PROTOCOL OF THE "ROUND TABLE CONFERENCE
ON STREPTOMYCES" ON THE OCCASION OF THE
7TH INTERNATIONAL CONGRESS OF
MICROBIOLOGY, STOCKHOLM,
AUGUST 4 and 5, 1958

List of Participants

1. Prof. Dr. E. Baldacci, Milano, Italy. 2. Prof. Dr. R.E. Buchanan, Ames, Iowa, USA. 3. Prof. Dr. R. Ciferri, Pavia, Italy. 4. Dr. R. Despois, Vitry-sur-Seine, France. 5. Prof. Dr. L. Ettlinger, Zürich, Switzerland. 6. Dr. W. Frommer, Wuppertal-Elberfeld, Germany. 7. Prof. Dr. G. Giolitti, Milano, Italy. 8. Prof. Dr. D. Gottlieb, Urbana, Illinois, USA (Chairman). 9. Dr. E. Grossbard, Haidenhead, Berkshire, Great Britain. 10. Prof. Dr. J. Horvath, Gödollo, Hungary. 11. Dr. R. Hütter, Zürich, Switzerland. 12. Dr. E. Katz, New Brunswick, New Jersey, USA. 13. Prof. Dr. N. A. Krasilnikov, Moscow, USSR. 14. Dr. E. Küster, Braunschweig, Germany (Convener and Secretary). 15. Dr. H. J. Kutzner, Braunschweig, Germany. 16. Prof. Dr. J. Malek, Praha, Czechoslovakia. 17. Dr. N. Pfennig, Göttingen, Germany. 18. Dr. S. Pinnert, Vitry-sur-Seine, France. 19. Dr. A. Sanchez-Marroquin, Mexico City, Mexico. 20. Dr. I. Scotti, Milano, Italy. 21. Dr. L. Silvestri, Milano, Italy. 22. Dr. P. Sneath, London, Great Britain. 23. Prof. Dr. Z. Tešić, Beograd, Yugoslavia. 24. Dr. V. Treccani, Milano, Italy. 25. Dr. G. A. de Vries, Baarn, Netherlands. 26. Dr. K.H. Wallhäusser, Ffm.-Höchst, Germany. 27. Prof. Dr. M. Welsch, Liège, Belgium. 28. Dr. L. Zeller-Celso, Milano, Italy.

The session was opened on August 4, at 2:15 p.m. by the chairman of the conference, Prof. Dr. D. Gottlieb. A short introductory report was made by the secretary (Küster) on the present status of the classification of streptomycetes (See Appendix 1). The individual participants then expressed their basic concepts of this taxonomic problem. Most of the contributions are in manuscript form. Some were already distributed with the letter of the 13th of June or during the proceedings.

Appended to this Protocol are copies of the papers presented (Küster, Hütter, Baldacci, Tešić, Krasilnikov, Welsch, Wallhäusser, and Kutzner).

Horvath referred to the great variability among streptomycetes. This he attributed at least partially to heterokaryosis. This phenomenon consists in the presence of various nuclei of diverse origin in a common mycelium. In the formation of spores, each conidium receives one only of the various nuclei, so that the colonies derived from these conidia exhibit properties differing among themselves as well as from the parent culture.

Frommer observed that a certain characteristic may be constant within one group but variable in another; this results in an inability consistently to classify a given characteristic as strictly constant or variable. Furthermore, he suggested the examination of the color of the substratum mycelia and of the soluble pigments he supplemented by use of simple chemical tests (solubility in different solvents, color change with change in pH, etc.).

Pfennig considered it important from the standpoint of ecological investigations to use a classification based on morphology. After all, the goal of such studies is ability to classify very many cultures on the basis of simply determined characteristics and to place them rapidly in the larger groups.

