0.4%); sucrose (20%); NaCl (4%) and horse serum (Gibco, previously checked for mycoplasma (10%) and adjusted to pH. 7.0: b) Middlebrook 7H9 broth (DIFCO) (4.7 gr./l.); glycerol (0.5%); Polyethylene glycol 4000 (4%) OADC supplement (DIFCO) (200 ml/l); and horse serum (10%) and adjusted to pH. 7.0. After inoculation, the media were incubated at 37 C either aerobically; in 2 to 3% CO_2, or anaerobically by using anaerobic jars (Gaspak, BBL) with hydrogen plus Carbon dioxide generator envelopes (Gaspak, BBL) and a palladium catalyst to ensure complete removal of oxygen.

Samples to be evaluated for the presence of L-Forms were obtained from 1 case of infection due to N. brasiliensis (case No. 18), 2 cases of A. madurae (cases No. 5, 23) and 1 case of S. somaliensis (case No. 13) (Table 1). After 1 week of incubation, both the BYE-L broth and the Middlebrook 7H9 L-form broth inoculated with samples from cases No. 5 and 23 (See Table 1) were positive for granules and spherical-like organisms (Fig. 2c). No conventional bacteria were isolated from these two cases following inoculation of these samples into standard laboratory media such as BHI agar, BHl broth, blood agar, Tryptic Soy agar or broth, and Mueller-Hinton agar or broth). Positive growth of granules and spherules were obtained from case No. 5 aerobically, in 2-3% CO_2 and anaerobically.

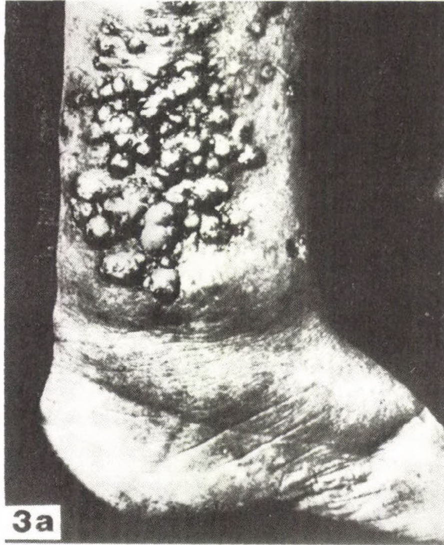
FIGURE 2a. Foot lesion, case No. 5 (Table 1). Actinomycetoma due to A. madurae, 25 years evolution of the disease.

2b. H&E of a biopsy (case No. 5). Showing a grain of A. madurae, multilobed with an irregular border, showing a peripheral band; deeply stained. The grain is surrounded by a nonspecific inflammatory reaction composed of mainly PMNs. Around the microabscess is an extensive infiltration of histiocytes ("foam cells").

2c. Granules and spherules obtained from cultivation of A. madurae grains in Middlebrook 7H9 L-Form media (hypertonic).

2d. Thin section of tissue sample from case No. 5. Note the presence of numerous granules and spherules, protoplast-like cells. Some of them show intracellular elementary bodies, also these bodies are observed free in the mass of the grain.

2e. Thin section of "L-Form"-like structures present in the tissues of patient No. 5. Note the presence of tubular structures inside of the cytoplasm of the cells. Mesosome-like structures can be observed in the large cell.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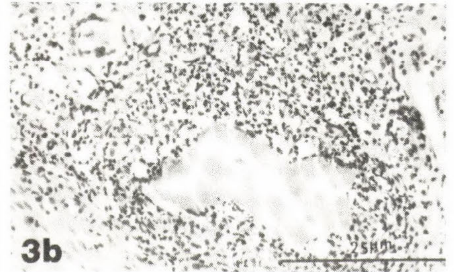
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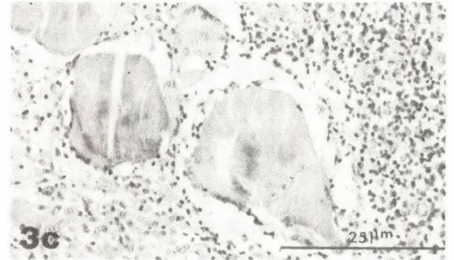
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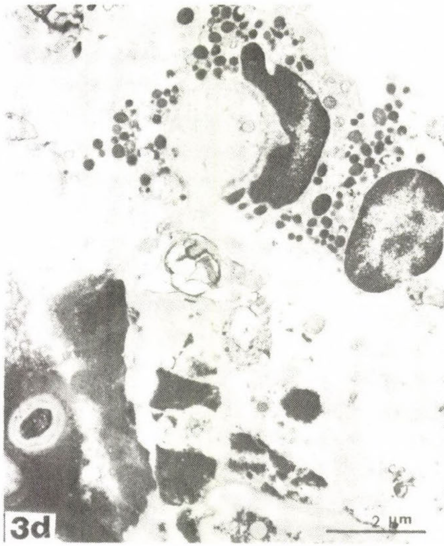
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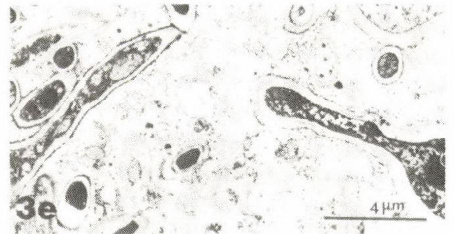
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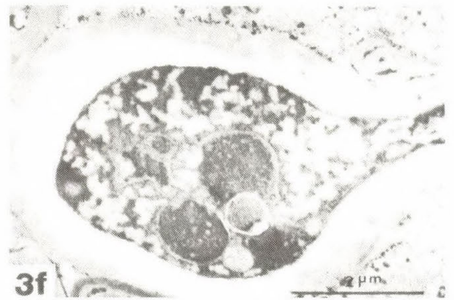
3f

Positive growth was obtained aerobically using the Middlebrook L-Form media incubated with tissue from case No. 23. The growth of L-Form-like cells obtained from either BYE broth or Middlebrook 7H9 L-form media was transferred to standard laboratory media; however, even after two months of incubation no growth of conventional bacteria was obtained.

Light and Electron Microscopy of L-forms

Tissues or granules obtained by cutaneous punch biopsy were fixed in 10% formalin for light microscopy and in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH. 7.2) for electron microscopy (E.M.). The E.M. samples were postfixed in osmium tetroxide, dehydrated, embedded in Epon and sectioned. Thin sections were observed and photographed using either a HITACHI 500 E.M. or a Philips-400 E.M.

The results of the study of tissue from case No. 5 (Table 1) are presented in Fig. 2e, and the results of case No. 13 are shown in Fig. 3e-f. In samples from case 5, we observed the presence of granules and spheres similar to type A "L-Forms" (28). In case 13, structures were observed that resembled type B "L Forms" (28). In both cases (No. 5 and 23), the tentative diagnosis of the etiologic agent was made based on the histological observation of the grain within the lesions. The ultrastructural studies revealed that the grains were primarily composed of granules and spherules, very similar to the L-form variants of *N. caviae* 112 observed by Beaman and Scates (29).

Thus far, positive cultures of "L-Form"-like structures, have been obtained from tissues and grains of 4 cases using hypertonic culture media. Electron microscopy of these "L Form"-like structures suggest the possible presence of "L phase variants" in many cases of chronic infections due to the Actinomycetes.

←

FIGURE 3a. Actinomycetoma of the leg due to *S. somaliensis*, case No. 13 (Table 1), after 15 years evolution of the disease.

3b,c. H&E of biopsy (case No. 13), showing a grain of *S. somaliensis*. Note the cracks as a result of sectioning. The hyphae are closely packed in a cement-like material. The grain is surrounded by a granuloma type tissue reaction composed of PMNs, lymphocytes, plasma cells, epithelioid cells and multinucleated giant cells with a peripheral fibrosis.

3d. Electron micrograph of the peripheral portion of a *S. somaliensis* grain. Some bacterial cells are observed, all of them surrounded by an outermost dense layer. The cells were embedded in a highly dense material. Active PMNs with several lysosomal granules dispersed around the outer portion of the cell were observed. These were in close association with the bacterial grain.

3e,f. Electron micrograph of the central portion of a *S. somaliensis* grain. Note the presence of bacillary and pleomorphic filamentous bacterial cells devoid of cell wall. Round elementary type bodies can be observed in the cytoplasm of these cells.

More studies are necessary in order to elucidate the possible role of these "L form"-like structures in the pathogenecity of these microorganisms. It should be noted that only in 12 of the 25 cases, the diagnosis was made by culture. It should be further emphasized that in cases No. 8 and 21 both patients had lesions in the foot and leg and when these were amputated the infection reappeared in the stump. Also in case No. 23 the patient was considered clinically cured after four years of treatment; but, the infection reappeared at the same site two years after the patient had stopped these treatments. Cultivation of the grains obtained from the lesions of these patients failed to yield growth in standard microbiological media, and growth of granules and spheres was obtained only in the hypertonic media designed for growing "L Forms". Furthermore, in case No. 5, which is still active, no growth of conventional bacteria has been possible by using standard culture media; but, organisms were obtained by using the hypertonic media for "L Forms". In case No. 18, a patient with an active mycetoma of the back, on only three occasions during almost 10 years of evolution of this disease was it possible to obtain positive cultures of *N. brasiliensis*. The cases described above strongly suggest that *N. brasiliensis*, *A. madurae*, and *S. somaliensis* may be present and active within mycetomatous lesions in an altered cellular state. Furthermore, these altered cellular forms appear to play a role in some of the chronic, progressive infections caused by the aerobic Actinomycetes. The possible role of altered cellular variants of *Nocardia* has been well demonstrated by Bourgeois and Beaman (30, 31), Beaman and Scates (29) and Beaman (32, 33).

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PATHOGENICITY AND IMMUNOLOGY

Mini-Symposium

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SECRETION OF 2,3-DIHYDROXYBENZOIC ACID BY VIRULENT NOCARDIA ASTEROIDES GUH-2

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The mechanisms of pathogenesis of N. asteroides (NA) are complex and not well understood. This bacterium is not readily killed by host phagocytes, and its ability to evade these defenses appears to be associated with inhibition of phagosome-lysosome fusion, alteration of macrophage lysosomal enzyme activity, and neutralization of the oxidative killing mechanisms of neutrophils (Beaman et al. 1985). In addition to cell-associated components, NA may produce soluble metabolic products important for its virulence. The aim of this study therefore was to screen for differences between metabolites excreted by NA-GUH-2 (virulent) and NA-10905 (less virulent), to identify the corresponding compound(s), and to correlate these findings with the above mentioned impairments of host defenses.

Both strains were grown in chemically defined broths, and the culture filtrates (CF) were analyzed for uv absorbing compounds. When sodium acetate was used as sole source of carbon, the CF from NA-GUH-2 displayed a uv absorbance maximum at 320 nm, which was almost absent with CF from NA-10905. The main causal agent could be isolated and purified via extraction on polyamide followed by anion chromatography on DEAE-Sephadex-A25. Proton nuclear magnetic resonance and electron impact

mass spectrometry (EI-MS) revealed 2,3-dihydroxybenzoic acid (DHB). The structure assignment was corroborated by GC/MS of the methylation products from authentic DHB and that of nocardial origin. Subsequently, an HPLC method, previously developed for polyphenolic compounds (Vande Casteele et al. 1983), was substituted for the initial isolation procedure. This method was directly applicable to CF, without prior purification, thus allowing quantification in a very straightforward manner. The specificity of the assays was increased through off-line coupling with EI-MS. During the course of our investigations it became clear that absorbance at 320 nm was due to several different compounds: indolyl derivatives eluted at longer, and what appeared to be salicoylserine even at the same retention time as DHB. DHB production could be stimulated by substitution of glutamic acid for sodium acetate and, as was expected for this well-known iron chelator, suppressed by the addition of ferric citrate to the growth medium. For NA-GUH-2, DHB production continued throughout the exponential growth phase (up to 10 µg/ml). Most important, however, was the observation that NA-10905 did not produce DHB under any condition tested (detection limit 100 ng/ml).

Since DHB is known to be an effective radical scavenger, it may assist nocardial superoxide dismutase and catalase in neutralizing the oxidative killing mechanisms of host phagocytes by converting superoxide into a resonance stabilized, less reactive free radical (Crichton 1979). Other bacteria produce DHB as well (Bryce and Brot 1972), but, as they are not known as intracellular pathogens, DHB apparently does not give them a similar protection like we are now suggesting for NA. It is conceivable that in these cases non-oxidative killing mechanisms (e.g. lysosomal enzymes) might be decisive, whereas with NA its extraordinary

lipophilic cell wall might prevent such action. Consequently degration of NA would require prior oxidative break-down of cell wall lipids, the primary target of which would be unsaturated hydrocarbons. The highest degree of unsaturation in nocardial cell wall lipids is found during its exponential growth phase (Beaman 1975). Thus, consistent with our hypothesis about the possible mode of action of DHB, the latter is obviously produced by NA-GUH-2 when it is needed the most. NA probably also gains other advantages from DHB, like iron sequestration from host transferrin for its own growth and, possibly, interference with phagosome-lysosome fusion through the high negative charge density of this molecule at physiological pH. However, with respect to the fact that DHB does not protect other bacteria equally well, the superoxide scavenging ability of DHB might be the most important advantage that NA acquires through its production.

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COMPOSITION AND TOXICITY OF LIPIDS FROM RHODOCOCCUS RHODOCHROUS GROWN ON MANNOSE

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INTRODUCTION

In an attempt to establish a relationship between the biological activity and the chemical constitution of the sugar moiety of mycoloyl esters several sugar derivatives have been prepared and, their activities investigated. It was demonstrated that methyl 6-mycoloyl-α or β-D-glucopyranoside induced the same pattern of functional damage in mitochondria as cord factor (3); 6-mycoloyl-α-mannopyranoside was only slightly toxigenic while 6-mycoloyl-derivatives of allopyranoside and galactopyranoside were inactive (1).

The semi-synthetic products have some disavantage because a small hydrophobic environment was created around the anomeric carbon atom due to the introduction of the methyl group. Therefore an attempt was made to produce a mycoloyl ester having no hydrophobic group at the anomeric carbon atom.

This report describes a preliminary result on the composition and toxicity of lipids extracted from R.rhodochrous grown on mannose.

MATERIAL AND METHODS

Rhodococcus rhodochrous (from Dr. J. Rozanis, University of Western Ontario, Canada) was grown at 37°C in a medium containing 0.5% yeast extract, 0.1% sodium chloride and 1.0% mannose at different incubation times: 36,72,144 and 192 h, in a rotary shaker at 100 rpm. The cells were harvested by centrifugation and washed with distilled water. The packed cells were extracted with diethyl ether-ethanol (1:1, by vol.) and the diethyl ether soluble lipids fractionated by column chromatography; elution was carried out with chloroform, 2% and 3% (v/v) ethanol in chloroform, acetone and methanol. The fraction containing glycolipids was repurified by preparative thin layer chromatography in chloroform-acetone-methanol-water (50/60/2.5/3, by vol). Assays for toxicity were carried out by inoculating into male swiss mice (weight, 18-2.5g) intraperitonially 0.1ml of a solution of lipid in mineral oil (500μg/ml) every 24 hs in a total

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of five injections. Body weight and survival rate were recorded. The control group received mineral oil according to the same schedule. Lipid fractions on thin layer chromatography (TLC) plates were detected by spraying 0.25% potassium dichromate in 10% (v/v) aqueous sulfuric acid and heating at 110°C. Quantitative evaluation was carried out by densitometry. Sugar content was determined by phenol/sulfuric (2). Other analytical procedures were similar to those described in ref. 4.

RESULTS AND DISCUSSION

The cultures of R.rhodochrous grown on mannose and withdrawn at 36,72,144,192 h gave: 47.9±23; 169.8±7.8; 158.0±45; 182.8±18.4mg total mass (diethyl ether-ethanol extract + cell residue) per flask, and 6.0±0.1; 3.1±0.3; 3.5±0.2; 3.9±0.8 mg diethyl ether soluble lipids per flask, respectively. According to Fig. 1, diethyl ether extracts of cells collected at 36 and 72 h were slighthy more toxigenic than those of 144 and 172 h. This difference may be accounted for by the changes in the lipid composition. All extracts contained a high polar glycolipid, HPL and a low polar glycolipid, LPL. The 192 h lipid extract gave by column chromatography combined with preparative TLC four fractions: neutral lipids plus fatty acids (NL+FA); LPL; HPL and phospholipids (PL). Only glycolipid fractions were toxigenic.The purified glycolipids (LPL and HPL showed the following characteristics: mp: 51-56°C; 36-40°C and $(\alpha)_D^{25}$= +24.1°; 57.5°; sugar

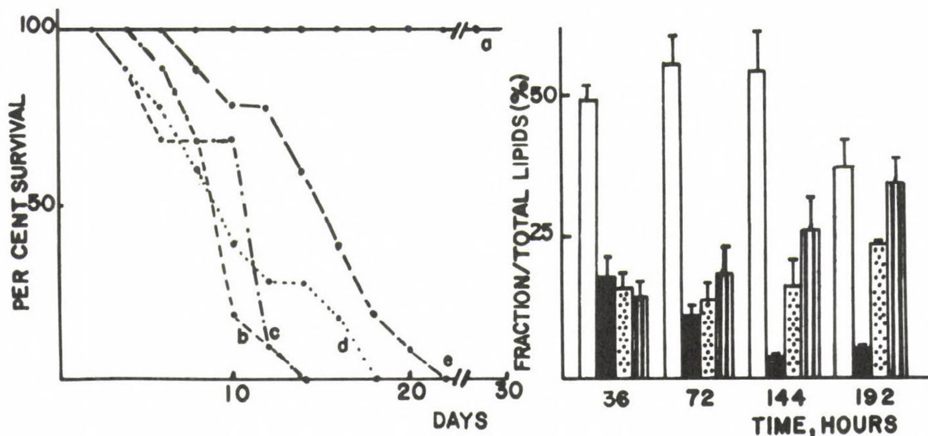

FIGURE 1 - A. Toxicity for mice of diethyl ether soluble lipids from Rhodococcus rhodochrous grown on mannose for 36(b), 72(c), 144(d) and 192(e) hours. Ten mice in each group were inoculated either with 0.1ml Nujol (control group, a) or 0.1ml lipid solution in Nujol (500μg/ml) intraperitonially every 24 h as indicated by an arrow. B. Per cent composition of lipid extracts from Rhodococcus rhodochrous grown mannose for 36,72,144,192 h. Phospholipids, □; high polar lipid, ■ ; low polar lipid; ▦ ;neutral plus fatty acids, ▥ .

content 22.7; 21.6%, respectively. These lipids released into the aqueous phase mannose and trehalose respectively; the fatty acid moiety contained ~C_{40}-mycolic acids. The infrared spectrum of LPL showed a profile distinct from that of other sugar mycolates while that of HPL corresponded to trehalose dimycolate. The 36- and 72 h extracts contained mannose- and trehalose lipids in appr. equivalent ratios, however this ratio from 142- and 192 h cultures was reduced to approx one fifth. It is suggestive that the lipid composition may play a fundamental role in the relationship between pathogenic microorganism and the host.

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GLYCOLIPIDS FROM RHODOCOCCUS RHODOCHROUS GROWN ON SUCROSE

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INTRODUCTION

Previous work on lipid composition of *R. rhodochrous* revealed that this organisms produces ~C_{40}-mycolic (2) therefore with a chain lenght between that of diphtherial mycolic acid (3) and that of *Nocardia asteroides* (1,5). The identification of 6,6'-dimycoloyl thehalose (2) as well as 1-monomycoloyl glycerol (6) has been reported. In a recent investigation, the isolation from *R.rhodochrous* of a glycolipid characterized as 6-monomycoloylglucose was described (8).

This paper reports a preliminary result of a study carried out on the glycolipid fraction of the diethyl ether soluble lipids from chloroform-methanol extracts of *R.rhodochrous* grown on sucrose.

MATERIAL AND METHODS

Rhodococcus rhodochrous (from Dr. Rozanis University of Western Ontario, Canada) was grown at 37°C for a week in a medium containing: 1% yeast extract (Difco), 0.1% sodium chloride and 1% sucrose. Lipids were extracted with chloroform-methanol (1:2, by vol.) and the material soluble in diethyl ether was fractionated by column chromatography as described in ref. 8, and a stepwise elution was performed with 5% (V/V) ethanol in chloroform (fraction I), acetone (fraction II) and methanol (fraction III). Thin layer chromatography of fraction II developed with chloroform-acetone-methanol-water (50/60/2.5,2.5,by vol.) revealed three glycolipid spots: GL1, GL2, GL3, in order of decreasing migration rate. These lipids were further isolated by column chromatography on 50% (W/W) silicic acid-silica gel H and elution was performed with 10, 20, 30, 40, 60, 70% acetone in chloroform. Eluates containing the same single component were pooled. Analytical procedures used were similar to those described in ref. 8.

RESULTS AND DISCUSSION

The crude lipids (~14.5% of cell dry weight) from *R. Rhodochrous* grown on sucrose contained a high proportion of

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glycolipids (~50%), representing about 7.5% of cell dry weight. The three glycolipids isolated GL1, GL2, GL3 represented ~37.5, 45.3 and 16.2% of the fraction and had the following characteristics: mp. 55-58°C; 67-70°C; 70-77°C; $(\alpha)_D^{22}$ +31°; +59°C; +37°, and the IR spectrum of each of glycolipids displayed a characteristic profile. These purified fractions released after alkaline hydrolysis, glucose, trehalose and sucrose, respectively. The methyl ester derivative of each fatty acid moiety revealed a migration rate similar to that of ~(40-mycolic acid methyl ester.

The results show a surprising and versatile ability of R.rhodochrous to develop acylation systems for various sugars such as arabinose (4), glucose (8), mannose (Almeida and Ioneda, accompagning paper), galactose at a lower extent (Breda and Ioneda, unpublished results), trehalose (2) and sucrose. In the case of Arthrobacter paraffineus Ky 4303 grown on sucrose, the production of glycolipids is limited to sucrose lipids (7). Therefore, the extraordinary capability of R.rhodochrous to grow up on different substrates and to synthesize distinct types of mycoloyl esters represents an exceptional advantage and a powerful tool for many purposes, such as: 1) to disclose the enzymic machinery which allows the synthesis of so wide range of glycolipids; 2) source of glycolipids for physico-chemical studies regarding the activity either on biological or artificial membranes, or on immunological and pathological investigations as well; 3) a tool for investigating the effects of antimycobacterial drugs on lipid composition and metabolism.

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CLONING AND EXPRESSION OF MYCOBACTERIUM DNA IN STREPTOMYCES LIVIDANS

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Mycobacterium tuberculosis and M. leprae are important human pathogens. Gene cloning could help the study of the biology of these organisms and may facilitate the preparation of diagnostic and therapeutic agents. Some antigenic determinants from both these mycobacteria have been expressed as fused proteins in Escherichia coli (Young et al., 1985a,b). It seems difficult, however, to produce useful amounts of entire Mycobacterium proteins in E. coli, probably because most promoters and translational start signals of mycobacteria are, at best, only weakly active in this organism (Clark-Curtiss et al., 1985).

Taxonomic relationship and also the known ability of Streptomyces lividans to express other bacterial genes (Bibb and Cohen, 1982) suggest that streptomycetes may be better equipped to express Mycobacterium genes from their own signals. We tested this by making gene fusions with the aminoglycoside phosphotransferase gene (nptII) from the transposon Tn5. A truncated nptII gene was incorporated into the multicopy Streptomyces plasmid vector pIJ688 (Fig. 1). The nptII gene in pIJ688 is inactive because it lacks a promoter, a ribosome binding site and a translational start codon. The gene can be activated when these elements are supplied in the correct reading frame by a cloned DNA fragment. Expression of the nptII gene confers kanamycin resistance on S. lividans 66 which was chosen as host strain. The level of kanamycin resistance depends on multiple factors including promoter strength and efficiency of translation.

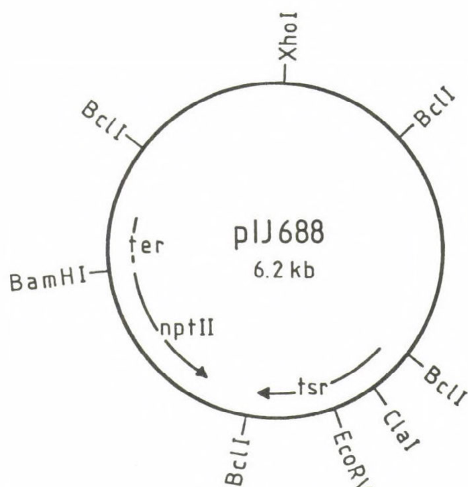

Fig. 1 The plasmid pIJ688 is derived from the Streptomyces plasmid pIJ486 (Ward et al., in preparation) and contains the pIJ101 replicon (Kieser et al.,1982), and the truncated aminoglycoside phosphotransferase gene (nptII) from pKM109-90, prepared by Reiss et al. (1984). The thiostrepton resistance determinant (tsr) is used for selection and the phage fd terminator (ter) reduces transcription from the vector reaching the nptII gene. The unique BamHI site is used for cloning.

We chose DNA from the non-pathogenic Mycobacterium bovis BCG for our experiment, digested it with BamHI and inserted the fragments into the unique BamHI site of pIJ688. For comparison we did a similar experiment using S. lividans DNA. The result (Fig. 2) suggests that many genes from M. bovis BCG are strongly expressed in S. lividans. The slight differences between the frequency of clones of a given level of kanamycin resistance may be due to the fact that the genes from M. bovis have been separated from their natural regulatory elements.

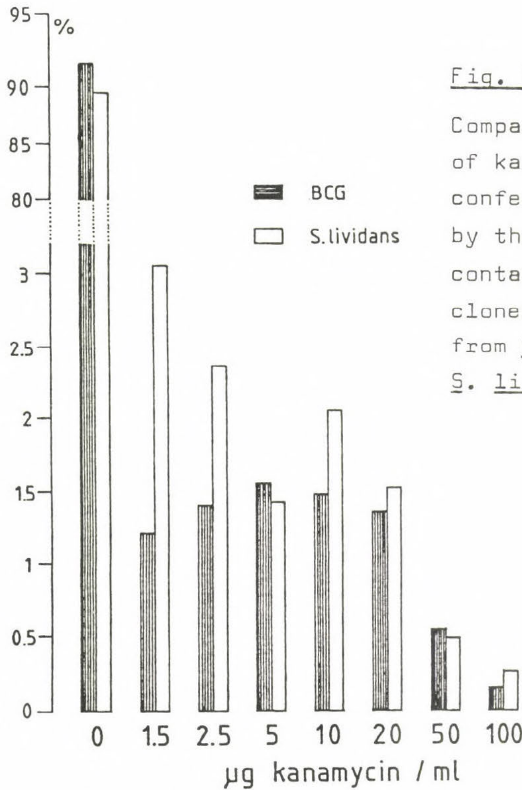

Fig. 2

Comparison of the level of kanamycin resistance conferred on *S. lividans* by the plasmid pIJ688 containing randomly cloned DNA fragments from *M. bovis* BCG or *S. lividans* total DNA.

From these data we conclude that *S. lividans* is a promising host for the expression of *Mycobacterium* genes.

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ACTIVATION OF MOUSE IMMUNE SYSTEM BY THERMOACTINOMYCES VULGARIS. ROLE OF ALVEOLAR MACROPHAGES

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INTRODUCTION

Allergic alveolitis or hypersensitivity pneumonitis is a syndrome caused by inhalation of a variety of organic dusts including fungal and actinomycetal spores and other material of microbial origin. Both nonimmunological and immunological effects of the inhaled material have a role in the development of the syndrome.

Alveolar macrophages (AM) have been studied both in humans and in different animals as modyfiers of different immunological functions in vitro. Their role as initiator cells of immune responses in lower airways is well established. We have shown earlier, that intra-nasal sensitization with a thermophilic actinomycete Thermoactinomyces vulgaris (THV) causes pulmonary reaction resembling histologically allergic alveolitis in mice (Seppä et al.1984, Jägerroos et al 1985). The aim of the present study was to investigate biological effects of THV on AM in normal mice.

MATERIALS AND METHODS

Inbred C57BL/6J, DBA/2 and BALB/c (Animal Center of the University of Kuopio, Finland) were used.

THV (Mycological Reference Laboratory, London) was grown in tryptic soy agar at 50 C. Ultrasonicated cells were treated with formalin, dialyzed and lyophilised and the dry weight of THV was measured.

AM were harvested with minor modifications as described by Sugar et al. (1983). The AM THV treatments were performed in glass tubes in a total volume of 1.0 ml in RPMI 1640. AM were pulsed for two hours with varying doses of THV and after 22 hours incubation with fresh medium supernatants (SNT) were collected. Indomethacin (IND) was used at final concentration of 1 microg. per ml in some stimulations.

Spleen cells were separated and cultivated as described earlier (Jägerroos et al. 1985) except that 5 % foetal calf serum and 3H thymidine as a label was used. The SNT were tested in spleen cell Concanavalin A (Con A) stimulation with a concentration of 1 microg. per ml of Con A. 25 % (v/v) concentration of the SNT was used.

RESULTS AND DISCUSSION

SNT from all strains of mice had a strong supressive effect on Con A-induced lymphocyte stimulation. The strongest supression was seen at concentration of 10 microg. per ml of THV (results not shown). The supressive concentrations of THV were in the same range as mitogenic concentrations of THV (Mäntyjärvi et al 1985). Products of arachidonic acid metabolism, especially prostaglandins (PG) E1 and E2 have strong supressive characteristics (Paul 1984) and AM have been shown to release them (Hayari et al. 1985). The partial restoration of the activity by a prostaglandin synthesis inhibitor IND (Table 1.) indicates that AM release considerable amounts of supressive PG following THV treatment. Since IND is capable of blocking almost completely synthesis of PG (Hayari et al. 1985), other mechanisms must also have role in the supression.

Table 1. Effect of the supernatants (SNT) in the spleen lymphocyte Con A stimulation

Strain	Con A		
	-	SNT	SNT + IND
C57BL/6J	7785 ± 655	984 ± 245	4156 ± 856
DBA/2	5123 ± 655	845 ± 329	3960 ± 544
BALB/c	6254 ± 433	1123 ± 259	3297 ± 469

The results are expressed as mean ± SD of the duplicates.

In the IL-1 assay a small increase in thymocyte proliferation was seen without IND treatment (Table 2.).

Table 2. Effect of the supernatants (SNT) in the thymocyte proliferation

Strain	PHA		
	-	SNT	SNT + IND
C57BL/6J	344 ± 201	674 ± 143	8754 ± 724
DBA/2	222 ± 54	434 ± 199	6254 ± 279
BALB/c	465 ± 320	566 ± 333	5987 ± 466

The results are expressed as mean ± SD of the duplicates.

Similar findings have been reported earlier when using the same method for IL-1 assay, the PHA induced thymocyte proliferation assay, and LPS as a stimulant (Hayari et al 1985). On the other hand in the presence of IND the thymocyte label take-up was almost ten fold (Table 2.). Because IL-1 is a potent initiator of immune responses and also endogenous pyrogen (Dinarello 1984) it has effects opposite of supressive PG, at least in vitro.

In the report by Hayari et al. (1985) the release of PG and IL-1 by AM was uncoupled. It was possible to separate two subpopulations of AM which were independent of each other. This is probably reflected in our finding that also THV treatment causes a multitude of actions in the AM.

In summary, treatment of AM with extracts of a thermophilic actinomycete, THV, induces the release of IL-1 and of supressive PG. Part of the supression cannot be explained on the basis of the data of this study. No gross differences between mouse strains in the secretion of IL-1 and supressive PG were detected in this study.

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ANTIBODIES OF DIFFERENT Ig CLASSES IN FARMER'S LUNG

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INTRODUCTION

Two thermophilic actinomycetes, Thermoactinomyces vulgaris and Micropolyspora faeni and fungi of the genus Aspergillus are considered to be the most important microbes associated with farmer's lung (FL) disease. The presence of circulating antibodies against these microbes has been used as a supporting evidence for exposure and sensitization in the diagnosis of the disease.
The traditional technique to detect these antibodies has been gel-precipitation. In recent years, however, enzyme-linked immunosorbent assay (ELISA) and radioimmuno assay (RIA)have been increasingly used. One of the advantages of these methods is the possibility to measure Ig class specific antibodies.
The sera of FL patients have higher levels of total IgG and also of IgA compared to control persons (Roberts et al.,1973).
They have also increased levels of IgG3 (Stokes et al.,1981).
Specific IgG have been measured with ELISA and RIA. Antibodies of IgG and IgA have been found against FL microbes (Bamdad, 1980, Huizinga and Perrens, 1985, Mäntyjärvi et al.,1980, Ojanen et al., 1980, Patterson et al.,1976). Microbe specific antibodies of different IgG subclasses have not been determined.
In the present study we have measured antibodies of IgG, IgA, IgM, IgE and of IgG subclasses IgG3 and IgG4 against FL microbes in the sera of FL patients.The antibody determinations were made during the follow up period to see if there would be any seasonal variation in the antibody levels. The association between antibodies and the disease was estimated by comparing the antibody levels with pulmonary functions.

MATERIAL AND METHODS

The study group comprised 13 persons, 9 women and 4 men, diagnosed as having FL disease by physiological, radiological and physical findings. The age of the patients varied from 29 to 62 years. They were included in the study when hospitalized because of an acute episode of the disease. Pulmonary functions, total diffusion capacity, specific diffusion capacity and partial oxygen tension of arterial blood were measured during the hospitalization and subsequently four to seven times during the follow up period of 18 to 27 months. Serum samples were obtained at each attendance. Antigens for ELISA were prepared from cells of T. vulgaris and M. faeni and also of two aspergilli A. fumigatus and A. umbrosus as described

previously (Ojanen et al.,1980). ELISA was performed on microtiter plates as previously described (Ojanen et al.,1980). IgG and IgM antibodies against all microbes and IgA antibodies against M. faeni were measured using peroxidase-conjugated anti-human IgG, IgM or IgA (Orion Diagnostica, Finland). The other antibodies were measured by an indirect modification of ELISA. Patient serum was followed by rabbit anti-human IgA (Miles, USA), IgE (Dako, Denmark) or sheep anti-human IgG3 or IgG4 (Miles, USA). The conjugate was peroxidase-conjugated anti-rabbit IgG (Orion Diagnostica, Finlamd) or anti-sheep IgG (Dako, Denmark).

RESULTS

Antibody analysis of the sera showed that at the acute phase of the disease all patients had antibodies of some Ig class against at least one of the microbes. Most patients had antibodies both against the actinomycetes and the fungi. Seven of them had antibodies against three microbes and five against all four microbes. Antibodies against T. vulgaris and A. umbrosus were most often detected, and IgG, IgA and IgG3 antibodies were most common. IgM antibodies were detected only against T. vulgaris. Antibodies of IgE were also found against all microbes but they were less common. IgG4 antibodies were detected only against T. vulgaris and A. umbrosus.
An analysis of antibody titers of different microbe-Ig class combinations of individual patients revealed that during the follow up period titers of the patients showed three types of behaviour. Seven of the patients had both stable and decreasing antibody titers. Two patients had stable and increasing antibody titers, and four patients had both decreasing and increasing antibody titers in addition to stable antibody titers.
The change was most often seen in T. vulgaris antibodies and they were all decreases. Generally decreases were observed in all Ig classes, increases were measured in IgG, IgM, IgE and IgG3. The decreases in antibody levels were generally gradual during the follow up period.
When antibody titers of all patients were compared, the titers against the actinomycetes showed highly significant correlation with each other. The same relationship was observed between the two fungi.
To estimate the relationship between the antibodies and the FL disease, correlations between the antibody titers and pulmonary functions were calculated. To compare the antibodies and pulmonary functions of all patients, the total diffusion capacity of each patient was expressed as a percentage of normal diffusion capacity value of the person of same age, sex and height. IgG3 antibodies against all microbes tested showed highly significant correlation with the pulmonary function. Highly significant correlation was also obtained between this normalized diffusion capacity and IgG4 antibodies against T. vulgaris. Significant correlations were obtained between the pulmonary function and T. vulgaris IgA and IgM, M. faeni IgA and A. fumigatus IgG4.

DISCUSSION

Pulmonary exposure to microbial material leading to FL disease is associated with antibody response. The role of antibodies in the pathogenesis of the disease is not clear but they provide evidence of exposure supporting the diagnosis. IgG and IgA antibodies against microbes have been measured in FL. Microbe specific subclass antibodies have not been determined previously.

In our study IgG, IgA, IgE and IgG3 antibodies were found against all microbes tested. Antibodies of total IgG correlated with IgG subclass antibodies but there were differences in antibody levels because only IgG3 antibodies against all microbes and IgG4 antibodies against T. vulgaris correlated highly significantly with the pulmonary function. Immunologic response to some type of antigen may be limited to one or few of the IgG subclasses and IgG3 response is usually detected against protein antigens.
According to our results antibody analysis in FL disease is relevant. Antibodies may not have a significant role in the pathogenesis of the disease but their level may indicate the severity of the disease. Generally antibody levels of a patient showed a decreasing trend when pulmonary functions improved but only IgG3 antibodies correlated highly significantly with the pulmonary function. IgG3 is therefore the antibody class which ought to be measured together with total IgG and IgA to obtain more precise information of the FL disease.

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PATHOGENICITY AND IMMUNOLOGY

Poster Abstracts

IDENTIFICATION OF MONOCLONAL IgG AND IgM ANTIBODIES SPECIFIC AGAINST VIRULENT NOCARDIA ASTEROIDES GUH2

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Infections in humans caused by N. asteroides (NA) are being recognized with increased frequency, especially within the compromised host. Specific therapy and diagnosis of these infections have presented significant problems. Monoclonal antibodies may represent powerful tools for use in immunoprophylactic augmentation, immunodiagnosis and determination of nocardial virulence factors. Therefore, by somatic hybridization, we began to generate a library of monoclonal antibodies (Ab) against virulent NA-GUH2. Thus far, 10 monoclones have been identified. Three clones produce antinocardial IgG and 7 clones produce antinocardial IgM. All of these monoclonal Abs appear to have high specific activity by both ELISA and agglutination assays. It was demonstrated that IgM Ab injected intravenously (IV) into mice 1 hr prior to IV challenge with 10^6 CFU NA-GUH2 resulted in significantly enhanced clearance of the nocardiae from the kidneys and brains. These data suggest that monoclonal Ab can be used both for immunoprophylaxis and as a probe to determine specific nocardial virulence factors.

PROPERTIES OF THE ANTIGENIC STRUCTURE OF BACTERIA BELONGING TO THE GENUS RHODOCOCCUS, CULTIVATED ON DIFFERENT MEDIA

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The influence of conditions of the cultivation of bacteria belonging to the genus Rhodococcus upon their antigenic structure has been studied. The specific functional antigens, pointing to the development of bacterial cells at the expense of concrete growth substrate - gaseous hydrocarbon (propane) have been found with the help of the double immunodiffusion reaction in agar gel in cells of the gasoxidated Rhodococcus in the conditions of transposition from carbohydrate nutrition to hydrocarbon one.

INTERACTION OF MYCOLIC AND OTHER CARBOXYLIC ACID ESTERS WITH NEUTROPHILS; STIMULATION OF SUPEROXIDE OUTPUT AND CHEMILUMINESCENCE

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Using light reflection photometry, superoxide formation in a low number (5000-10,000) of neutrophils has been determined. The method allows investigations on interactions between neutrophils and water-insoluble compounds such as mycolic acids (C_{80-88}-3-hydroxy-alfa-branched carboxylic acids) or other carboxylic acid derivatives (e.g. 3-OH-C_{14}-myristic acid butylester of lipopolysaccharides in Gram-negative bacteria). Mycolic acid methylesters and 3-OH-C_{14}-butylester stimulated superoxide output strongly; myristic acid methylester or its 2- and 3-hydroxylated derivatives were inactive in this respect. Chemiluminescence determinations using glass reagent tubes coated with mycolic acids or other compounds showed a high activation by both mycolic-methyl- and carboxylic acid butylesters (lauryl-, myristyl- and stearyl-esters). In comparison, the respective methyl-, propyl- and ethylesters of straight-chain or substituted carboxylic acid (myristic acid) were inactive in respect to superoxide output and stimulation of chemiluminescence as well.

