RULES FOR THE CLASSIFICATION OF ANTIBIOTIC-PRODUCING ACTINOMYCETES

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One of the most important tasks in our present day science of antibiotics is the establishment of a completely rational taxonomy and nomenclature for antibiotic-producing organisms. A purposeful search for antibiotic substances is possible only with good knowledge of the species which produce them.

A great many antibiotic-producing actinomycetes have been described recently. Investigators often give new species names without a strict taxonomic analysis, ignoring the existing rules of nomenclature and classification, and without adequate analysis and appropriate comparison of a culture with previously described ones. One important deficiency of many publications in microbial taxonomy is the extremely poor description of cultures. Many authors often confine themselves to two or three insignificant phrases or do not give any information on the antibiotic-producing abilities of species described. Comparison of described species is complicated by the fact that published investigations characterize species under unlike conditions. Some investigators use only rich organic media, others use simple defined media with an inorganic source of nitrogen, etc. Great confusion is created as a result of such neglect of classification rules; it becomes more and more difficult to evaluate published material and often it is even impossible to make use of it.

Therefore frequently different species of organisms are described under the same name, or the same species designation is given to cultures belonging to different species. We have encountered such inhomogeneity and species mixtures in the study of strains received from different laboratories in America, England, Japan, and other countries under the name of Actinomyces griseus, Actinomyces lavendulae, Actinomyces fradiae, Actinomyces erythreus, Actinomyces olivaceus, and Actinomyces globisporus. Among 14 strains designated as A. lavendulae, only 6 actually belong to that species; the remaining 8 strains belong to 3 other independent species. We detected 4 distinct species among 9 strains named as A. olivaceus. Similar data were obtained in the study of strains of the other mentioned species.

Such extreme confusion in the nomenclature of actinomycetes is caused to a great extent by the complexity of species determination and establishment. The understanding of species and of methods for their determination appear to be the basic conditions in systematics. If the first condition represents a purely biological problem, the second must respond to practical aims.

How is a species defined, which principles and characteristics should be considered fundamental in distinguishing and subdividing species, what technical methods should be applied in revealing leading species criteria? Each investigator solves the proposed questions in his own way, in conformity with his opinion and preference, his specialty, working conditions, etc. It is quite natural that under such circumstances data accumulate which are difficult to compare. It is essential that different countries cooperate in the elaboration of unified rules for classification and nomenclature obligatory for all workers in the given field.

Different authors use various criteria for determination of actinomycete species. Some construct their classification on physiological and cultural characteristics (Waksman and Lechevalier, 1953; 1957), others on coloring of cultures—i.e., of their colonies, aerial hyphae and medium (Baldaic et al., 1953; 1954). Some investigators subdivide actinomycetes according to their ability to assimilate certain carbon sources (Kurosawa, 1948; Benedict et al. 1955, Zähner and Ettlinger, 1957) or nitrogen sources (Okami, 1952; Burkholder et al., 1954). Further, the
specificity of actinophages, serum reactions, and other peculiarities of the microbial cell are proposed for the establishment of species. There is also an attempt to differentiate species according to the chemical composition of cell walls (Cummins and Harris, 1958), etc.

It is needless to prove that it is impossible to establish species by means of a single characteristic, whatever it might be. Only the sum of characteristics—morphological, cultural, physiological, biochemical, etc.—can be used to characterize and differentiate species.

Although each organism has numerous characteristics, they are far from having equivalent taxonomic significance. Some do not have great importance in species differentiation, others appear valuable and significant. Consequently, it is important, from the standpoint of biological analysis also, to detect the most significant characteristics.

Primary importance in the classification of actinomycetes, as well as of all other groups of organisms (higher and lower), should be given to morphological characteristics, especially of fruit-bearing organs. The latter structures are particularly significant, since they reflect the biological nature of the organism and are taken as a basis for taxonomy of higher plants and fungi. There is no reason to ignore these criteria in the classification of actinomycetes.