Despois noted as a serious defect that, in the newer systematic work, essentially only organisms capable of producing antibiotics are considered. The greater problem in systematic work with the streptomycetes is, in his opinion, careful definition of species. He sees further difficulties due to the absence of designated type cultures of species described earlier; this lack makes almost impossible the identification of new isolations with them. Designation of neotypes and their adequate description would eliminate much of this deficiency. His further observations concerned the standardization of experimental conditions and the description of the characteristics. The morphology and the color of the aerial mycelium he considers the most important properties for characterization.

Krassilnikov supplemented his previously submitted and distributed comments, which may be summarized as follows: He views the definition of the species as the greatest problem. As a result of the broadening of our knowledge, species which were once considered uniform are now divided into several species. For example, the old species Actinomyces globisporus becomes as a result a group of 10 species which

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differ from one another by the formation of different antibiotics, for example:

<u>Actinomyces streptomicini</u>	=	<u>Streptomyces griseus</u> Waksman : streptomycin
<u>A. levoris</u>	:	levorin
<u>A. toxicus</u>	:	strongly toxic antibiotic with a toxicity that is 10 times greater than that of KCN
<u>A. bacillaris</u>	:	

In this group the differentiation of the species is based primarily on the production of different antibiotics.

Similar groups are represented by Actinomyces albus (now divided into 12 to 13 species) and A. coelicolor characterized by the formation of several pigments and again divided into three subordinate groups:

a) 6 species: spiral	[	blue anthocyanin pigment
b) 2 species: straight		pH dependent
c)		blue pigment, not pH dependent

In all Krassilnikov differentiates ten species-groups.

Reference was made to the phenomenon that the organisms which produce antibiotics are not usually influenced by their own products. It is thereby often possible to identify producers of antibiotics by testing one against the other. This rule, however, is not valid in every case because there are some species which inhibit themselves. Krassilnikov also considers important the customary morphological criteria for the characterization of the species.

Gottlieb reported on a meeting of the "Committee on Taxonomy of the Actinomycetes" of the Society of American Bacteriologists. During the meeting, which took place in May, 1958, problems similar to these being considered here were discussed. At 11 different institutes in the United States, characteristics suitable for use in a classification are to be worked out in a common study under specified experimental conditions. If the results of the test of a character are in agreement at all the institutes participating, this specific character will be considered suitable for characterization.

For the further discussion the separate items of the proposed agenda were treated in the order suggested by Gottlieb and with special consideration of the points A₂, B₂, and C.

On A₂: Difficulty of reproducing some media. Standardization difficulties are often encountered in the production of complex culture media. This seems, however, to be less applicable to culture media whose components have not experienced industrial reconditioning. This was shown to be true in the use of potato or oatmeal agar in various laboratories, as well as by use of potatoes of various types and origins (Baldacci, Küster). Media whose names indicate a similar composition, but whose components have experienced dissimilar treatment can naturally not be compared with one another. For example, several laboratories reported that Difco-potato-agar, compared with potato-agar, is (according to Baldacci) a poor substratum for streptomycetes. Wallhäusser stated that in the preparation of synthetic media the source of the chemical ingredients must be considered because of the variable proportions of trace elements and similar substances. According to Scotti, the age of a medium at the time of inoculation may also exercise a certain influence on the development of cultures. In choosing the media for streptomycetes, one must differentiate between substrata for the maintenance of cultures and those used exclusively for characterization. Media for maintenance must guarantee, above all, satisfactory development of aerial mycelia (Ettlinger).

On B₂: Discussion of criteria.