ECOLOGY AND EPIDEMIOLOGY

Plenary Session

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ACTINOMYCETE ECOLOGY - A CRITICAL EVALUATION

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INTRODUCTION

The actinomycetes are a widely distributed group of bacteria which are predominantly saprophytic but also include species forming parasitic or mutualistic associations with plants, animals and humans. They occur in a wide range of natural and man-made environments including soil, plant litter manure compost, biodeteriorated materials, fresh and salt water, and the live tissues of plants, animals and humans. The most widely distributed genera are coryneforms, Mycobacterium, Nocardia, Rhodococcus and Streptomyces, but other genera such as Frankia and Thermoactinomyces occupy important, clearly defined ecological niches. Detailed reviews of actinomycete ecology have been provided by Goodfellow & Williams (1983) and Williams et al. (1984). Therefore, the aim here is to assess the current state of actinomycete ecology, to identify developing areas and hence to assess future prospects. In the "age of biotechnology" there is a rapidly developing interest in the manipulation and exploitation of useful isolates from, and processes in the natural environment. Developments in actinomycete ecology are therefore not solely a matter of academic interest. There are, however, still many gaps in our basic knowledge and it is appropriate to first consider these.

THE IDEALS OF ACTINOMYCETE ECOLOGY

Microbial ecology may be defined as "the study of the distribution, activities and interactions of the microbe in its natural habitat". These objectives are not easily achieved and a more realistic or cynical definition is "microbial physiology studied under the worst possible conditions". To meet the ideals of microbial ecology, it is necessary to obtain some or all of the information listed in Table 1. These criteria can be used to assess our current basic knowledge of actinomycete ecology.

Table 1. Basic information needed for the study of microbes in their natural habitats

1. Detection and location.
2. Identification.
3. Measurement of activity.
4. Assessment of interactions with other microbes, plants and animals.
5. Responses to physical and chemical environmental factors.

Detection and location

Detection of actinomycetes is routinely achieved by the dilution plate technique which gives little or no information on the precise location of isolates within the sample and is dependant upon the selectivity (intentional or not) of the medium employed. Occasionally selected particulate substrates have been plated directly to obtain isolates from, for example, dead root fragments (Mayfield *et al.* 1972), live root surfaces (Watson & Williams, 1974) or root nodules (e.g. Callaham *et al.* 1978). All isolation procedures demonstrate the existence of an actinomycete in a habitat but provide only circumstantial evidence of its activity *in vivo*. Direct evidence for the presence of mycelial growth *in vivo* necessitates microscopic examination of the substratum, which is rarely attempted. Among the few examples are the studies of growth of streptomycetes in soil by transmission electron microscopy (Szabo *et al.* 1964), and scanning electron microscopy (Mayfield *et al.* 1972), and examination of the fine structure of the *Frankia* endophyte within root nodules (e.g. Gardner, 1976).

Thus there is generally little information about the precise microhabitat or substratum from which isolates are derived or their status as active members of, or passive aliens in the natural population. Characterization of the gross habitat from which the sample is taken is relatively easily achieved. Unfortunately it is not always provided, particularly when the isolate obtained produces a potentially useful metabolite. Hence the influence of geographical factors, such as climate, vegetation or soil type, on the distribution of actinomycetes is still obscure.

Identification

Accurate identification of isolates is clearly essential to many ecological studies, particularly in those attempting to characterize a natural population. Ideally this involves (i) random or directed selection of colonies on isolation plates; (ii) pre-screening of isolates before detailed taxonomic examination to avoid and note duplication; (iii) identification of selected isolates by reference to an appropriate taxonomic system. Step one is dependant on the aims of the study but can be relatively easily achieved by an experienced observer. The second step is essential in general ecological studies to minimize the numbers of strains subjected to detailed study. Such studies face a conflict between the needs of ecology, requiring data based on as many strains as possible, and identification which can be a time-consuming process. An example of a preliminary grouping scheme for streptomycetes

was provided and tested by Vickers et al. (1984). The final step is dependant on the existence of workable informative identification systems. Although these are available for some genera (e.g. Streptomyces, Williams et al. 1983; coryneform bacteria, Seiler, 1983; Streptoverticillium, Williams et al. 1985), in many cases the comprehensive data in numerical classifications have not been used to construct identification systems.

Faced with the problems of identification, some workers understandbly give up or obtain an incorrect diagnosis. Another, more confusing alternative is to invent a new taxon which is not validly published. This approach is typified by companies with strains producing novel metabolites and by countries isolated from the mainstream of taxonomic literature. Thus many new taxa have recently been isolated and proposed by Chinese workers (e.g. Liu et al. 1983; Yan et al. 1983) and remain to be evaluated.

Measurement of activity

Assessment of overall or specific activities of actinomycetes in the environment has been made by direct or, more frequently, by indirect means. The approaches taken may be categorised as (i) direct measurement of activity in situ; (ii) determination of specific activities of isolates obtained from selected, well characterized environments; (iii) determination of activities of isolates obtained at random from the environment.

The first objective meets the ideals of ecology but is difficult to achieve and has seldom been attempted for actinomycetes. Among the few examples are the determination of growth rates of streptomycetes in soil by fluorescence microscopy (Mayfield et al. 1972) and the measurement of acetylene reduction to assess nitrogenase activity of Frankia in root nodules (e.g. Schwinter & Tjepkema, 1983). In the second approach, appropriate isolation procedures are applied to selected habitats which are likely to contain strains having desirable, environmentally related activities. When used on clearly defined habitats or substrates, this approach has been quite successful, as exemplified by the isolation of thermostable enzymes from thermophilic actinomycetes (e.g. thermitase from Thermoactinomyces vulgaris, Broemme & Kleine, 1984) and the selective isolation of streptomycetes degrading lignocellulose from soil and plant litter (Crawford, 1978). The third approach involves screening of randomly obtained isolates for any of a wide range of activities. Although it has little or no ecological rationale, this approach has been widely adopted to obtain useful strains, such as those producing antibiotics and other useful secondary metabolites.

Assessment of interactions with other microbes, plants and animals

The degree of specificity of such interactions ranges from high to non-existent and the extent and accuracy of our ecological knowledge generally follows the same pattern. Thus information on interactions between actinomycetes and plant roots extends from the increasingly well documented Frankia endophytic association to the still equivocal role of free-living saprophytes in the rhizoplane and rhizosphore (Williams, 1978). Similarly with actinomycete-animal and human interactions, there is an increasing amount of ecological and

epidemiological information on pathogens such as Actinomadura spp., Actinomyces spp. and Nocardia spp. In contrast, very little is known of the interactions between saprophytic actinomycetes and animals or humans. Exceptions are the conclusive evidence for the role of actinomycete spores as causal agents of allergic alveolitis (Lacey, 1981) and the detection of taxonomically homogeneous populations of Promicromonospora sp. in the intestinal tract of millipedes from plant litter (Dzingov et al. 1982).

Actinomycetes are well known as producers in vitro of antibiotics and enzymes which seem eminently suited to facilitate their competition with other microbes in the natural environment. However, while there is much circumstantial evidence for the role of antibiotics and enzymes in natural interactions such as the biological control of root-infecting fungi, there is still little convincing proof of the production or role of these metabolites in the natural environment (Williams, 1978; 1982).

Responses to physical and chemical environmental factors

Most information on this topic has been derived from correlations between gross measurements of factor(s) in the environment from which isolates originated and their responses to variation of the factor(s) in laboratory culture. Ideally such studies should involve determination of the limits and optima for growth and the limits for viability. The most clear cut correlations are obtained when the environmental factors are relatively extreme, as for example, with thermophiles from self-heated materials (Lacey, 1978), acidophiles from acid soils (Flowers & Williams, 1977) and alkalophiles from alkaline soils (Mikami et al. 1982).

However, there has been little study of the potentially more subtle effects of spatial and temporal variations of factors within environments. This reflects the frequently random and destructive nature of sampling procedures, together with the lack of micro-scale measurement of environmental factors. Also, continually fluctuating factors which are difficult to measure, such as aeration and moisture tension, have received comparatively little study. Most attention has been given to soil actinomycetes, as exemplified by the demonstration of the effects of micro-scale variations in soil pH on streptomycetes (Williams & Mayfield, 1971).

SOME DEVELOPING AREAS IN ACTINOMYCETE ECOLOGY

It is clear from the above discussion that many of the ideals of actinomycete ecology remain unfulfilled. Nevertheless the topic is by no means moribund and it is possible to identify several currently developing areas. All of these are of direct or potential applied significance and rely largely on the exploitation or control of natural populations rather than study of their basic ecology (Table 2). As most of these topics are considered in detail by other symposium contributors, a brief assessment of an example of each is given here.

Table 2. Some developing areas in actinomycete ecology

1. Study of defined ecological niches.
2. Control of defined ecological activities.
3. Exploitation of defined ecological activities.
4. Exploitation of random isolates for secondary metabolite production.

Study of defined ecological niches

Clearly defined, specific associations provide relatively attractive subjects for ecological investigation. Hence, one of the most rapidly developing areas of actinomycete ecology is the study of the *Frankia* endophyte. Although the nitrogen fixing ability of the nodules and their inclusion of an actinomycete-like microbe was known for many years, it was the initial isolation of the endophyte (Callaham *et al.* 1978), followed by many others, that stimulated research in this field. To date several hundred isolates have been grown in pure culture often on defined media, known host plants have increased to over 170 species and nitrogen fixation has been demonstrated *in vitro* (Tjepkema *et al.* 1980). Concurrently there have been advances in nodule physiology and the ecology of actinorhizal plants. Such developments, together with improvements in strain identification and specifity, will enhance the practicality of inoculation programmes for economically important forest trees such as *Casuarina*. It is perhaps inevitable that relatively little is yet known about the behaviour of *Frankia* when it is free in the soil. The detection and characterization of plasmids from several *Frankia* isolates (Normand *et al.* 1983) raises the possibility of strain improvement by genetic engineering.

Control of defined ecological activities

Although microbial activity in the natural environment occurs in a myriad of micro-habitats which are difficult to demarcate, it is sometimes possible to exert an overall control of their activities by effecting gross changes in the habitat. An example of this approach is provided by the attempts to control the production of undesirable taints and odours in potable water.

Streptomycetes have long been implicated in this problem. The compound most frequently associated with biologically induced flavours is geosmin, which is produced by a wide range of streptomycetes in culture. The provision of a pure standard of geosmin facilitates the detection of the compound at low but effective concentrations in both natural and laboratory systems. The studies of Wood *et al.* (1985) on reservoirs in the United Kingdom indicated that geosmin contamination was more likely to originate from streptomycetes growing in exposed sediment, plant debris on the banks or in the surrounding soil than in the water mass. Hence odour problems could be predicted if water levels drop and control enhanced by prevention of plant growth on banks and adequate sealing of the banks from the surrounding soil.

Exploitation of defined ecological activities

Screening of isolates from appropriate habitats or substrata provides a convenient means of obtaining strains with specific activities *in vitro*. This approach is based on the logical assumption that the required activities also occur in the selected natural environment.

This is well illustrated by the studies of ^{14}C lignin-labelled lignocellulose degradation by streptomycetes and other actinomycetes selectively isolated from soils and plant litter. Much information on streptomycetes has been provided by the studies of Crawford and co-workers (see Crawford & Crawford, 1984; and this symposium). Similar studies have been made on other genera, such as *Thermomonospora* (McCarthy & Broda, 1984) and *Nocardia* (Trojanowski *et al.* 1977). The results have provided convincing evidence that the ability of actinomycetes to degrade woody materials in the natural environment approaches that of the well known wood-rotting fungi. The practical value of these data is underlined by the increasing interest in the potential of the controlled degradation of waste lignocellulose materials for production of useful by-products. Pure cultures may be genetically manipulated to alter the regulation of lignolytic activity and to achieve the levels of degradation and ranges of transformations found in the natural environment (McCarthy & Broda, 1984). The production of lignin degradation intermediates was enhanced in recombinants derived from protoplast fusions between *Streptomyces viridosporus* and *S. setonii* (Pettey & Crawford, 1984).

Exploitation of random isolates for secondary metabolic production

The effort devoted by pharmaceutical organizations to isolate actinomycetes producing useful metabolites far exceeds that of any other microbiologists. Although a wide range of useful products are now sought, the rationale of this approach is well demonstrated by the strategies used to isolate actinomycetes producing novel antibiotics.

The diversity of isolates producing antibiotics has been largely achieved by a random approach, involving the large-scale, indiscriminate sampling of the environment and the subsequent screening of most or all isolates. While the efficiency of the latter step is being improved, the approach to isolation remains largely subjective. The main reasons for this are (i) lack of knowledge of the ecological significance, if any, of antibiotics; (ii) inadequate information about the distribution of producing strains within and between habitats defined on a micro-environmental, macro-environmental or geographical scale; (iii) lack of correlation between antibiotic production and the taxonomic status of the strains. Despite these problems, the supply of isolates producing novel antibiotics remains unabated. Development of more logical classification and identification schemes for streptomycetes also provide a basis for improving the objectivity of procedures designed to isolate and select streptomycetes producing antibiotics (Vickers *et al.* 1984; Williams *et al.* 1984). Thus there is still considerable scope for improving our knowledge of actinomycete ecology and applying it in the search for new metabolites.

CONCLUSIONS

Some of the fundamental problems and developing areas of actinomycete ecology have been discussed. While the former present some apparently intractable difficulties, the latter indicate the potential practical benefits to be gained by future developments in this field. The many remaining challenges of fundamental research in actinomycete ecology should not therefore be ignored in the "age of biotechnology".

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ON THE ECOLOGY OF NOCARDIOFORM INTESTINAL ACTINOMYCETES OF MILLIPEDES (DIPLOPODA)

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Actinomycetes are often characteristic but in many respect less or completely unknown members of both aerobic and anaerobic indigenous gut microfloras of arthropods, worms etc. (Hungate 1946; Vályi-Nagy et al.1954; Szabó and Marton 1966; Szabó et al.1967, 1976; Szabó 1974; Bignell et al. 1980; Bignell 1984, etc.).

Some years ago we revealed (Dzingov et al. 1982; Szabó et al. 1983) that yellow coloured smearable bacteroid nocardioforms of oxidative and partly also fermentative metabolism can attain large population sizes in the gut of millipedes. On studying them and describing their first species as Promicromonospora enterophila (Jáger et al. 1983; Szabó et al. 1985) it became obvious that they are very closely related to certain, from ecological point of view almost unknown genera and groups of actinomycetes such as Oerskovia, Promicromonospora nonmotile Oerskovia-like-(NMOs; Lechevalier and Lechevalier 1981) and other related organisms, isolated randomly, presumably as disseminating propagules, from laboratory- and clinical-materials, soils, etc. (Krassilnikov et al. 1961; Lechevalier 1972; Sottnek et al. 1977; Yevtushenko et al. 1982, etc.). Accordingly, dealing with such intestinal partners of millipedes we can contribute to our knowledge also on the biology of the above-mentioned nocardioform genera and groups, which together with this gut actinomycetes will be designated below simply as OPNs (Oerskovia-Promicromonospora-NMO-type microbes).

First of all, we touch upon the definition of "nocardioforms", then outline the millipede gut as an environment for them.

NOCARDIOFORM ORGANISMS: A VERY HETEROGENEOUS GROUP OF ACTINOMYCETES

Nocardioforms (Prauser 1967) are intermediates between coryneform bacteria and sporoactinomycetes (Prauser 1981), which reproduce only by fragmentation of their hyphae. It might be supposed that among them Promicromonospora is a minor intermediate towards sporoactinomycetes, and Cellulomonas

connects them with coryneforms. This supposition could, however, be confirmed only with difficulty because both coryneforms (Goodfellow and Minnikin 1981a; Goodfellow et al. 1982; Seiler 1983) and nocardioforms (Goodfellow and Minnikin 1981b) are very heterogeneous groups comprising different genera. Besides, according to the results of numerical phenetic surveys, analyses of DNA bases, cell walls, lipids, etc. it seems to be more reasonable to study these organisms on the basis of new groupings, not respecting the earlier proposed nocardioform, coryneform, etc. distinctions (Goodfellow and Minnikin, 1981a).

Without doubt, the taxonomic inconsistencies may considerably be responsible for our limited knowledge also about their ecology. At present the correct diagnostic descriptions on invertebrate gut nocardioform isolates seem to be more important than their taxonomic identification using the nomenclature of one of the proposed systems.

THE MILLIPEDE GUT AS AN ENVIRONMENT FOR OPNs

The main characteristics of the forest litter consuming millipedes' gut environment are:

a, The foregut is only poorly utilized by microorganisms.

b, The midgut represents the absorptive surface and continuously secretes the semipermeable peritrophic membrane which is perhaps a barrier to microorganisms. This encloses the solid food materials - preventing their direct contact with the epithelium - into the endoperitrophic space where the enzymatic degradation of macromolecules is taking place, while the hydrolysis of dimers and oligomers proceeds in the ectoperitrophic space.

c, The cuticle-lined hindgut is strongly developed and bears both flat cuticular surfaces and ornaments such as spines of various shapes, which assist in shredding the peritrophic membrane and provides sites for microbial colonization. It contains a rich and complex flora: common enterobacteria (*Klebsiella* etc.), nocardioform actinomycetes, selected soil bacteria (*Micrococcus*, *Bacillus* etc.), unknown filamentous procaryotes and presumedly eucaryotes observed otherwise already more than 130 years ago (Leidy 1850) and others.

d, Malpighian tubules generate a fluid containing a wide range of organic and inorganic anions, nitrogenous compounds, etc. (Maddrell 1981), mostly easily utilizable by nocardioforms. These secreted materials and the fluid input to the gut via salivary glands (containing mucous components and digestive enzymes) provide suitable environment for intense multiplication of many other microbes too.

e, In the millipede gut conditions are far from anaerobic.E.g. the redox potencials (E_h) vary in the midgut of *Glomeris marginata* between +267, and +307 mV and in its hindgut between +167 and +277 mV (Bignell 1984). Accordingly,

in this gut environment also obligate aerobic nocardioforms may be good competitors with bacteria.

OCCURRENCE OF OPNs IN THE FREE SOIL MATRIX

Regarding the data of Soviet and American workers (Lechevalier and Lechevalier 1981; Yevtushenko et al. 1982, etc.), OPNs may be ubiquitous organisms widely distributed in soils all over the world. The occurence of them was detected by us also in Hungarian soils. But in our opinion they do not belong to the true indigenous terrestrial microbes residing in the free soil matrix.

It might be supposed that there are particular genetic soil types, varieties or at least soil horizons, subhorizons, microhabitats, etc. preferred and colonized by them, but such milieus have not been revealed so far. Also in samples of litter layers (A_{00}-A_0) and A_F-horizon matter of brown forest soils where millipedes occur and feed in relatively dense populations and lay their excrements, OPNs are present only sporadically or not at all.

E.g. from a composite sample of A_F-horizon matter rich in old millipede faecal pellets collected in the feeding habitat of different millipede species, among them _Chromatoiulus projectus_, in November when the feeding activities of them reduced already strongly, altogether 1523 isolates were obtained in the following distribution: _Bacillus cereus_-type isolates 118; _Bacillus subtilis_-type isolates 26; green fluorescent bacteria 79; a heterogeneous group of different bacterial isolates 640; _Streptomyces spp_ about 17 types or species 267; sterile actinomycetes (their hyphae did not show fragmentation) 371; other not OPN-type actinomycetes 22; OPNs 0.

Another composite A_F-horizon sample obtained from a _Chromatoiulus_-nest in a more favourable time point was predominated by _Streptomyces spp_ (more than 10 species), members of other actinomycete genera (more than 6 species) and many types of bacteria, contained OPNs in less than two per cent of all isolates.

At present it could be said that the probability of finding OPNs in a soil sample is increasing with the amount of fresh millipede (or other, OPNs-bearing invertebrate-) faecal matter incorporated in it.

OCCURRENCE OF OPNs ON THE EXOSKELETON OF MILLIPEDES

Millipedes play an important role as animal vectors in the passive dispersal of soil microbes (Szabó et al. 1983) transporting them, mostly adhered to the projections of the tarsal region of legs, and inoculating with them the freshly exponed, still uncolonized dead organic matter surfaces present in the soil. According to the results of our electron-microscopic observations on different regions of the millipede's exoskeleton and bacteriological analyses on the leg

surface-flora, OPNs do not belong to the external partners of these animals at all. The overwhelming majority of the leg-isolates proved to be Streptomyces spp (more than 17 by legs transported species were studied by us; Dzingov et al. 1982; Szabó et al. 1983). The strongly hydrophobic negatively charged streptomycete propagules (see Ruddick and Williams 1972) are attracted in large numbers by the hydrophobic cuticle of the millipede's exoskeleton, from which OPNs were not isolated.

OCCURRENCE OF OPNs IN THE GUT OF DIFFERENT SOIL INVERTEBRATES

Promicromonospora enterophila proved to be a stable and only in its population densities varying member of the hindgut-community of Chromatoiulus projectus. OPNs (physiologically more or less different of P.enterophila; Márialigeti and Contreras, unpublished) regularly occur also in the gut of other Diplopoda such as Leptoiulus proximus, Cylindroiulus luridus, C.boleti, Unciger foetidus etc., as it was revealed in our Department. Fernandez isolated (unpublished) a lot of Promicromonospora-type strains from the hind gut of the millipede Polydesmus complanatus.

From the intestine of specimens of Protracheoniscus amoenus (Isopoda) OPNs are missing completely, although this woodlice feeds frequently together with Ch.projectus on fallen leaves of the same trees in very same litter-environments where they can presumedly contaminate each other with their faecal bacteria (Márialigeti et al. 1984).

A detailed numerical phenetic survey carried out by Jáger in this year on the platable microflora of fresh faecal matter of the springtail Tomocerus longicornis (Collembola) schowed that OPNs are lacking also in this soil animal belonging similarly to the forest litter biota.

OPNs were not found in the gut contents of adult specimens of the earthworms Eisenia lucens, Octolasium montanum, Fitzingeria platyura depressa, Allolobophora rosea and the juvenile specimens of Lumbricus polyphemus collected from the A-horizon of a brown forest soil either. But surprisingly, it has succeeded to show (Ravasz et al. 1985a,b) a large homogeneous, facultatively anaerobic Oerskovia-type OPN-population from the gut contents of adult specimens of the earthworm Lumbricus polyphemus. According to Ravasz, all of the selected and thoroughly studied 25 representative strains of this OPN-population proved to be identical with each other. As regards the stability of the partnership between these OPNs and Lumbricus polyphemus we have at present no data, but it does not seem very close.

Taking all of these findings into consideration, some important questions may be exposed: How large can be the host range of these nocardioform gut colonizers? Does it involve a few or many different taxonomic groups of invertebrates? Are these gut colonizers different at species, generic or even familial levels? Is the gut of specimens of the very same animal species colonized consistently by the same type of nocardioforms or not? etc.

As regards the first question, our experiences have lead us to believe that their host range is very large. OPNs adapted themselves, during their evolutionary history, not exclusively to millipedes at all. In this respect it might be interesting to note that in the past many workers detected in the faeces of different invertebrates not only Corynebacterium (as e.g. Baleaux and Vivares in the case of the millipede Schizophyllum sabulosum var. rubripes, in 1974), but also simply as coryneform bacteria which in several cases might be nocardioforms in accordance with OPNs. Khambata and Bhat (1955) e.g. reported on the occurrence in tropical earthworms of yellow coloured, doubtfully motile, short rods and filaments partly with swollen end called coryneform bacteria.

As regards the questions on the systematic relationship of these gut nocardioforms, a pregnant answer was given by the results obtained on studying (Chu Thi Loc 1985) the gut-OPNs of the millipede Glomeris hexasticha.

THE NOCARDIOFORM GUT-PARTNERS OF CHROMATOIULUS PROJECTUS AND GLOMERIS HEXASTICHA

Many problems have arisen already at the identification (Jáger et al. 1983) of P.enterophila, the gut-partner of Ch.projectus. Its identity at generic level with Promicromonospora was questioned by some workers suggesting that it belongs perhaps to a new genus. On the other hand, Prauser /personal communication/ showed that its type strain is sensible to a highly specific Oerskovia-phage. Consequently, it might belong to oerskoviae, so much the more because it also has fermentative abilities. Also the adequacy of the name Promicromonospora which was introduced by Krassilnikov et al. in 1961, as P.citrea, for some randomly isolated identical strains is questionable if we are considering the results of Kuimova et al. (1983), according to which in P.citrea spore formation, similar to that in Micromonospora, it does not exist at all.

In 1980 Anderson and Bignell supposed that in the faeces of the millipede Glomeris marginata multiply rather soil bacteria than the specific gut symbionts. But what kind of gut symbionts? In the hindgut of this animal Professor Anderson has observed with the aid of electronmicroscope among others unidentified monosporic actinomycetes (personal communication, 1982).

In 1983 in our Department Chu Thi Loc isolated such microbes from fresh faeces of another Glomeris species G.hexasticha, common in Hungary. These behaved as it was supposed by Anderson and Bignell (1980) and as P.enterophila. They originated from the hindgut, quickly dissappeared from the faeces and occurred in the soil sporadically at most. On their identification many at present hardly resolvable problems have arised.

On the first approach, the selected representative strains, which were identical with each other showing that also G.hexasticha possesses its own homogeneous nocardioform

gut population, seemed to be the members of Promicromonospora. Their growth and multiplication have proceed according to a very complex life cycle including all morphological elements which were revealed in P.citrea by Kuimova et al. (1983): rod and coccus shaped vegetative cells or hyphal bits, chlamydospores, oval or sphaerical spore-like lateral bodies, swollen vesicles, subterminal vesicular bodies, unseptated extensively branched substrate hyphae which rapidly broke up into fragments, etc. Also the biochemical characteristics of these obligately aerobic strains showed a very close similarity to those of P.citrea. But in the cultures of the Glomeris-strains a few actively moving flagellated cells were also observed among the many others showing only Brownian movement. This was the first contradiction to the authentic description of Promicromonospora, but not in general to the summarized characters of OPNs.

Our nocardioform Glomeris-strains proved to be photochromogenic organisms: their colonies became yellow only after 1-2 days cultivation on agar media in illuminated surroundings. They produced besides also diffusible red-coloured pigments in certain agar-media. These properties, however, are completely lacking in any one true Promicromonospora species or strains. In contranst to the strains of P.enterophila, the Glomeris--nocardioforms did not show any sensibility against Oerskovia-,Promicromonospora-phages /Prof. Prauser, Jena: personal communication/. Finally, one of our coauthors, I.Szabó /Debrecen/ has detected differences in the major cell-wall constituents of strains of P.enterophila and those of Glomeris-nocardioforms.

At present it might be supposed that the individual invertebrate species, genera or larger taxa may harbour (if they have at all) their own nocardioform species, genera or groups, but still many further data would be necessary to confirm this supposition.

SURVIVAL CAPABILITY OF P.ENTEROPHILA IN SOIL

As we have repeatedly stated, OPNs disappear quickly from the laid faeces of their host animals and do not participate in the community metabolism of the soil biota. We did try to clarify the nature of environmental stresses responsible for this phenomenon.

In the framework of a series of laboratory experiments we compared the survival of P.enterophila in untreated and heat sterilized A_F-horizon material of an eutrophic brown forest soil. Soil samples in Erlenmayer flasks were inoculated with a standard cell-suspension of P.enterophila and incubated at $28^{0}C$ for a year. Moisture content of the samples was about pF 2 and water loss was continuously replaced by sterile water. The presence of alive P.enterophila propagules in the samples (at least in two parallels) was detected at different time points by random

reisolations using the plate-count technique and nutrient-furthermore starch-agar as plating media.

Reisolations were carried out on six occasions. Altogether 1930 P.enterophila isolates were obtained from sterile soil samples, among them 1200 ones already after a whole year incubation. From the untreated soil samples, after two weeks incubation we obtained altogether 1773 isolates, but among them we did not find P.enterophila at all. This gut actinomycete disappeared in the presence of the complex soil microbiota extraordinarily quickly. The result of this experiment was identical if we used for inoculation of the samples a suspension containing P.enterophila in tenfold germ number. This nocardioform survived, however, in sterile soil samples a year without changing the growth activity of its reisolates. This experiment was later repeated using other forest soil matter for cultivation.

During the second series altogether 1488 isolates were obtained among them only 8 P.enterophila, from untreated soil samples of a water content of about pF 0 and 2, respectively. Also in this series P.enterophila dissappeared from the samples quickly i.e. within two months. Its elimination was more rapid at pF 2 than at pF 0, which provides less advantageous physical conditions for microbial activities.

The highest limit of the duration of its survival in sterile soil we could not be clarified, but we suppose that it can remain alive for a very long time. All these render probable that biotic factors may be responsible for the disappearance of this microbe from the soil.

ARE THE PROPAGULES OF OPNs SUFFICIENTLY DURABLE STRUCTURES FOR AERIAL DISPERSAL?

Although Oerskovia has motile flagellated forms which can migrate actively by swimming, and this stage is presumedly implicated in its dispersal, OPNs are subjected more frequently to dissemination by wind and air. Freshly laid millipede pellets dry quickly up and disintegrate on the surfaces of fallen leaves in the litter layer where these animals regularly feed. From laboratory materials or from anywhere randomly isolated OPNs may derive from windblown litter or soil matter. Cells, bits of hyphae etc. of OPNs may adhere to minute dry excrement fragments, easily raised by wind and they may travel as airborne particles for long distances. But are their propagules sufficiently durable for such travels? Those of P.enterophila remained in our laboratory alive for more than six months in air-dry condition in sterile dry A_F-horizon matter-, quartz sand- and clay-samples, without decreasing their viability (which was checked on 968 reisolated cultures).

It is very likely that millipedes did not develope any particular mechanism for the transmission of their OPNs gut partners to their offsprings. The digestive canal of the latters may be contaminated by these microbes by oral uptake on feeding on organic particles bearing disseminating nocardioform propagules. We did not detect the presence of

P.enterophila within or on the surface of eggs laid by Ch.projectus into the soil at all.

COMPETITIVENESS AND RESISTANCE TO SOIL MICROBES

After it became obvious that OPNs do not possess traits which would endow them with the capacity to cope with biological stresses of the free soil surroundings, it seemed to be interesting to evaluate the range of tolerance of P.enterophila against different biotic influences. Already on studying the influence of changing water conditions in non--sterile with P.enterophila inoculated soil samples checking the alteration in the composition of the microbiota on 1023 isolates it was found that at about pF 6, when owing to the low moisture content the microbial antagonistic activities strongly decrease, P.enterophila survived >10 months in large population sizes.

On the other hand, stimulating the activities of indigenous microorganisms in nonsterile with P.enterophila inoculated A_F-samples, at pF 2, with adding into them pepton, cellulose, starch, glucose or yeast extract, respectively, in 1 per cent concentrations or ammonium-phosphate in a tenth one, the disappearance of this nocardioform, in comparison with the rate of its elimination from the unamended control samples, was accelerated (to a few weeks or even less). This was checked by studying altogether 5041 isolates in the overwhelming majority soil bacteria and actinomycetes.

If the A_F-horizon matter was heat sterilized, but after this procedure reinoculated simultaneously with P.enterophila and a dense suspension in tap water of an alkali soil sample (solonchak) containing the complex original microflora the alien soil biota proved to be unable to destroy the nocardioform population exponed to it. After 9 months incubation (at pF 2) still 52 per cent of all isolates (382) belonged to P.enterophila. From non-sterile solonchak and acid peat soil-samples (pF 2) however, P.enterophila disappeared within two months.

In sterilized A_F-matter synchronously reinoculated with a suspension of A_F-material of the same origin, but heated only at 70°C for 10 minutes or with a suspension containing the complete original A_F microbiota and P.enterophila, the latter was eliminated more slowly than in the untreated non-sterile A_F-samples.

Consequently, it can be concluded that P.enterophila can survive, or at least prolong its existence, in presence of a complex microflora only if the soil surroundings are alien and still uncolonized for/by the latter.

Another interesting finding was that in sterile samples of A_F-horizon matter (pF 2) the survival of P.enterophila was not limited, or at least not considerably, in mixed populations with a lot of soil microbes. Among those which were cocultivated with it there were also common soil bacteria and fungi possessing prominent community positions

under natural soil circumstances and also in the original A_F-samples used for these experiments.

During several months P.enterophila and a strain of Aspergillus niger showed in soil cocultures only slow changes in the ratio of their population densities checked by the aid of plate count estimates and reisolations. After 1 month incubation their ratio was in previously heat-sterilized A_F-matter 24:7, two ones 15:4, five ones 8:4, etc. Having cocultivated it under such conditions with a Penicillium sp. strain the results were similar.

With inoculation into sterile A_F-samples introduced, the density of population of P.enterophila practically did not change during a year incubation if these samples were simultaneonsly inoculated with one of three Streptomyces spp. strains (No 11/658, 15/107 and 22/181, respectively) or three Bacillus spp. ones (1/635, 2/858 and 5/123, respectively) which proved to be codominant organisms in the untreated A_F-samples. This statement was confirmed by studies on altogether 7900 reisolated cultures of them.

P.enterophila also maintained its approximative initial inoculation density during a year when we cultivated it in previously sterilized A_F-samples as a member of an artificially created community composed of the above-listed six soil strains. During this experiment, on checking the survival of this nocardioform, from this complex seven members population altogether 2640 reisolated cultures were obtained. At the end of the cocultivation, P.enterophila constituted still 29 per cent of the mixed population.

All these results indicate that biological factors may be responsible for the low rate of survival of P.enterophila in natural soils, and in this respect there exists a definite range of growing environmental stresses: sterile soil + pure or mixed bacterial-and actinomycete-populations < sterile soil + reinoculation with moderately heat treated soil suspension< sterile soil + reinoculation with untreated soil suspension < natural untreated soil.

It is also without doubt that P.enterophila possesses a certain grade of competitive ability which, however, is not enough to compete succesfully with the members of an established complex natural soil microbial population, and to replace or displace only a single one of them.

Finally, we observed that in sterile A_F-samples survival and viability of P.enterophila were not limited or influenced at all if these samples were wetted with sterile filtrates of suspensions or water-extracts of freshly collected A_F-horizon material.

P.ENTEROPHILA AS A MEMBER OF THE MILLIPEDE'S HINDGUT COMMUNITY

Different interspecific metabolic relationships (on which no data are available) may exist among the members of in its composition less studied microbiota in the also

poorly researched chemical environment of the millipede's hindgut lumen.

Contreras (1985), who studied the species composition and biochemical properties of the hindgut flora of Ch.projectus by the aid of numerical phenetic analyses, has isolated and selected a lot of Klebsiella-, Pseudomonas-, Micrococcus- etc. strains representing the codominant platable gut bacteria of this millipede. Ten different strains of them were contrasted by Heydrich with nine P.enterophila strains on nutrient- and synthetic glucose-agar plates using the cross streaking method to clarify the frequency and types of their mutual interactions. Table 1 shows the number and distribution of antagonistic and stimulative interactions between them.

As can be seen, this gut microflora might generally be considered in the relation of these media (!) a neutral community characterized by low numbers of detectable interactions among its members also on synthetic agar. Strains of P.enterophila produce otherwise no antimicrobial substances acting in interspecific, -generic, etc. relations, as this was detected in several tests using Escherichia coli, Bacillus subtilis, other bacteria, yeasts, fungi, etc. as test organisms. This behaviour is very characteristic of many other strains of different species of OPNs studied by us so far. This neutrality observed among the representatives of the Ch.projectus hindgut flora was not copletely confirmed on contrasting bacterial strains isolated from the gut of specimens belonging to different millipede species. E.g. a Bacillus cereus strain (No 24) isolated from Cylindroiulus boleti antagonized some P.enterophila strains, and many other gut strains isolated both from Ch.projectus and C.boleti.

It is interesting to note that some P.enterophila strains proved to be able to antagonize slightly some other strains of their own species. Does perhaps any bacteriocinogenesis exist in the population of this species? At present we cannot give answer on this question as well as on many others concerning the ecology and biology of the gut flora of millipedes.

OPNs studied so far at our Department seem to be only moderately adapted to the animal's gut milieu. They are easily cultivable on laboratory media, have simple nutritional requirements and can multiply with inorganic N-sources and simple organic compounds such as glucose, arabinose, fructose, glycerol, lactose, raffinose, acetate, malate, succinate, etc. as sole source of carbon. They do not decompose cellulose and a lot of complex organic compounds, about which we suppose that are hardly available for their host animals. In our opinion, they multiply in the hind gut on the expense of simple hydrolytic products of polysaccharides, furthermore on proteins. It is possible that the host animals can utilize the cell-materials of OPNs. Such a phenomenon was showed by Reyes and Tiedje (1976) on studying the terrestrial isopod Tracheoniscus rathkei, which can intensively digest the resident gut microorganisms particularly under conditions of starvation.

Table 1. Number, distribution and types of mutual interactions among 9 P.enterophila- and 10 Pseudomonas-, Klebsiella-, Micrococcus- etc. strains representing the gut flora of Ch. projectus on nutrient- and synthetic glucose-agar plates

	Nutrient-agar		Synthetic glucose-agar	
	Total	%	Total	%
Number of contrastings	361	100	361	100
Detected antibiotic actions	3	0,8	30	8,3
Detected stimulations	0	0	22	6,1
Promicromonosporae among themselves contrastings	81	100	81	100
(antibiotic) interactions	3	3,7	7	8,6
stimulations	0	0	0	0
Promicromonosporae←→other bacteria contrastings	180	100	180	100
antibiotic interactions	0	0	15	8,3
stimulations	0	0	14	7,7
Bacterial strains among themselves contrastings	100	100	100	100
antibiotic interactions	0	0	8	8
stimulations	0	0	8	8

PERSPECTIVES

OPNs and closely related organisms are widely distributed in Nature. According to Williams and Wellington (1982), Oerskovia easily isolated from soil, but its frequency of ocurrence is still questionable. Prauser (1976) remarked that "The relative small number of strains of Nocardioides, Oerskovia and Promicromonospora isolated in different laboratories seems to be incompatible with the ease of getting the respective phage from soil". This dilemma does not seem to be insoluble if we are taking into consideration that they are not true soil microorganisms, but intestinal ones of soil invertebrates.

The yellow coloured, quickly fragmenting mycelia, chlamydospores, single monospore-like lateral structures,

swollen vesicles, subterminal vesicular bodies, nonmotile or flagellated cells producing oxidative or facultatively fermentative organisms of simple nutritional requirements characterized by type VI. or eventually other cell wall composition are perhaps the members of a less known and so far only sporadically studied large and variable taxon which now might provisionally be designated as a group of "enteroactinomycetes". The host-range of these organisms is presumably broad, involving very different taxonomic groups of invertebrate animals.

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THE ROLE OF ACTINOMYCETES IN THE DECOMPOSITION OF LIGNOCELLULOSE

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SUMMARY

Actinomycetes play a significant role in the decomposition of lignocellulose in soil. In particular, Streptomyces appears to occupy an important degradative niche. While they may not be true wood decay organisms, certain Streptomyces readily decompose grass, hardwood, and softwood litters in soil. Accumulated evidence shows that they catabolize lignin by a unique pathway that sets them apart from the white-rot, brown-rot, and soft-rot fungi. Streptomyces degrade this recalcitrant polymer by oxidative depolymerization, involving the introduction of phenolic hydroxyl and carboxylic acid groups and cleavage of lignin's β-ether intermonomeric linkages. This generates water soluble, polymeric lignin fragments. Once liberated, these soluble fragments are more available to other soil microbes. Streptomyces are, in effect, lignin solubilizers, which remove lignin in order to access polysaccharides which they degrade with active cellulases and xylanases. Streptomyces further metabolize lignin fragments, but metabolism is generally slow, and lower molecular weight intermediates are more readily degraded than are highly polymeric ones. Metabolism of single ring aromatic lignin degradation intermediates also varies considerably between different Streptomyces strains. Though there may be exceptions with certain highly ligninolytic Streptomyces which more efficiently mineralize lignin, or in lesser studied genera of actinomycetes which may metabolize lignin differently, it appears that actinomycetes accentuate the rate of turnover of lignin in soil by means of their lignin solubilizing enzymes.