1. Morphological characteristics

a) Colony: Consideration of the general morphology of the colony is desirable in characterization. For strains that do not form aerial mycelia on any medium, the nature of the substrata mycelium should be appraised.

b) Mycelia: As a result of a preliminary discussion as to the designation of the several kinds of mycelia; the following definitions were agreed upon:

Substratum mycelium: the totality of the hyphae growing in the agar or solution

Aerial mycelium: the totality of the mycelium parts which rise above the surface of the substratum

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Vegetative mycelium: the totality of the mycelium not dividing into spores (substratum mycelium + sterile parts of the aerial mycelium)

Sporophores: the parts of the mycelium dividing into spores

In general the morphology of the substratum mycelium does not play an important part in the description of the strain.

c) Sporophore structure: There was complete agreement on the importance of the description of the morphology of the aerial mycelium. The division into seven morphological types, which was recently (1958) suggested by T. G. Pridham and associates, was discussed and recommended for general application. Baldacci suggested the following minor changes, among others:

	<u>Pridham</u>	<u>Baldacci</u>
<u>Sectio rectus flexibilis</u>	straight flexuous fascicled	straight
<u>Sectio retinaculum-apertum</u>	primitive spirals open loops	flexuous open loops
<u>Sectio spira</u>	open spirals closed spirals	spirals

Kutzner showed some photomicrographs and discussed the way in which he described the aerial mycelium. Special emphasis was laid upon the arrangement of the spirals on the aerial mycelium, as well as their form and the number of coils. The constancy of the direction of rotation (sinistro, dextro) which he advocated was not approved by all and was therefore considered to be unessential for characterization. It was suggested that the description of the aerial mycelium be supplemented by a photograph. Uniformity of spiral morphology accompanied homogeneous development of aerial mycelia on various media, but only when these media favor the formation of aerial mycelia: for example, Frommer found the same aerial mycelium morphology on five different culture media.

d) Method of spore formation: A differentiation is made between segmentation and fragmentation as methods of spore formation. Within the genus Streptomyces fragmentation is generally assumed, while segmentation is typical for Nocardia. According to Krassilnikov, some Streptomyces do, however, show segmentation; in this respect, they are similar to Nocardiae. The method of spore formation should be used more for a differentiation of these genera than for species determination.

e) Spore morphology: The observation of the spores by use of a light or a phase-contrast microscope (Giolitti, Ciferri, Küster) allows too limited a recognition of morphological differences that might be used as reference criteria in the identification of species. Unquestionable morphological differences became apparent for the first time with the use of the electron microscope. The fine structure of the spore surface which is recognizable here (smooth, spinous, hairy) is viewed as a very constant characteristic and therefore as an important property for characterization (Hütter, Baldacci). As in the judgment of the morphology of the aerial mycelium, a culture medium should be used for this criterion which guarantees a good formation of aerial mycelia and spores, for, on an unsuitable medium the characteristic appendages, which are otherwise apparent, may be absent (Küster). The preparation should be made by the print-process, as the spores may lose their appendages by suspension (Küster).

A description of the spores as shown by the electronic microscope is considered essential for an adequate species description. The use of this characteristic in a key for determination, as Ettlinger and Hütter suggested, is probably justified, but the use of such characters in a key makes difficulties for the student, however, because electron microscopes are not available everywhere. This deficiency may be overcome by an exchange of strains.

2. Color characteristics

All three components of a streptomycete culture, aerial mycelium, substratum mycelium and medium (substratum), may develop colors which can be used in characterization.

a) Color of the aerial mycelium: The color of the aerial mycelium was defined as the color of the mature aerial mycelium on a culture medium which is optimal for

the production of aerial mycelium and of spores. This color is generally regarded as constant and its description essential for characterization. Baldacci suggested that in addition to a description of the mature stage, there be evaluation of intermediate stages. On the basis of an observation on the color change of the aerial mycelium of Streptomyces griseus subjected to iodine fumes, de Vries pointed to the possibility that the color of the aerial mycelium may be characterized by chemical reactions.

b) Color of the substratum mycelium: This color evaluation was considered less important for characterization, since it is more variable than the color of the aerial mycelium and, in most cases, is not characteristic. For those strains that show a characteristic substratum-mycelium-color, e.g., red, blue, violet, this color should be recorded. In such cases, the dependency of the coloring on the composition and the pH value of the medium should be especially noted (Frommer, Giolitti).

c) Color of the medium: The color of the medium, also commonly designated as soluble pigment, is with a few exceptions, not important, since it is either uncharacteristic or, when strikingly colored, it has the same color as the underside of the substratum mycelium, though less intense. Moreover, the color of the medium is observed to be more variable than that of the substratum mycelium. After several transfers, the pigmentation of the medium may frequently be lost, but may be regenerated by a single passage through soil (Wallhäusser). It must be expressly pointed out that the coloring of the medium, here under discussion, does not include the melanoid pigmentation of culture media containing peptone. In the discussion on color characteristics, it was repeatedly expressed that a distinction must be made between "pigment" and "color." Krassilnikov defined these terms as follows:

Pigment = the chemically uniform metabolic product which can be extracted and is not liable to change by external factors, e.g., 3 types of pigment in Streptomyces violaceus: an anthocyanin, a carotinoid and a lipochrome.

Color = subject to influence by external factors in the culture (trace elements, pH value etc.)

The color of the mature aerial mycelium (spores en masse) in all cases should definitely be described. In some cases, the color of the substratum mycelium or, occasionally, the color of the medium may be noted; for these the specific medium, pH changes, and similar data must be described.

3. Growth response to temperature

Most of the streptomycetes are mesophilic and have very similar temperature requirements, only a few deviate and are thermophilic. Only in the cases in which the optimal temperature lies substantially above that of the mesophilic species should the temperature requirement be used as a characteristic in the differentiation of the species.

4. Metabolic characteristics

a) Hydrogen sulfide production: testing for the formation of H_2S was not considered necessary.

b) Reduction of nitrates: Only limited experience with this test was available, but as far as the test applied, it represented a good characteristic. It is recommended.

c) Gelatin liquefaction: Differences are noted solely in the rate of the liquefaction. This test is applicable in the description, when the length of time for liquefaction to occur is recorded.

d) Milk peptonization: In light of the experience of several participants, this test seems to be strongly dependent on the external conditions of the experiment. For example, de Vries observed a distinct influence of the temperature and length of time of sterilization, as well as of the origin of the milk on decrease in peptonization. This result may also depend on the size of the inoculum (Hütter). A further difficulty is present in the variability in the description of the results: for example, in one case only a thick flocculation may be designated as coagulation, while in another case a "more bilious" condition is also designated as coagulation (de Vries). Because of the difficulties in carrying out such a test with fluid milk, de Vries and Wallhäusser suggested the use of milk agar or, under some circumstances, casein agar. Ciferri called attention to a method of Gorini by which the old agar cultures are covered with a layer of milk; in comparison to the customary milk test in tubes, coagulation is observed much more frequently.

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Despite the difficulties indicated, the milk test is given a certain value, although its application presupposes an exact adherence to standardized experimental conditions. All participants who occupy themselves especially with this test are asked to communicate their experiences to Prof. Gottlieb.

e) Carbohydrate utilization: The lively discussion on this test can be summarized as follows:

1. This test is viewed by many participants as useful (Wallhäusser, Sanchez-Marroquin, Pfennig, Pinnert, de Vries, among others) but it must be limited to a few carbohydrates, since many do not yield results which are reproducible (Pfennig, Sanchez-Marroquin). When others are tested they reveal few differences among most of the streptomycetes.

2. Hütter and Ciferri, on the basis of their studies, reject this test completely; Hütter, for example, found that single spore cultures of a single strain may possess different C-source spectra. The observations of Pinnert are somewhat similar. She found that, following a reaction generally regarded as negative, individual positive colonies appear which she designates as mutants. Difficulties in the exploitation of this test are seen by Giolitti in the fact that some strains develop on the basal medium without the addition of carbohydrates.

3. It was noted concerning the methodology of this test that it should be carried out either on agar or in a fluid medium in a submerged culture. Cultures floating on solutions lead to unsatisfactory results (Küster). Wallhäusser utilized successfully the submerged culture; he found that the amount of mycelium can be determined quantitatively after centrifugation.