INTRODUCTION

The recycling of lignocellulosic residues by soil microorganisms is an incompletely understood process. Fungi, bacteria, and actinomycetes are all involved to one degree or another in the ultimate mineralization of both cellulosic polysaccharides and lignin [1]. However, it has been generally believed until recently that fungi played the dominant role in recycling lignocellulose, because of their unique ability to degrade the complex aromatic polymer lignin [2,3]. Bacteria were though to play a lesser role in lignin decomposition, though many species were known to decompose low molecular weight aromatic compounds structurally similar to the basic phenylpropane subunits of lignin [4]. It was thought that

bacteria were generally unable to significantly degrade the recalcitrant intact lignin macromolecule. The participation of actinomycetes in the degradation of lignin was open to conjecture, though limited evidence indicated some species might have ligninolytic ability. For example, an early report on lignocellulose degradation by actinomycetes came from Waksman's laboratory [5], and numerous other reports prior to 1970 indicated that Streptomyces and other mesophilic and thermophilic actinomycetes decomposed lignocellulose [1,6]. Evidence was conclusive of the involvement of actinomycetes in the degradation of cellulosic and hemicellulosic components of lignocellulose, but not in their ability to degrade lignin. Cross and Goodfellow concluded in 1974 [7] that "the actinomycetes play a minor role in the decomposition of the total litter added to soil, but form an intergral part of a balanced biological community which is invariably characterized by an extremely diverse flora." Kirk [2] had pointed out earlier that proof of bacterial involvement in lignin degradation was equivocal because of weaknesses in experimental techniques used previously to assay for lignin. In 1978, Ander and Eriksson [3] concluded similarly that available evidence indicated limited role for bacteria in the decomposition of lignin. They felt that while bacteria might be involved in a certain amount of lignin degradation, their true abilities were unclear. Presumably, the authors of these papers lumped actinomycetes into the broader bacterial group.

If the actinomycetes truly have but a limited ability to attack lignin, this would have major implications relative to their role in the recycling of lignocellulosic plant residues in soil. In nature lignin acts as a physical barrier that protects cellulosic polysaccharide components of the lignocellulose complex from attack by cellulolytic organisms [6]. Non-ligninolytic microorganisms would be unable to efficiently access the cellulosic components in decomposing plant residues because of this lignin carrier. They would, therefore, be relegated to minor roles in the decomposition process, such as competing for readily available metabolites and/or the polysaccharides least tightly associated with lignin in the residues. In fact, this conclusion agrees with the basic view summarized by Goodfellow and Cross [7].

In 1978, we began an intensive study of Streptomyces and conclusively established their abilities to degrade the lignin and cellulosic components of intact lignocellulosic substrates [8-12]. We isolated and screened over 500 soil isolates using ^{14}C-isotopic techniques that provided one of the first truly sensitive assays to show whether Streptomyces were attacking lignin and/or polysaccharide components of lignocelluloses [13-15]. ^{14}C-lignocelluloses were prepared which were specifically ^{14}C-lignin or ^{14}C-glucan labeled, and decomposition was followed by monitoring the ability of pure cultures to degrade the labeled lignin or cellulose to $^{14}CO_2$ and ^{14}C-labeled water soluble products. We confirmed unequivically that certain Streptomyces significantly degraded lignin as well as cellulose within intact lignocelluloses prepared from Douglas fir or blue spruce [8,10,16]. Both $^{14}CO_2$ and ^{14}C-water soluble products were formed from polymeric lignin. Since then, we have shown that strains such as Streptomyces viridosporus T7A and Streptomyces setonii 75Vi2 more efficiently decompose lignocelluloses derived from grasses as opposed to hardwoods or softwoods [17]. Lignocellulose decomposition was followed by monitoring substrate weight loss, lignin loss and carbohydrate loss over time. Grass lignocelluloses were

decomposed to weight losses ranging between 49.2 and 56.7% after 12 weeks incubation in aerobic solid state fermentations. Lignin and carbohydrate losses ranged from 39.0 to 44.2% and 58.7 to 68.8% respectively. Based upon these findings, we and others reconsidered previous conclusions on the importance of actinomycetes in the decomposition of lignin rich plant residues. Goodfellow and Williams [18] reviewed our work in 1983, and concluded that we had convincingly demonstrated that actinomycetes play a role in lignocellulose degradation.

About 1980 we instituted a study of the chemistry of lignin degradation by selected Streptomyces strains. Over the next several years we pieced together sufficient chemical data to propose a pathway for the metabolism of lignin by strains such as S. viridosporus and S. badius [19]. We have also discovered a group of enzymes produced by ligninolytic Streptomyces which we believe may be involved in the initial depolymerization and oxidation of lignin to the level of modified, water-soluble polymeric fragments [19,20], although we are still uncertain as to their precise role in lignin metabolism. We have not yet detected these enzymes in extracellular form. We have also found that the lignin degrading ability of S. viridosporus can be enhanced by ultraviolet irradiation mutagenesis and by protoplast fusion [20,21].

Based upon the accumulated chemical and enzymological evidence, we can now propose a specific role for S. viridosporus, S. badius, and related ligninolytic Streptomyces in the recycling of lignocellulose in soil. These actinomycetes transform lignin by a unique metabolic pathway that is distinct from the pathways described for the classic white-rot and brown-rot fungi. The uniqueness of the pathway is supported primarily by chemical characterizations of lignin degradation intermediates. Streptomyces degrade lignin by oxidative depolymerization involving the cleavage of intermonomeric β-ether linkages, and the introduction of phenolic hydroxyl and carboxylic acid groups into the lignin polymer as it is depolymerized. These reactions generate water soluble, polymeric fragments which we have named Acid Precipitable Polymeric Lignin (APPL). APPLs are the principal initial lignin degradation intermediate produced by Streptomyces [19,22]. Once liberated, these soluble APPLs are more available to other soil microorganisms. Streptomyces are in effect, lignin solubilizers, which remove lignin from lignocellulose as they degrade it, probably in order to gain more complete access to the polysaccharide components which are degraded with active extracellular cellulases and xylanases. Lignin solubilizing actinomycetes further metabolize APPLs, but only slowly, and our recent data indicate that APPL metabolism by Streptomyces may involve preferential attack on the lowest molecular weight fractions, particularly single-ring aromatics released from APPLs by either enzymatic attack or by chemical hydrolysis.

We have studied only a limited number of organisms, but our findings have recently been collaborated by others [23,24]. Still, there are probably as yet uncharacterized actinomycetes which are exceptions to the pattern described above, in that they may mineralize lignin more efficiently. Lesser studied genera of actinomycetes may also contain species which metabolize lignin differently. Lignin degrading activity has, for example, now been conclusively shown for certain Micromonospora and Thermononspora species [23]. The lignin degrading metabolism actinomycetes clearly needs much additional study. Never-the-less, it

appears that a distinct group of lignin solubilizing actinomycetes, such as S. viridosporus, accentuate the rate of lignin turnover in soil by means of their lignin solubilizing enzymes. Though these species may not be true "wood decay" organisms, they do play an important role in lignocellulose degradation in soil and/or aquatic habitats.

In the remainder of this paper, I will summarize the key chemical and enzymological data which lead us to the conclusions discussed above. I will also point out areas where additional research is needed to better define the role of actinomycetes in lignocellulose biodegradation.

The Biochemistry of Lignin Degradation by Streptomyces

As shown in Tables 1-2, a variety of soil Streptomyces have been shown to metabolize lignocellulosic substrates to substantial weight losses. Chemical analyses of partially degraded residues show that both lignin and polysaccharide components are degraded extensively (Table 1). Further studies have confirmed that these strains produce active extracellular cellulases and xylanases, and they also produce a β-ether-cleaving enzyme system thought to be involved in lignin degradation by Streptomyces [20]. Several strains have been compared in their abilities to attack hardwood, softwood, and grass lignocelluloses, and they show a clear preference for decomposition of lignocelluloses derived from grasses, although hardwood and softwood substrates are also attacked significantly (Table 2).

There are two types of lignin degradation intermediates formed as Streptomyces decompose lignocellulose. They include low molecular weight single and dimeric ring aromatic intermediates [16] and highly polymeric water soluble modified lignins [22]. With S. viridosporus single ring aromatic compounds detected in culture filtrates after 4 weeks of growth on corn stover lignocellulose include p-hydroxyphenylacetic acid (PHPA), p-hydroxybenzoic acid (PHB), vanillic acid (4-hydroxy-3-methoxybenzoic acid; VA), protocatechuic acid (3,4-dihydroxybenzoic acid ; PCA), syringic acid (3,5-dimethoxy-4-hydroxybenzoic acid; SA), p-coumaric acid [3-(4-hydroxyphenyl)-2-propenoic acid; CA], ferulic acid (4-hydroxy-3-methoxycinnamic acid; FA) and several phenylpropanoid ketols [16]. Substituted benzoic acids predominate (PHB, VA, SA), but none were shown to accumulate in amounts greater than 1% of the initial substrate lignin. Another culture, S. setonii, generates a similar range of compounds from lignin but degrades them to undetectable levels after several weeks incubation [19, and unpublished data]. It is possible that these Streptomyces do not accumulate significant amounts of low molecular weight intermediates in part because they are metabolized almost as rapidly as they are produced. However, we have isolated mutants of S. setonii which are blocked in the metabolism of certain key intermediates (VA, FA). They still attack lignin, but do not accumulate significantly increased amounts of the intermediates [19]. We think that these metabolites are produced in small amounts from peripheral monomeric units in the lignin or by release of aromatic acids naturally esterified to the lignins. Their release in turn induces their own metabolism by induction of separately regulated catabolic pathways in those species possessing the independent ability to metabolize the compounds [19]. The ability of any specific culture to attack lignin is, therefore, not obligately tied to its ability to catabolize the single-ring aromatic intermediates.

Table 1. Lignocellulose degrading abilities of ligninolytic *Streptomyces* as shown by their abilities to cause substrate weight loss and to deplete both lignin and carbohydrate components of a lignocellulose prepared from corn stover (*Zea mays*).[a]

Culture	Reference Source	Lignocellulose Weight Loss (%)	Carbohydrate Loss (%)	Lignin Loss (%)[d]
S. viridosporus T7A[b]	16,17	56.7	68.8	44.2
S. setonii 75Vi2[b]	17	49.2	58.7	39.0
S. badius 252[c]	19	15.3	17.4	15.3
S. sp. 523[c]	19	53.2	69.8	28.8
S. sp. 225[c]	19	47.2	67.0	18.4

[a] Cultures were grown on lignocellulose using a solid state (dampened lignocellulose) culture system (22).

[b] Results are for cultures incubated aerobically at 37°C for 12 weeks.

[c] Results are for cultures incubated aerobically at 37°C for 6 weeks.

[d] See reference 22 for details on specific methods used to determine each value.

Table 2. A comparison of the abilities of selected ligninolytic *Streptomyces* to attack softwood, hardwood, and grass lignocelluloses.[a]

Culture	Lignocellulose Weight Loss (%)			Carbohydrate Loss (%)			Lignin Loss (%)[b]		
	Spruce	Maple	Corn	Spruce	Maple	Corn	Spruce	Maple	Corn
S. viridosporus T7A	18.8	23.0	56.7	65.1	45.9	68.8	30.9	32.0	44.2
S. setonii 75Vi2	20.8	19.3	49.2	47.7	37.1	58.7	34.1	29.5	39.0

[a] From results published in reference 17. Cultures were grown for 12 weeks in solid state fermentations (dampened lignocellulose culture) at 37°C. Lignocelluloses were prepared from: softwood spruce (*Picea pungens*); hardwood maple (*Acer platenoides*); and a grass (*Agropyron repens*).

[b] Specific methods for the determination of each value are reported in reference 17.

Table 3. Correlation of lignocellulose degrading ability with Acid Precipitable Polymeric Lignin (APPL) production in selected Streptomyces.[a)]

Organism	Substrate Weight Loss (%)	Lignin Loss (%)[b)]	Carbohydrate Loss (%)[b)]	APPL (mg)	APPL Yield % Initial Lignin
S. viridosporus T7A	36.2	19.7	44.4	48.8	39.0
S. badius 252	15.3	14.7	17.4	33.7	27.0
S. setonii 75Vi2	14.3	16.8	14.5	23.6	18.9
S. sp. 523	47.2	18.4	67.0	20.6	16.5
Coriolus versicolor	39.2	23.6	41.0	11.8	9.4
Phanerochaete chrysosporium	61.3	44.5	64.4	5.1	4.1

a) Results are summarized from data previously reported (19). The growth substrate was corn stover lignocellulose and incubations were carried out in solid state fermentations under optimal conditions for lignin degradation by each organism. Assays were performed as previously described (19), based upon a lignin content of 25% for the lignocellulose substrate used.

b) From the insoluble lignocellulose residues.

Since S. setonii degrades lignin primarily by attacking peripheral units of the lignin polymer, its overall ability to degrade lignin is lessened by its limited ability to depolymerize the polymer and produce modified, water soluble polymeric lignin fragments [21]. Those cultures which do substantially degrade lignin (e.g., in excess of 30% of the lignin in a lignocellulose substrate) also produce large amounts of the polymeric intermediate, Acid Precipitable Polymeric Lignin (APPL) [19,22,25]. The data shown in Table 3 and in Figures 1 and 2 demonstrate the quantitative importance of the APPL intermediate for highly ligninolytic Streptomyces. As shown in Table 3, ligninolytic Streptomyces produce amounts of APPL sufficient to account for essentially all of the lignin removed from the insoluble substrate. In some cases (e.g., S. viridosporus) APPL production appears to be greater than lignin loss from the substrate. However, this can be explained by the fact that the Klason lignin assay used to measure insoluble lignin is insensitive and underestimates actual lignin loss due to the presence of acid soluble lignin [22]. In addition, the APPLs contain up to about 10% of nonlignin contaminants [22,25]. The data also shows that these Streptomyces extensively degrade the polysaccharide fraction of the substrates. In contrast to these Streptomyces, two white-rot fungi even more extensively degraded the lignin component of the lignocellulose, but they did so without producing substantial amounts of APPL. Either APPL is not an intermediate in lignin metabolism by these white-rot fungi, or it is degraded almost as rapidly as it is produced. It should also be noted that we have not yet adequately characterized the APPL-like fungal

products to determine how chemically similar they are to the actinomycete produced APPLs. Formation of a water-soluble polymer from aspen wood lignin by P. chrysosporium has also been reported by Reid et al. [26].

The close correlation of Streptomyces mediated APPL production with lignin loss from substrates is shown in Figures 1 and 2. In these experiments, S. viridosporus T7A and S. badius 252 were grown on corn stover lignocellulose under conditions optimal for lignin degradation by each strain [25]. Lignin degradation and APPL production were followed over time. They correlated quite closely for both cultures. The principal differences between S. viridosporus and S. badius are in the cultural conditions optimal for lignin degradation and in the chemistry of the APPLs produced by each. S. viridosporus grows optimally on lignocellulose in solid state fermentations and produces APPLs at a constant rate over a 6-8 week period. S. badius grows optimally in liquid shake cultures after a period for establishment of growth on dampened substrate, and it produces APPL over a 7-8 day period. The chemistry of the APPLs produced by each strain also differs. The S. viridosporus APPL is chemically more like natural lignin than is the S. badius APPL. These data have been discussed in detail in a previous publication [25]. Recently, we have determined that the S. badius APPL is contaminated by a moderate amount (∿25%) of protein (unpublished data), therefore, the correlation seen in Figure 2 is actually not quite so close. However, these data do show an overall pattern where the lignin lost from lignocellulosic substrates is recovered in large part as modified, water-soluble polymeric lignin (APPL).

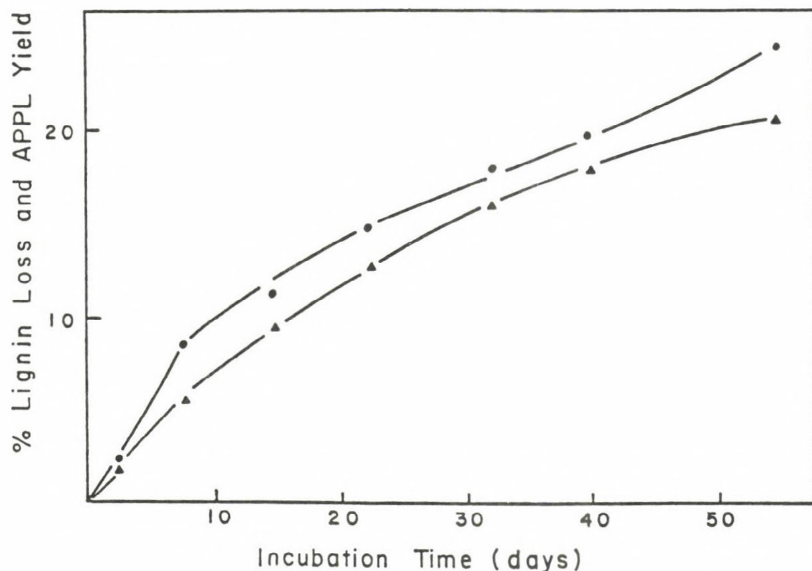

Figure 1. Rate of lignin degradation and APPL accumulation from corn lignocellulose by S. viridosporus T7A in dampened lignocellulose cultures. Symbols: ● , % lignin loss; ▲ , % APPL yield.

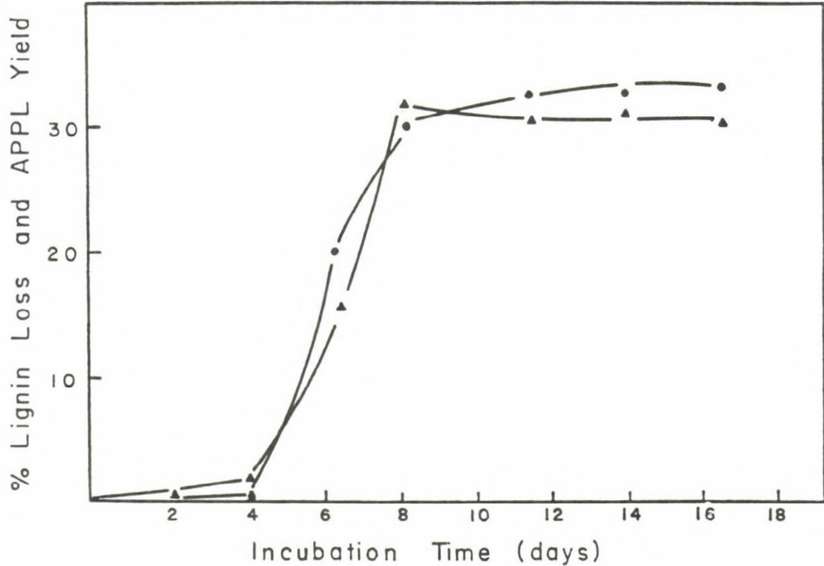

Figure 2. Rate of lignin degradation and APPL accumulation from corn lignocellulose by S. badius in liquid shake cultures. Symbols: ●, % lignin loss; ▲, % APPL yield.

Once produced as a water soluble catabolite, APPLs are only slowly metabolized further by Streptomyces. Data in Table 4 show how S. badius, S. setonii. and S. viridosporus catabolize a ^{14}C-APPL, as compared with the white-rot fungus P. chrysosporium. For this experiment, ^{14}C-APPL was prepared by growing S. viridosporus on a ^{14}C-lignin labeled corn lignocellulose for 4 weeks [27]. Then, the ^{14}C-APPL that had accumulated was extracted from the residues with 0.1 M NaOH, precipitated from the aqueous extract by acidification, washed thoroughly, and air dried. The ^{14}C-APPL was then fed back to each of the organisms listed above as a component [0.05% (w/v) final concentration; 4,680 DPM per culture vessel] of a liquid medium optimal for lignin degradation by the specific Streptomyces [27] or white-rot fungus [28]. Cultures and sterile controls were incubated aerobically for 3 weeks, using a bubbler tube culture system previously described [16]. After 3 weeks incubation, S. setonii was the best lignin mineralizer of the three actinomycetes, but it was not as efficient as P. chrysosporium (6.4% and 10.2% of the ^{14}C-APPL recovered as $^{14}CO_2$ respectively). A significant percentage of the APPL was recovered in the water soluble phase of culture media (Table 4, column 2). The better APPL mineralizers tended to deplete the amount of water-soluble APPL more than the cultures which metabolised ^{14}C-APPL to $^{14}CO_2$ more slowly. The cells of S. setonii, S. viridosporus, and P. chrysosporium also tended to absorb significant amounts of APPL (Table 4, column 4). Absorption of APPL to the cells correlated positively with APPL-mineralizing ability.

Table 4. Catabolism of ^{14}C-[lignin] APPL by the three lignin degrading Streptomyces and Phanerochaete chrysosporium after 3 wk incubation in bubbler tube cultures at 37°C.[a) b)]

Culture	% $^{14}CO_2$ Recovered	% ^{14}C-Water Soluble Recovered[c)]	% ^{14}C-APPL Recovered[d)]	% ^{14}C-Cell Mass Recovered[e)]
S. badius	3.2	42.6	50.4	3.8
S. setonii	6.4	39.3	40.5	13.7
S. viridosporus	4.0	43.0	35.1	17.0
Control	2.2	45.9	51.8	----
P. chrysosporium	10.2	31.2	48.2	10.5
Control[b)]	0.4	45.2	54.5	----

[a)] Each Streptomyces culture was run in triplicate in 0.05% (w/v) ^{14}C-[lignin]-APPL (4,680 dpm), 0.1% (w/v) yeast extract, and 0.1% (w/v) glucose in mineral salts solution (27). Values are averages of the three replicates.

[b)] For Phanerochaete chrysosporium, the medium of Kirk et al. (28) was used with 0.05% ^{14}C-[lignin]-APPL (4,680 dpm). Values are averages of three determinations.

[c)] Percentage remaining in solution after acid precipitation of the APPL. The high values for controls indicate a significant amount of low molecular weight APPL fragments may have been generated during medium sterilization by autoclaving.

[d)] Percentage precipitating from culture filtrate upon acidification to pH ~2.

[e)] Percentage recovered with cell mass when culture supernatants were filtered directly after harvest.

APPLs can be precipitated from aqueous solutions by acidification to pH $\leq$5 [22]. In the present study, uninoculated controls, however, showed an approximately equal distribution of counts between acid soluble and acid precipitable fractions (Table 4, columns 3 and 4). These values indicate that the medium sterilization procedure and/or long term aerated incubation caused significant solubilization of APPL. HPLC analysis has also shown that control culture supernatants contained significant amounts of p-coumaric acid and lesser amounts of other single-ring aromatic compounds [27]. The p-coumaric acid was probably derived from p-coumaric acid naturally esterified to native lignins and released from the corn lignocellulose derived APPL [25]. S. setonii and P. chrysosporium rapidly

degrade these soluble single ring compounds, whereas S. viridosporus metabolizes some of them. S. badius degrades none, but some new compounds appear in the same region of HPLC chromatograms. A more complete discussion of this data is presented elsewhere [27].

Cumulative data indicate that APPLs are degraded, but at a quite slow rate by Streptomyces. Most APPL mineralization to CO_2 is probably at the expense of the most water soluble, lowest molecular weight components of this lignin degradation intermediate. P. chrysosporium, which is capable of the total degradation of lignin [28], attacks APPL rather slowly, but at a much more significant rate than any of the Streptomyces. Thus, Streptomyces appear to generate APPL as an initial lignin degradation intermediate that tends to accumulate as it is slowly metabolized further. In contrast P. chrysosporium steadily mineralizes APPLs to CO_2.

The Enzymes of Lignocellulose Degradation by Streptomyces

There is much to learn concerning the enzymes utilized by lignocellulose degrading Streptomyces. We have examined one culture, S. viridosporus T7A, for its production of cellulases, xylanases and for enzymes of the β-etherase pathway [20]. As shown in Table 5, S. viridosporus and two S. viridosporus mutants known to be stablily enhanced in lignin solubilizing ability [20] produce active cellulases and xylanases. The two mutants produce approximately 50% more APPL when growing on lignocellulose than does the parental stain T7A under similar cultural conditions [20]. The data in Table 5 indicate that they also

Table 5. Cellulase and xylanase specific activities of culture supernatants from Streptomyces viridosporus T7A cultures and selected S. viridosporus lignin degradation enhanced mutants growing on lignocellulose.[a]

Culture	Specific Activity[b]		
	CMCase (μmol/min/mg protein)	Filter Paper Activity (μmol/hr/mg protein)	Xylanase (μmol/min/mg protein)
T7A	0.36(±0.07)	0.35(±0.05)	0.46(±0.02)
T7A-81	0.75(±0.04)	0.28(±0.03)	0.91(±0.17)
T7A-138	0.46(±0.02)	0.28(±0.02)	0.41(±0.26)

[a] Cultures were grown on lignocellulose and enzymes were assayed using five-day (for cellulases) or three-day (for xylanase) culture supernatants as the source of enzymes. Growth conditions and assay procedures have been described (20).

[b] Measured as μmol reducing sugar as glucose formed either per minute or hour per mg extracellular protein, ± standard deviations for 3 replicates (20).

overproduce endoglucanases (CMCase), but not exoglucanase (filter paper activity), but the data is inconclusive regarding xylanases. We feel that overproduction of these polysaccharidases by the mutants is indirectly linked to regulatory mutations which effect ligninases, but that overproduction of cellulase or xylanase is probably not primarily responsible for the enhanced production of APPL by the mutants [20], since some, but not all APPL-overproducing protoplast fusion recombinants, also overproduce cellulase and/or xylanase. The regulatory linkage between cellulases, xylanases and APPL producing enzymes definitely needs to be further examined. The principal point to be made in the present discussion is that S. viridosporus is an active polysaccharide decomposer which degrades both cellulosic and hemicellulosic components of lignocellulose while simultaneously solubilizing the lignin component.

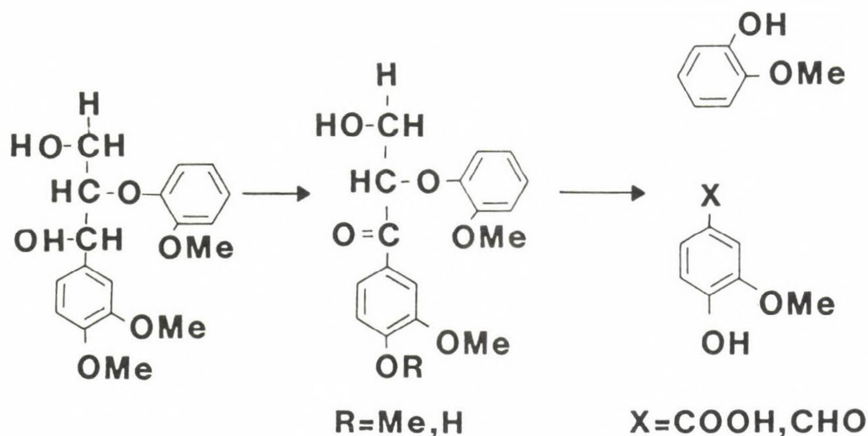

Figure 3. The pathway of veratrylglycerol-β-guaiacyl ether metabolism by ligninolytic, APPL producing Streptomyces.

Previous work has shown that S. viridosporus and S. badius metabolize the lignin substructure model veratrylglycerol-β-guaiacyl ether (VE) by a sequence of reactions involving: 1) demethylation of the p-methoxyl group of the veratrylglycerol unit, 2) oxidation of the α-hydroxyl group on the propane side chain to the α-carbonyl, followed by 3) β-ether cleavage resulting in release of guaiacol from the guaiacyl unit [20]. We have termed this pathway the β-etherase complex (see Figure 3). β-ether cleavage is a lignin depolymerizing reaction, and we have evidence to indicate that ligninolytic, APPL-producing Streptomyces produce the enzymes when metabolizing lignin , synthesizing them as a cell-associated activity [19,20]. Currently we feel that this enzyme system is part of the ligninolytic system of Streptomyces, and that it is involved in the depolymerization of lignin, and at least partly in the production of APPL [19,20]. However, we still expect to eventually discover extracellular

enzymes with depolymerizing activity. If so, the cell associated β-etherase may ultimately be shown to be involved primarily in the oxidation and cleavage of low molecular weight lignin fragments. Yet, this system is almost certainly a part of the lignin degrading system of these Streptomyces. Our work with the wildtype strains of S. viridosporus, S. badius, S. setonii, and with APPL-overproducing S. viridosporus mutants and protoplast fusion recombinants, shows that the ability of strains to produce APPL correlates with their ability to produce the β-etherase complex, which is inducible by lignocellulose or APPLs [20]. All APPL-overproducing mutants and recombinants also overproduce β-etherase [20,21]. Despite these preliminary findings, the make-up and cellular locations of the ligninolytic enzymes of Streptomyces remain unclear. Yet, the accumulated data thus far indicates that Streptomyces ligninases are involved primarily in lignin solubilization, not lignin mineralization.

FINAL REMARKS

It seems clear from what we now know of the biochemistry and enzymology of lignocellulose degradation by actinomycetes that a novel group of Streptomyces, of which Streptomyces viridosporus T7A is the best studied example, decompose lignocellulosic plant residues by mineralizing and assimilating cellulosic and hemicellulosic components while simultaneously solubilizing the lignin component by oxidative depolymerization. The solubilized lignin is slowly metabolized by these Streptomyces and is probably also attacked by other soil microorganisms. These modified lignins (APPLs) may also be incorporated into humus fractions in soil. It appears that there are considerable variances in lignin metabolism between individual Streptomyces with regard to how well APPLs are further metabolized, though the rate of metabolism is quite slow in all strains thus far examined. It is also evident that the chemistry of APPLs produced by different lignin solubilizing Streptomyces may also vary. There is a need for further research to delve into these differences with an emphasis on identifying and characterizing more ligninolytic enzymes, particularly extracellular ones. Differences in ligninases and aromatic compound oxidizing enzymes between different strains may be considerable. It is important, too, that a wider variety of actinomycetes be characterized to determine whether lignin solubilizing species are dominant within the group, or whether some actinomycetes are able to more completely degrade the lignin polymer. It is also important to characterize more organisms with respect to their efficiencies of decomposition of grass, hardwood and softwood lignocelluloses. Thus, we have only scratched the surface of potential physiological ecology research on the actinomycetes. Yet, what we have learned in recent years is intriguing.

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ECOLOGY AND EPIDEMIOLOGY

Mini-Symposium

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ACTINOMYCETE SCUM IN SEWAGE TREATMENT PLANTS

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BIOLOGICAL WASTEWATER TREATMENT

Biological wastewater purification is a continuous-flow process where biomass is recycled. This recycling allows a mean cell residence time longer than the wastewater residence time which is necessary for an elimination of up to 95% of the organic load of the primary effluent. The efficiency of a sewage treatment plant is not only depending on high degradation efficiency but also on the complete separation of activated sludge microorganisms from the purified secondary effluent. In most treatment plants separation is achieved by sedimentation. The two main problems connected with this process are sludge bulking and sludge scumming. The first problem is mostly caused by a high biomass of filamentous microorganisms such as Sphaerotilus spec. or fungi but never by actinomycetes (Eikelboom 1975). The filamentous structure of these sludge bulking organisms prevents proper settling. Because of high viscous drag sludge is hanging in the bulk of water which can cause sludge losses into the secondary effluent. In contrast sludge scumming is caused by high densities of nocardioform actinomycetes in 90% of the reported cases. The branched hyphae of these organisms form a net between the sludge flocs which entraps grease and oil droplets or gas bubbles which make the sludge float on the surface of the secondary clarifier (Pipes 1978).

The aim of our research was scum problem troubleshooting in sewage treatment plants by the knowledge of physiological and ecological characteristics of scum actinomycetes.

CHARACTERIZIATION OF.SCUM ACTINOMYCETES

From 14 isolates 11 scum actinomycetes belong to the Rhodococcus rhodochrous group, 2 to the Gordona aurantiaca group, 1 is Nocardia amarae (Lemmer and Kroppenstedt 1984).

The main physiological characteristics of these scum actinomycetes are the versatility in utilizing carbon-, nitrogen-, phosphorus- and sulphur-sources, relatively long generation times even under optimal growth conditions (3-14h depending on the species), the linear dependence of cell yield on the substrate concentration up to values which are a 100times higher

than those typical for primary effluents and the high hydrophobicity of the cell surface (Lemmer 1985).

Their most important ecological feature is the ability to switch between two growth strategies: K-strategy is used if substrate is scarce. This strategy is characterized by a high substrate uptake efficiency at low substrate concentrations, low K_s-values and relatively low cell yield. μ_{max}-strategy is used if substrate is abundant. This strategy is characterized by a high biomass production with growth rates exceeding those in K-strategy.

BEHAVIOUR OF SCUM ACTINOMYCETES IN SEWAGE TREATMENT PLANTS

In scumming activated sludge actinomycetes use μ_{max}-strategy increasing cell counts up to a 100times the values found in normal sludge where actinomycetes maintain a stable background population by using K-strategy. Actinomycetes are able to compete successfully with other sludge organisms under various environmental conditions (Lemmer and Popp 1982). By several means they are provided with high substrate concentrations as are necessary for μ_{max}-strategy growth. In treatment plants with low organic load, i.e. low food to microorganism ratio they achieve sufficient substrate concentration by living saprophytically on dead cells. In plants with a high load of hydrophobic wastewater ingredients such as grease and oil they are selectively supplied with food by the adherence of this substrate to their hydrophobic cell surface (Matsché 1980). In plants with a high load of easily degradable substrate competition pressure by other μ_{max}-strategists is strong so that actinomycetes have little chance to survive. However, bench scale experiments showed that actinomycetes outgrow other sludge bacteria if detergents are present in concentrations of 7mg/l. Such values are also found in primary effluents of sewage treatment plants. Surface-active materials accumulate dissolved substrate at gas/water-interfaces (Wangersky 1976). Surface-active molecules serve as ionic samplers by orienting at the interface and secondarily trapping water ingredients. By the enhanced adherence of gas bubbles at the hydrophobic cell surface actinomycetes achieve selective supply with accumulated substrate. By the excretion of surface-active material (Lechevalier et al. 1976) actinomycetes are able to trigger such accumulation mechanisms.

The stable adherence of gas bubbles and grease particles to the hydrophobic cell surface of the actinomycetes makes the sludge float to the surface of the secondary clarifier. Because of its density gas is by a factor of 1000 more effective in causing buoyancy than grease and oil. This is the reason why oil never was reported to be the only floating agent in scum events.

Within the sludge blanket on the secondary clarifier actinomycetes have selective advantages over other sludge bacteria as for instance resistence against UV-radiation by pigmentation, resistence against dehydration and the possibility to store reserve material as poly-ß-butyrate and polyphosphates. Thus they cannot only survive but even increase biomass in the sludge blanket.

Most important for fighting scum production in sewage treatment plants is therefore the continuous and complete removal of this sludge blanket where actinomycetes are accumulated. After dehydration and degassing by for instance centrifugation this sludge fraction should be transferred to the anaerobic digester.

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GENOME IDENTITY OF DIFFERENT NOCARDIA AUTOTROPHICA ISOLATES FROM ALNUS spp. ROOT NODULES AND RHIZOSPHERE

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Eleven actinomycete strains identified as *Nocardia autotrophica* [1] were isolated from root nodules and the rhizosphere of two alder species (*Alnus incana* and *A.glutinosa*) from different geographical regions. The isolates are characterized by the unusual life cycle (Fig.1), wall chemotype IV and the absence of mycolic acids [2]. All the strains were shown to be very similar in many other phenotypic properties [1,3,4] displaying only insignificant differences in a few cultural and physiological characteristics.

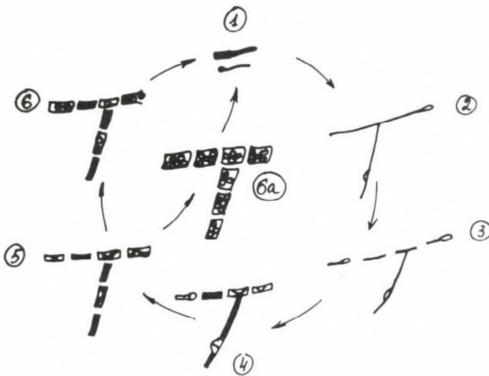

Fig.1. Schematic life cycle of a *N.autotrophica* strain isolated from an alder association, showing fragmentation of hyphae and formation and maturation of non-motile spore-like cells within the fragments.

The genome structure was studied in three arbitrarily chosen strains of this group, 3LS and 11LS having been isolated from root nodules of *A.incana* in Moscow region, while 6LS having been isolated from the *A.glutinosa* rhizosphere in the Caucasian Reservation. Restriction analysis of total cellular DNAs by seven site-specific endonucleases revealed absolute identity between the strains 3LS and 11LS. DNA restriction banding patterns of the strain 6LS produced by *Bgl*II (Fig.2,B), *Eco*RI, *Eco*RV and *Xho*I (data not shown) were also identical to those of the former two strains. The occurrence of one or several higher molecular weight extra fragments in the *Bam*HI (Fig.2,A), *Sma*I (Fig.2,B) and *Sal*I (data not shown)

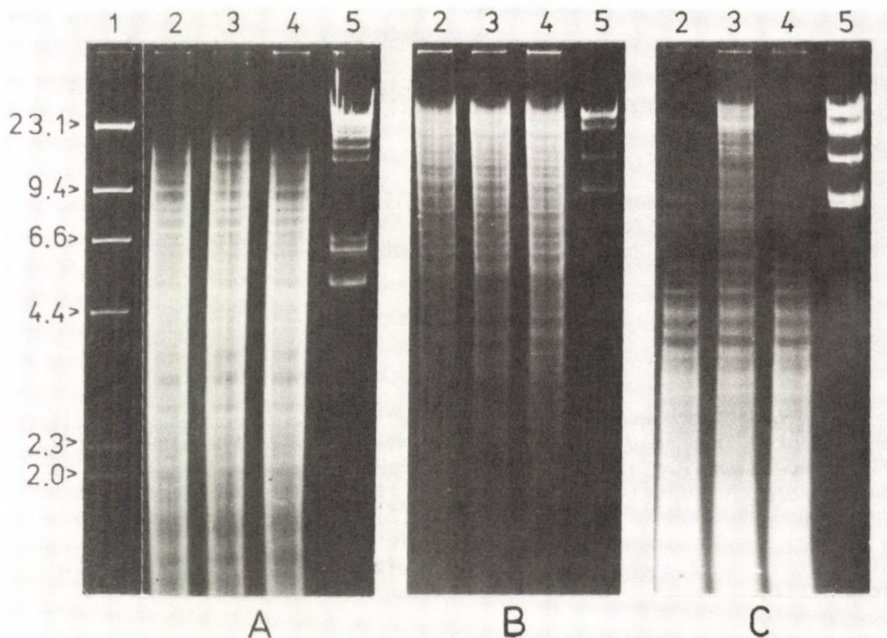

Fig.2. Agarose gel electrophoresis of *N.autotrophica* 3LS (2), 6LS (3) and 11LS (4) DNA fragments as well as those of bacteriophage λ (5) produced by the restriction enzymes *Bam*HI (A), *Bgl*II (B) and *Sma*I (C). λ DNA *Hind*III restriction fragments were used as a molecular weight marker (the sizes in kilobase pairs are indicated on the left).

DNA digests from the strain 6LS as compared to 3LS and 11LS can be attributed to peculiarities of the DNA preparations used but not to any strain differences in the DNA sequences or DNA methylation.

The structural relationship between the genomes was further confirmed by the identity of the plasmid profiles in the strains 3LS, 6LS and 11 LS [5]. Each of them harbours two large plasmids (ca. 80 and 120 Md, Fig.3). The results permit concluding that these strains have the same genotypes and, therefore, can be referred to as isogenic subcultures of the same organism.

The further investigations (S.V.Dobritsa, to be published) indicated that the alder-associated strains differed from collection *N.autotrophica* strains both in DNA restriction banding patterns and in plasmid sets and, hence, represented a special population.

Thus, the rhizoplane and rhizosphere of nitrogen-fixing actinorhizal plants seem to be a natural habitat of *N.autotrophica*, which may be due to hydrogen autotrophy of this species [6]. Alder trees may be supposed to create a specific ecological background promoting selection of strains having this particular genotype. Better understanding of the ecology of

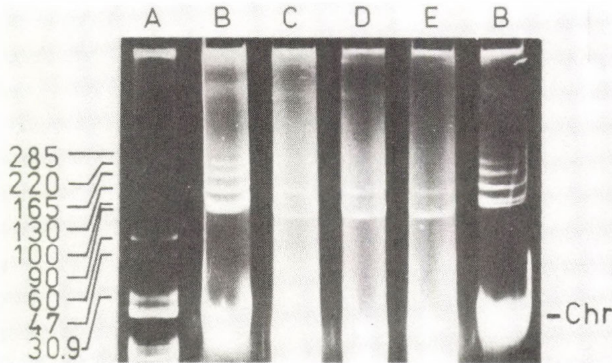

Fig.3. Agarose gel electrophoresis of cleared lysates from *N.autotrophica* 3LS (C), 6LS (D) and 11LS (E). M_r - values ($\times 10^{-6}$) of the marker plasmids from *Bacillus megaterium* 216 (A) and *Rhizobium leguminosarum* T3 (B) are the same as in [5].

these actinomycetes and specifically their interactions with plants may ultimately clear up the remarkable similarity between the independent isolates.

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STUDIES ON ACTINOMYCETES ISOLATED FROM AUSTRALIAN SOILS

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Actinomycetes are one of the outstanding groups of soil microorganisms, similar to bacteria and fungi. As is well known, the microflora in soil is greatly affected by environmental conditions such as climate, vegetation and mineralogical properties. Therefore, we have studied actinomycetes in various kinds of soil including marine sediment. We collected 305 soil samples from various areas of Australia having very different environmental conditions from Japan; Darwin(43) Cairns(44), Brisbane(69), Sydney(35), Canberra(20), Melbourne(25), West Australia(8) and Tasmania(61).

Generally speaking, Australian soils are very immature and give a very different appearance from Japanese soils. Physical characteristics of soils in Australia and Japan are shown in Table 1.

Table 1. Physical characteristics of soils in Australia and Japan

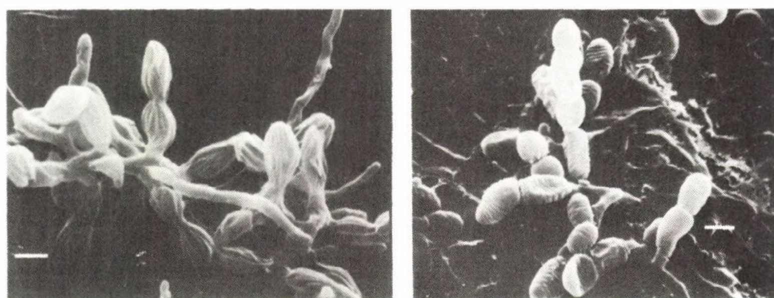
	pH		Nutrient (%)		Moisture (%)	
	Australia	Japan	Australia	Japan	Australia	Japan
$\tilde{x}$	6.808	6.079	9.470	18.583	2.958	11.605
d	0.208	0.561	3.623	8.361	0.603	4.401

$\tilde{x}$; average (N=48), d; standard deviation

The pH of Australian soil is mostly neutral and the nutrient ratio is about half that of Japanese soils. Also, moisture in Australian soils is extremely low as compared with Japanese ones. These characteristics of Australian soil do not suit actinomycetes, because most of them are fond of rich nutritious soils. However, we have devised isolation conditions relating to the environment where the strain were isolated. PCA and SEA media containing novobiocin were used for micromonosporas and actinoplanetes, and UGS and NGS with tetracycline for nocardioform and micropolysporas. If no actinomycetes appeared, some organic materials and/or dist. water were added to the soil and preserved 1 - 6 months at room temperature. Afterward, we tried reisolation of actinomycetes from the enriched soils. Thus we have isolated 4,286 actinomycetes; *Streptomyces* 2,762, *Streptoverticillium* 22, *Chainia* 3, *Actinoplanes* 247, *Streptosporangium* 21, *Ampullariella* 4, *Plimelia* 1, *Micromonospora* 96, *Microbispora*

1, Microtetraspora 5, Micropolyspora 11, Nocardioform 448 and other actinomycetes 665. These isolates contained not only taxonomically interesting strains but also industrially important ones.

Strain SANK 62681 was isolated from an acacia forest soil collected in Brisbane. It is characterized by good sporulation, pinkish white mass color of aerial mycelium, long axial hyphae and longitudial paired spores with a unique surface having vertical rugose. The cell wall contained only meso-A_2pm as a major constituent. Glycine, arabinose and galactose were not detected. The strain can be considered to have a Cell Wall Type III. These taxonomical results place strain SANK 62681 in the genus Microbispora. Among known species of Microbispora, M. rosea most closely resembles strain SANK 62681. However, the surface of spores of strain SANK 62681 showed vertical rugose, whereas one of the type strains of M. rosea showed smooth or rarely horizontal rugose as shown in Photo 1.



Strain SANK 62681 M. rosea KCC A-0030

Photo 1. Scanning electron micrographs of spores of strain SANK 62681 and M. rosea KCC A-0030 (on inorganic salts-starch agar, 18 days at 28°C). A mark equals 1 μm

Therefore, strain SANK 62681 is considered to represent a new subspecies of the M. rosea for which the name M. rosea subsp. laenispora subsp. nov. is proposed (Enokita et al. 1981).

Strain SANK 60384 was isolated from a soil sample collected in Melbourne. From its taxonomic studies, the strain was determined to be Streptomyces olivaceus. This strain was found to produce a new antibiotic, deoxycephamycin B, as well as known antibiotics, cephamycin B and diumycin B group antibootics. Deoxycephamycin B was active against Gram-positive and negative bacteria but inactive against yeast and fungi. Mice tolerated intravenous administration of 100 mg/kg of the antibiotic. The structure was determined by its physico-chemical properties and chemical degradation studies as shown in Fig. 1 (Takahashi et al. 1984).

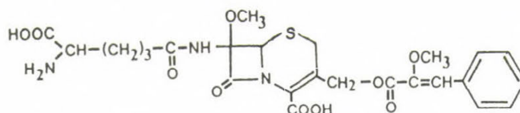


Fig. 1. The structure of deoxycephamycin B

Three actinomycetes capable of 3β-hydroxylation of ML-236B (compactin), a competitive inhibitor of HMG-CoA reductase, were isolated from soils at Canberra (Fig. 2). 3β-hydroxy ML-236B is expected to be used as hypo-lipidemic drug (Serizawa et al. 1983).

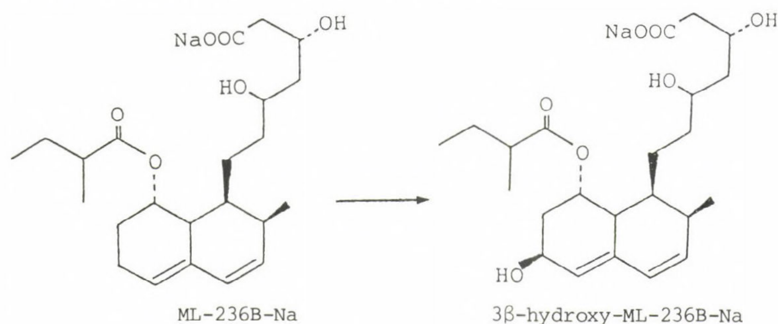


Fig. 2. Microbial transformation of ML-236B-Na

From these taxonomical studies, strain SANK 62781 isolated from eucalyptus forest soil was identified as Nocardia autotrophica. Strains SANK 62881 and SANK 62981 isolated from lake-side sand and lake bottom sediment were identified as new subspecies of Nocardia autotrophica for which the names N. autotrophica subsp. canberrica (ATCC 35203) and N. autotrophica subsp. amethystina (ATCC 35204), respectively, were given (Okazaki et al. 1983).

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ACTINOMYCETES OF THE CARPOSPHERE OF VITIS VINIFERA

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Istituto di Patologia Vegetale, Università
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Milano, Italy

INTRODUCTION

The carposphere microflora of grapevine has been the subject of several investigations, but the actinomycete fraction has, to our knowledge, never been studied. This investigation has been undertaken in order to establish the developmental dynamics of actinomycete microflora throughout the growing season and to study the potential role of actinomycetes as potential antagonists of Botrytis cinerea. (Bisiach et al.; 1981, Ciccarone; 1970).

MATERIALS AND METHODS

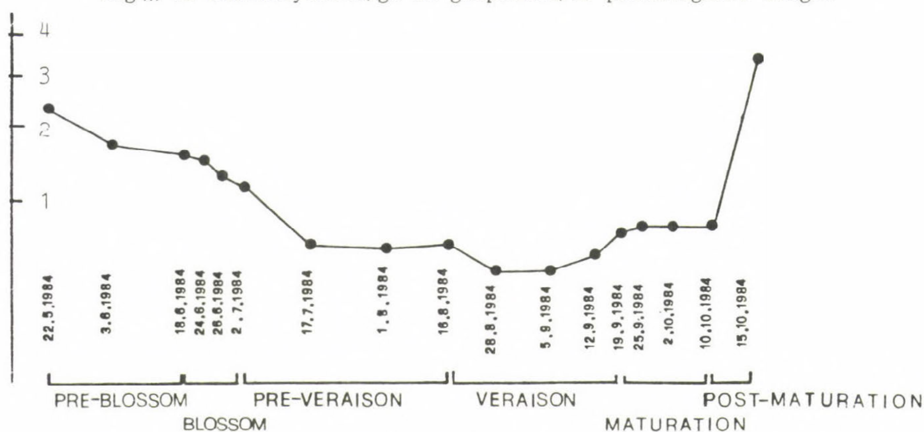
Samples of Vitis vinifera, cv.Barbera, were collected in the Oltrepò Pavese at the following phenological stages: Pre-blossom, Initial blossom, Full blossom, Post blossom, Pre-veraison, Initial veraison, Full veraison, Post veraison, Maturation, Post maturation. Ten clusters were collected, washed in an amount of water sufficient to cover the samples then were shaken for ten minutes and plated. After the blossom stage berry and rachis were washed and plated separately.

Counting and isolation were carried out by dilution techniques on Küster and Williams agar (1964) at 27°C. Selected colonies were transferred to potato dextrose agar and identified according to Küster Key (Küster; 1972),(KK),and the ISP results (Shirling and Gottlieb; 1968, 1969). Spore surface was determined using a Cambridge Stereoscan 250. Antibiotic activity was tested against E.coli, B.subtilis, S.cerevisiae, A. niger, B. cinerea.

Chemical characterization was carried on liquid cultures extracted with organic solvents separatly for mycelium and broth.

RESULTS

The fluctuation in the actinomycete population during the growing season is shown in Graph 1.



Graph 1: Distribution of actinomycetes with reference to phenological stages.

Actinomycete microflora is very low up to the maturation stage. A total of 3000 colonies were counted, but of these 2578 were found in the last sampling. 35 strains were selected and identified as streptomycetes species. According to KK they could be related to the species listed in Table 1.

Table 1: Identification of isolates according to KK

color of aerial MY	distinctive Riv side pigment	melanin R X N	soluble pigment	sporophores	spores	* Sugar utilization								Defined strain
						RH	RF	I	M	X	F	A	S	
GY	D	—	—	SP	SP		—							S. arabicus (ipv-2654 2684)
GY	D	—	+	SP	SM	—						—		S. tubercidicus (ipv-2666,2668,2669,2672,2679,2692)
GY	no D	—	—	SP	SP	+	—							S. viridodiastaticus (ipv-2659,2660,2673,2675,2676)
GY	no D	—	+	SP	SP									S. canus (ipv-2667, 2685,2682)
GY	no D	—	—	SP	SP	+	+	+						S. malachiticus (ipv-2674)
GY	no D	—	—	SP	SM	+							+	S. parvullus (ipv-2694)
Y	D	—	—	F	SM							+		S. citreus (ipv-2677)
Y	D	—	—	F	SM							—		S. varsoviensis (ipv-2681)
Y	D	—	—	ST	SM							+		S. rubiginosohelvolus (ipv-2688, 2691)
Y	no D	—	—	ST	SM	+							+	S. autotrophicus (ipv-2678)
Y	no D	—	—	ST	SM	—						+		S. levoris (ipv-2680, 2693)
Y	no D	—	—	F	SM	+			+	+	+	—		S. microflavus (ipv-2686)
Y	no D	—	+	F	SM	—								S. somaliensis (ipv-2695, 2696)
Y	no D	—	—	SP	SM	+		—	+					S. spheroides (ipv-2687, 2689)
Y	no D	—	—	F	SM					—				S. albohelvatus (ipv-2670, 2671)
B	no D	+	+	SP	SP									S. bicolor (ipv-2697)
W	D	—	+	ST	SM									S. griseoalbus (ipv-2683)
R	no D	—	—	F	SM					—	—			S. roseolilacinus (ipv-2690)

* Abbreviations: RH: Rhamnose, RF: Raffinose, I: Inositol, M: Mannitol, X: Xylose, F: Fructose, A: Arabinose, S: Sucrose

They were randomly distributed throughout the all phenological stages without any dominance. The percentage of active strains is shown in Table 2. No active metabolite could be characterized.

Table 2: Percentage of active strains isolated during the phenological stages

phenological stages	E. coli	B. subtilis	S. cerevisiae	A. niger	B. cinerea (1) (2)*
pre-blossom	10%	40%	10%	30%	80%
blossom	87,5%	75%	25%	37,5%	100 %
pre-veraison	83,3%	83,3%	—	—	66,6%
veraison	40%	40%	100 %	80%	100 %
maturation	33,3%	33,3%	66,6%	—	100 %
post-maturation	66,6%	—	66,6%	33,3%	66,6%

* B.cinerea (1) and (2) differ from each other in their virulence

CONCLUSION

As shown actinomycetes are not abundant on the grapevine surface during growth, but there is a very large increase during post maturation. This can be explained by the composition of berry surface which allows their development. The activity against S.cerevisiae and B.cinerea is interesting with particular reference to their ecological and phytopathological implications.

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DISTRIBUTION OF RHODOCOCCI IN THE ENVIRONMENT

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The bacteria of the genus Rhodococcus play an important role in nature and human life. Rhodococci decompose compounds weakly assimilated by other microorganisms: hydrocarbons, xenobiotics etc.; they can produce amino acids, enzymes, polysaccharides, organic acids, vitamins, antibiotics. Among them there are species pathogenic for man, animals and plants.

Information on the distribution of rhodococci in nature is rather limited. This work is devoted to this problem.

METHODS

Rhodococci from Ukrainian soils were isolated at 26-28°C with the help of nutrient enrichment methods. Enrichment cultures were obtained on mineral salt media containing various nitrogen sources and 1-2% of mixture of n-alkanes C_{12}-C_{22} (Nesterenko et al. 1978, 1984). Munz medium was the best one. Winogradsky's nitrite agar was almost of the same value. Various species of rhodococci demand different media: R. flavus, R. longus and R. rubropertinctus are better isolated on Munz medium, but R. maris and R. ruber on nitrite medium. R. erythropolis is isolated on all test media with the same frequency.

Munz and nitrite media are effective for isolation of rhodococci from sludge of salinized and fresh water estuaries, from Dnieper river water, mineral water like "Naphtusya". As rhodococci grow well on individual n-alkanes from C_{13} to C_{18} hydrocarbon mixture in the above-mentioned media may be replaced by one of n-alkanes. For isolation of rhodococci from fresh water reservoirs we also successfully used some other mineral salt media with n-alkanes (Kvasnikov et al. 1981).

For isolation of rhodococci utilizing propane and butane from ground waters of oil-bearing deposits, enrichment cultures were obtained on liquid and agarized mineral salt medium K in an atmosphere of propane (or butane) - air (1:1) (Ivshina et al. 1981). Rhodococci from stratal salinized waters and diluted stratal waters of oil-bearing deposits were isolated according to described methods (Berdichevskaya 1982; Berdichevskaya et al. 1984).

RESULTS

We have established that rhodococci are widely distributed in nature. They were isolated from all 305 soil samples taken from various soil-climatic zones of the Ukrainian SSR including samples of oil-bearing soils of western and eastern Ukraine. In soils which do not contact with oil from different soil-climatic zones of the Ukraine (woodlands, forest-steppe, Cis-Carpathians), R. erythropolis was isolated more often. Other species - R. longus, R. luteus, R. maris, R. opacus, R. rubropertinctus and particularly R. flavus and R. ruber - were present less often. The poorest ones for rhodococci are soils of Cis-Carpathians (Nesterenko et al. 1978).

In soils impregnated with oil in western and eastern regions of the Ukraine the most numerous were R. erythropolis, R. luteus, R. opacus and R. ruber, R. aquosus, R. rhodochrous, R. terrae have been isolated only from oil-bearing soils; the amount of these species was negligible. Soils impregnated with petroleum from eastern Ukraine were more abundant in strains of many species of rhodococci than similar soils of western Ukraine.

Rhodococci have also been detected in soils from the South of the USSR (Krasnodar, Stavropol Territories, Georgian SSR, Armenian SSR). From these soils R. erythropolis is often isolated. Considerable amounts of strains of R. aquosus which were rarely registered only in oil-bearing soils of eastern Ukraine have been isolated from southern soils. R. flavus, R. maris, R. rubropertinctus were isolated less often.

Rhodococci were found in sludge of salinized estuaries (R. flavus, R. opacus, R. rubropertinctus and particularly

often R. aquosus, R. erythropolis, R. ruber), in sludge of fresh water reservoirs (R. aquosus, R. erythropolis).

Rhodococci are widely distributed in waters of Dnieper river ports (Kvasnikov et al. 1981). More frequent were R. erythropolis, R. luteus, R. rubropertinctus (which were common for soils, of Ukr. SSR) and also R. terrae, R. opacus, R. rhodochrous, and R. ruber have been recovered very seldom. Rhodococci have been detected more often in waters of those ports, where oil pollution was higher. In waters of Dnieper fair-way only R. erythropolis was registered.

In curative water of "Naphtusya" type we have revealed R. erythropolis, R. luteus, R. ruber, R. rubropertinctus.

We have established that rhodococci capable of assimilating propane and butane are common for ground water of oil-bearing deposits (Ivshina et al. 1981). Their amount was 10^3-10^4 c.f.u./ml of water. No propane- or butane-oxidizing bacteria were isolated from water originating from the drilled-out nonproductive ("empty") structure. The majority of isolated rhodococci was identified as R. rhodochrous, considerably less as R. ruber.

Table 1. Rhodococci recovered from different sources

Species isolated	Sample
R. aquosus	Soil, sludge
R. erythropolis	Soil, sludge, river water, oil strata
R. flavus	Soil, sludge
R. longus	Soil
R. luteus	Soil, river water, fish, mineral water of "Naphtusya" type, oil strata
R. maris	Soil, algae, fish, water of salt industry, oil strata
R. opacus	Soil, sludge, river water
R. rhodochrous	Soil, sludge, river and ground waters, oil strata
R. ruber	Soil, river and ground waters
R. rubropertinctus	Soil, sludge, river water
R. terrae	Soil, river water

Rhodococci (R. erythropolis, R. luteus, R. maris, R. rhodochrous) may be isolated readily from stratal salinized waters and diluted stratal waters with different salinity (0-267 g/l) and different content of H_2S (0-278 mg/l) (Berdichevskaya 1982).

We have detected rhodococci in waters of salt industry (R. luteus, R. maris), on skin and in intestinal tract of fishes (R. luteus, R. maris), on surface of algae Dunaliella viridis and Nephrochloris salina) (R. maris), in sediments from mud-boxes of alcohol plants (R. aquosus, R. rubropertinctus), in marine sediments (R. terrae).

Thus, different species of rhodococci are widely distributed in nature (Table 1). For preferential isolation of rhodococci from different sources media with n-alkanes are recommended.

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ON THE PROBLEMS OF ECOLOGICAL-TAXONOMIC ANALYSES OF COMPLEX NATURAL MUD-MICROMONOSPORA POPULATIONS

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As regards its frequency of occurrence in many habitats of the world, Micromonospora occupies the second place among the most common genera of sporogeneous actinomycetes behind Streptomyces. In contrast to this, it represents a taxonomically very unsatisfactorily studied, otherwise very large group of microorganisms which has only been described on the basis of a few recognized species. This is the mean reason why at present all efforts to analyse complex natural Micromonospora communities at specific level encounter great or even insoluble difficulties. Below I try to demonstrate this statement by quoting two typical examples.

RESULTS OF A NUMERICAL TAXONOMIC ANALYSIS ON THE MICROMONOSPORA POPULATION OF THE BOTTOM MUD OF LAKE BALATON (W. HUNGARY)

A total of 120 representative strains of Micromonospora selected from among our Balaton's mud isolates were subjected to 172 cultural-morphological and physiological tests (Szabó and Fernandez 1984), the results of which were numerically analysed. 20 authentic (type) strains of recognized Micromonospora spp. were also involved in these studies. The data were analysed by a Control Data B-300 type computer for calculating the similarity values according to Sokal and Michener (1958) and clustering the strains into groups of high level of similarity according to Ferenczy (Szabó et al. 1967).

20 percent of Balaton-strains (among them gentamicin--producing ones; Szabó 1984) were identified as representatives of the following species: Micromonospora coerulea, M. echinospora, M. inositola, M. parva, M. purpureochromogenes and M. sagamiensis.

Two large clusters representing 30 and 10 %, respectively, of our mud strains were numerically sharply separated from each other, and from all synchronously studied authentic strains of the Micromonospora species known so far. They were designated by us as Micromonospora balatonica sp.nov. and M. nigra sp. nov., respectively, and their descriptions is published elsewhere.

COMPOSITION OF MICROMONOSPORA COMMUNITIES IN THE BOTTOM MUD OF SOME PONDS IN THE HUNGARIAN ALKALI SOIL REGION

In the second series of our studies, altogether 117 representative strains were selected from among our *Micromonospora* isolates obtained from some ponds of the Hungarian alkali soil region. For numerical analyses 122 properties were tested. In these studies we included 23 authentic strains.

At a similarity level of 81.1 %, all of the 140 strains formed a single group (Fig. 1). 100 strains were separated into 4 clusters defined at between 89.9 and 90.8 % similarity levels, whereas 40 strains, among them all of the authentic strains, remained unclustered. Consequently, the common *Micromonospora* types proved to be unidentifiable also in this case.

The largest cluster (No. 2.; Fig. 1) comprised 69 strains isolated from different ponds. It represents an undescribed species of very broad range of phenotypic variability.

Two further relatively larger clusters (Nos. 1. and 3.; Fig. 1) comprised 11 and 15 mud isolates, respectively. They can be considered assemblages of the members of two distinct new species, easily separable also by conventional methods (Table 1). We designated them as *Micromonospora alcalophila* sp. nov. and *M. hungarica* sp. nov., respectively. Their original descriptions are presented below.

DESCRIPTION OF MICROMONOSPORA ALCALOPHILA SPEC. NOV.

alcalophila (liking alkaline media). Sporulation is limited, spores are spherical and single, dispersing evenly on the hyphal filaments ("open web" type sporulation). Sporophores arranged monopodially. Fair to good growth on yeast extract-malt extract agar (ISP Med. 2), inorganic salts-strach agar (ISP Med. 4), Czapek-sucrose agar and glucose-asparagine agar. Poor growth on glycerol-asparagine agar (ISP Med. 5) Colonies raised and folded, characteristically yellowish or pale orange colored and covered sometimes with minute dark gray spots. A pale yellowish diffusible pigment occasionally observed surrounding colonies on some media. Aerobic. Good growth on and positive utilization of L-arabinose, D-xylose, L-rhamnose, D-fructose, D-galactose, D-glucose, mannose, cellobiose, β-lactose, maltose, sucrose, trehalose and dextrin. Growth with sorbose, α-melibiose, raffinose, inulin, adonitol, dulcitol, glycerol, D-mannitol and i-inositol was not better than on the negative control medium. Good growth on acetate, citrate and pyruvate. No growth or only in traces on oxalate and benzoate. Cellulose, aesculin, arbutin and starch are hydrolyzed, but allantoin and hippurate not. Milk is digested. Nitrate and tellurite reduction tests are positive. Poor or no growth at 40 $^{\circ}$C temperature. Growth on potato slices without calcium carbonate (pH 5.8 - 6.0) is fair. Maximum tolerated level of NaCl is 5 %. Isolated from the mud of ponds in the Hungarian alkali soil area. Type strain: Z-86.

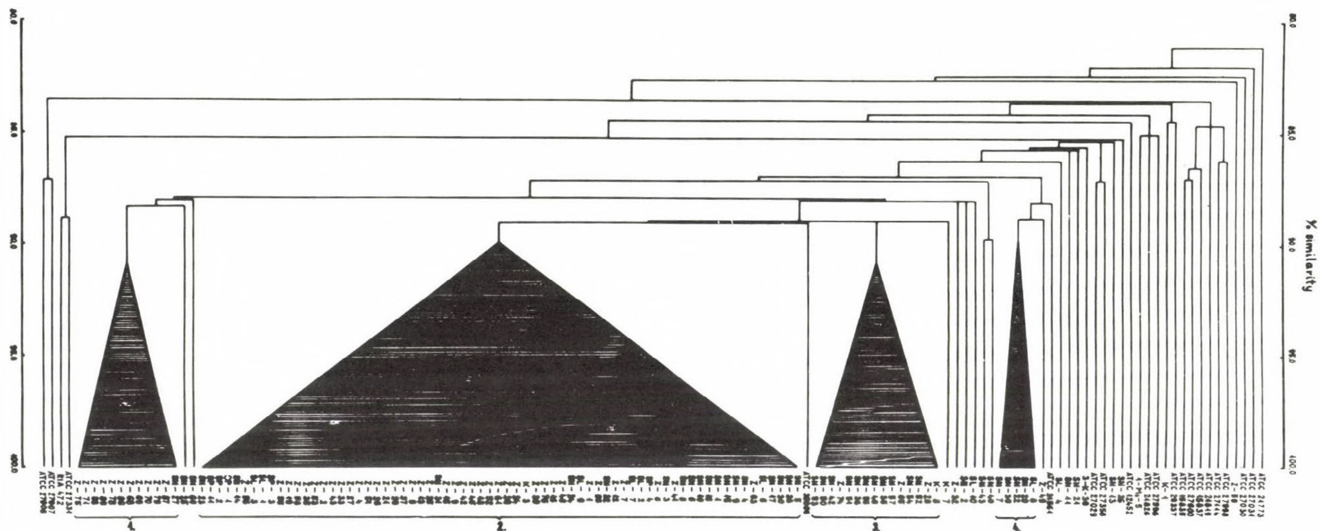

Fig. 1 A simplified dendrogram based on simple matching coefficients for 117 Micromonospora strains isolated from mud-samples obtained from some ponds in the Hungarian alkali soil area and for 23 authentic (mostly type) strains of Micromonospora spp.

	M.alcalophila[1]	M.aurantiaca[2]	M.balatonica[3]	M.carbonacea[4]	M.chalcea[5]; M.narashino[6]	M.chalcea ssp.izumensis[7]	M.coerulea[8]	M.echinospora ssp. echinospora[9]	M.fusca[10]	M.halophytica ssp. halophytica[11]	M.heviziensis[12]	M.inositola[13]	M.inyoensis[14]	M.lilacina[15]	M.megalomicea ssp.nigra[16]	M.nigra[17]	M.olivasterospora[18]	M.parva[19]	M.purpurea[20]	M.purpureochromogenes[21]	M.rosaria[22]	M.rubra[23]	M.sagamiensis[24]	M.hungarica[25]
Utilization of L-Arabinose	+	+	+	+	+	+	-	+	+	+	+	+	-	+	+	+	-	+	+	-	+	+	+	-
L-Rhamnose	+	-	-	-	-	+	-	+	-	-	+	-	-	+	-	-	-	-	-	-	+	+	-	-
D-Fructose	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
D-Galactose	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+
β-Lactose	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	-	+	-	+	+	+	-	+
α-Melibiose	-	+	+	+	+	+	+	-	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-	-
Trehalose	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
Raffinose	-	+	+	-	+	+	+	-	+	-	+	+	-	+	-	-	-	-	-	+	-	+	-	-
D-Mannitol	-	-	+	-	-	+	+	-	+	-	+	-	-	+	-	-	-	-	-	-	+	+	-	+
Cellulose decomposition	-	-	+	+	+	+	-	+	+	+	+	-	-	-	+	+	-	+	+	-	+	-	-	+
Hydrolysis of allantoin	-	-	-	+	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+
Reduction of NO_3 to NO_2	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	+	-	-	-	+	+	-
Reduction of tellurite	+	+	-	+	+	+	+	-	+	-	+	+	-	+	-	-	-	-	-	+	-	+	-	+
Tolerance to 5 % NaCl	+	-	-	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Growth on potato slices without $CaCO_3$	+	+	+	+	+	+	+	-	+	-	+	-	-	+	+	-	+	+	-	+	-	+	-	-

DESCRIPTION OF MICROMONOSPORA HUNGARICA SPEC. NOV.

Hungarica (Hungarian). Spherical spores found singly on the monopodially branching hyphae. On some places cluster-like formation of sporulating hyphae were also observed. Fair to good growth on yeast extract-malt extract agar (ISP Med. 2), inorganic salts-starch agar (ISP Med. 4), Czapek-sucrose agar and glucose-asparagine agar. Poor growth on glycerol-asparagine agar (ISP Med. 5). Colonies raised and folded, pale to vivid orange colored and covered, sometimes, with brownish spots. Aerobic. Good growth and positive utilization of D-xylose, D-fructose, D-galactose, D-glucose, mannose, β-lactose, maltose, sucrose, trehalose, dextrin and D-mannitol. Growth with L-arabinose, L-rhamnose, sorbose, α-melibiose, L-melezitose, raffinose, inulin, adonitol, dulcitol, glycerol, sorbitol and i-inositol was not better than on the negative control medium and what is more L-arabinose had an expressively impeding effect. Aesculin, arbutin, allantoin and starch are hydrolyzed, but hippurate is not. Milk is digested. Tellurite reduction test is positive. Cellulose digestion and nitrate reduction tests are variable. No growth on potato slices without calcium carbonate (pH 5.8 - 6.0). Maximum tolerated level of NaCl is 2 %. Source: mud samples obtained from ponds in the Hungarian alkali soil region. Type strain: SN-49.

DISCUSSION

Our presented results clearly show that the majority of Micromonospora isolates obtained from mud-samples were both numerically and traditionally unidentifiable with the species described so far. On the other hand, also the standard descriptions of the recognized species of Micromonospora show inconsequencies and scantiness, which make them less suitable

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Table 1 A comparison of selected diagnostic properties of type strains of Micromonospora alcalophila sp.nov. (Z-86) and M. hungarica sp.nov. (SN-49) with those of authentic (mostly type) strains of Micromonospora spp.

[1]Z-86; [2]ATCC 27029; [3]3-K-39; [4]ATCC 27114; [5]ATCC 12452; [6]ATCC 27331; [7]ATCC 21561; [8]ATCC 27008; [9]ATCC 15837; [10]RIA 472; [11]ATCC 27596; [12]ATCC 35005; [13]ATCC 21773; [14]ATCC 27600; [15]ATCC 27030; [16]ATCC 27598; [17]1-M_3-5; [18]ATCC 21819; [19]ATCC 27358; [20]ATCC 15835; [21]ATCC 27007; [22]ATCC 29337; [23]ATCC 27031; [24]ATCC 21826; [25]SN-49

for extensive taxonomic-ecological work. Taking all of these facts into consideration, we suggest for the ICSB Subcommittee on Taxonomy of Actinomycetales to organize an international working group for the redescription or description of all available type strains and other selected ones of particular importance of Micromonospora.

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DEGRADATION OF THE HERBICIDE BROMOXYNIL IN STREPTOMYCES FELLEUS

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Bromoxynil [BX] (4-hydroxy-3,5-dibromobenzonitrile) is the effective component of the herbicide Labuctril 25 used for the control of broad-leaved weeds.

This herbicide applied in forest soil decreased the number of actinomycetes by 18-50%, depending on the length of the post-treatment period and on the type of soil horizon (1). Under laboratory conditions, BX (10-80 µg/mL) inhibited growth and, at sublethal levels, affected morphology and pigmentation of most soil streptomycetes (2).

A strain P-14 resistant to 400 µg of BX per mL and supporting the growth of sensitive strains on a BX-containing medium was isolated and classified by ISP method as Streptomyces felleus.

Investigation of interaction of the strain P-14 with BX gave the following results:

1) Solid and submerged cultures exhausted about 95% of the initial amount of BX from the medium; from this about 50% was degraded and 45% was accumulated in cells in nondegraded form till the end of cultivation. The decrease of BX was brought about by cometabolism at presence of utilizable sources of nutrients and energy. No products of BX degradation were detected in cultures of the strain P-14, while at least two chromatographically distinguishable compounds, probably corresponding to those described previously (3), were present under conditions of parallel experiments with nonsterile soil. No detectable degradation occured both in noninoculated laboratory medium and in sterile soil.

2) About 90% BX accumulated in cells of the strain P-14 retained in cytoplasm and about 10% in cell wall. The presence of KCl increased the affinity of BX to cell wall considerably.

3) Variants sensitive to <100 µg BX per mL were present in natural populations of the strain P-14 with an average frequency 0.6%. Their yields increased by 5-times after UV irradiation and 47-times after acriflavine treatment. It may be supposed that extrachromosomal DNA is involved in genetic control of the resistence to BX.

4) The degradation of BX in cells of the strain P-14 was catalyzed by a partially inducible Fe^{2+}-dependent decyclizing dioxygen-

ase. This activity was not detected either in a BX sensitive mutant of this strain (resulting from acriflavine treatment) or in other BX sensitive strains of streptomycetes.
5) The addition of the strain P-14 to nonsterile soil increased the degradation of BX considerably, so that already after 1-week incubation no detectable amount of BX was found in the samples.
6) Although the strain P-14 was growing at several times higher concentrations of BX than those found for other streptomycetes, BX affected its growth rate, morphology and physiological activity. The onset of the growth was delayed by 1-2 days and the maximum dry mass was by 50% lower in the medium with BX as compared to control experiments. The formation of aerial mycelium was decreased and sporulation suppressed completely on the solid medium with BX (Fig. 1). Under shaken-flask conditions, control cultures formed compact oval-shaped pellets, while irregular more loose formations were present in the medium with BX (Fig. 2). Study of cell ultrastructure indicated more frequent occurence of vesicules and irregular septation; in addition, great number of electron-dense bodies was found in some samples, but not in control (Fig. 3). Production of an antibiotic and a brown pigment as well as a dehydrogenase activity of the culture were reduced by BX.

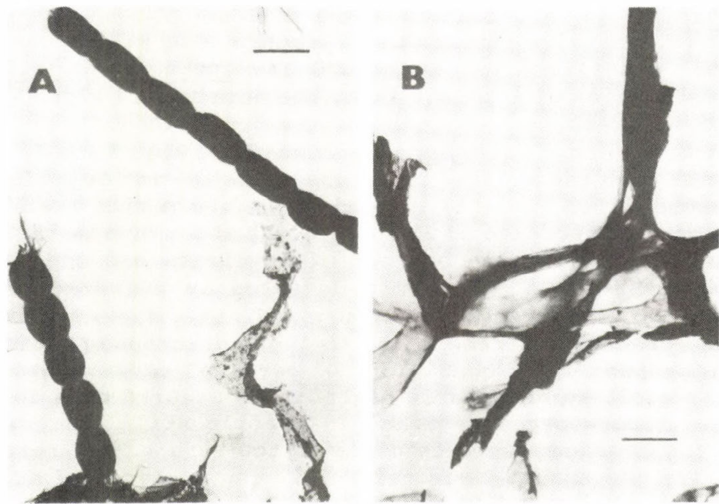

Fig. 1. Aerial mycelium of Streptomyces felleus P-14. **A** - control; **B** - culture with BX (100 μg/mL). Yeast extract-malt extract [YM] agar, 28°C, 10 days. Bar represents 0.5 μm.

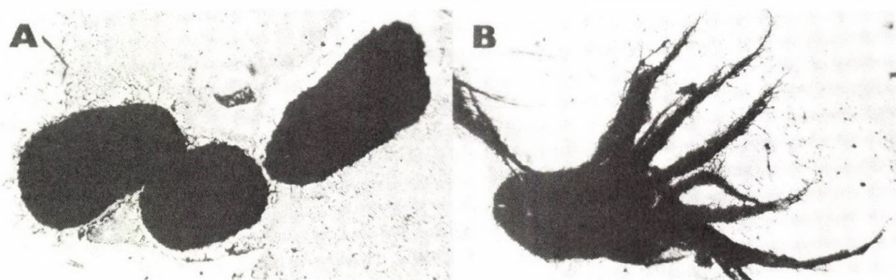

Fig. 2. Submerged mycelium of Streptomyces felleus P-14. **A** - control; **B** - culture with BX (100 µg/mL). Liquid YM medium, reciprocal shaker (1.6 Hz), 28°C, 5 days.

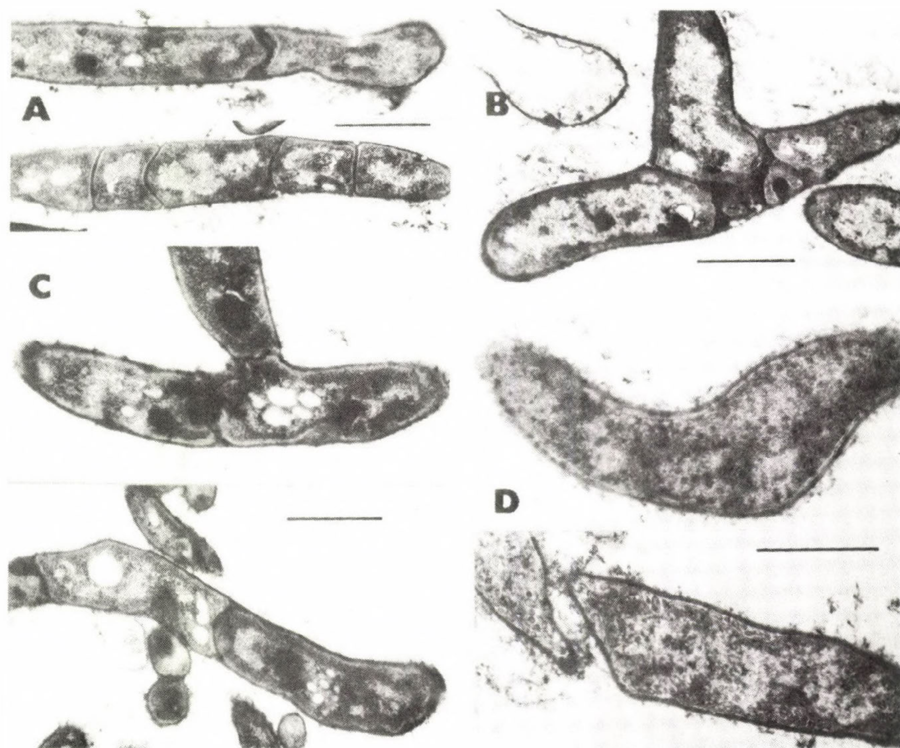

Fig. 3. Cell ultrastructure in 5-day solid cultures of Streptomyces felleus P-14 grown on control (**A**) and BX containing YM agar (**B, C, D**). **B** - irregular septation; **C** - vesicules; **D** - electron-dense bodies. Bar represents 0.5 µm.