Despite the critical comments, the use of the C-test is recommended for the characterization of a strain. Pinnert is asked to send to Küster an exact description of the method of this test which she used. Likewise, all participants are asked to communicate their experiences with this test, particularly with reference to the selection of C-sources, to Küster.

f) Nitrogen utilization: As in the test for C-source, the utilization of various N-sources by strains was evaluated very differently by the participants. Pinnert reached good results with such tests, while Ciferri considers the N-source spectrum uncharacteristic. Too little experience is available to arrive at a final judgment.

g) Production of antibiotics: The formation of antibiotics is rejected as a criterion for the characterization of a species for the following reasons:

1. This property is not considered constant, because a loss of antibiotic formation can frequently be established under laboratory conditions.

2. There are numerous species of streptomycetes which contain inactive strains, as well as strains that produce antibiotics.

3. In several cases more than one antibiotic was formed by one strain.

4. The same antibiotic can be formed by different species. Kutzner established that, in many cases, strains which were very similar to their morphological and cultural characteristics on different culture media also exhibited a corresponding antibiotic spectrum with five different test organisms. On the basis of this observation, he comes to the conclusion that reference can be made to the antibiotic spectrum for classification of subgroups. The question remains unanswered, however, to what extent the spectrum can be brought about by the same antibiotics or, as the case may be, by the same mixture of antibiotics. It once more turns out that the formation of antibiotics or, more exactly, the antibiotic spectrum, is useful only for a description of a strain and not for the definition of a species.

h) Sensitivity to antibiotics: Only very limited experience with this test is available. Krassilnikov uses it for diagnostic purposes.

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5. Serology

Those present had had no experience in this method of investigation.

6. Phage Specificity

Kutzner reported on his investigation with five different phages in his group I (corresponding approximately to the griseus group). Two phages proved to be quite specific, that is: only a few sub-groups of streptomycetes are sensitive; the remaining three, among which was the phage specific for Streptomyces griseus, had a very wide spectrum. This differing specificity of the actinophages must be considered in evaluating their usefulness in diagnosis of streptomycetes. Baldacci has worked with phages that were specific only for the griseus group but for none of his other series.

On C: Speciation

Baldacci presented in the following diagram his conception of the characteristics which he suggests for a species and infrasubspecific division:

Species	sporophore structure color vegetative mycelium color aerial mycelium spore morphology (electron microscope)
Infraspecific	metabolic characteristics soluble pigments antibiotics (?)

Sneath explained his ideas on speciation and called attention to his account already published (Jour. Gen. Microbiol. 17: 184, 201, 1957). Gottlieb suggested that the various laboratories exchange strains in order to establish through use of uniform record cards the stable criteria suitable for characterization. A plan was developed to carry out a common experiment somewhat similar to the study being made by a taxonomic committee of the Society of American Bacteriologists. Participating in this experiment will be 14 individuals using the same strains (from Baldacci's collection) under standardized experimental conditions, including observation and judgment as to the morphology of the aerial mycelium

and spores and the color of the aerial and substratum mycelia as well as the intensity of the melanoid pigment. In this way, the reproducibility of these characteristics should be co-ordinated. The specific arrangement for the experiment will be worked out and forwarded by Küster, who will then assume the task of the final evaluation after conclusion of the experiment.

Earlier in the session Buchanan had suggested the possibility that it might be desirable and advantageous for the conference to seek recognition as a continuing taxonomic subcommittee of the International Committee on Bacteriological Nomenclature under the auspices of the International Association of Microbiological Societies. The suggestion met with the approval of all participants. It was formally resolved to form a "Subcommittee on Streptomycetes" within the International Committee on Bacteriological Nomenclature. Gottlieb was elected chairman, and Küster secretary of the Subcommittee. After 3 half-day sessions, Gottlieb closed the meeting on August 8, 1958, at 6 p.m. with a few words.

Dr. E. Küster
Secretary