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GRASS LIGNIN DEGRADATION BY ACTINOMYCETES*

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Degradation of [^{14}C] lignin-labelled lignocellulose preparations provides unequivocal evidence for activity against lignin, and has been used by D. L. Crawford and coworkers to study ligninolytic Streptomyces strains (reviewed in Crawford & Crawford 1984). By adopting a similar approach, we have sought to identify other actinomycetes involved in lignin solubilisation, with emphasis on the degradation of grass lignocellulose. Progress towards understanding this process could provide insights into natural humification and reveal the potential of actinomycetes for the bioconversion of lignocellulosic substrates such as cereal straw.

By screening for the ability to produce $^{14}CO_2$ from [^{14}C] lignin-labelled wheat lignocellulose, we identified several ligninolytic actinomycetes including representatives of the genera Actinomadura, Thermomonospora and Streptomyces (McCarthy & Broda 1984). In contrast to the well-studied white-rot fungus, Phanerochaete chrysosporium, strains of Thermomonospora mesophila and Streptomyces collinus degraded [^{14}C] lignin during primary growth and their activity was unaffected by nitrogen concentration, elevated oxygen tension, and culture agitation. A mixture of acid-soluble and insoluble [^{14}C] labelled products of high molecular

* This work was part of a programme supported jointly by British Petroleum's Venture Research Unit and the Agricultural and Food Research Council.

weight (> 5000 daltons) were produced in addition to comparatively small amounts of $^{14}CO_2$ (up to 8% of total ^{14}C).

[^{14}C] lignin solubilising activity was also exhibited by culture supernatants of T. mesophila grown on straw or xylan, but not glucose or ferulic acid. Degradation of xylan, which is covalently bound to lignin in the plant cell wall, could have a role in lignin solubilisation by T. mesophila. However, highly active endoxylanase preparations from thermophilic actinomycetes did not significantly solubilise [^{14}C] lignin, indicating that other enzymes must also be involved. To some extent this was confirmed by analysis of the acid-insoluble product of T. mesophila growth on ball-milled straw. The product was identified as a lignocarbohydrate complex in which the carbohydrate component (ca. 20%) was mainly hemicellulose-derived pentose. In addition, the presence of nitrogen (ca. 4%), originating from microbial protein, in an acid-insoluble complex suggests a relationship to humic acid. A similar complex is produced by Streptomyces viridosporus (Crawford et al. 1983) and may be a general feature of actinomycete attack on lignocellulose, contributing to humus formation in soils.

The structural complexity and variable nature of plant lignins places a constraint on the methods which can be applied to elucidate chemical structure and the mechanisms of enzymic attack. We have used solid-state ^{13}C-NMR spectroscopy to study both grass lignins and the acid-insoluble material produced by T. mesophila. Milled straw lignin, produced by dioxane extraction, and the T. mesophila product were similar in that signals from aromatic structures predominated, in contrast to the untreated straw spectrum which largely comprised signals from carbohydrate groups. The spectra also suggested that T. mesophila had not extensively demethylated the lignin but had significantly increased the carbonyl

content. Esterified phenolic acids are an established structural feature of grass lignins and probably accounted for the comparatively weak carbonyl signals detected in the milled straw lignin spectrum. Oxidation of the C_α of lignin monomer side-chains could account for the intensity of this signal on the *T. mesophila* product spectrum and is a modification which would lead to increased lignin solubility. This reaction is also part of a proposed pathway for lignin degradation by *S. viridosporus* (Crawford & Crawford 1984) and we have evidence for increased carbonyl contents in ^{13}C-NMR spectra of straw degraded in actinomycete-rich mushroom compost.

Clearly, identification of the extracellular proteins responsible for the solubilisation of grass lignin by actinomycetes will be an important step towards elucidating the degradative mechanism. Although the biochemistry of lignin degradation by actinomycetes remains poorly understood, there is a sound basis of general observations which illustrate their ecological importance and biotechnological potential.

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ECOLOGY AND EPIDEMIOLOGY

Poster Abstracts

POPULATION ECOLOGY OF ACTINOMYCETES

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Population approach in ecology is based on the combination of laboratory and field studies. Both aspects (i.e. in vitro and in situ) of population ecology of S. olivocinereus are covered in our study. First, the growth of colonies is linearly dependent on the time. The maximum of the radial growth rate K_r in vitro was observed at a low glucose concentration, but strong antibiotic production (heliomycin) was detected at a high concentration (0.1 g/l). The advantage of such strategy is that at low nutrient concentration the population must search resources. The production of antibiotic and antagonism can occur in favourable soil micro-environments. Second, this concept was examined in situ by immunofluorescence and autofluorescence methods. The population density oscillated convergently, there was a tendency for stabilization. If equilibrium structure is expressed as a percentage, then 0.4-1% is attributed to mycelium. It is sufficient to balance death and detect (autofluorescence) antibiotic granules in situ by direct methods.

MORPHOLOGICAL AND PHYSIOLOGICAL CHARACTERISTICS OF ACTINOMYCETES ISOLATED FROM IRAQI SOILS

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The cultural, morphological and physiological characteristics of 25 actinomycetes isolated from Northern, Central and Southern Iraq have been studied. The isolates obtained could be classified into 3 main families: Streptomycetaceae (18 strains), Actinoplanaceae (6 strains) and Nocardiaceae (1 strain). All the isolates were tested for antibiotic sensitivity. In general the actinomycetes were salt tolerant to about 100 g/l.

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DISTRIBUTION OF ACTINOMYCETE ANTAGONISTS IN SOILS OF NORTHERN KAZAKHSTAN

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One thousand actinomycete strains were isolated from soil samples on selective media containing rubomycin, tavromycitin and streptomycin. 565 strains proved to be antagonists. They inhibited the growth of Gram-negative (50.2%) and Gram-positive (64.0%) bacteria as well as phytopathogenic fungi (26.4%). Solonchaks and chestnut soils were the most rich in antagonists and rarely occurring actinomycetes (Actinomadura, Streptosporangium and others), while typical chernozems and dark-brown soils were less rich in them. Strains Nos 4 and 1425 (Streptomyces) furthermore No. 2036 (Actinomadura) producing antibiotics, which were of interest in chemical and biological aspects, have been studied separately.

A COMPARISON OF THE RESULTS OF ISP-TYPE AND NUMERICAL ANALYSES ON A COMPLEX SOIL STREPTOMYCETE POPULATION

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Using the methods, criteria and redescriptions of the International Streptomyces Project for analysing a natural Streptomyces population of a forest soil, strains of at least 25 species were isolated and selected. The numerical analysis, however, resulted only 3 large groups of soil strains. These might be identified with the members of E_{1-2}, F_{1-2} and H_{2-3} Streptomyces species groups, respectively, of a system of Kurylowicz et al. (1975) created by using the centrifugal correlation method. It may be that these 3 groups of strains represent 3 soil populations of only 3 variable Streptomyces species.

THE INTERACTION OF STREPTOMYCETES AND THEIR PHAGE IN A NATURAL ENVIRONMENT

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The interaction of streptomycetes and their phage is readily demonstrated under laboratory conditions, the result usually being complete lysis of the host. However, the interaction in natural environments has received little or no attention, work available mainly concentrating on individual aspects of the interaction, e.g. the effect of soil pH and soil colloids on actinophages. An investigation was therefore undertaken to examine the interaction of two streptomycete-phage systems in soil over a protracted period of time in order to:

a) determine whether an interaction can occur at population densities corresponding to those observed in soil,

b) determine the size and extent of any interaction,

c) determine if there is any evidence for alternative hosts for added phage and vice versa.

THE INTESTINAL STREPTOMYCETE MICROFLORA OF EARTHWORM-SPECIES BELONGING TO DIFFERENT "ECOLOGICAL GROUPS"

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Differences in compositions of streptomycete communities of soil and earthworm gut habitats were found. Only few streptomycete types predominating in the soil itself were isolated from earthworm gut contents and, on the other hand, in the faecal matter there occurred only certain types of streptomycetes. The composition of gut streptomycete communities of earthworm species belonging to different "ecological groups" proved to be also different. Food selection tests with specimens of Lumbricus terrestris were also made, in which a preference for paper discs inoculated with selected gut streptomycete isolates were shown.

ASSOCIATION OF STREPTOMYCETES AND GREEN ALGAE

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Cultivation of streptomycetes and Chlorella vulgaris together resulted in the formation of association resembling the thallus of nature lichens. Electron microscopic investigations of experimental thallus showed that algae cells within single thallus undergo the normal cycle of development, while the streptomycete lack to form spores. At the cellular level there were some kinds of interactions typical for lichens: simple wall to wall contact with close fibrous attachment between hyphae and algae cells, or sometimes the penetration of hyphae through algae cell wall being adjacent to algal plasmalemma.

The physiological properties of algobiont and streptobiont in thallus differ from axenic cultures specifically in antimicrobic properties. Under low moisture conditions components of association preserve viability of a larger degree than the axenic cultures.

NUMERICAL TAXONOMIC STUDIES ON NOCARDIOFORM INTESTINAL ACTINOMYCETES

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Up till the latest times the identification of the members of actinomycete genera characterized by cell-wall type VI such as Promicromonospora and Oerskovia, furthermore the NMO-s was based on certain key characters. Owing to increasing number of isolates belonging to these organisms and taking into consideration the broad range of their natural variability, a more precise taxonomic arrangement of them might be achieved by computer aided numerical analyses.

The results of comparative numerical studies carried out on more than 200 intestinal nocardioform isolates obtained from millipedes and closely related authentic Oerskovia and Promicromonospora strains are presented and discussed.

THE COMPOSITION OF ACTINOMYCETE COMMUNITIES IN THE BOTTOM SEDIMENTS OF THE ZALA RIVER AND LAKE BALATON (HUNGARY)

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In the mud of the Zala River more than 50 species of Streptomyces form a large and predominant fraction within the actinomycete population. This fraction has a relatively constant composition with S. viridogenes as predominant member. In the mud of Balaton among actinomycetes Micromonospora with about 10 species predominates, but streptomycetes play a subordinate role. In the Balaton's mud at the mouth region of Zala the decreasing frequency of S. viridogenes indicates the streaming river's water influence. The validity of the statement that streptomycetes are terrigenous organisms is questionable.

ECOLOGICAL ROLE OF DISSOCIATION OF RHODOCOCCUS

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The growth and dissociation into R-, S- and M-variants of Rhodococcus rubropertinctus under the influence of some environmental factors characteristic of their habitats were studied: temperature, pH, UV-radiation and high NaCl concentrations. R-variant gains a selective advantage under the influence of UV-irradiation and high temperature; S-variant decreases the pH of medium. The frequency of S and M variants also changes under the influence of changing pH, NaCl concentration and temperature of growth. Therefore dissociates, arising at frequencies of 10^{-3}-10^{-2}, increase the heterogeneity of the bacterial population, enlarge limits of the survival of species; and this is probably the biological role of dissociation.

ASSAY OF ENZYMATIC CLEAVAGE OF THE HERBICIDE BROMOXYNIL BY CELL-FREE EXTRACTS OF STREPTOMYCES FELLEUS

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A method of spectrophotometric monitoring of degradation of herbicide bromoxynil [BX] by cell-free extracts of *Streptomyces felleus* was developed. This method involves decrease of absorbance at 286 nm (absorption maximum of BX) which can be ascribed most probably to the cleavage of benzene ring of BX molecules. Conditions necessary for measuring this degradation as well as other aspects indicate that the reaction(s) could be catalyzed by enzyme(s) belonging into the group of decyclizing dioxygenases.

ISOLATION FROM SOILS AND PRESERVATION OF NATURAL PRODUCT-PRODUCING ACTINOMYCETES

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Actinomycetes exist in soils in clusters. Screening for useful products has become repetitive and inefficient. We found that soil location influences significantly the types present (Microb. Ecol. 10, 123-136, 1984). Therefore, highly unusual soils were plated at or near the sampling sites.

Many cultures sporulated poorly and were labile. Up to 50% were dead after 18 months at 4°C. Primitive laboratory facilities at some sites prevented use of sophisticated culture preservation techniques. A procedure in which 3 gm sterile soil is poured unto 1 ml liquid culture, grown in a 13 x 100 mm tube, followed by air drying, yielded good viability and preserved the natural product productivity of actinomycetes.

BIOSYNTHETIC POTENTIAL OF STRAINS OF STREPTOMYCES HYGROSCOPICUS, ISOLATED FROM BULGARIAN SOILS

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Investigation of biosynthetic potential of 16 strains of S. hygroscopicus, isolated from different regions of this country, has shown that in spite of some differences in morphological, cultural and physiological properties all strains produce antibiotics, close in their chemical structure. Strains studied have accumulated in their micall antibiotics with antimicotic effect with nonpolien macrolid structure of the type of Niphimycin, polien antibiotics (tetraens and hexaens) antibacterial ones, identical to elyophilins (asalomycin B). Antibiotics, isolated from the broth, have shown a great diversity. They have mainly antibacterial action.

DETERIORATION OF THE PARCHMENT OF CODICES FROM THE LIBRARY OF KING MATTHIAS OF HUNGARY BY STREPTOMYCES FIMICARIUS

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From some of the old very frequent and mostly lifeless reddish coloured spots of pH indicator character on strongly damaged parchment materials of King Matthias' codices (15th century) typical strains of the griseorubin producing Streptomyces fimicarius were isolated. These strains caused on sterile parchment identical symptoms of biodeterioration. S. fimicarius can hinder other biodeteriogens with its diffusible, red exopigment of indicator character and antimicrobial activity.

GROWTH PATTERNS IN STREPTOMYCETES

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Parameters of potential use in defining growth patterns in prokaryotes, with special reference to the size-shape complex, are examined. In the particular case of streptomycetes their special position, as branching prokaryotes capable of forming aerial growth, is illustrated and the ecological significance of actinomycete diversity is discussed.

GROWTH OF STREPTOMYCETES WITH CARBON SOURCES AS LIMITING FACTORS

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In natural habitats nutrient paucity is the rule and therefore one of the factors in species survival.

Development under starvation conditions, with particular reference to carbohydrate availability may shed some light on the phenomenon.

Data related to morphogenetic responses of streptomycetes to carbon concentration are illustrated and ecological implications of the findings are discussed.

DIFFERENTIATION

Plenary Session

Sixth Int. Symp. on Actinomycetes Biology, 1985
G. Szabó, S. Biró, M. Goodfellow (eds)

THE LIFE CYCLE OF STREPTOMYCES: GERMINATION AND PROPERTIES OF SPORES AND REGULATION OF SPORULATION

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The genus Streptomyces is comprised of bacteria unique amongst the procaryote in many important ways. Growth does not involve the entire cell itself, but just the tip region of the lengthening hyphae. Reproduction occurs by multiple fragmentation division of apparently differentiated regions of hyphae. The reproductive units, conidiospores, are dormant and capable of germinating when properly stimulated to begin anew the life cycle. Finally, the organisms are notorious for the numbers and varieties of their extracellular products: pigments, enzymes, antibiotics and odors.

Despite the importance of streptomycetes to industry and human health, and probably also to the web of nature, little is known of their developmental bio!›gy at the physiological and molecular biological level. The subject of development of streptomycetes was reviewed recently [1,2]. This paper focuses on our ongoing studies of spore properties, spore germination and sporulation.

METHODS

Growth conditions

For most experiments, S. viridochromogenes NRRL B1551 and S. griseus NRRL B2682 were grown in a semi-defined medium (DM-1) containing 25 mM morpholino-propanesulfonic acid buffer pH 7.0 (MOPS), 5 mM $(NH_4)_2SO_4$, 0.1 mM $MgSO_4 \cdot 7H_2O$, 50 mM glucose, 10 mM K_2H-KH_2PO_4 buffer pH 7.0, 0.02% casein hydrolysate (Sigma Chemical Co.) and 5 ml per liter of the trace salts previously described [3]. Solid media contained 1.5% Difco agar. Incubations were at 30°C. Spores were obtained, activated and germinated as previously described for S. viridochromogenes [4] and for S. griseus [3]. Microcycle sporulation of S. viridochromogenes followed the procedure of Koepsel and Ensign [5].

Cell fractionation

Mycelia stage cells of S. griseus were disrupted mechanically in a French pressure cell at 4°C or by osmotic lysis of protoplasts prepared with 1.0 mg/ml lysozyme in 15% sucrose. Spores were disrupted by vigorous agitation with 100 µM glass beads with a Vortex mixer. Cells were disrupted in 50 mM Tris 3 mM EDTA buffer (TE) pH 7.5. Where necessary,

viscosity of extracts was eliminated by 1-30 sec of sonication with a microtip (Branson Sonifier, power setting of 5). Large cell debris was sedimented by centrifugation at 3,000 x g for 15 min at 4°C. Membranes were sedimented from the slow speed supernatant fraction by centrifugation at 10,000 x g for 20 min and then washed twice in TE. The 10,000 x g supernatant fluid, crude extract, was used for most subsequent enzyme assays.

Nucleic acids were precipitated from the crude extracts with polymin P [6]. The polymin P precipitate was sedimented at 10,000 x g, washed once with TE buffer, followed by extraction with 2.0 M NaCl in TE buffer. Nucleic acids were also precipitated from cell extracts with either (final concentrations) 0.045 M $MnCl_2$, 0.8% streptomycin SO_4 or 10% polyethylene glycol 6000.

Enzyme assays

Trehalose was assayed by the procedures of McBride [7]. Nicotine-adenine dinucleotide (NAD-NADP) glycohydrolase was assayed by the cyanide method [8]. A unit of activity is defined as a decrease of 0.001 $O.D._{325\ nm}$ after 15 min incubation at 35°C. Adenosine diphosphate (ADP) ribosylation was determined by incubating the test sample with 5 mM NAD containing 1.0 μc of ^{3}H-adenine labeled NAD (New England Nuclear Corp.). At 30 and 60 min incubation, 50 μl samples were added to 50 μl of 10% cold trichloroacetic acid (TCA). After incubation on ice for 30 min, precipitates were collected on glass fiber filters (Whatman GF/A), then washed with cold 5% TCA and ethanol. Radioactivity was counted in Aquasol using a Beckman scintillation counter. Proteins were determined by the Lowry procedure [9].

Nucleotide pool levels were determined using a Vydac 301TP anion exchange column and a Perkin-Elmer series 3B HPLC apparatus. A gradient of 0.007 M KH_2PO_4 to 0.25 M KH_2PO_4-0.5 M KCl pH 4.0 was used to elute nucleotides. Detection was by absorption of fractions at 254 nm. Cells were poured from growth flasks onto 0.4 mM pore size membrane filters and the fluid removed by vacuum. The filters containing cells were quickly immersed in cold 1 N HCOOH and kept on ice for 30 min. The pellet was washed once with cold 1 N HCOOH and the combined filtrates were lyophilized, resuspended in water and analyzed.

RESULTS

Properties of spores

A summary of the properties of spores of *S. viridochromogenes* and *S. griseus* is shown in Table 1. Spores of both organisms are dormant by virtue of the low levels of endogenous metabolism. The QO_2 values are less than 5% of those for 24 hr mycelia (QO_2 of 3.2 for *S. viridochromogenes* and 2.1 for *S. griseus*; data not shown). The spores maintain detectable pool levels of nucleotides. The levels of ATP and GTP in *S. griseus* spores are higher than *S. viridochromogenes* despite their lower rate of endogenous metabolism. The level of cyclic adenosine monophosphate (c-AMP) is more than 10-times greater in *S. griseus* spores. Spores of both organisms contain NAD but not NADP (detection limit 1-10 pmol). It is interesting that ADP-ribose, a product of NAD glycohydrolase hydrolysis of NAD, is present in *S. griseus* spores. No

PADP-ribose (from NADP hydrolysis) was detected. Non-germinating spores of both organisms oxidize glucose to CO_2. The pattern of oxidation of C-1, C-3,4, and C-6 carbon atoms show that the Embden Meyerhof and pentose phosphate pathways are functional but the Krebs cycle is not. Oxidation of glucose does not appear to be coupled to phosphorylation since no increase in ATP was detected (these data not shown). Spores of both organisms contain similar levels of trehalose which, as shown later and by McBride [7] is a source of energy during germination. Also, non-germinating spores are capable of incorporating glucose into trehalose. Macromolecules are, however, not synthesized by the dormant spores since we could detect no incorporation of leucine, uridine or thymidine during incubation in buffer. We detected no protease activity in crude extracts of the spores.

Table 1. Summary of spore properties

Property	Streptomyces viridochromogenes	Streptomyces griseus
QO_2 endogenous[1]	0.09-0.10	0.05-0.08
Nucleotide pools[2]		
ATP	0.15	0.48
ADP	0.06	<0.01
AMP	0.06	<0.01
c-AMP	<0.01	0.12
GTP	0.06	0.15
NAD	0.12	0.06
NADP	0	0
ADP-ribose	0	1.25
Trehalose (% dry wt)	9.7	9.0
Calcium (% dry wt)	0.28	-
Heat activation[3]	Yes	Yes
Germination[4]	3 hr	5 hr
Krebs cycle[5]	No	No
Protease activity[6]	0	0
Macromolecule incorporation[7]		
Leucine	0	0
Uridine	0	0
Thymidine	0	0
Heat resistance[8]	+	+
Desiccation resistance[8]	+	+

[1] μM O_2/mg dry weight/hr.

[2] nmol/mg dry weight.

[3] 10 min incubation at 55C except S. griseus where temperature was 45C.

[4] Time when >90% of spores possess germ tubes.

[5] Based on radiorespirometric conversion of C_6 and $C_{3,4}$ labeled glucose to $^{14}CO_2$.

[6] Azocoll assay at pH 5.5, 7.0, 8.5.

[7] 10% TCA insoluble label from ^{14}C-leucine, ^{3}H-uridine or ^{14}C-thymidine following incubation for 1 and 2 hr at 30C.

[8] CFU surviving heat at 65°C for 10 min and desiccation for 7 da, relative to vegetative mycelia.

Spores of both organisms germinate synchronously and the rate is increased by heat shock. The heat shock treatment for S. viridochromogenes spores of 55° for 10 min is too harsh for S. griseus; a treatment at 45°C for 15 min is optimal for spores of the latter. Germination of S. viridochromogenes spores is absolutely dependent on calcium ions but this is not the case for S. griseus spores. The spores of both organisms are more heat and desiccation resistant than are the corresponding vegetative mycelia. These data and those of Hardisson et al. [review, 10] establish that streptomyces spores are a differentiated cell form and that the nature of dormancy and germination is not necessarily the same for spores of different species of the genus.

Germination of spores

We have made an extensive study of initiation of germination of S. viridochromogenes spores [4,11,12]. Initiation is triggered by calcium ions and the calcium must be present during the entire initiation period lasting 40-60 min. Most of the calcium appeared to be located exterior to the spore protoplast membrane. The spores release approximately 20% of their carbon-containing compounds and a low molecular weight germination inhibitor during the initiation period. The inhibitor specifically inhibits membrane bound ATPase activity and a case can be made for a role of the inhibitor in maintenance of dormancy and initiation of germination.

Our recent studies have focused on the nature and role in dormancy and germination of the other matter released during germination, the mechanism of action of calcium ions, and the role of the major energy reserve in spores, trehalose, in dormancy and germination.

We have recently found that the calcium transport inhibitors (and local anasthetics) dibucane and tetracaine, at 0.3-0.5 mM concentrations, inhibit initiation of germination of S. viridochromogenes spores. Buffer washing of the spores does not restore germinability but addition of calcium ions at 10 times the level normally used for germination (6.75 versus 0.675 mM) causes germination to resume. Adding the inhibitors at 10 and 20 min after initiation of germination arrests the process immediately. Thus, initiation of germination seems not to be an irreversible trigger. A calcium transport system must be operative. The last idea is reinforced by the report of calcium release from germinating spores of S. antibioticus [13]. Calcium transport may be the initial source of ATP during germination.

The results of analyses of the material released during germination of S. viridochromogenes spores are shown in Table 2. Significant amounts of amino acids, ammonia and trehalose were excreted. More than half of the amino acid component was accounted for as glutamate and γ-amino butyrate. Analyses of pool levels of the dormant spores showed that glutamate and γ-aminobutyrate were present at 184 and 20 nmol/mg spore dry weight and the ammonia content was 3.4% of the level found in the supernatant fluid (data not shown). We believe glutamate may play a role in resistance and dormancy of Streptomyces spores similar to that of dipicolinic acid in Bacillus spores.

During our study of microcycle sporulation of S. viridochromogenes, we observed that the spores formed in media with a low level of glucose contained much lower levels of trehalose than spores formed on solid media

Table 2. Composition of material released during germination of *S. viridochromogenes* spores

Component	Spore dry weight (nmol/mg)
Amino acids	105
Amino sugars	35
γ-Aminobutyrate	20
Glutamate	53
Hexoses (glucose)	200
Pentoses	247
Phosphate	55
RNA	0
Ammonia	530

Analyses of supernatant fluid after germination of spores in calcium medium for 60 min. Amino acid and amino sugar analyses were of acid hydrolysed samples.

containing a high level of glucose [5]. The trehalose content of *S. griseus* spores varies over a wide range depending on the growth conditions (Table 3). Large amounts of trehalose are present in spores formed by organisms grown on nitrogen or phosphate limiting conditions and with a high concentration of glucose. The level of trehalose is much lower when glucose is limiting or replaced by another carbon source. McBride [7] reported that trehalose-poor spores accumulate trehalose to normal levels when incubated under non-germinating conditions. He showed a progressive increase in heat and desiccation resistance and refractility of the spores as they accumulated trehalose. The endogenous respiration rates were nearly the same in the low and high trehalose containing spores, however the spores became less germinable as they accumulated trehalose.

Table 3. Effect of growth media on trehalose content of spores of *S. griseus*

Medium[1]	Trehalose % dry weight
DM-1, 50 mM glucose	11.8
DM-1, low phosphate (0.5 mM)	19.2
DM-1, low nitrogen[2]	24.9
DM-1, low glucose (2.0 mM)	0.8
DM-1, 100 mM glycerol[3]	5.3

[1]Normal DM-1 contains 50 mM glucose, 5 mM $(NH_4)_2SO_4$, 10 mM K-phosphate, 0.05% casein hydrolysate.

[2]No $(NH_4)_2SO_4$ added, casein hydrolysate at 0.02%.

[3]No glucose in medium.

Non-germinating spores degrade trehalose very slowly. This is probably the substrate for endogenous metabolism of the spores (Table 1). As shown in Fig. 1, the spores begin to degrade trehalose rapidly upon initiation of germination and essentially deplete their reserves of the

sugar when germination is complete. Extracts of dormant spores contain trehalase and the level of activity remains essentially unchanged during germination. The mechanism of controlling trehalose degradation in dormant spores is an interesting question. McBride (unpublished data) has shown that trehalase is located inside the dormant spore protoplast while trehalose may be located outside. This permeability barrier between substrate and enzyme is presumably altered immediately upon initiation of germination.

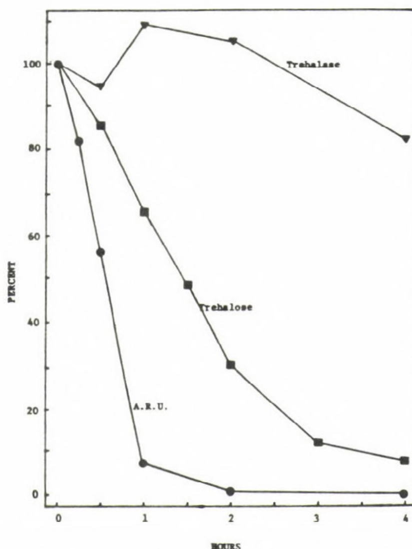

Figure 1. Trehalase activity and trehalose degradation during germination of S. griseus spores. Spores were germinated in a complex medium. Samples were removed at the times indicated and tested for resistance to 0.05N HCl for 5 min at 25°C as an index of germination. Trehalose was determined with hot water extracts of the spores. Trehalase activity was measured following mechanical disruption of spores. Initial trehalose level was 524 μg/mg protein. Initial trehalase activity was 60 nm trehalose hydrolyzed/mg soluble protein/min at 33°C.

Sporulation

We have been searching for a manageable system for studying the sequence and control of processes involved in sporulation of streptomyces. A study of aerial mycelium formation and sporulation of S. viridochromogenes during growth on a defined solid medium showed sporulation to be repressed reversibly by some amino acids [14]. The repression was reversed by phosphate limitation and by adenine. These observations led us to suspect that nucleotide metabolism might play an important role in sporulation. Consequently a study of the complete nucleotide pool levels was made of the organism growing on solid media under sporulating and repressed conditions and in the subsequently developed microcycle system [5]. The conclusion reached was that there was no clear cut correlation between changes in pool components and sporulation (unpublished data). Highly phosphorylated and cyclic adenylates and guanylates were detected but their levels did not fluctuate prior to or during sporulation.

More recently, we are focusing our studies on S. griseus for several reasons. The organism appears to be genetically stable, conditional sporulation mutants affected by A-factor [15], C-factor [16] and carbon source (unpublished) are easily isolated. Also strain NRRL B2682 sporulates predictably in submerged culture [3] allowing experiments to be done with a more homogenous population than can be achieved on solid media (personal opinion).

The prevalent dogma is that streptomycetes sporulate when nutrient deprived. This is not the case for S. griseus NRRL B2682. Sporulation occurs at approximately 27 hr during growth in DM-1 medium at 30°C while submerged and shaking (Fig. 2).

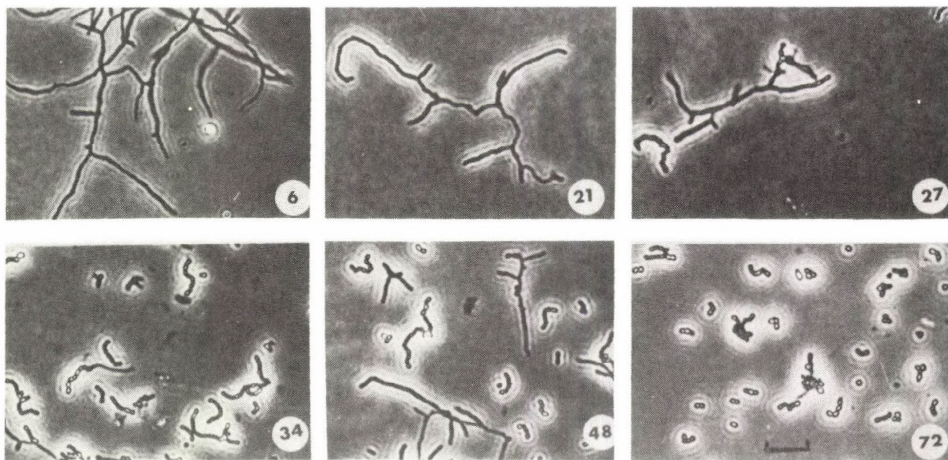

Figure 2. Phase contrast photomicrographs of S. griseus growing in DM-1 medium. Spores were inoculated into DM-1 and incubated while shaken at 30°C. At times indicated (hours), samples were removed and photographed. Bar = 10 μM.

Sporulation occurs between 26 and 28 hr (several hours earlier when inoculum is germinated spores) in media with wide ranges of concentrations of carbon, nitrogen, phosphate and trace mineral sources (Table 4). Varying the inoculum size from 5.2×10^5 to 1.3×10^7 spores/ml did not alter the time of sporulation. We consistently find that too large an inoculum, i.e. 10^8 spores/ml, results in acid production by the culture, (pH 5.2 at 18 hr), and spores are not formed even after 7 da incubation. Perhaps more convincing is the observation that shifting the entire culture from DM-1 medium to the same volume of fresh DM-1 medium at 4 hr intervals did not alter the time of sporulation. The possibility that accumulation of A-factor in the medium is a signal for sporulation was tested by adding the factor at different times. This did not change the time of sporulation.

Table 4. Time of sporulation of S. griseus is independent of nutrient levels

Variable tested[1]	Spores first evident[2]
A. Glucose concentration (mM)	
5-150	22-28
1	None (poor growth)
B. NH_4 concentration (nM)	
0.5-50	21-28
0	None (poor growth)
C. PO_4 concentration (mM)	
0.1-50	21-28
0	None (poor growth)
D. Trace elements (Mg, Mn, Fe, Zn, Co, Ca, Ni	
0.1-5X normal	26-28
E. Inoculum size (spores/ml)	
1.3×10^7	25-26
8.0×10^6	24-25
2.6×10^6	25-26
8.0×10^5	25-26
5.2×10^5	24-26
1.1×10^8	None (acid pH)
F. Transfer to fresh medium at	
4 hour	28
8 hour	28
12 hour	28
16 hour	28
20 hour	28
every 4 hr to 24 hr	28
G. A-factor added (10μg/ml)	
0 hour	28
4 hour	28
8 hour	28
12 hour	28

[1]DM-1 medium amended as shown.

[2]Time in hours when spores are first evident microscopically and by acid resistant or sonication resistant counts.

NAD-glycohydrolase activity is not found in dormant spores and first appears in cell extracts at 9 hr (Table 5). At this time the cells are in the early exponential growth phase. Activity appears in the growth liquor at 16 hr. The enzyme appears to be exocellulàr as well as intracellular because of its early appearance in the medium and the large amount of activity excreted. Lysis is not visibly evident until about 30 hr of growth. A stable spore minus mutant, bald 10, does not produce glycohydrolase when grown on various media but does so following addition of A-factor. The time of appearance of activity is similar to the parent. Sporulation occurs on schedule, 28 hr, in presence of A factor.

Table 5. Activity of NAD-glycohydrolase during growth of S. griseus

Hours	Units/ml[1] (X10^4)		Specific Activity[2] (X10^4)
	Liquor	Extract	
A. Parent			
0-6	0	0	-
9	0	0.24	1.14
12	0	11	18
16	0.6	68	65
20	6.1	216	142
24	9.4	240	185
28	18	156	87
30	29	124	73
36	21	156	173
50	24	152	150
72	28	48	77
B. Bald 10			
0-72	0	0	-
C. Bald 10 + A factor[3]			
0-8	0	0	-
10	0	17	24
16	6	110	76
24	11	142	129
36	28	176	194

[1]Change of O.D. 325 nm x 10^3.

[2]Units activity/mg protein (cell extract).

[3]10 μg/ml added at 0 time.

The S. griseus strain produces A-factor and some of its spore minus (bald) mutants respond to the factor (unpublished results). A-factor has been shown by several investigators to induce or stimulate synthesis of NAD(P)-glycohydrolase [15,17,18]. There has been speculation that the glycohydrolase, and thus A-factor, is involved in regulating antibiotic production by affecting metabolic flow of glucose due to the effect of glycohydrolase on pyridine nucleotides and various dehydrogenases [15,17, 18]. A similar role in sporulation was considered [15,17]. A consistent observation that we made was that NAD-glycohydrolase appears in cell extracts of cells grown under some of the conditions described in Table 4 at the same time, 9-10 hr. How, and if, this enzyme might be involved in sporulation is an intriguing question on which we have decided to focus some attention. NAD(P)-glycohydrolases are widely distributed in both eucaryotic and procaryotic organisms. The enzyme carries out two potentially important reactions: hydrolysis of NAD and NADP to ADP-ribose and PADP-ribose and nicotinamide, and transfer of ADP-ribose to acceptor proteins effecting post-translational modifications [review, 19]. One such modification involves ADP-ribosylation of RNA polymerase of Escherichia coli and causes a shift in promoter specificity to phage genes [20]. Even if NAD-glycohydrolase is not involved in regulation of development, the question of why an organism like S. griseus produces the enzyme is worth considering.

A study of the distribution of glycohydrolase activity in 12 and 24 hr cells showed the enzyme to be bound to membranes and DNA, and also to be present in the supernatant fluid following centrifugation at 105,000 g (unpublished data). The enzyme is removed from membranes by digestion with DNase I and by a 2.0M NaCl wash, but not by a 0.5M wash.

The observation that glycohydrolase binds to DNA and membranes may be significant. A variety of agents known to precipitate nucleic acids and associated proteins from cell extracts were tested for ability to remove glycohydrolase from a crude extract. Each of the agents precipitated an appreciable amount of the enzyme activity and in each case the enzyme was eluted from the precipitate with a 2M salt wash (Table 6). Preliminary analysis of proteins eluted from the precipitates using SDS-gel electrophoresis shows that many proteins were eluted from the streptomycin precipitate and a virtually identical pattern of approximately 15 proteins eluted from the other precipitates (unpublished data).

Table 6. Precipitation of NAD-glycohydrolase by nucleic acid precipitating agents and elution of activity by 2M NaCl

Treatment[1]	% Activity		ADP
	Precipitated	Eluted[2]	Ribosylation[3]
Polyethylene glycol	45	30	126
$MnCl_2$	57	39	140
Polymin P	60	23	13,900
Protamine SO_4	85	50	156
Streptomycin SO_4	75	54	56

[1]Crude extract (2.5×10^6 units/ml NAD-glycohydrolase) incubated 15 min with precipitating agent at 4°C followed by centrifugation at 10,000 g.

[2]Activity in 2M NaCl wash.

[3]CPM label from ^3H-adenosine NAD incorporated into cold 10% TCA precipitate.

We have made an extensive effort to detect ADP-ribosylation of proteins in cell extracts with a poor level of success. Incorporation of label from adenine labeled NAD into 10% TCA precipitable material has been observed in some extracts but without convincing consistency. The eluates of precipitated material, Table 6, were tested. As shown, a low level of counts were found in all cases except for polymin P where a significant amount of label was incorporated. Attempts to locate the labeled proteins in the polymin fraction using SDS gels have thus far failed. The ADP-ribose bond is labile in some proteins and this may be the cause of our problem. Success in demonstrating ADP-ribosylation of membranes of _S. griseus_ is reported elsewhere in this volume by Barabas et al. [21].

Theoretically, an organism containing high levels of NAD(P)-glycohydrolase activity cannot carry out respiratory activities. The enzyme both destroys NAD(P) and the product of hydrolysis, (P) ADP-ribose, is a competitive inhibitor of dehydrogenases [15,17]. We were unable to detect any dehydrogenase activities in extracts of cells after the time of glycohydrolase appearance at 10 hr (unpublished results). We tested intact cells for ability to oxidize glucose using radiorespirometry to determine the metabolic pathways involved. A summary of the data is presented in Table 7. The column labeled C_1-C_6 reflects activity of the

Table 7. Evaluation of glucose catabolism pathways during growth cycle of S. griseus

Hours	% Label Converted to CO_2 [1]		
	C_1-C_6	$C_{3,4}$	C_6
Dormant spores	6	28	2
2	6	55	18
4	12	51	19
8	4	63	20
12	–	–	18
16	–	–	20
24	5	50	3
48	3	71	2

[1] Spores incubated in buffer (dormant) or DM-1 base medium (growth) while shaking at 30°C. At times indicated, cells were harvested, washed four times and inoculated into tubes containing 5 ml DM-1 base medium containing 0.25 μmoles glucose labeled (0.5 μCi) in the 1, 3, 4 or 6 carbon atoms. The tubes were sparged with air, the CO_2 evolved was collected and the level of radioactivity counted. Data reported after 95% of labeled was consumed (cell pools and respired).

Pentose Phosphate pathway. Glucose is catabolized by this route at a slow rate at all ages. Evolution of CO_2 from carbon atoms 3 and 4 of glucose is an index of the Embden-Meyerhof (glycolysis) pathway. This pathway is operative at all cell ages. The Krebs (tricarboxylic acid) cycle is very low or absent in spores and in 24 and 48 hr (sporulated) cultures. However, significant conversion of C_6-glucose to CO_2 occurs at 12 and 16 hr, when the cells contain appreciable levels of glycohydrolase. These results are best interpreted as evidence for some sort of compartmentalism separating NAD(P) from the hydrolytic enzyme.

DISCUSSION

Spores of S. viridochromogenes and S. griseus are cryptobiotic and resistant (Table 1). Germination of Streptomyces spores, primarily S. antibioticus, has been studied extensively by Hardisson and co-workers [summarized in 10]. The overall conclusions reached by ourself and the Hardisson group are basically the same. However, there are some important differences in observations. Garcia Diaz et al. [22] reported respiratory rates (QO_2 values) for S. fradiae, antibioticus, coelicolor and viridochromogenes of 1.1, 10.8, 15.6 and 31.2, respectively. The QO_2 values we observed for S. griseus were 1.1-1.8 and for S. viridochromogenes 2.0-2.2 (values shown in Table 1 converted from μM O_2 to μl O_2). The level of dormancy that we observe, except for S. fradiae, are much lower. The S. viridochromogenes strains studied by both groups apparently come from the same source. The levels of ATP that we measured in spores are similar to those of Garcia Diaz et al. [22] but the levels of AMP and ADP that we measured are much lower. We detected little or no Krebs cycle activity, no protease activity in spore extracts and no macromolecular synthesis in dormant spores while Hardisson and co-workers observed significant amounts of all three activities in S. antibioticus spores. It is evident that spores of all Streptomyces are not identical in their properties.

Streptomycetes are generally viewed as terrestrial organisms uniquely adapted to growth and development on solid surfaces. Sporulation involves two distinct sequential processes; formation of aerial hyphae followed by their differentiation into spores. Differentiation of vegetative mycelia into aerial hyphae appears to be signalled by nutrient limitation and the growing aerial hyphae obtain some substrates from the lysing vegetative mycelia below [23, review 7]. Sporulation of S. griseus in submerged culture is clearly not related to nutrient limitations. The spores of this organism are virtually identical when produced on solid or in liquid submerged culture [3]. The usual definition of aerial hyphae is rather crude: powdery growth on colony surfaces. Until a more precise definition is found, hopefully based on unique cytochemical or physiological processes, it is not known if structures equivalent to aerial hyphae are formed during submerged sporulation.

Nutrient independent sporulation of S. griseus may be unique to this species. Nutrient limitation is clearly involved in submerged sporulation of S. viridochromogenes in the microcycle system [5]. Germination of S. griseus spores (which in our hands is synchronous: 90-100% of spores possess germ tubes 5 hr after initiation) may set a clock mechanism in motion. Sporulation events are triggered irregardless of limitation (within growth limits) or excess of nutrients. This hypothetical trigger may occur as early as 8-10 hr following spore germination. Commitment to sporulate occurs at that time [3]. This is the approximate time when we first detect formation of A-factor (unpublished data) and NAD-glycohydrolase activity. We are tempted to think that these molecules are involved in regulation of sporulation in S. griseus. It is admittedly difficult to resolve this theory with the observations that NAD(P)-glycohydrolase and A-factor are produced by many other streptomycetes [unpublished data; 18], some or possibly all of which do not sporulate in submerged culture. Another possible obstacle to the theory is that many bald mutants of S. griseus produce NAD-glycohydrolase, but at much lower levels than the parent strains [24,25]. Some of these mutants do not respond to A-factor with increased formation of the glycohydrolase and yet do sporulate. Possibly the low level of NAD-glycohydrolase activity in these mutants is sufficient to fulfill an involvement in sporulation. The bald 10 mutant that we study produces no detectable NAD-glycohydrolase activity in the absence of A-factor but responds to presence of the factor by producing the enzyme and sporulating.

The question of survival of an organism with a cytoplasm containing high levels of an enzyme capable of degrading NAD and NADP is intriguing. The coenzymes are absolutely essental both for energy production and for DNA replication. Reason dictates that some sort of cell compartmentalism must separate the enzyme and its substrates. The enzyme is found in virtually all cell fractions following cell breakage. Yet, the organism grows and maintains NAD(P) dependent metabolic processes. The soluton to this apparent puzzle that we currently favor is that the S. griseus cell is an elongated tube of multinucleate cytoplasm comprised of an old possibly senescent region and a young, vigorous growing tip. The glycohydrolase is expressed only in the older regions and is not found in the hyphal tips. Respiration and DNA replication can thus continue unimpaired in the mycelial tip regions.

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ACTINOMYCETE AUTOREGULATORS

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About 4 years ago I have read on International Symposium on Overproduction of Microbial Products in Hradec Králové a lecture about microbial autoregulators of different origin (Khokhlov, 1982). Now I have a pleasure to survey here autoregulators of actinomycetes alone. The necessity of a more specialized report shows quick accumulation of new materials in this field of science. In this review I shall try to remind the data which were in the previous lecture very briefly to have a possibility to acquaint you with new materials.

For the last years the research in the field of actinomycete products underwent deep changes. When during rather a long previous period the main attention was paid practically only to antibiotics, now a lot of other products are investigated along with them - specific enzyme inhibitors, immunomodulators, stimulators of cell growth and many others including actinomycete autoregulators, from which the first - cosynthetic factor I - was described about twenty five years ago (1960). Its story is rather unusual. In the course of studies of the mechanism of tetracycline biosynthesis by means of mutants a strain S-1308 was obtained which produced small quantities of chlortetracycline along with great amounts of its 5a(11a)-dehydroanalog. When it was grown in mixed fermentation with other low active mutant W-5 large quantities of chlortetracycline were produced. From cultural broth of strain W-5 a substance - cosynthetic factor I - was isolated. By addition to the culture of mutant S-1308 it was ensuring transformation of 5a(11a)-dehydroanalog into 7-chlortetracycline. The activity of this bioregulator was very high - by addition of 1 microgram it catalysed production of 50.000 micrograms of chlortetracycline. For bioregulator an empirical formula $C_{19}H_{22}N_4O_9$ and flavin or pteridin nature were supposed. During the following 20 years there were no new data about this compound. However, in 1982 quite unexpectedly the identity of cosynthetic factor I and the product of partial hydrolysis (FO-fragment) of coenzyme F-420 was discovered (McCormick, Morton, 1982). The coenzyme F-420 is produced by different methanobacteria and Streptomyces griseus. According revised data the cosynthetic factor has the formula $C_{16}H_{17}N_3O_7 \cdot H_2O$. Its direct comparison with previously prepared synthetic 7,8-didemethyl-8-hydroxy--5-deazariboflavin has shown their doubtless identity. There-

fore not only the structure was elucidated, but also the synthesis of cosynthetic factor was achieved.

Among actinomycete autoregulators A-factor (autoregulating factor) is the best studied in different aspects. It was discovered by us in 1967 as a result of systematic studies of a great number of mutants, obtained from the streptomycin producer - actinomycete Streptomyces griseus (according to another system of terminology - Actinomyces streptomycini) (Khokhlov et al., 1967). In the beginning A-factor (or "autoregulating factor") was found in a mutant, which produced very small amounts of streptomycin. This substance was shown to secure a high level of antibiotic synthesis by inactive mutants, when it was added into medium. Later A-factor was thoroughly investigated by us. I may mention the following aspects (For bibliography see Khokhlov, 1982).

Table 1. Effect of A-factor on decrease of distinctions between parent strain 773 and mutant 1439

Properties	Parent strain 773	Mutant 1439	
		without AF	in presence of AF
Formation of			
Streptomycin (MCG/ML)	3000	0	3000
Streptidine	+	-	+
O-Phosphorylstreptidine	+	-	+
NADP-Ase	+	-	+
Dark pigment	+	-	+
Aerial spores	+	-	+
Intracellular structures:			
Membranes	high	low	high
Polysomes	high	low	high
Tubular organelles	+	-	+
ATP content	low	high	low
Streptomycin resistance	high	low*	high*
Streptomycin-6-phosphotransferase activity	high	low*	high*
Transaminase activity (units pro 1 mg of protein)	3.9	0.12	2.0
G6PDH activity	low	high	low
Mycelium basophility after 96 H	low	high	low
Release of spores from hyphae	+	-	+

*In these experiments A mutant FT-1 (A-factor$^-$) was used.

1) A-factor was detected in a great number of Streptomyces griseus strains which produced streptomycin and also in many actinomycetes of different species (Anisova et al., 1984).

2) The A-factor was shown to influence very strongly not only streptomycin biosynthesis and related biochemical processes, but also to change the enzymatic properties, for example, the activity of glucose-6-phosphatedehydrogenase and morphological processes in actinomycetes. In this period of

time Gräfe and coworkers have published interesting materials about glucose-6-phosphatedehydrogenase and other dehydrogenases of Streptomyces griseus and related actinomycetes. (For bibliography see Gräfe et al., 1983-84, 1984).

As one can see in Table 1, the mutants deficient in A-factor have a number of alterations of biochemical and morphological processes; these alterations can be prevented, when A-factor was introduced into the medium. To give a complete picture, the new data of the author and other scientists were included into this Table.

3) A difficult stage of the whole investigation was isolation of A-factor in highly purified state due to its small contents in cultural broth. The next step was the elucidation of its structure as 2-isocapryloyl-3-hydroxymethyl-γ-butyrolactone. This formula was later confirmed by other authors. A synthesis of racemic A-factor and a great number of its analogs was carried out. The studies of the biological properties of analogs had demonstrated very high specificity of the A-factor molecule. Practically any change of its structure resulted in strong decrease or even in total disappearance of biological activity.

4) An unusual group of secondary mutants of Streptomyces griseus was discovered, which produced no A-factor, but were able to sporulate and synthesize streptomycin.

For the last four years after the Symposium in Hradec Králové the A-factor studies proceeded rather intensively. In these investigations besides us many other scientists took part. For example, the Japanese scientists Hara and Beppu (1982a) have found that A-factor deficient mutants of Streptomyces griseus and Streptomyces bikiniensis originated rather often not after the chemical mutagen treatment but also under UV-irradiation.

Some new processes were discovered on which the A-factor had a strong influence. According to our data (Vasilenko et al., 1983) one of the important effects of the A-factor is its influence on the level of adenylates (especially ATP and ADP) in different strains of Streptomyces griseus.

Hara and Beppu (1982b) showed the deficient in A-factor mutants to be more sensitive to streptomycin than parent strains producing great quantities of streptomycin. However, cultured in A-factor presence the deficient mutants increased the streptomycin stability and appeared to be more similar in this respect to the parent strains. The main reason of the increased stability was shown to be the induction of streptomycin-6-phosphotransferase biosynthesis. In A-factor presence the level of this enzyme in A-factor deficient mutants increases sharply and becomes more similar to the original strains.

Interesting data were published about a synergistic effect of cobalt ions and A-factor on formation of aerial mycelium and anthracyclines in a blocked Streptomyces griseus mutant (Gräfe et al., 1983-1984; 1984).

A number of chemical studies of the A-factor were published by a Japanese scientist Mori (1981, 1982, 1983). The author developed a method of synthesis of natural optically active A-factor and its stereoisomer on the basis of optically active S- and R-paraconic acids.

Although the natural A-factor and its stereoisomer were synthesized from optically active compound, determination of their absolute configuration was a rather complicated problem due to the discrepancy of published data on stereochemistry of paraconic acids themselves. Therefore in the beginning Mori (1981, 1982) ascribed to the natural A-factor the absolute configuration 3S, but later (Mori, 1983) changed it for 3R. The last conclusion seems to be rather trustworthy.

Comparison of biological activity of synthetic optically active A-factor (3R) and its stereoisomer (3S) demonstrated that unnatural stereoisomer (3S) had also rather high biological activity, which corresponds to about 40% of pure natural A-factor activity.

We have described a method for preparation of the A-factor labelled with tritium at the level of 0.25 - 4 Cu/mol (Neiman et al., 1984). The labelled sample could play an important role in the investigation of bioregulator interactions with microbial cells.

Recently a number of communications appeared which were devoted to genetics of A-factor biosynthesis (Dekhtyarenko et al., 1982; Hara et al., 1983; Horinouchi et al., 1983), but it is still rather difficult to generalize them. Soviet scientists used various strains of Streptomyces griseus (both doubtless A-factor producers and deficient mutants). On the basis of obtained data the autoregulator synthesis supposed to be controlled by chromosome genes but not by a plasmid. Simultaneously there is a possibility that in some actinomycetes biosynthesis is controlled by chromosome genes while in others by plasmids (Dekhtyarenko et al., 1982; Hara et al., 1983).

Considering materials on genetics obtained using Streptomyces coelicolor A3(2) it is necessary to take into account the fact that this actinomycete produces not A-factor as Japanese scientists supposed (Hara et al., 1983; Horinouche et al., 1983), but a mixture of its analogs (Anisova et al., 1984). These compounds have the composition $C_{13}H_{22}O_3$ (Acl 1) and $C_{14}H_{24}O_3$ (Acl 2) and are very similar, but non-identical with A-factor.

It must be stressed that insufficient attention to the isolation of supposed new bioregulator in a pure state and its chemical identification can lead to serious errors. Thus, it is impossible to suggest that all the actinomycetes, which stimulate streptomycin biosynthesis by A-factor deficient mutants produce just this substance namely, but not its biologically active analogs. The last possibility was proved in the case of Streptomyces coelicolor A3(2) (Anisova et al., 1984).

On the other hand, the neglect of careful proof of obtained samples homogeneity can lead to erroneous conclusions of another type. In such cases crude preparations of biologically inactive substance, containing small amounts of known bioregulator (for instance A-factor), are described as a new bioregulator. As an example of such misunderstanding the description of the so-called L-factor from Streptomyces griseus JA 5142 can be mentioned (Gräfe et al., 1982a). After eluci-

dation of its structure as stereoisomeric lactones of 4,5-dihydroxy-n-decanoic acid its synthesis was realized (Stamatatos et al., 1984). When pure synthetic samples of 4,5-dihydroxy-n-decanoic acid lactones were tested on their biological activity, it was clear, that they had no biological properties ascribed to L-factor. In fact, the regulating action was rendered by small amounts of the A-factor, which was present in crude preparations (Gräfe, Eritt, 1983). Thus it was proved that there is no such natural bioregulator as L-factor.

Therefore the appearance of many papers devoted to L-factor (Eritt et al., 1984; Gräfe et al., 1983-1984a), after the evidence of its inactivity, i.e. after July 1983 seems very strange.

Some years ago the same group of scientists described the isolation from Streptomyces viridochromogenes ZIMET 43683 of a new inducer of anthracycline biosynthesis (Gräfe et al., 1982b). Its structure was elucidated as an A-factor analog, which had a hydroxyl group instead of carbonyl group of the A-factor. This compound was synthesized earlier by us and turned out to be biologically inactive in our exoeriments (Onoprienko et al., 1979). Dr. Gräfe and coworkers for explanation of these contradictions supposed that they may be attributed to differences in the indicator strains used. A simpler explanation seems to be the presence of A-factor in crude preparations of the so-called "inducer of anthracycline biosynthesis". As wrote the authors themselves (Gräfe et al., 1984b) their samples of this supposed bioregulator were only 90% purity. In all the papers devoted to the hydroxy analog of A-factor there was no convincing evidence of its own biological activity (Gräfe et al., 1983, 1984).

A necessity arises of critical evaluation of modern state of studies of "factor IM" from Streptomyces virginiae. It is especially important in connection with discovery of a number of actinomycetes producing A-factor. According to the last available information (Yanagimoto, 1983) this substance is a lactone $C_{12}H_{22}O_4$. This autoregulator may be identical with A-factor. Therefore it seems very interesting to study the pure A-factor on biological models previously used in the course of factor IM investigations.

In Streptomyces griseus, better to say, in one of its mutants H-45 an interesting bioregulator - the factor C - was discovered many years ago by Prof. G.Szabo and coworkers. Under some conditions it influences rather strongly the cytodifferentiation of another mutant strain No. 52-1. Factor C was shown to be a protein with molecular weight of approximately 34.500 and a rather high content of hydrophobic amino acids (Biro et al., 1980), but its structure, as I know, is not elucidated. I hope, professor Szabo and his coworkers will tell us in detail the recent achievements in the studies of this interesting substance.

Recently a new very active bioregulator (B-factor) was discovered and studied; it induces rifamycin biosynthesis (Kawaguchi et al., 1984). Mutants of rifamycin producing microorganism (Nocardia sp. KB-993), which did not synthesize antibiotic, were obtained during cultivation at elevated tem-

perature (42°C) or after long storage of the cultures on agar. A mutant strain has lost also the ability to form aerial mycelia. When an extract of active strain cells was added to the culture of the deficient mutant the ability to synthesize the antibiotic and to form aerial mycelia was restored. The bioregulator was isolated and its structure was elucidated - it turned out to be 3'-(1-butylphosphoryl)adenosine. The structure was confirmed by its synthesis.

Stimulation of rifamycin biosynthesis in deficient mutant was observed in the liquid medium at a bioregulator concentration above 2 ng/ml and was increased till the concentration of 30 ng/ml. A number of compounds structurally related to B-factor showed no regulatory effect. These data demonstrated the high specificity of the B-factor.

There were no new data published about studies as bioregulators such substances as methylenomycin, pamamycin, sporulation pigment of Streptomyces venezuelae, inducer of oxytetracycline biosynthesis and others.

Recently there were described some new properties of known interesting inhibitor of spore germination from Streptomyces viridochromogenes (Grund, Ensign, 1985). The activity of samples was increased approximately fifty times after purification. The substance is soluble in water and alcohol, its molecular weight is less than 1000. Inhibiting activity remains after treatment with ribonuclease, deoxyribonuclease, lipase and various proteolytic enzymes. The substance has rather unusual antibiotic spectrum: it inhibits strongly Bacillus cereus, Bacillus subtilis, Streptomyces viridochromogenes, Streptomyces parvulus, Streptomyces griseus, but doesn't suppress Staphylococcus aureus, Nocardia carolina and a number of gramnegative bacteria. The substance inhibits specifically ATPase. I hope prof. Ensign will tell us some new materials about this interesting substance.

Completing my short survey of modern state of actinomycete bioregulator research, I wish to draw some general conclusions. The studies of natural bioregulators are developing not so quickly as investigations of other types of actinomycete biologically active substances (antibiotics, enzyme inhibitors and so on). The main reason of this relative slowness is connected with their very high biological activity - thus, the majority of pure bioregulators are by two-three orders of magnitude higher than the activity of usual actinomycete antibiotics. Due to the high activity the extremely low content of bioregulators is, as a rule, in the cultural broth. However, these difficulties may be overcome by combined use of modern biological and physico-chemical methods of research. If such broad complex investigations were organized, the study of a new bioregulator proceeded very quickly - as an example may be mentioned B-factor. On the contrary, when the proper degree of cooperative scientific work was not achieved, the investigations proceeded very slowly. In some cases they led to erroneous conclusions, when crude samples of inactive substances (which contained small amounts of well-known bioregulators) were described as new bioregulators.

None the less, the great principal importance of actinomycete bioregulator research studies allows to await the quick

progress in this field. Such investigations open us a possibility to understand the intimate developmental mechanisms of these unusual microorganisms and give the hope to find novel scientifically based methods of their control. I hope that at the time of our next meeting elsewhere we shall be able to see and hear the new bright discoveries in this important branch of knowledge.

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EFFECT OF FACTOR C ON THE DIFFERENTIATION OF STREPTOMYCES GRISEUS

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INTRODUCTION

In our previous work (Vitális et al. 1984, Valu et al. 1984) we attempted to describe the development of *Streptomyces griseus* (S.griseus) strains: No.45H (the well differentiating mutant) and No.52-1 (which does not conidiate in submerged culture) by studying their qualitative and quantitative protein composition with the aid of pulse labeling and by one dimensional polyacrylamide gel electrophoresis (PAGE). We obtained characteristic changes of the protein patterns during the life cycle of both strains, and the protein patterns of the two closely related *S.griseus* mutants differed significantly from each other.

In the present study we examine the effect of factor C on the protein composition of *S.griseus* No.52-1. The specific activity of some bands from the factor C treated culture was found to become very similar to that of the well differentiating mutant, showing that factor C influences - probably indirectly - the expression of genes taking part in differentiation.

In this publication we have to correct some of our earlier results (Szabó et al. 1984), namely that factor C inhibited *in vitro* transcription and translation. Our previous factor C preparations contained a minor component (less than 5 %) responsible for inhibition *in vitro* translation. We have succeeded in separating the two types of molecules responsible for the cytomorphological effect and the *in vitro* translation inhibitory activity. The effect of the purest factor C and the minor component on the *in vitro* transcription has not been examined yet.

MATERIALS AND METHODS

Strains were described in previous publications (Szabó et al. 1961, Vitális et al. 1963). *S.griseus* No.52-1 does not conidiate in submerged culture, *S.griseus* No.45H profusely sporulates even in submerged culture and produces factor C (Szabó et al. 1962, Szabó et al. 1967, Biró et al. 1980), a protein which when added to submerged culture of strain

No.52-1, induces the production of reproductive branches (Szabó et al. 1967, Vitális and Szabó, 1969).

Cell-free extracts were prepared as follows: cultures were washed twice with buffer I [0.01 M Tris (Aristar, BDH)-HCl, pH 7.5, 0.01 M $MgCl_2$, 1.0 M KCl, 5.0 mM Mg-Titriplex (Merck Co), 0.01 M mercaptoethanol (Merck Co.)], once with buffer II [buffer I with the KCl concentration reduced to 0.05 M], and once with buffer III [0.01 M Tris-HCl, pH 7.5, 0.01 M $MgCl_2$, 0.06 M NH_4Cl, 5.0 mM Mg-Titriplex, 5.0 mM mercaptoethanol and 3.0 mM phenylmethanesulfonyl fluoride (Merck Co.)]. Washed cells were broken with Alumina (Sigma Co., Type 305), then after a low speed centrifugation DNase I (Worthington) was added to the supernatant, then it was centrifuged once at 10 000 x g, twice at 30 000 x g, this supernatant is the S-30 fraction. Soluble or S-100 fraction was prepared from the S-30 extract by centrifugation at 110 000 x g for 3 h. Applying this breaking method both the vegetative and the reproductive hyphae have been disrupted but conidia have remained intact.

Subcellular fractions were prepared also from ultrasound sensitive (vegetative) and resistant cells (reproductive hyphae and spores) of the same culture of strain No.45H, separately: washed culture (72 h) was suspended in buffer III, ultrasonicated for 2 min, then centrifuged at 6000 x g for 30 min. The supernatant was fractionated into S-30 and S-100 fractions, these served as the subcellular fractions of the ultrasound sensitive hyphae. The sediment which contained ultrasound resistant hyphae and the spores, was washed five times with distilled water, twice with buffer III, resuspended in the same buffer, then disrupted in a cell homogenizer (MSK, B. Braun). After a low speed centrifugation the supernatant was fractionated as described above, resulting in the S-30 and S-100 extracts of the ultrasound resistant cells.

Pure, mycelium-free spores and their cell-free extracts were prepared as published before (Valu et al. 1984).

For pulse labeling, harvested cells were washed and resuspended in filtered soy-bean medium diluted fourfold with phosphate buffer (0.05 M, pH 7.1), and completed with 1.0 % of glucose. Cells were labeled for 40 min with (^{14}C)protein hydrolyzate at 30° C. The incorporation of amino acids was stopped by cooling at 4° C and adding chloramphenicol. Labeled mycelia were thoroughly washed, then after breaking with Alumina cell-free extracts were made as described above.

Polyacrylamide gradient (4-20 %) slab gels (200 x 135 x 1 mm) were prepared according to O'Farrell (1975) with some modifications (Vitális et al. 1984). Following electrophoresis gels were immersed in 25 % isopropanol and 10 % acetic acid as described by Irie et al. (1982). Gels were stained with Coomassie brilliant blue G-250 in TCA for 16-24 h. Photos and diapositives were taken of the stained gels. The diapositives were scanned in a Kipp and Zonen densitometer DD2. On the average this method is able to resolve about 150-200 bands.

In the case of radioactive labeled samples stained gels were soaked with sodium salicylate solution according to Chamberlain (1979), dried at 60° C in vacuum, exposed to Forte Medifort RP x-ray film at -70° C, then films were scanned.

The densitograms and fluorograms were processed in a computer.

Specific activities were expressed in arbitrary units: OD values of the scanned autoradiogram were divided - point by-point - with the OD values of the same stained gel.

Protein concentrations were determined by the Lowry method (Lowry et al. 1951), using bovine serum albumin fraction V (Sigma) as standard.

RESULTS AND DISCUSSION

The alternative gene expressions are best reflected in the cells by the synthesis and appearance of new proteins, by their quantities and relative proportions. In order to demonstrate contrasting differences during differentiation, the protein patterns of cell-free extracts (S-30 fractions) derived from separated vegetative hyphae and reproductive forms of strain No.45H were compared (Fig. 1).

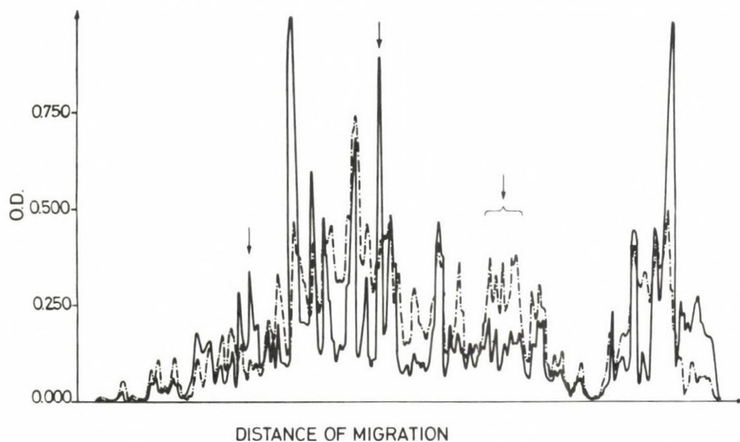

Fig.1. Densitograms of S-30 fractions from separated vegetative hyphae (-·-·-·), and reproductive forms (——) of strain No.45H cultured for 72 h.

Densitograms show that about one quarter of the bands is more or less identical, the rest of bands is of different intensity. Comparing the protein pattern of S-30 fractions from the ultrasound resistant forms to that of the purified spores (not shown here), they are almost identical, indicating that sporulation and protein composition are related.

The protein patterns of the No.52-1 mutant also change with the age of the culture. Strikingly the changes are more pronounced than in the well differentiating strain (Vitális et al. 1984). It may be concluded that the block of development does not lead to a freeze at a given stage, does not stop changes of gene expressions. This observation is in concor-

dance with results of our cytomorphological studies (Szeszák et al. 1967, Szabó et al. 1969, and unpublished results) which showed that morphological markers of spore development (accumulation of polysaccharides, condensation of nucleoids, stainability of cell wall, densely packed ribosomes in the cytoplasm) do appear also in the blocked mutant but in a haphazard way: in different order, in different hyphae and in different relationship to each other.

Studying the effect of factor C on development, S-100 fractions of the control and the factor C treated cultures of strain No.52-1 were used for SDS-PAGE. (10 ng per ml of factor C was added at the inoculation of spores, cultures were harvested at 40 and 64 h.) When the protein patterns of S-100 fractions from the control and the factor C treated mycelia (40 h) are compared, almost every band is altered qualitatively or quantitatively. On Fig. 2 the differences of OD values are depicted in the range of 35-75 kD.

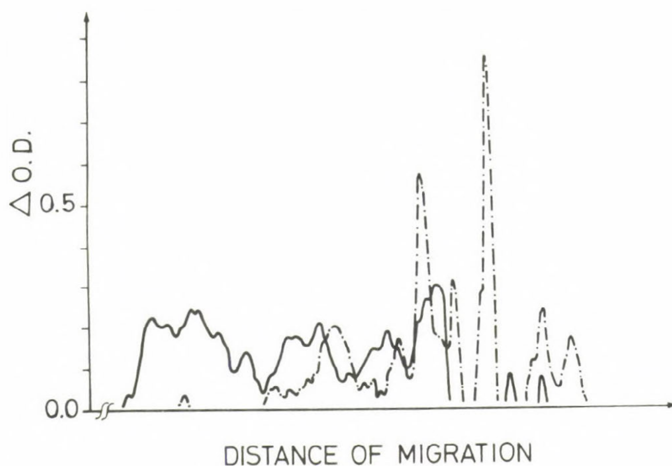

Fig.2. The differences of OD values of densitograms of S-100 fractions isolated from factor C treated and control cultures (No.52-1) at 40 (———) and 64 h (-·-·-) of cultivation. (OD values of the control densitograms were subtracted from that of the factor C treated cultures.) Differences higher than zero are shown in the range of 35-75 kD.

More informative results were obtained when the gene expression at a given stage was followed by a 40 min pulse labeling with (^{14}C)amino acids. After electrophoresis, protein patterns were developed both with staining and autoradiography. These data enabled us to calculate the specific activity of each band and have them portrayed.

We have found conspicuous differences between specific activities of bands of S-100 fractions isolated from the control and the factor C treated 52-1 cultures at an age of 40 h. The changes are mainly characteristic of the region of large molecules over 50 kD (Fig.3).

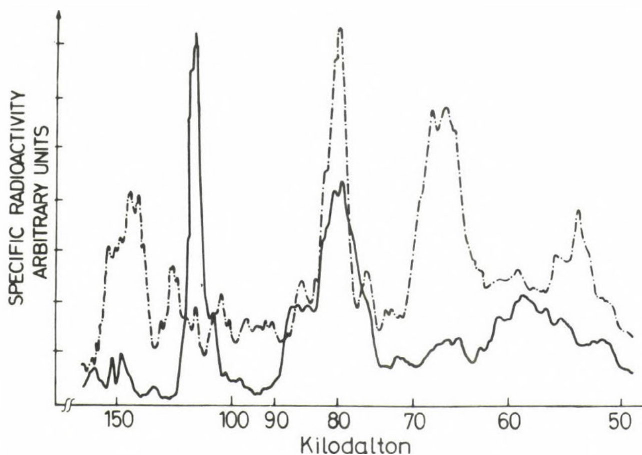

Fig.3. Specific activities of S-100 fractions from control (——) and factor C treated (-·-·-) cultures (40 h) of strain No.52-1, over 50 kD.

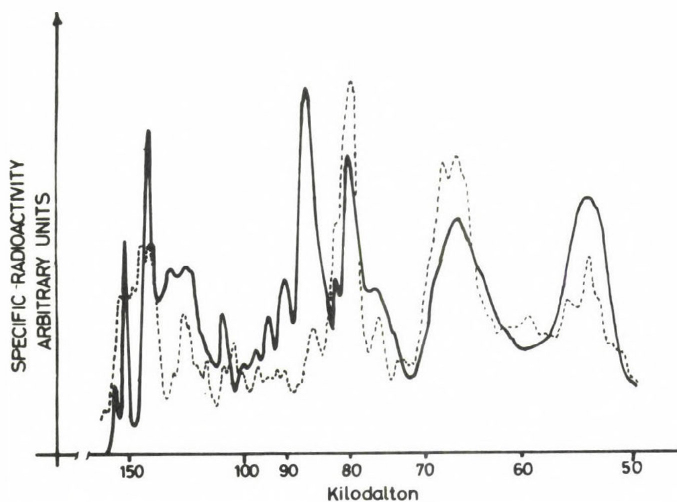

Fig.4. Specific activities of S-100 fractions isolated from the well differentiating strain No.45H (——) and from factor C treated 52-1 (- - -) at the same time of cultivation (40 h).

Fig. 4 shows the specific activities of S-100 fractions derived from factor C treated 52-1 culture and from the well differentiating strain No.45H at the same time of cultivation (40 h). The two graphs are very similar to each other.

To see if the changes of specific activities of S-100 fraction from the factor C treated culture are only a consequence of a shift in age of the culture, we have compared specific activities of S-100 fractions from old and young, control and factor C treated cultures in every possible combination. There were no indications which would speak for a shift in the age of the mycelia cultured with factor C. On Fig. 5 it can be seen that the changes of specific activities of S-100 extracts made from 40 and 64 h old control mycelia of strain 52-1 are different compared to each other, but these changes are not similar to the factor C induced ones (confer Fig. 4 with Fig. 5).

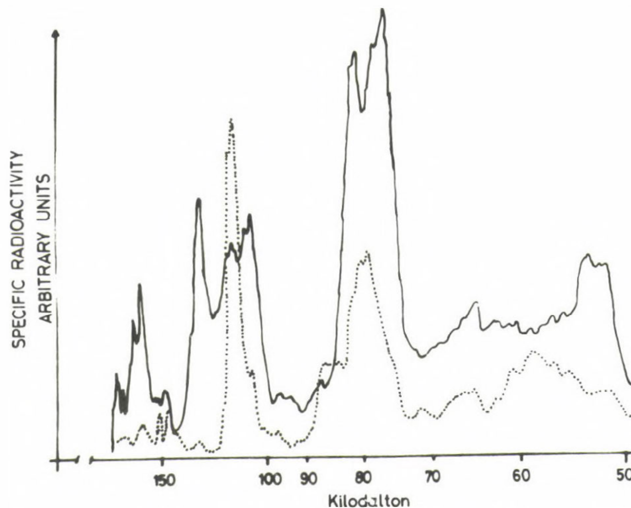

Fig.5. Specific activities of S-100 fractions from 40 (·····) and 64 h old (———) control cultures of *S.griseus* No.52-1.

Summarizing our results it can be concluded that during cultivation of *S.griseus* No.52-1 with factor C, the protein composition and protein synthesis of cells characteristically change, i.e. the specific activity of some bands of S-100 fraction has become similar to that of the well differentiating strain. We are aware of the fact that bands are not individual proteins but families of molecules of nearly identical molecular mass. Thus we demonstrate only a part of existing differences in the S-100 fractions.

We suppose that the effect of factor C on gene expression is indirect. It exerts one of its primary effects probably on

the cell membrane (Szeszák et al., in this volume). The relationship between permeability changes of cytoplasmic membrane and gene expression waits for future elucidation.

Contrary to our earlier reports factor C does not inhibit *in vitro* protein synthesis. Further purification of factor C resulted in separating a minor component (less than 5 %) responsible for inhibition *in vitro* translation.

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DIRECTED SELECTION OF DIFFERENTIATION MUTANTS BY AID OF CHEMOSTAT CULTIVATION

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Genetic studies with Streptomycetes revealed phenotypic alterations pointing to genetic instabilities mainly with respect to the secondary metabolism, which in the last few years has been demonstrated for example by Schrempf (1982a), Yoshikawa et al. (1982) and Ochi et al. (1984). Several authors were able to show correlations between the altered phenotype and structural changes in the chromosomal DNA (Hintermann et al. 1981, Ono et al. 1982, Crameri et al. 1983, Fishman and Hershberger 1983, Altenbuchner and Cullum 1984). The participation in this alteration of transposons has been suggested by Nakano et al. (1984) and Sermonti et al. (1983). Also plasmids have been shown to be able to interact with chromosomal DNA (Bibb et al. 1981, Hopwood et al. 1984, Schrempf 1982b).

For quantitative analysis of genetic segregation processes the method of continuous cultivation in chemostat is the most favourable one. In our laboratory the chemostat has been improved allowing the continuous cultivation of mycelialy growing Streptomycetes (Roth and Noack 1982) and the study of genetic segregation kinetics concerning the loss of antibiotic forming capacity (Noack et al. 1982, Roth et al. 1982a). In parallel experiments the maintenance of the recombinant plasmid pIJ2 in Streptomyces lividans has been followed (Roth et al. 1985).

In order to combine genetic segregation studies with both chromosomal and extrachromosomal DNA of an antibiotic producing Streptomyces strain, the chemostat cultivations were started with both plasmid-free and plasmid-containing strains of the species Streptomyces noursei (for reference Friedrich et al. 1984) which is the producer of the streptothricin antibiotic nourseothricin. Six types of differentiation mutants were selected after the chemostat culture reached a stationary state with respect to their genetic composition. These mutants were qualitatively and quantitatively characterized with respect to the control of nourseothricin formation and resistance to this antibiotic as well as to the neomycin resistance encoded by the recombinant plasmid pIJ385.

MATERIALS AND METHODS

Bacterial strain and plasmid: Streptomyces noursei JA3890b NG13 was received from the culture collection of this institute (for reference Friedrich et al. 1984). The plasmid pIJ385 confering neomycin and thiostrepton resistance was provided by D. A. Hopwood.
The chemostat and the culture media for continuous cultivation of Streptomyces strains are described by Roth and Noack (1982). Growth limitation was accomplished either with glucose (0.5 g/l) or with ammonium chloride (0.08 g/l).
For simultaneous limitation with both ammonium chloride and maltose the culture medium contained ammonium chloride 0.08 g/l and maltose 0.5 g/l instead of glucose.

Determination of nourseothricin formation: A loopful of surface mycelium of the respective strain was inoculated in 15 ml mineral salts medium with ammonium chloride and glucose in excess and cultivated 2 days in a rotatory shaker at 28 °C. Dropletts of about 2 µl of the culture suspension were placed in a distance of about mm onto the surface of antibiotic test agar. After 2 days incubation in 28 °C these plates were overlayed with Bacillus subtilis tester strain. After 16 hrs incubation at 37 °C the diameter of inhibition zones surrounding the colonies grown out of the dropletts were measured (e.g. see Fig. 1).
Content of antibiotic test agar (g per l):
AL53: Saccharose, 3; dextrin, 15; urea, 0.1; bacto peptone, 5; yeast extract, 1; NaCl, 0.5; KH_2PO_4, 0.5; $FeSO_4 \cdot 7 H_2O$, 0.01; agar, 0.15.
M79: Glucose, 10; bacto peptone, 10; casamino acids, 1; yeast extract, 2; NaCl, 6; agar, 0.2.
MM: Mineral salts medium as for chemostat culture with NH_4Cl in excess and without glucose; agar, 0.2.
MY: MM supplemented with 0.2 % yeast extract.
MYO: MY supplemented with KH_2PO_4 to a final concentration of 0.06 molar.
MG: MM supplemented with 1 % glucose.
MGO: MG supplemented with KH_2PO_4 to a final concentration of 0.06 molar.

Determination of antibiotic resistance: Liquid cultures as for determination of nourseothricin formation were spread onto M79. After incubation 4 hrs at 28 °C wells of 8 mm diameter were punched and filled with 0.05 ml of solution of antibiotic neomycin (Nm), viomycin (Vm), thiostrepton (Ts) and nourseothricin (Nt) with concentration 1000 µg/ml, 1000 µg/ml, 3000 µg/ml and 10 000 µg/ml respectively. After additional incubation for 3 days at 28 °C the diameter of inhibition zones surrounding the wells were measured (e.g. see Fig. 2).

RESULTS

Continuous cultivation

The nourseothricin producing Streptomyces noursei 3890b NG13 was continuously cultivated in a chemostat mainly character-

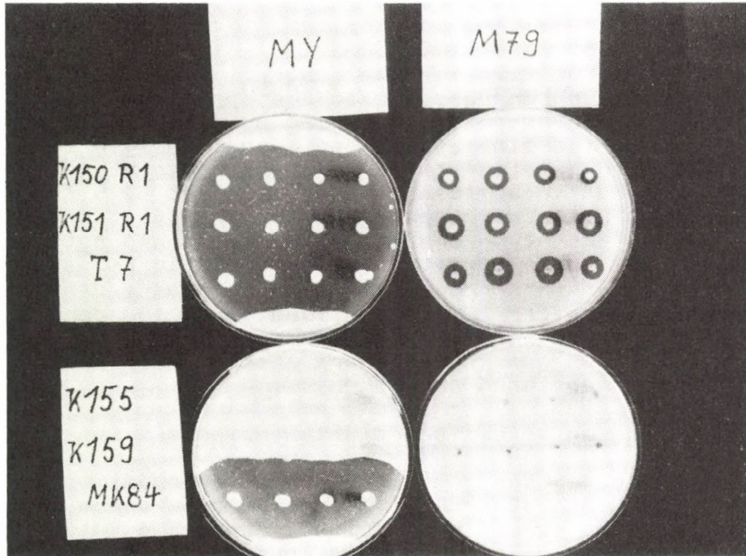

Fig. 1. Inhibition zones in the tester lawn surrounding Streptomyces colonies were used as measure for antibiotic productivity. The strains K155/159 and K150/151R1 are subclones of strains D129 and D12R1 respectively.

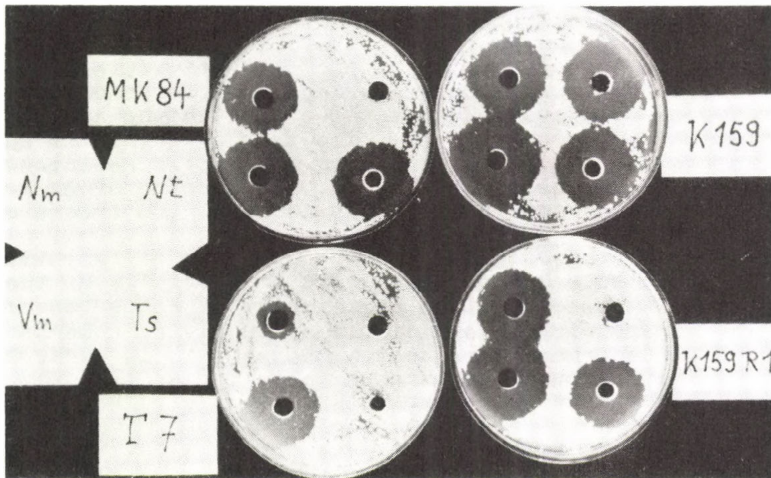

Fig. 2. Inhibition zones in the Streptomyces lawn surrounding wells were used as measure for resistance to the antibiotics filled in. The strains K159 and K159R1 are subclones of strains D129 and D12R1 respectively.

ized by a high-speed stirrer giving rise to the fractionation of mycelium and resulting in mycelium pieces of an overall mycelial length of about 100-200 μm.
During prolonged cultivation the genotypic composition of the mycelial population changes due to the enrichment of preexisting and/or spontaneously appearing mutants having a growth advantage under the limitation conditions employed in the chemostat. The population tends to a genotypic steady state which is proposed to be reached when at least 99.9 % of the colony forming units withdrawn from the chemostat culture exhibit the same phenotype characterized by growth pattern, control of antibiotic formation and antibiotic resistance. In all cases studied this population genetic steady state has been established after 100 generations, i.e. 100 doublings of biomass. Colony forming units withdrawn from the chemostat after at least 100 generations were checked with respect to the properties mentioned above.

Directed selection of differentiation mutants

Fig. 1 demonstrates the genealogy of differentiation mutants selected after chemostat cultivations in dependence on limitation conditions marked with solid arrows. The dotted arrows represent transformation with plasmid pIJ385 resulting in Nm^R and Ts^R clones marked by letter "T".
In the first step the strain NG13 was cultivated under ammonium chloride limitation at T = 28 °C and D = 0.15 h^{-1} in order to select differentiation mutants adapted to chemostat cultivation. After a cultivation time corresponding to 150 generations an altered phenotype appeared which has been shown not to be able to form aerial mycelium and spores (spo^-). This altered phenotype did not revert to the original one after several passages both in liquid and on solid medium. The properties of one representative clone, MK84, are listed in Tab. 1. The amounts of antibiotic productivity are calculated from inhibition zones appearing on antibiotic test agar and listed as relative values with respect to the highest one characterized by percentage 100. The same principle is applied to the calculation of relative antibiotic resistance level. All the values listed in Tab. 1 are means of at least 6 independent experimental data and include mean errors of ± 2 %.
With respect to the original strain NG13, the differentiation mutant MK84 shows a higher antibiotic productivity on the test medium MG and MY, but is drastically depressed on M79 agar. The sensitivity of antibiotic productivity towards high phosphate concentrations on medium MGO and MYO is markedly enhanced. The amount of resistance to Nm and Nt is not changed. By separate experiments (Krügel et al. 1985) it could be shown that the differentiation mutant MK84 got several properties to make him suitable as excellent recipient for recombinant DNA.
The segregation kinetics concerning the transition of the original population to the derivative one enriched in the chemostat can be described by two parameters, the segregation rate δ and the selection pressure σ (Noack et al. 1984).

Table 1. Properties of differentiation mutants selected from chemostat cultures

Strain	MG	MGO	MY	MYO	M79	Nm^R	Nt^R	Spo	Type
NG13	70	55	90	80	65	30	100	+	A
MK84	85	35	100	70	5	30	100	-	B
MK84T7	55	20	85	40	5	100	100	-	B'
MK84T7S1	75	35	100	65	12	30	100	-	E
NW253	10	5	15	10	10	30	65	-	C
NW380	55	45	80	65	10	30	100	-	D
NW380T2	45	35	65	55	8	100	100	-	D'
C5	0	0	0	0	15	30	20	-	F
D128	0	0	0	0	15	30	20	-	F
D129	0	0	0	0	15	30	20	-	F
C5T55	0	0	0	0	10	70	25	-	F'
D12T63	0	0	0	0	10	70	25	-	F'
C5R1	85	35	100	75	15	30	100	-	G
D12R1	85	35	100	75	15	30	100	-	G
C5T55R2	80	30	95	70	10	70	100	-	G'
D12T63R3	80	30	95	70	10	70	100	-	G'

The values of δ in relation to generation time τ can be seen from Tab. 2 and 3.

Table 2. Characteristic parameters of segregation kinetics concerning chromosomally encoded determinants of differentiated functions

Strain inoculated	Limiting substrate	Substrate in excess	Segregation rate δ	Selected strains
NG13	NH_4Cl	Glucose	$3.2 \cdot 10^{-2}$	MK84
MK84	Glucose	NH_4Cl	$2.5 \cdot 10^{-3}$	NW253
MK84	NH_4Cl	Maltose;PO_4	not determ.	NW380
MK84T7	Glucose	NH_4Cl	$5.6 \cdot 10^{-4}$	D128;D129
NW380T2	Glucose	NH_4Cl	$2.0 \cdot 10^{-4}$	C5
MK84T7	NH_4Cl	Glucose	0	
NW380T2	NH_4Cl	Glucose	0	
MK84T7	Malt.+NH_4Cl	Phosphate	0	
NW380T2	Malt.+NH_4Cl	Phosphate	0	

All further selection procedures and the properties of differentiation mutants obtained can be seen from Fig. 3 and Tab. 1, 2 and 3 respectively. A representative kinetics is shown in Fig. 4 summarizing the segregation of both the chromosomal markers and the plasmid pIJ385 DNA out of the differentiation mutant Streptomyces noursei MK84.

Table 3. Characteristic parameters of segregation kinetics concerning plasmid encoded neomycin resistance

Strain inoculated	Limiting substrate	Substrate in excess	Segregation rate δ	Selection pressure σ
MK84T7	Glucose	NH_4Cl	$2.3 \cdot 10^{-5}$	$7.4 \cdot 10^{-2}$
NW380T2	Glucose	NH_4Cl	$2.5 \cdot 10^{-2}$	$4.5 \cdot 10^{-2}$
MK84T7	NH_4Cl	Glucose	0	-
NW380T2	NH_4Cl	Glucose	0	-
MK84T7	Maltose+NH_4Cl	Phosphate	$5.6 \cdot 10^{-3}$	$1.7 \cdot 10^{-2}$
NW380T2	Maltose+NH_4Cl	Phosphate	$8.8 \cdot 10^{-3}$	$1.3 \cdot 10^{-2}$

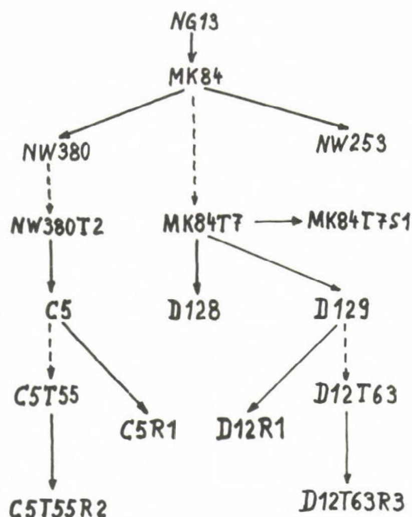

Fig. 3. Relation scheme of selectants mutated in differentiated functions.

DISCUSSION

The nourseothricin producing species Streptomyces noursei was continuously cultivated in a chemostat under different limitation conditions. After at least 100 generations corresponding 100 doublings of biomass six types of differentiation mutants called B to G in Tab. 1 could be isolated and distinguished from another by their control character-

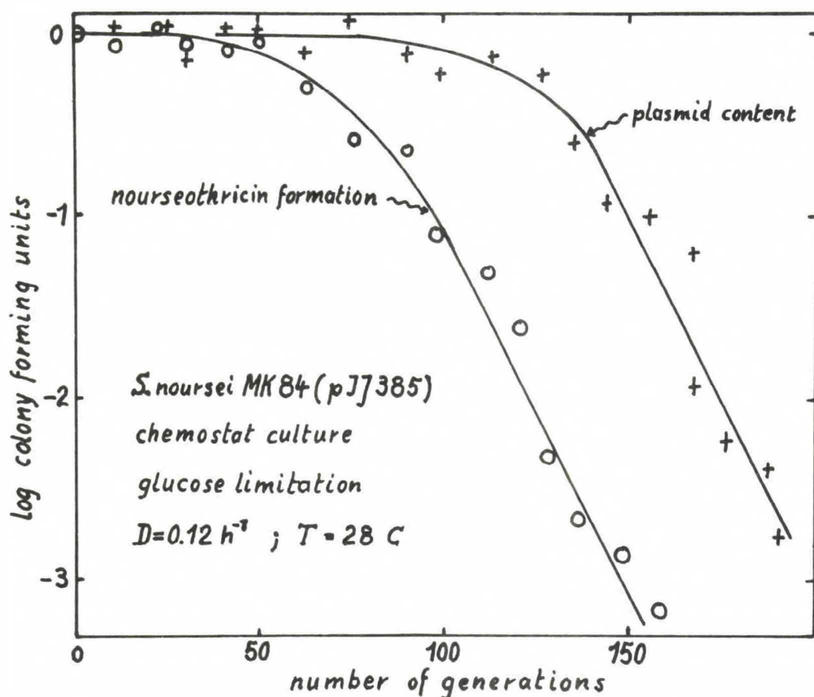

Fig. 4. Segregation kinetics of *Streptomyces noursei* MK84 (pIJ385) with respect to nourseothricin formation (o) and neomycin resistance (+).

istic of nourseothricin biosynthesis and antibiotic resistance. The phenotype of these differentiation mutants could be shown to be stable over at least 6 subcultures in liquid and/or on solid media without any selection pressure. Therefore they are designated as to be altered genotypes. The differentiation mutants obtained are in strong correlation to the selection pressure realized in the chemostat. From these results it can be postulated that a directed selection and enrichment of desired differentiation mutants can be achieved but that these mutants exhibit several additional properties which are not expected and predicted. These uncertainties result from our limited knowledge about the genetical and physiological reaction of the strain under study upon the selection pressure established within the chemostat.

This principle is also illustrated by the differentiation types C and F. Both of them were selected after glucose limitation. Type C retained a small nourseothricin forming capacity onto all media tested, but the type F strains show

nothing but a very low activity onto media M79. By separate experiments (Haupt et al. 1985) it could be shown that these derivatives lost the ability to enzymatically acetylate the nourseothricin molecules. These properties are not changed after transformation with plasmid pIJ385 resulting in strains C5T55 and D12T63 respectively. It can be speculated that these difference in the segregation principle may be due to the plasmid pIJ385 originally present in the parental strain MK84T7 and NW380T2 respectively. This proposition may be supported by the fact that the plasmid pIJ385 is eliminated out of MK84 and NW380 respectively with a quite different segregation rate (lane 1 and 2 of Tab. 3) in spite of the same limitation condition realized in the chemostat. In addition this fact and the observation that pIJ385 is eliminated out of MK84 and NW380 with nearly identical but intermediate rate when cultivated under simultaneous limitation with maltose and ammonium chloride demonstrate that the elimination kinetics of plasmids out of *Streptomyces* strains depend on both the selection pressure employed within the chemostat and the genotype of the host strain.
As it could be predicted from earlier experiments, the limitation with ammonium chloride did not result in the segregation of both chromosomally and extrachromosomally encoded determinants for both the control of antibiotic synthesis and antibiotic resistance.
The differentiation mutants of types F and F' respectively are obviously not absolutely stable but segregate clones of types G and G' respectively which received both the nourseothricin forming capacity and nourseothricin resistance with a degree somewhat higher then that of the parental types B' and D' respectively. This observation may be a hint for a strategy concerning the improvement of industrial antibiotic producing strains. The appearance of types G and G' revertants is obviously restricted to spontaneous processes in batch cultures with complex media because in chemostat cultures the antibiotic producing clones have a growth disadvantage with respect to those not able to form antibiotic.
In conclusion it can be postulated that desired differentiation mutants of streptomycetes can be selected with the aid of chemostat but that these mutants exhibit additional properties which exactly can not be predicted till now. However these additional properties seem to depend strongly upon the interrelation between the limitation condition realized in the chemostat and the genotype of the strain under study. The better the respective knowledge is the better is the prediction of desired differentiation mutants to be selected in the chemostat.

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DIFFERENTIATION

Mini-Symposium

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PLEIOTROPIC REGULATORY SYSTEMS INVOLVED IN THE CYTODIFFERENTIATION OF STREPTOMYCETES

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INTRODUCTION

Cytodifferentiation of the streptomycetes enables adaptation to changing environmental situations. Involved in this process there are endogenous signal molecules which trigger the onset of a series of sequential metabolic events leading to formation of aerial hyphae, spores, and secondary metabolites. Elucidation of the chemical structure and mode of action of such effectors can supply new information concerning the metabolic regulation of morphological changes in this industrially important group of microorganisms. But a major obstacle is presented to all the efforts aimed at exploration of the proper sequence of metabolic events involved in the expression of the complex phenotype of the streptomycetes: the initial effect of the signal molecule will be integrated in a highly interwoven regulatory network. Special reference will be given here to the results of our recent work on the role of A-factor [2-(6'-methylheptanoyl)-3-hydroxymethyl-4-butanolide] (Khokhlov 1982) and its derivatives as inducers of the formation of spores and anthracyclines by blocked mutants of Streptomyces griseus.

BIOLOGICAL EFFECT OF THE A-FACTOR

Similar to blocked Amy^-Str^- mutants of a streptomycin-producing strain of S. griseus (Khokhlov 1982), some of the aerial mycelium and anthracycline-negative mutants (Amy^-Ant^-)

such as strain 86 of the ancestral S. griseus strains JA 5142 and JA 3933 (Amy^+Ant^+) regained both wild-type morphology and the production of anthracyclines (leukaemomycin) during either submerged or surface cultivations when the A-factor or its derivatives have been added to starting cultivations (Gräfe et al. 1984) (Fig. 1).

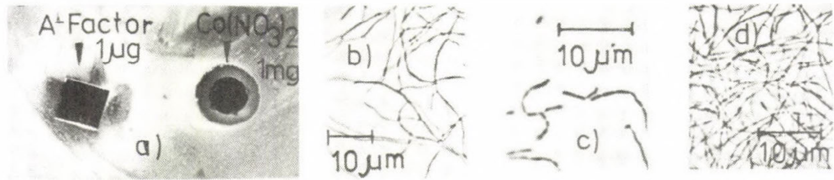

Fig. 1. a) Induction of aerial mycelium formation by A-factor on surface cultures and stimulation of this effect by cobalt. Morphology of submerged mycelia of the parental strain S. griseus JA 5142 (b) and blocked mutant 86 (c). d) Mutant 86, medium supplied with 0.3% KH_2PO_4.

INTEGRATION OF THE INITIAL EFFECT OF A-FACTOR INTO AN INTERWOVEN REGULATORY NETWORK

In an attempt to throw some light on the mode of action of A-factor or is derivatives such as trans-2-(6'-methylheptanol-1'-yl)-3-hydroxymethyl-4-butanolide obtained from S. viridochromogenes, we studied the biochemical changes induced by the presence of these effectors in submerged cultures of mutant 86 and other blocked strains grown on Hickey-Tresner's medium. The results of this work which have been reported elsewhere in detail (Gräfe et al. 1984). led to the following conclusions:

- A pleiotropic gene may control formation of A-factor by the parental strain concomitant with other metabolic activities such as formation of NADP-glycohydrolase, a green pigment, and particular fatty acid precursors. Addition of A-factor did not reconstitute the expression of these functions, and the parental protein pattern as revealed by SDS-PAGE was regained incompletely.

- Presumably, the A-factor affects cellular processes through its complexing properties. This is suggested by the

synergistic effect of cobalt (Fig. 1a). It seems also likely that A-factor interferes with the membrane, as a polar molecule.

- Through the activity towards still unidentified target sites, the A-factor provokes enhancement of the synthesis of lipid material and proteases.

- These changes can be expected to induce a plethora of subsequent alterations at various cellular levels. Alteration of neutral proteinase activity may give rise to changed pattern of proteolysis. Otherwise, alterations of lipid composition could interfere with membrane-dependent processes such as the secretion of enzymes.

- The latter idea is supported by the finding that mutant 86 and other blocked Amy$^-$Ant$^-$ strains cannot efficiently secrete alkaline phosphatase (Table 1). Table 2 reveals that most of this enzyme was bound to subcellular particles. These observations agree with reports on Bacillus strains which are unable to secrete the alkaline phosphatase, probably due to impaired function of a signal peptidase (Kumar et al. 1983). Growth of mutant 86 in the presence of A-factor restored partly enzyme secretion (Table 1). Due to the reduced secretion of the alkaline phosphatase by mutant 86, acquisition of inorganic phosphate from the complex medium should be greatly hampered. The observed nocardioform-like growth of strain 86 (Fig. 1c) thus can be ascribed to severe phosphate limitation. In fact, transition of mycelium morphology from the fragmentized type of hyphae (Fig. 1c) to dense aggregates as shown in Fig. 1d has been induced by adding phosphate to Hickey-Tresner's medium.

Thus, the effect of the A-factor on susceptible blocked mutants of anthracycline-producing strains of S. griseus may show that signals of cytodifferentiation are embedded in general into interwoven, pleiotropic, regulatory systems. Obviously, transformation of the initial signal input occurs at many subsequent levels of cellular control involving changes of the architecture of the hyphae.

Table 1. Specific activity of alkaline phosphatase in the culture medium (CM; nmol/min/ml) and supernatants of 2000 g centrifugation of sonic extracts (ME; nmol/min/mg protein). Cultivation on Hickey-Tresner's medium

	Specific activity of enzyme					
	24 hr		48 hr		72 hr	
	CM	ME	CM	ME	CM	ME
S. griseus JA 5142	150	15	200	20	280	30
Mutant 86	30	110	30	150	35	210
Mutant 86 grown in presence of 0.5 µg A-factor	50	50	100	80	110	100

Table 2. Subcellular localization of alkaline phosphatase in sonic extracts from 24-hr mycelium of mutant 86 (specific activity; nmol/min/mg protein)

Crude sonic extract		160
5000 g centrifugation (3 min)	sediment	200
	supernatant	50
22,000 g centrifugation of the 5000 g supernatant (30 min)	sediment	350
	supernatant	25
100,000 g centrifugation of the 22,000 g supernatant (45 min)	sediment	25
	supernatant	25

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PRODUCTION OF A-FACTOR TYPE REGULATORS BY MICROMONOSPORA spp.

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Substances close by their chemical structure and biological action to A-factor, a regulator of Streptomyces griseus differentiation, are frequently detected in Streptomyces spp. We studied production of A-factor type regulators in 13 strains belonging to 11 Micromonospora species (Table 1). An A-factor deficient strain of S. griseus 1439 was used as a test culture for the presence of such regulators. It was shown that 7 out of the 13 cultures produced substances inducing spore formation in mutant 1439. This meant that by their action the substances were close to the differentiation regulator, i.e. A-factor. The majority of the 7 strains excreted the regulators into the medium on the 8th to 14th day of the surface growth, though duration of the growth before the beginning of the regulator excretion into the medium could be different in the same strain and depended on the cultivation medium. Three agar media were used in the study; 3 out of the 7 strains produced the A-factor-like regulators only on one of the three media. Therefore, for the investigation of strains belonging to different species for their ability to produce regulators of the A-factor type it was necessary to vary the cultivation conditions and in particular the composition of the nutrient media and growth time.

Correlation between production of A-factor-like substances and differentiation was studied in Micromonospora fusca var. sisomycini RIA 1572. The submerged culture of strain 1572 was coloured black on the third day. This colour was preserved till the end of the culture growth. The highest levels of sisomicin were also detected on the third day. Later its concentration

Table 1. Production of A-factor type regulators by *Micromonospora* spp.

Cultures	Regulator production
1. *M. atratovinosa* RIA 1573	+
2. *M. brunnea* RIA 1659	-
3. *M. brunnea* RIA 1660	-
4. *M. chalcea* RIA 1662	-
5. *M. chalcea* RIA 1668	-
6. *M. coerulea* RIA 1663	+
7. *M. echinospora* subsp. *echinospora* RIA 951	+
8. *M. fulvopurpurea* RIA 1664	-
9. *M. fusca* var. *sisomycini* RIA 1572	
10. *M. olivoasterospora* RIA 1656	+
11. *M. purpurea* RIA 1634	+
12. *M. purpurea* var. *violaceae* RIA 1535	-
13. *M. purpureochromogenes* RIA 472	+

markedly decreased. Regulators of the A-factor type were detected in the culture fluid on days 6 to 8. Since the time of maximum production of sisomicin and A-factor-like regulators in strain 1572 was different it could be suggested that these substances did not directly participate in the biosynthesis of sisomycin. The highest number of separate viable spores was, on the contrary, detected in the submerged culture on days 6 to 8, which suggested that there was a correlation between the presence of the A-factor-like substances and sporulation. In this study we investigated the action of A-factor added to the submerged culture simultaneously with the seed material on biosynthesis of sisomicin and sporulation in strain 1572. It was shown that in concentrations of 0.1, 1, 5 and 10 μg/ml, A-factor had no effect on the biosynthesis of sisomycin, which was confirmed by the suggestion that there was no correlation between this process and production of the A-factor-like regulators in strain 1572. The time of sporulation under the effect

of A-factor did not change. However, the titre of separate viable spores increased at least 4 times. Therefore, there was significant intensification of the spore formation in strain 1572 under the effect of A-factor, a regulator of S. griseus. At the same time the regulator (or regulators) from M. fusca var. sisomycini 1572 had a regulatory effect on the A-factor-deficient strain of S. griseus 1439. This effect was similar to that of A-factor. Subsequently, strain 1572 produced A-factor or a substance close to A-factor. In the latter case the A-factor and regulator of strain 1572 had a cross effect on the strains of the species producing them.

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ADP-RIBOSYLATION IN STREPTOMYCES GRISEUS

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The process of ADP-ribosylation consists of two steps: NAD is split by NAD-glycohydrolase (NADGH) resulting nicotinamide and adenosinediphosphoribose (ADP-R). The last subtance is bound to specific acceptors by enzymatic transfer reaction producing posttranslational proteins (1).

Many of the enzymes catalyzing mono (ADP-ribosyl)ation are microbial toxins like cholera-, diphteria-toxins, E. coli enterotoxin, although their targets are eukaryotic proteins. On the other hand poly (ADP-ribosyl)ation includes almost solely enzymes and acceptors of eukaryotic cells (1). There are only few cases showing in vivo ADP-ribosylations in prokaryotes in E. coli (5, 6) and Rodosphillum rubrum (4), none has hitherto been reported in Streptomyces. It is known, however, from the works of the other groups (2, 3) that NADGH activity, the first step of ADP-ribosylation, is present in some Streptomyces, though a cytoplasmic origin had been attributed to this enzyme (2). We detected both the NADGH and ADP-ribosylation activity as membrane-bound processes. Membranes were isolated in the usual way by protoplasting. After detecting the NADGH activity in the isolated and purified membrane of S. griseus (No. 52-1) (7), we elaborated a procedure for detecting membrane-bound ADP-ribosylation (Fig. 1).

20 µl purified membrane /19.2 mg protein/ml/
20 µl 0.025 M TRIS•HCl /pH=7.3/
5 µl mercaptoethanol
1 µl ^{3}H-NAD /spec. activity: 2.8 Ci/mM/

↓

incubation for 15 min, at 37 °C

↓

5 µl NAD /120 mg/ml/
20 µl dissolving buffer
10 µl 40 % SDS

↓

boiling for 2 min, at 100 °C

↓

redissolved in 10 µl 1% TRITON X-100 and 8 M urea

↓

gel electrophoresis: PAGE gradient slab gel /5%-15%/
350 V, 20 mA, 5 hours

Fig. 1. Conditions for ADP-ribosylation.

After showing the presence of this reaction, it was also proved that one substance was ADP-ribosylated with a mol.weight (Mw) of 30 kD (Fig. 2).

To prove that the radioactivity can be attributed to an ADP-ribosylated product, the isolated membranes after the incubation with tritiated-NAD, were treated with ethylamine which specifically releases ADP-ribose from ADP-ribosylated proteins, and also with NaOH, and the released substances were then analyzed (Fig. 3).

Fig. 2. Fluorogram of the membrane sample incubated with ^{3}H-NAD.SDS-polyacrylamide gel electrophoresis.

Since the detectable ADP-ribosylation was in some cases very low, we had been looking for the explanation of it and found that there was phosphodiesterase activity in the membrane of our strain which could split the ADP-ribose resulting in the release of AMP. Inhibitors of phosphodiesterase were employed in the incubation mixture which markedly increased the incorporation of ADP-R into the acceptor molecule (Fig. 4).

Both NADGH- and ADP-ribosylation activity are membrane bound in _S. griseus_, the way of binding was, however, obscure. The membranes were treated with DNase and their NADGH-ase (see also J.C.Ensign's paper in this volume) and ADP-ribosylation activity were compared with those of untreated samples.

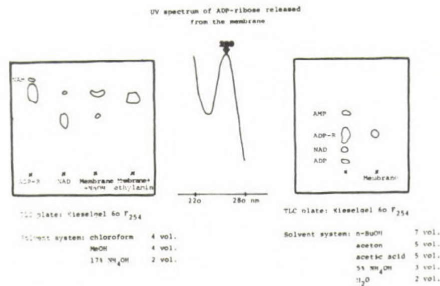

Fig. 3. Detection of ADP-ribose on TLC.
Right: membrane treated with ethylamine.
Spots were detected by UV light. Spot released by ethylamine (left plate) was scraped off the plate and analyzed (see the Fig. in the middle).

Both activities were higher in the membranes not treated with this enzyme. This allows the suggestion that the ADP-ribosylated compound, possibly a protein with a Mw (30 kD) close to histone-like proteins in prokaryotes, is near or at the repli-

Sample	Radioactivity incorporated
control	3oo2 cpm
lo^{-2}M gluthathion	832o cpm
lo^{-2}M cystein	7o43 cpm
lo^{-2}M ascorbic acid	75o2 cpm

Fig. 4. Effect of phosphodiesterase inhibitors on ADP-ribosyltransferase activity.

cation points (membrane-bound DNA) of S. griseus. It is tempting to postulate that ADP-ribosylation is involved in DNA-correlated events.

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SOME EARLY EFFECTS OF FACTOR C ON STREPTOMYCES GRISEUS MYCELIUM

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The factor C is a 30,000 dalton molecular mass protein isolated earlier in our institute /Biró et al. 1980/. Its biological activity can be measured by cytomorphological test which is a long-term assay /Biró et al. 1980/. Therefore it is not suitable for studying the mechanism of action of factor C. We had to look for some early effect. We had some previous observations such as the clump-formation of the mycelium, liberation of nucleotides and labeled amino acids within 30-60 min after the addition of factor C. These observations called our attention to the possible importance of the early permeability changes in the action of factor C.

In order to get information on the permeablity changes we worked out an experimental system /Fig.1/ in which we are able to measure extracellular K^+ concentration by means of an ion-selective membrane electrode produced by the Hungarian firm Radelkis. The method is suitable for measuring rapid changes in the concentration range down to 10^{-6} M K^+ in suspensions of living mycelia.

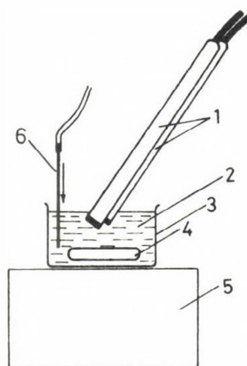

Fig.1: K^+ concentration measuring system. Mycelia of *S. griseus* strain No 52-1 were washed by filtration and suspended in 32 ml buffer A /15 mM $NaPO_4$ buffer, pH 7.2; 680 µM $CaCl_2$; 200 µM $MgSO_4$; 13 µM $CuSO_4$; 1 µM $FeSO_4$; 2 µM $MnCl_2$; 1,5 µM $ZnSO_4$; 1% glucose/ and measured by an ion-selective membrane electrode /Gale, 1974/. mV values were measured after 1 min equilibration. Supplements were added in 50 µl. 1/ electrodes; 2/ vessel; 3/ magnetic flea; 4/ stirrer /heater; 5/ air inlet.

The factor C has a marked effect on the K^+ efflux /Fig.2/. In the presence of the compound, the equilibrium between the mycelium and the buffer is shifted toward a higher concentration of external K^+. The effect can be completely abolished by

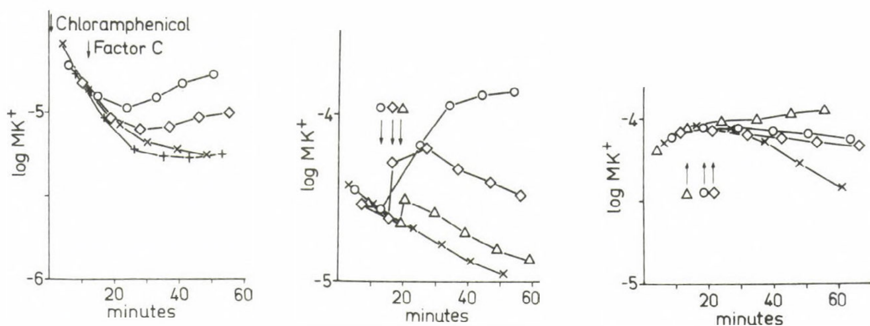

Fig.2: Increase of K^+ efflux of the mycelium after the addition of factor C. Effect of chloramphenicol on the factor C induced K^+ efflux. 4 samples were run in parallel. Aeration and stirring went on continuously at 25 °C and the electrodes were transferred from sample to sample. x: control; o:+28 U/ml factor C; +:+ 5 µg/ml chloramphenicol; ◇:+ 5 µg/ml chloramphenicol + 28 U/ml factor C.

Fig.3.: Comparison of the effect of factor C, histone F_1 and F_{2a} on the K^+ efflux of the mycelium. x: control; o: + 28 U/ml factor C; ◇: + 5 µg/ml histone F_I; △: + 5 µg/ml F_{2a} /histone preparations were from Sigma/.

Fig.4.: Concentration dependence of the effect of factor C on the K^+ efflux. x: control: △:4 U/ml factor C; o: 1.5 U/ml; ◇:0.7 U/ml.

heat-denaturation of factor C. /The changes of K^+ concentration observed in these studies ensuing after the addition of factor C mount to less than 10% of the total mycelial K^+/.The effect is partially resistant to chloramphenicol. The external K^+ concentration of the sample containing both factor C and chloramphenicol is obviously higher than that of the control, but lower than that of the sample containing factor C only. We have not yet examined systematically more details of the chloramphenicol action. The effect of factor C was also resistant to actinomycin D, thus we can tell that the early effect of factor C is basically not dependent on the presence of newly synthesized macromolecules.

The effect of factor C is not abrupt /Fig.3/. It becomes apparent when compared to the effect of histone F_1 or F_{2a}. Adding histones to the system, the increase of the K^+ concentration takes place within milliseconds. Then the curves start to run parallel with that of the control. For the administration of factor C, as a rule, no effect can be observed within 3-4 min, the characteristic latency period of the response being about 7 min. The progressive increase of the K^+ efflux is another characteristic of the factor C response, i. e. the curves of samples treated with factor C do not run parallel with the control, on the contrary, they diverge more and more with time.

We tried several other compounds such as hitone F_{2b}, F_3, poly-L-lysine, protamine, cytochrome C and polyanions like heparin and poly-L-glutamic acid. The polycations had similar but weaker effect than the histone F_1 had, while cyto-

chrome C and polyanions were without any effect. No proteins and polymers tested resulted in any change reminding in the least of the effect of factor C. Thus, the latter seems to be specific for the K^+ efflux of *S. griseus* mycelium.

Decreasing the concentration of factor C /Fig. 4/ we have found it effective down to 0.7 U/ml. It corresponds to about 0.7 ng/ml of factor C. We have calculated that no more than 10 molecules per cell of factor C were present in our mycelial suspensions.

CHANGE OF PROTEIN SYNTHESIS AND AMINO ACID UPTAKE UPON THE EFFECT OF FACTOR C

Age of culture /h/	Protein synthesis /incorp. amino acids, dpm x 10^{-3} x mg^{-1}/		Acid soluble fraction /per cent of synthesized proteins/	
	Control	+ Factor C	Control	+ Factor C
17.5	-	-	3.27	-
40	224	463	48.0	76.3
64	122	235	-	-

Table 1: Factor C was given at the inoculation, cultures were harvested, washed at the times indicated and labeled for 60 min with ^{14}C -protein hydrolysate

To draw conclusions from the K^+ concentration measurements one has to consider the effect of factor C on the protein synthesis too /Table 1/. Adding factor C at zero time we can see that it does in fact double the protein synthesis in 40 or 60 h old mycelia. The relative amount of the acid soluble fraction, which can be taken as a measure of the amino acid uptake, increases with the age of the mycelium and it is further increased by factor C. Thus as a late effect of factor C an increased amino acid uptake is also relevant.

We suspect, and try to study in the future that by some mechanism the factor C changes the permeability of the cytoplasmic membrane. This would lead to an increased transport of amino acids, increased rate of macromolecular synthesis and cytodifferentiation. The increased K^+ efflux would be a well-measurable side-effect of the change of permeability.

Summarising our results, the factor C

- increases the K^+ efflux of washed mycelia of *S. griseus* strain No. 52-1 at concentrations down to 0.7 ng/ml
- its effect cannot be prevented either by chloramphenicol or actinomycin D
- no other proteins and polymers tested had similar effect to that of factor C.

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METABOLIC CONTROL OF DIFFERENTIATION IN STREPTOMYCES FRADIAE

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In *Streptomyces* there seems to be some unexplored relationship between the arginine metabolism and the control of differentiation. We isolated an Arg^- mutant from *S.fradiae*. Its biosynthetic block was localized to the ornithine carbamoyltransferase step [1]. Grown on an arginine-supplemented synthetic medium the colonies were bald, but when arginine was replaced with citrulline, aerial mycelia were produced [1].

It turned out that methionine-sulfoximine (MS), a specific inhibitor of glutamine synthetase (GS), could promote aerial development on arginine-supplemented medium. Then we suggested that a too high GS activity could inhibit aerial growth [2].

In this paper, data on GS activity and some other nitrogen assimilatory functions in *S.fradiae* are reported.

MATERIALS AND METHODS

S.fradiae St 3110 mutant, sucrose nitrate (SN) media, and cultivation conditions were reported earlier [1].

Cell homogenates were prepared from liquid cultures. Mycelia were cooled, washed with 0.9% NaCl, and 2500*g* supernatants of sonic homogenates were used for the enzyme measurements.

Protein content was determined by the Lowry method.

Glutamine synthetase (GS), NADPH-dependent glutamate dehydrogenase (GDH), and NADH-dependent alanine dehydrogenase (ADH) activities were measured according to Gräfe *et al.* [3].

For determination of nitrite content 0.5 ml H_2O; 0.25 ml sulfanilic acid, 1% in 3N HCl; and 0.25 ml N-(1-naphthyl)-ethylenediamine dihydrochloride, 0.02% in H_2O were added to an agar block of 0.3 ml cut from a surface culture, and after 30 min of incubation at room temperature the absorbance of the 1600*g* supernatant was determined at 560 nm. The nitrite content was calculated using a calibration curve.

RESULTS AND DISCUSSION

The first question was wether MS really inhibited GS activity of our strain, or not. An inhibition of 65% was observed by MS at 5 mM concentration, while NH_4Cl caused only a 23% inhibition at 10 mM concentration. Arginine was not inhibitory (Table 1).

Table 1. Inhibition of GS activity in homogenates of *S.fradiae* St 3110 grown in SN + 1 mM citrulline medium for 48 h

Test compound	GS activity (%)
None	100
DL-Methionine-DL-sulfoximine, 5 mM	35
NH_4Cl, 10 mM	77
Arginine, 20 mM	104

Next the effect of different supplementations on GS activity was investigated. In arginine-supplemented media GS activity was low (at higher arginine concentration even lower) during the whole cultivation period, but in citrulline-supplemented medium the enzyme activity was relatively high (Table 2). Therefore, since the presence of citrulline was favourable for aerial development on solid medium [1], the assumption that high GS activity was inhibitory to differentiation [2] seems unlikely.

Table 2. GS activity during growth of *S.fradiae* St 3110

Medium	GS activity (nmol/min/mg protein)		
	24 h	48 h	72 h
SN + arginine, 1 mM	12	11	12
SN + arginine, 10 mM	11	5	5
SN + citrulline, 1 mM	133	126	133

The NADPH-dependent assimilatory GDH activity behaved essentially similary to the GS activity. In cells grown in arginine-rich media there was less enzyme activity than in cells grown in the presence of citrulline (results are not shown).

The medium used contained nitrate as inorganic nitrogen source. Reduction of it to nitrite should have been the first step of its utilization. When nitrite was measured in the medium, it turned out that cells produced much less nitrite in arginine-supplemented than in citrulline-supplemented media (Fig. 1).

A major nitrogen assimilatory enzyme in cells fed with arginine seemed to be the NADH-dependent ADH, since its activity was relatively higher when exogenous arginine was present (Table 3).

Table 3. ADH activity of 42 h old *S.fradiae* mycelia

Medium	ADH activity (nmol/min/mg protein)
SN + arginine, 1 mM	30
SN + arginine, 10 mM	167
SN + arginine, 20 mM	195
SN + citrulline, 1 mM	25

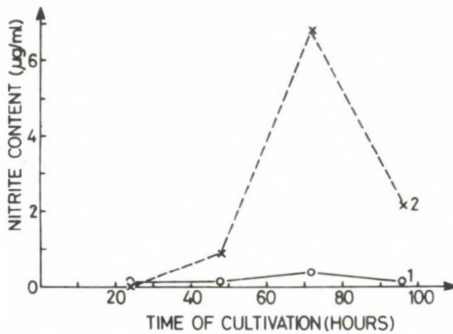

Fig. 1. *S.fradiae* was grown on SN agar plates supplemented with 1 mM arginine (1), or 1 mM citrulline (2). At intervals samples were removed for nitrite determination.

It became clear that the way of nitrogen assimilation in the cells grown on arginine was quite different from that when citrulline was added instead of arginine. We suggest that this nutritional difference may cause the observed developmental peculiarities.

On the basis of these data we can conclude that since the way from the nutritional signals to the regulatory mechanisms of cell differentiation (sporulation) should go through the cell metabolism, this way may involve steps that are sensitive to cellular levels of certain amino acids or other metabolites. This approach may be fruitful for exploration of regulatory mechanisms of differentiation and also of antibiotic production [4,5].

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SOME BIOCHEMICAL ASPECTS OF THE FORMATION OF AERIAL MYCELIUM WITHIN STREPTOMYCETES

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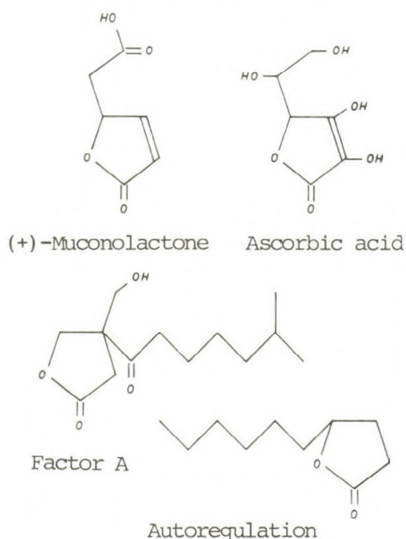
Aerial mycelium differs from the vegetative mycelium by its structure and particularly by its function. It serves as the place of sporulation and multiplication. It can be therefore assumed that another kind of metabolism takes place in aerial mycelium. The factors which induce and support the formation of aerial mycelium are not completely known. Some years ago we isolated a fungal metabolite which causes the formation of aerial mycelium within streptomycetes (Küster a. Attaby 1982). The chemical composition of that compound is not known, however, the structure of phenolic compounds, amino acids, sugars and sugar derivatives could be excluded.

Recently we examined the effect of various sugars on the formation of aerial mycelium of Strept.lavendulae (Kim 1984). Glucose, mannose, maltose, dextrin and starch proved to be the best C-sources. Higher concentrations of glucose, 2-3 %, retarded the beginning of aerial mycelium (AM)-formation for 1-2 days. This was due to the greater production of lactic acid combined with a clear decrease of the pH-value. A small decrease of the pH (about 0.5), on the other hand, stimulated or made even possible the AM-formation. This is explained by the uptake of NH_4.

Salicin as C-source is not suitable for the AM-formation, even if the pH decreases a little and NH_4 is uptaken. Salicin is a ß-glucosid which is split by ß-glucosidase to salicylalcohol and salicylic acid. Na-salicylate inhibits the AM-formation at very low concentrations. A metabolite of salicylic acid, (+)-muconolactone, contains a γ-lactone structure, similar to ascorbic acid which inhibits the cell division (Kang et al.1982). Ascorbic acid suppresses the AM-formation at 0.1 %, but stimulates it at low concentrations of 0.0001 %. It is suggested that the inhibiting effect of salicylic acid is due to the presence of (+)-muconolactone. Two stimulating substances, Factor A (Khokhlov et al.1973) and an Autoregulator (Eritt et al.1982), contain a γ-lactone ring, they are effective at very low concentrations.

Substances with γ-lactone structure



They are considered to be positive regulators of the genes responsible for the AM-formation. It is possible that the Na-salicylate analogous to Factor A and Autoregulator stops their effects resulting in the lack of aerial mycelium formed. Another stimulating substance, Factor C, was obtained from *Strept.griseus* (Biró et al.1980) which does not show a γ-lactone-structure, as far as I know.

Furthermore, the effect of 10 amines was examined with regard to a stimulation of AM-formation and compensation of the inhibiting effect of Na-salicylate and ascorbic acid resp. Spermidine and spermine only showed a positive effect. These substances are formed from ornithine and arginine via putrescine. It seems to be possible that the uric acid metabolism plays a role in the AM-formation.

In another study 10 different species of *Streptomyces* were examined with regard to their content of sugars and amino acids in the cell wall and whole cell hydrolysates of aerial, substrate, and submerged mycelium (Neumeier 1984). At first a method had to be developed which allows a separate harvesting of aerial and substrate mycelium. In general, the content of the amino acids was quite high in the aerial mycelia, in particular that of glutamic and aspartic acids. This means that a great proportion of peptidoglycans occurs in the cell wall of the aerial mycelium. On the other hand, the sugar content was distinctly lower in the aerial mycelium in com-

parison with the substrate and submerged mycelium. The submerged mycelium takes an intermediate position between aerial and substrate mycelium; it is neither equal to aerial nor to substrate mycelium. The complete lack of galactose in the aerial and submerged mycelium was striking, it was present in the substrate mycelium in considerable amounts. Galactose is considered to be a suitable substrate for the formation of ascorbic acid. The absence of galactose also explains that of ascorbic acid, the importance of which was already mentioned.

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REGULATORY NUCLEOTIDES IN STREPTOMYCETE DIFFERENTIATION

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Nucleoside polyphosphates (or highly phosphorylated nucleotides, HPN) are mediators of pleiotropic response of cells to changes in their environment. Formation of HPN takes place after some metabolic stress, e.g. amino acid starvation. HPN synthesized under starvation conditions inhibit RNA synthesis; this is the well-known stringent control. The signal for HPN synthesis is the binding of deacylated tRNA to the acceptor site on the ribosome when an amino acid is absent. Other signals for HPN formation have been described: lack of a carbon source, temperature shift, salt shock, etc.

HPN participate in changes of transcriptional specificity of RNA polymerase and in the control of transcription of some operons as positive or negative effectors. Thus, some metabolic reactions are stopped, and some new syntheses start to effectuate new metabolic pathways.

The pleiotropic response of the stringent control mediated by HPN is reported mainly from E.coli. This type of HPN synthesis is the most extensively studied one. Besides, non-ribosomal synthesis has also been described. It takes place e.g. in relaxed mutants of E.coli or in Bacilli. This system does not require activation by ribosomes. The enzyme catalyzing the synthetic reaction, nucleotide pyrophosphotransferase (NPTase), transferring the pyrophosphate group from ATP to GDP or GTP to yield (p)ppGpp, is present in the high-speed supernatant fraction (Fehr and Richter 1981). The analogous enzyme of the ribosomal type synthesis, so-called stringent factor, is exclusively found in the ribosomal fraction.

We studied non-ribosomal HPN formation in streptomycetes. ppGpp was found in streptomycetes by Murao et al. (1974) who isolated and characterized NPTase from the streptomycete cultivation medium. Several authors observed the incidence of HPN in streptomycetes and made attempts to correlate the level of these effectors with metabolic processes in cells, especially with antibiotic production.

We have found relatively high levels of HPN in Streptomyces cells as well as in the medium. Testing for the activity of NPTase in various subcellular fractions we have not detected any in the ribosomal wash but we found it in the membrane

fraction (Fig.1). It means that the non-ribosomal type of HPN synthesis occurs in streptomycetes similarly like in Bacilli.

There are various ways of measurement of HPN levels but each brings complications in determining the absolute amounts of the nucleotides. Because of the low accuracy in estimation of the absolute HPN levels and impossibility to compare them in different media we have introduced estimation of the ratio of ATP or GTP to ppGpp. It seems to be a very important characteristic showing the physiological state of the culture: well growing cultures reveal a high ratio, cultures somehow inhibited (either by starvation or a metabolic block in mutants) are characterized by a low ATP:ppGpp ratio (Table 1; see also Fig.2).

Table 1. Ratio of nucleotides in different cell types of S.granaticolor

	vegetative cells		spores
	rich media	poor media	
ATP:ppGpp	9.39	1.83	2.11
GTP:ppGpp	3.24	1.16	0.57

Fig.1

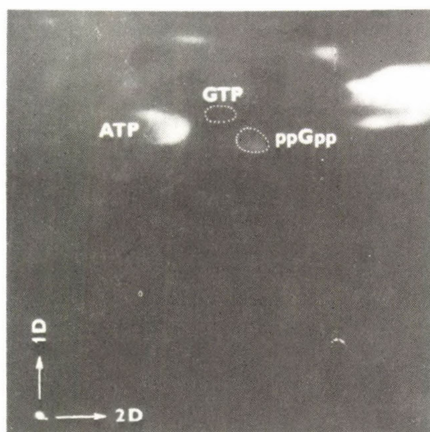

Fig.2

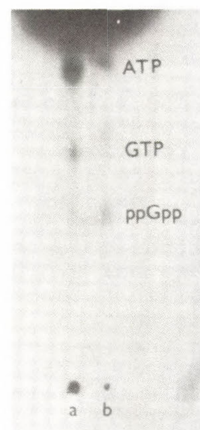

Fig.1. Activity of S.granaticolor NPTase from the membrane fraction. Two-dimensional chromatography on PEI-cellulose: first dimension (1D), 4.5 M ammonium formate/0.68 M ammonium borate (pH 7.0); second dimension (2D), 1.5 M KH_2PO_4 (pH 3.4).

Fig.2. Thin layer chromatography of nucleotides from vegetative cells of S.granaticolor. a, The parental strain; b, a mutant strain with a metabolic block. The separations in Figs.2 and 3 were done on PEI-cellulose in 1.5 M KH_2PO_4 (pH 3.4).

We have followed the level of nucleotides during submerged fragmentation preceding sporulation. The amount of GTP decreases quickly while that of ppGpp rises (Fig.3A). There-

fore, the ratio of these nucleotides changes very markedly. Similar pattern was found in the early sporulation phase of Bacilli when the content of GTP diminishes (Freese et al. 1979). Streptomycete aerial spores labeled with ^{32}P during sporulation possess some ppGpp. It is interesting that there is almost no detectable GTP in the spores (Fig.3B, lane c). During germination of the labeled spores in a medium with ^{32}P, GTP appears roughly after 1 hour, and the ATP:ppGpp ratio increases (Fig.3C).

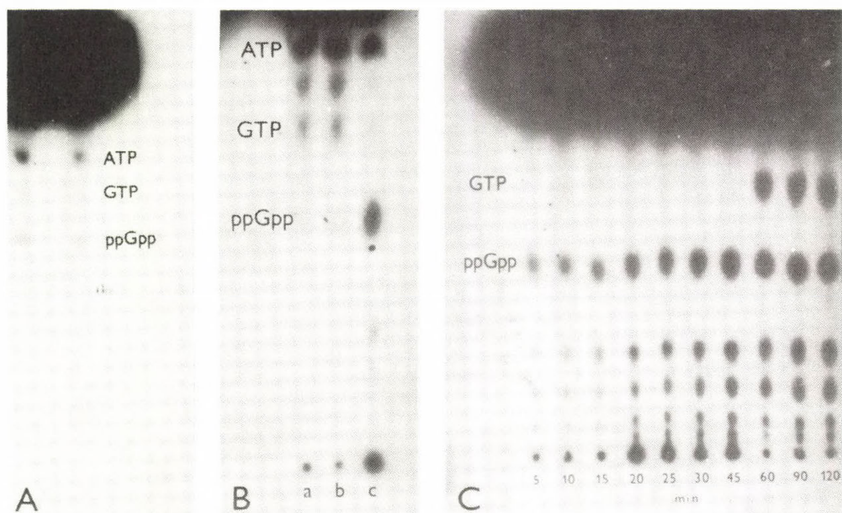

Fig.3. In vivo synthesis of nucleotides in S.granaticolor. (A) Submerged fragmentation. (B) a,b, Vegetative cells; c, dormant spores. (C) Time course of spore germination.

Our results suggest that HPN play a regulatory role in streptomycetes. However, more information is needed to elucidate their mode of action and involvement in cell differentiation.

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THE EFFECT OF TREHALOSE CONTENT ON STREPTOMYCES GRISEUS SPORES

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INTRODUCTION

Trehalose is an important storage compound of the actinomycetes [1]. This sugar is particularly abundant in actinomycete spores [2]. This paper describes the effect of intracellular trehalose content on a variety of properties of the spores of S. griseus.

METHODS

Organism and culture conditions. Streptomyces griseus NRRL - B-2682 was maintained as described previously [3] except that the medium used for obtaining spores consisted of 25 mM morpholinopropane sulfonic acid (MOPS) - 10 mM KH_2-K_2HPO_4 buffer (pH 7.0), 5.0 mM $(NH_4)_2SO_4$, 0.5 mM $MgSO_4$, 50 mM glucose, 0.5 g/L casein hydrolysate, 15 g/L Difco agar, and 2.5 ml of a concentrated trace salts solution per liter. The concentrated trace salts solution consisted of 27.2 mM $CaCl_2$, 7.19 mM $FeSO_4$, 11.8 mM $MnSO_4$, and 0.174 mM $ZnSO_4$ in 0.1N HCl. This medium (DM-1) was used for growth and sporulation of S. griseus in liquid (agar omitted) and solid culture. This medium was modified for particular experiments as described in the text. Cultures were incubated at 30°C. Spores were harvested from plates after 7 days of incubation.

The nitrogen-free medium used in experiments involving trehalose accumulation consisted of 50 mM glucose, 0.5 mM $MgSO_4$ and 10 mM KH_2-K_2HPO_4 (pH 7.0). MP buffer was identical to GMP buffer except that glucose was omitted. Spores were incubated at 33°C with shaking at 150 rpm to allow trehalose accumulation. The spores were harvested at various times by centrifugation, washed 3 times with 15 ml of distilled water and resuspended in distilled water.

Analytical methods. Trehalose was extracted from spores by boiling for 30 minutes. Trehalose was hydrolyzed to glucose by a partially purified trehalase obtained from a bacterium originally isolated from soil. The glucose produced was measured enzymatically by the glucose oxidase-peroxidase method. Total spore protein was determined by a modification of the method of Lowry [4].

Measurement of spore properties. Heat resistance was measured by injecting 0.1 ml of a spore suspension into 9.9 ml of heated 10 mM potassium phosphate buffer (pH 7.0). Samples were removed at various times, cooled rapidly, diluted in cold buffer and plated on complex media.

To measure desiccation resistance spore samples were lyophilized. The lyophils were incubated for 10 days in chambers containing various salt solutions to regulate the humidity. A saturated solution of $NaNO_2$

was used to maintain a relative humidity (RH) of 65%. To measure viability the spores were resuspended in 10 mM phosphate buffer containing 0.01 g/L of the detergent Triton-X100 by 2 minutes of gentle sonication in a sonic water bath. The samples were diluted and plated on complex media.

Germinability was measured by inoculating spores into a complex germination medium (CGM) at a final concentration of 3 x 10^8 spores per ml. CGM consisted of 5 g of Difco yeast extract and 1 g of casein hydrolysate in 1 liter of 10 mM potassium phosphate buffer (pH 7.0). The percentage of spores with germ tubes was determined after 3 hours of incubation on a shaker at 33°C.

Respiration was measured as the rate of O_2 consumption with a Clark electrode (Yellow Springs Instruments Co.).

RESULTS

Culture conditions were varied to determine their effect on trehalose accumulation. The level of trehalose in spores was dependent on the concentration of glucose in the medium. Spores formed on DM-1 medium containing 5 mM glucose had 44 μg trehalose per mg protein. Spores formed on DM-1 medium containing 100 mM glucose had 670 μg trehalose per mg protein. Spores formed under conditions of nitrogen or phosphorus limitation contained large amounts of trehalose.

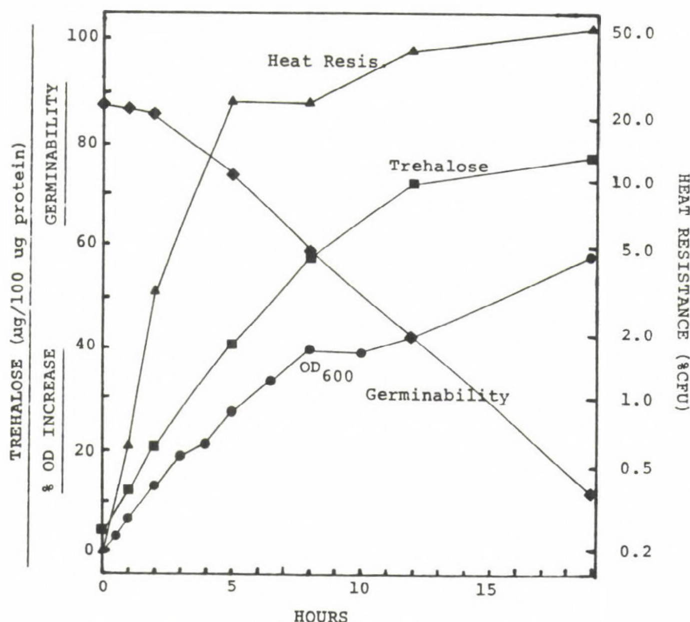

Fig. 1. Synthesis and accumulation of trehalose by spores
■ Trehalose (μg/100 μg protein)
● Percent increase in optical density (600 nm)
◆ Germinability (percent germ tube formation after 3 hours in CGM at 33°C)
▲ Heat resistance (percent CFU) after incubation at 60°C for 10 min)

Spores containing low levels of trehalose synthesized and accumulated trehalose during incubation in GMP buffer (Fig. 1). The spores did not germinate in this buffer. When we incubated spores for 8 hours in buffer containing ^{14}C-glucose, greater than 90% of the incorporated label was in

trehalose. As the trehalose content of the spores increased they became more refractile as observed with a phase contrast microscope and as measured by increased optical density. The spores containing large amounts of trehalose germinated more slowly and with less synchrony than spores containing low levels of trehalose. Spores became more resistant to heat as they accumulated trehalose. Spores containing 49 µg trehalose/mg protein had a decimal reduction time at 65°C (D_{65}) of 0.7 minutes. Spores which had increased their trehalose pools to 588 µg trehalose/mg protein during 12 hours of incubation in GMP buffer had a D_{65} of 3.0 minutes.

Spores with low or high trehalose content were resistant to lyophilization followed by incubation at relative humidities of 0, 32, 90, or 100 percent saturation. It has been previously demonstrated that spores are sensitive to incubation at 65% RH [5]. In preliminary experiments spores with low levels of trehalose were more sensitive to storage at 65% RH than were spores with high levels of trehalose. Spores had low endogenous respiration rates regardless of their trehalose content.

Table 1. Properties of S. griseus spores containing different levels of trehalose

Growth conditions (mM glucose)	µg trehalose/ mg protein	Heat resistance[c]	Desiccation resistance[d]	QO_2[e]	Germinability[f]
2.0	39.4	0.41	-	0.71	94
5.0	41.4	0.14	0.12	0.59	90
20.0	158.0	14.0	-	0.56	71
100.0	668.0	66.0	19.0	0.81	1.3
5.0[a]	46.6	0.12	-	0.59	91
5.0[b]	721.0	40.0	5.5	0.71	42

[a] Spores harvested from solid media, washed and incubated in MP buffer for 12 hr.
[b] Spores harvested, washed and incubated in MP buffer containing 50 mM glucose for 12 hr.
[c] % survival after 10 min at 60C.
[d] % survival after 10 d incubation at 65% relative humidity.
[e] µM O_2/mg protein/hr.
[f] Percent germ tubes after 3 hr incubation in complex medium.

The results summarized in Table 1 suggest that trehalose accumulation may be important in the formation or maintenance of the dormancy and resistance properties of the streptomycete spore.

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POSITIVE REGULATION OF ANTIBIOTIC PRODUCTION WITH CITRULLINE

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According to Vargha et al. (1983) and Ochi et al. (1984), it was suggested that citrulline, not arginine, involves in the induction of aerial mycelium formation or secondary metabolite production in actinomycetes. Recently, we found that a kasugamycin(KSM)-nonproducing strain derived from Streptomyces kasugaensis MB273 restores KSM productivity by addition of citrulline to a medium containing peptone, but such a effect was not detected in arginine. For the purpose of making clear the function of citrulline on KSM production, we investigated its function from biochemical and genetic points of view.

MATERIALS AND METHODS

S.kasugaensis MB273 and its mutant 18a-C2219 were used in the experiments. The 18a-C2219 is the strain purified by regeneration of protoplasts of strain 18a which was naturally isolated from MB273. C2219 has no plasmid detectable, and grows well in a minimal medium in the presence of citrulline, arginosuccinate or arginine but not in ornithine. This suggests that C2219 is deficient in ornithine carbamoyl-transferase activity. On the other hand, citrulline is the sole effector for KSM production in an agar medium containing peptone, but the production was not inhibited by addition of arginine in a strain MB273. C2219 is also lacking in the formation of aerial mycelium.
In order to detect a very small amount of KSM produced, super-sensitive test organism (designated M212) was prepared from Psendomonas fluorescens IMC B-0376. Minimal detectable amount of KSM in a disc assay was 3μg/ml in the M212 and the resistance to aureothricin which is produced together with KSM was more than 100μg/ml. S.kasugaensis was incubated for 7days at 27°C on the small agar cylinder punched from KSM-producing agar medium. Amount of KSM produced in the agar cylinder was assayed using the super-sensitive M202 as a test organism.

RESULTS AND DISCUSSION

KSM production and Citrulline Concentration

Effect of citrulline concentration on KSM productivity was tested using strain C2219. About 20mM citrulline showed the highest production indicating 70µg/ml and more concentration than 20mM inhibited the productivity. Growth of C2219 on the production medium was irrespective of the addition of citrulline.

Exploration of the Natural Products Indicating the Similar Function with Citrulline

Effect of possible metabolites derived from citrulline on KSM production was tested. Every substance tested was added at 5mM to the agar medium. Arginosuccinate showed slight effect but other metabolites, such as arginine, agmatine, r-guanidinobutyric acid, urea, spermidine, spermine, guanidinoacetic acid, creatine and creatine phophate did not show any effect.

Some microbial products are known to have activity as inducer of aerial mycelium formation or secondary matabolite production. The effect of these substances on KSM production was examined. Slight increase was detected in 0.5mM ppGpp and 0.1mM S-adenosylmethionine, but GTP, ATP, A-factor and vitamin B12 did not show any increase. The experimental results described above suggest that only citrulline has significant activity as the inducer or positive regulator for KSM production. Slight effect in arginosuccinate seems to be caused with citrulline formed by the reverse reaction of arginosuccinate synthetase. However, we don't know the reason why ppGpp or S-adenosylmethionine indicates slight activity.

Experiments using radioactive citrulline

We examined whether the carbon atoms of citrulline are incorporated into kSM molecule. Strain C2219 was incubated on the KSM-producing agar mediun containing C^{14}-labeled citrulline. After incubation, the juice of the agar medium was taken out, and both KSM and citrulline in the juice was isolated using Amberlite IR120. The sample, after concentration, was subjected to TLC to separate citrulline and KSM. The labeled citrulline used was C^{14}-ureido-citrulline and 5 carbon-labeled citrulline which was prepared from uniformly labeled ornithine using ornithine carbamoyltransferase.

The tested samples showed two ninhydrin positive spots indicating KSM but the spot corresponding citrulline was not detected. However, radioactive spot in the sample was found only in the site of citrulline but never in the site of KSM. These results indicate that the carbon of citrulline molecule are not incorporated into KSM molecule.

Clonig of Cit gene

Genetic studies were attempted to analyze the relation of KSM production and citrulline. The complemental gene of citrulline mutation in strain C2219 was cloned to C2219. Total DNA of MB273 was partially digested with BclI. Plasmid pSK21-B5 was digested with the same BclI and treated with alkaline phosphatase. Both DNAs were ligated and the ligation mixture was introduced into C2219 protoplasts. Initial transformants were detected by their thiostrepton resistance, and citrulline-nonrequiring colonies were selected on a minimal agar plate. Two transformants indicating Cit^+ were selected from 2000 initial transformants. The recombinant plasmids isolated from the transformants carried 8.0Kb DNA inserted which had 2 BamHI, 1 BglII and 2 SmaI sites. The plasmid made C2219 retransform to Cit^+ but faild to complement arginosuccinate synthetase-deficient strain of S.lividans I10. So, the plasmid seems not to carry arginosuccinate synthetase gene. Trimming experiments of the MC1 plasmid indicate that the Cit^+ gene and its promotor locate in 4.3Kb region consisting of BclI SmaI BglII sites.

KSM productivity of the retransformants carrying MC1 plasmid was tested. Most strain carrying MC1 showed KSM production of more than 40µg/ml. Some strains carrying pSK21-B5 produced small amount of KSM less than 20µg/ml, though 68% of the transformants did not produce it. The reason why B5 plasmid indicates slight increase of KSM is not known yet. It is apparent that cloning of Cit^+ gene as well as addition of citrulline make C2219 produce KSM. Moreover, Cit^+ revertant of C2219 also changed to the strain producing KSM. All of the experimental results apparently suggest that citrulline has a function as an inducer or positive regulator for KSM production. On the other hand, Ochi et al. reported that the metabolic turnover of citrulline is very rapid in the microbial cells he studied. Therefore, I think it may be important to make the citrulline accumulate in a cell.

*

We express our thanks to Dr. H. Ogawara for providing the S. lividans I10 strain.

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MORPHOLOGICAL DIFFERENTIATION OF ACTINOMYCES sp. 0234, A PRODUCER OF INSECTICIDAL AND ANTIBACTERIAL ANTIBIOTICS, IN SUBMERGED CULTURES

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In our search for new antibiotics useful for agricultural practice, we investigated also a strain described previously by Samoukina (1983) as Actinomyces (sensu Krasilnikov) sp. 0234. The strain produced two types of antibiotics (**A** and **B**) differing in their biological activity. The antibiotic **A** (a complex of water soluble, dialyzable components of low molecular mass) was active against Gram-positive bacteria. It was isolated from the fermentation broth filtrate by adsorption on Amberlite XAD-2 and elution with water-acetone 9:1; after purification by dialysis the components were separated on Sephadex G-15 in water. The antibiotic **B** (soluble in water or methanol) was inactive against microorganisms but exhibited an insecticidal effect as demonstrated previously by Samoukina (1983) in experiments with Colorado Potato Beetle (Leptinotarsa decemlineata). We assayed this activity by using caterpillars of Wax Moth (Galleria mellonella), a honeybee pest, from laboratory breeding on the artificial Haydak's medium. The caterpillars (at the 6th growth stage) were narcotized over dry ice and injected with aqueous or methanolic solutions of a crude antibiotic **B** preparation (methanolic extracts of lyophilized fermentation broth); every sample was applied in two concentrations using 10 caterpillars for each. Control caterpillars received the same amount (0.002 and 0.01 ml) of sterile apyrogenic water or methanol. The mobility, paralysis, and mortality of caterpillars were evaluated after 1, 24, and 48 hours.

Structural studies on the antibiotics are in progress. In this paper we summarize preliminary findings on the morphology of the producer in relation to its biosynthetic activity.

Solid cultures of the strain 0234 grown on ISP media at 28°C for 10 days (Fig. 1) exhibited streptomycete morphology features: aerial hyphae fell into the category "rectus-flexibilis", smooth spores occured in long chains of more than 10 (Fig. 2); according to the color of aerial mycelium mass, the strain belonged to the "yellow" series.

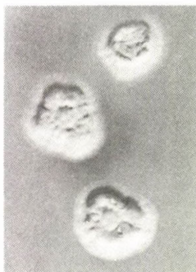

Fig. 1. Solid culture of Actinomyces sp. 0234. Yeast extract-malt extract agar, 28 °C, 10 days.

Fig. 2. Electron micrograph of spore chain of the strain 0234. Bar represents 1 µm.

Under shake flask conditions favourable for antibiotic biosynthesis (a complex medium containing starch, distiller's solubles and autolyzed yeasts; initial pH 7.0; high aeration; 28 °C), the following developmental stages were observed by optical microscopy of both stained and native preparations (Figs. 3,4): 1) formation of long, multiply branched hyphae; 2) formation of spore-like bodies [SLB] (oval-shaped, of larger size and thicker wall than aerial spores) on the surface of mycelium; 3) maturation of SLB resulting in an increase in their refractility; 4) disintegration of mycelium into long cylindrical fragments [LCF] and releasing of SLB into medium; 5) germination of SLB followed by new mycelial growth. The antibiotic **A** biosynthesis was closely associated with the growth of mycelium; it ceased at the stage 2 (i.e., after 24 h of cultivation) and was restored at the stage 5 (after 120 h). The antibiotic **B** was produced during the stage 4 (i.e., between 48 and 120 h). An enrichment of medium with soybean meal and molasses stimulated considerably the SLB formation but decreased the biosynthesis of both antibiotics. On the other hand, under conditions inhibiting antibiotic biosynthesis (poor media containing glucose or glycerol as carbon sources, reduced aeration, or 37 °C), a rapid fragmentation of mycelium to LCF (without SLB formation) occured (Fig. 5).

Ultraviolet-induced bald mutants of the strain 0234 (bearing no aerial mycelium and spores) retained the ability to form SLB under shaken conditions. In solid cultures of the strain 0234 (grown in a thin layer of agar medium) the SLB did not occur. The formation of SLB was not observed also in shaken cultures of 10 other strains (belonging to tetracycline, anthracycline, macrolide, or peptide antibiotic-producing streptomycetes) growing under the same conditions as the strain 0234.

Similarly to LCF, SLB were sensitive to lysozyme (25 mM Tris-HCl, pH 7.4, containing 0.45 M sucrose, 0.1 mM dithiothreitol and 1 mg of lysozyme [Fluka, 22400 units/mg] per ml, 30 °C, 90 min; after Erban et al., to be published) and to heat (60 °C,30 min). In contrast to LCF, they were resistant against ultrasonic oscillations (Labsonic 2000, 20 kHz, 5 min).

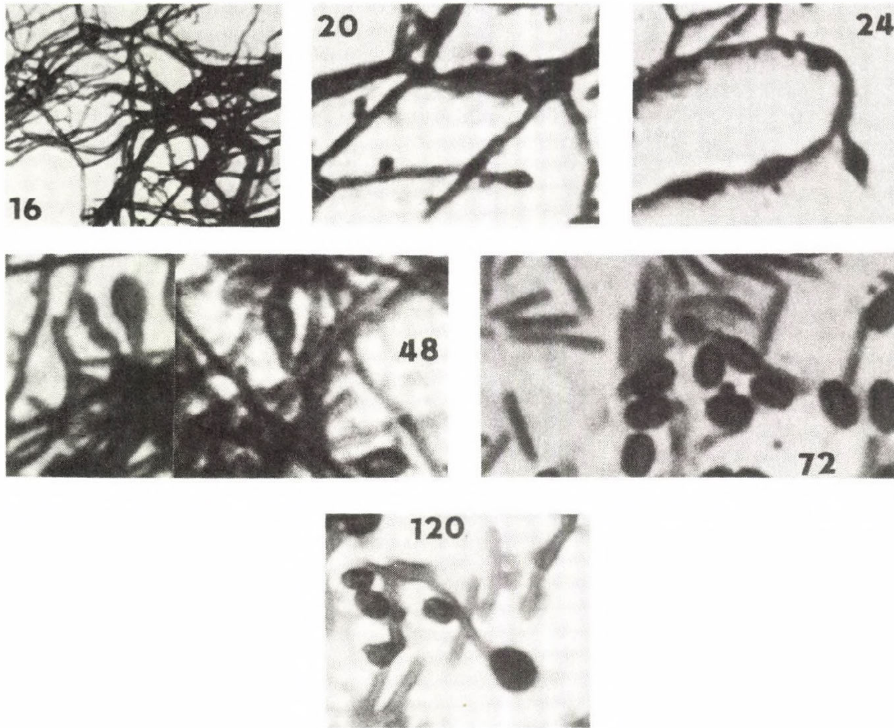

Fig. 3. Morphological differentiation in submerged cultures of the strain Actinomyces sp. 0234. Medium: starch, distiller's solubles, autolyzed yeast, pH 7.0; a rotary shaker (3.67 Hz). Preparations were stained with carbol fuchsin. Numbers refer to incubation time (h). **16-48**, development of spore-like bodies [SLB]; **72**, fragmentation of hyphae, release of SLB; **120**, SLB germination. The sterility of cultures (absence of contaminations with bacilli) was confirmed by cultivation tests.

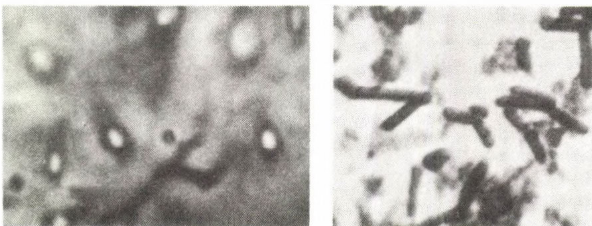

Fig. 4. Phase-contrast micrograph of SLB in a native culture of the strain 0234 (72 h).

Fig. 5. A 24 h culture of the strain 0234 grown at 37 °C (suppressing SLB formation). For other conditions see Fig. 3.

To explain the phenomena observed, more detailed studies are needed, aimed not only at clarifying the taxonomical position of the strain 0234 but also at elucidation of biochemical mechanisms that participate in the environmental control of the SLB formation and in the observed relations between SLB and antibiotic biosynthesis in this organism.

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DIFFERENTIATION

Poster Abstracts

RECONSTITUTION BY THE FACTOR A OF THE PARENTAL PROTEIN PATTERN IN AN ASPOROGENOUS MUTANT OF STREPTOMYCES GRISEUS

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The asporogenous anthracycline-negative mutant 86 ($Amy^{-}Ant^{-}$) of *S. griseus* was distinguishable from the parental strain JA 5142 ($Amy^{+}Ant^{+}$) by an altered composition of the mycelial proteins as shown by SDS polyacrylamide gel electrophoresis. Addition of the autoregulatory factor A or similar molecules to submerged cultures caused the adaptation of protein pattern to the parental picture concomitant with the restoration of anthracycline biosynthesis and morphological changes. In addition to the many other effects of the factor A on the metabolic flux, the observed changes could be caused by alterations at the level of the intramycelial proteolysis. But during growth of mutant 86 in the presence of factor A [2-(6'-methylheptanoyl)-3-hydroxymethyl-4-butanolide] some of the intramycelial proteins remained unchanged supporting the view that a pleiotropic regulatory gene could be involved in the synthesis of this endogenous effector by the parental strain *S. griseus* JA 5142.

THE METABOLISM OF ENDOGENOUS TREHALOSE BY SPORES OF STREPTOMYCES GRISEUS

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The disaccharide trehalose is accumulated as a storage product during sporulation of S. griseus NRRL B-2682. Dormant spores utilize their trehalose reserves slowly during extended incubation under starvation conditions. In contrast the spores rapidly deplete their trehalose pools during the first hours of germination. Extracts of dormant spores contain a high specific activity of the enzyme trehalase. The level of trehalase activity remains relatively constant throughout germination. Compartmentalization of trehalose and trehalase appears to be involved in the regulation of trehalose utilization by S. griseus spores. Two lines of evidence suggest a cytoplasmic localization of trehalase: (1) The trehalase activity of intact spores is resistant to acid treatment; (2) a high specific activity of trehalase is found in isolated protoplasts of S. griseus spores. In contrast trehalose is not released by a short acid treatment but is released into the buffer during protoplast formation.

THE EFFECT OF LOCAL ANESTHETICS ON THE INITIATION OF GERMINATION OF STREPTOMYCES VIRIDOCHROMOGENES AND MICROMONOSPORA ECHINOSPORA SPORES

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Calcium ions are strictly required for the initiation of germination of S. viridochromogenes spores. The requirement for Ca is not as stringent for M. echinospora spore germination. Each of the divalent cations Mg, Sr, and Mn will replace Ca in the germination medium, but none are as effective as Ca in stimulating germination. A Ca-ATPase inhibitor released during the germination of S. viridochromogenes spores is a potent inhibitor of the germination process in both organisms. Germination of S. viridochromogenes and M. echinospora spores is inhibited by the local anesthetics tetracaine and dibucaine. These compounds inhibit ATPase activity and active Ca transport, and cause dissipation of proton gradients in some organisms. Concentrations of 300 μM dibucaine and 500 μM tetracaine completely inhibit germination of unactivated and heat activated spores. The anesthetics interrupt germination (as measured by OD decrease) when added at any time during germination. The anesthetics are not removed from spores by washing. However, the inhibitory effect is reversed by adding 6.7 mM Ca. Current studies are underway to determine the effect of the anesthetics on other germination processes such as respiratory activity, spore carbon release and Ca transport.

THE RELEASE OF SPORE CARBON DURING THE INITIATION OF GERMINATION OF STREPTOMYCES VIRIDOCHROMOGENES AND MICROMONOSPORA ECHINOSPORA SPORES

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Sporulation of S. viridochromogenes and M. echinospora appear to be very different processes. Yet, the events accompanying germination of spores of both organisms are similar. In both cases, germination is accompanied by a decrease in optical density of spore suspensions and loss of spore refractility. The respiration rate of spores of both organisms is low and increases during germination. Each release approximately 25% of the total spore carbon during germination. The composition of the material released was analyzed. In each case the material is composed primarily of hexose sugars, peptides and amino acids. Analysis of the amino acids reveals that S. viridochromogenes spores release low levels of a variety of amino compounds with glutamic acid present at more than ten times the concentration of any other constitutent. The M. echinospora spores release a low level of a limited number of amino acids, but did release a large amount of γ-amino butyric acid, a decarboxylation product of glutamic acid. The boiling water extractable pools of dormant and germinating spores of both organisms contain low levels of free amino acids and glutamate-rich peptides. The pools also contain a high level of hexose sugars, primarily trehalose.

COMPARATIVE LIPID ANALYSIS AND FREEZE-FRACTURE MICROSCOPY OF CYTOPLASMIC MEMBRANES FROM STREPTOMYCES HYGROSCOPICUS AND ITS STABLE L-FORM

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Comparative investigations of the cytoplasmic membrane isolated from stable L-form cells and freshly prepared lysozyme protoplasts revealed characteristic differences in the lipid moiety. L-form membranes showed higher contents of extractable lipids, phospholipids, phosphatidylethanolamine, ornithinolipid and squalene. They did not contain C 18:2 fatty acids, and they have a changed ratio of C 15 and C 17 isomers. Furthermore, characteristic structural changes were obtained by freeze-fracture electron microscopy. Components of the phospholipid are responsible for these alterations in the membrane structure.

HETEROGENEITY OF STREPTOMYCETE POPULATIONS

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The stabilization of antibiotic biosynthesis depends on the population resistance of producers. Heterogeneity of the populations is easily detected on special media when their growth is prolonged to 2-4 weeks. The heterogeneity is uniformly detected in cultures belonging to different species and is expressed as a regulatory formation of secondary colonies. Cultures from secondary colonies have a simplified cycle of growth (they do not form the aerial mycelium and spores) and metabolic peculiarities (changed antibiotic and enzymatic activity). The secondary growth is recommended to be used for selection studies and while working out methods for the limitation of the heterogeneity.

CHARACTERIZATION OF *FRANKIA* VESICLES

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Frankia isolates fix N_2 when incubated aerobically in defined growth medium. Appearance of N_2 fixation following transfer of *Frankia* from a NH_4 to a N_2 medium coincides with formation of a specialized cell structure, the vesicle. Evidence suggests that the function of the vesicle is to protect the nitrogen fixation system from inactivation by O_2. In order to determine the role of vesicles during nitrogen fixation, we developed procedures for their isolation from strain EAN1. Vesicles are purified by passing a culture through a french pressure cell under an argon atmosphere. This completely disrupts the mycelia while vesicles remain intact. Vesicles are separated and purified by differential centrifugation or by isopynic gradient centrifugation in Renografin. The purified vesicles require a low potential reductant and an ATP regenerating system for acetylene reduction under anaerobic conditions. They do not reduce acetylene when incubated in air. The vesicles do reduce acetylene when incubated at reduced O_2 partial pressures using various carbon compounds as sources of energy and reductant.

Ca^{+2} transport blocking agents and the Ca^{+2} chelator EGTA inhibit acetylene reducing activity. This suggests that energy for N_2 fixation may be coupled to Ca^{+2} transport.

EFFECT OF NUTRIENT LIMITATION ON SPORULATION OF *STREPTOMYCES GRISEUS*

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Streptomcyces griseus MRRL B-2682 spores were highly resistant to a short acid treatment. We used this property to selectively quantitate spores, and thus monitor sporulation during growth in liquid culture under various conditions. The onset of sporulation was marked by an exponential increase in numbers of acid-resistant units while total colony-forming units increased only slightly. In glucose-NH_4-salts medium, the time of sporulation remained constant at about 23-25 hours of incubation. Sporulation onset time was not significantly altered by varying inoculum size 25-fold, or independently varying [PO_4] from 0.1 to 50 mM, [glucose] from 5-150 mM, or [NH_4] from 0.5-20 mM. Repeatedly transferring either liquid- or solid-grown cultures to fresh medium during the first 20 hours of incubation had no significant effect on the time of sporulation. These data suggest that sporulation of *S. griseus* is not initiated by nutrient limitation, as has generally been assumed.

When *S. griseus* cultures were exposed to conditions of nutrient limitation or excess by a variety of methods, there was no significant change in the time at which they began to sporulate. This implies that sporulation was not initiated in response to a change in external nutrient concentration. A similar observation has been made for conidiation of the fungus *Aspergillus nidulans*. This organism is "internally programmed" to reach developmental competence at a certain age, regardless of the concentration of limiting nutrients in the culture medium. We are exploring the possibility that *S. griseus* development may show similar features.

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