The structure and development of fruit-bearing apparatus in actinomycetes is well expressed and sufficiently characteristic for species and whole groups or subgroups. The fruit-bearing organs are not only significant in these organisms, but also constitute a stable characteristic, genetically determined and unvarying under experimental conditions. The actinomycetes preserve their fruit-bearing characteristic in spite of great variability and a capacity for forming numerous and diverse variants (Krasilnikov, 1938).

Among the morphological characteristics deserving of attention are peculiarities of cell wall construction in aerial spores (smooth, spiny, toothed, hairy, etc.), formation of special chlamydsospore-like cells on the threads of substrate mycelium in some species, the manner of branching (monopodial, whorled, etc.) of aerial mycelium, and formation of sclerotia, coremia, and other possible bodies. The taxonomic significance of these structures is not yet sufficiently clarified; further observations and investigations are necessary before such criteria can be utilized. The specificity, stability, and regularity of these formations in particular species must be established.

Among cultural characteristics having taxonomic significance, pigments and the coloring of cultures due to pigments attract attention. It is known that actinomycetes differ significantly from each other in the coloration of colonies, growth medium, and aerial hyphae. Such a feature, especially the color of colonies and of media, is rather characteristic and, to a certain degree, specific. Less specific and stable is the color of aerial hyphae. However, the color of colonies and of medium does not always accurately reflect the species nature of the culture. The color of cultures can be caused by substances of nonpigment character, such as occasional intermediate metabolic products. Apparently in establishment of species it is necessary to take into consideration only that coloring of cultures which is caused by true pigments. This necessitates comprehensive studies of pigments.

Although the ordinary fermentative properties, liquefaction of gelatin, peptonization of milk, and starch hydrolysis are insignificant or not at all characteristic, nevertheless they should be taken into consideration in establishment of actinomycete species at least for general biological evaluation of cultures.

There is no doubt that assimilation properties of cultures have great significance. The ability to assimilate carbon and in some cases nitrogen compounds can be a good species differentiation characteristic within determined groups of actinomycetes.

The manifestation of antagonism deserves special attention among biochemical criteria of actinomycetes. Its specificity makes possible the differentiation and distinguishing of species fairly accurately and quickly as do the antibiotic substances produced by the species.

The possibility of revealing other biochemical characteristics which could be useful in distinguishing and subdividing actinomycete species is not excluded.

All previously mentioned criteria, such as morphological, cultural, as well as physiological and biochemical, appear in greater or lesser constancy only under definite growing conditions and on defined media. As is known, actinomycetes
are extremely variable; their external characteristics, as well as their physiological and biochemical properties, vary to a great extent depending on the composition of nutritional media and on external conditions. The wide polymorphism of actinomyces cultures compels investigators to study them under various growing conditions and on different media, in order to establish boundaries of species polymorphism. The more thoroughly and comprehensively the culture is studied and the better it is characterized, the easier the establishment of its species status.

However, the selection of nutritional media and of growing conditions of cultures should be strictly standardized. As mentioned before, in order to establish species relationships, cultures can be compared only when the diagnostic characteristics are obtained from growth on media of the same composition and under similar conditions. It is impossible to compare cultures when one of them is characterized on protein media and the other on defined media.

The species structure of a group of cultures is determined not only by the described morphological, cultural, physiological, and biochemical characteristics, but also by their ecology, their habitat, and their prevalence in nature. Therefore in describing the studied cultures it is desirable to have information on substrata from which they were isolated, on microbial associations in which they occurred in these substrata, etc.

In connection with the above-mentioned statements, we suggest that the following basic data should be obligatory in descriptions of new cultures and in the establishment of their species relationship.

1. **Habitat of cultures.** Where the strains were isolated and the degree of their distribution in soils. Characteristics of the soil from which the strain studied was isolated. The soil type: Chernozem, Podzol, Krasnozem (red soils), Chestnut soils, etc.; content of humus, pH, total nitrogen content, degree of cultivation (cultivated or virgin soil), plant cover, and other properties (very concise).

2. **Data of soil sampling and date of treatment.** If the soil samples were not treated at once, their storage conditions should be indicated. Repetition of soil sampling and number of microbiological analyses of the same soil.

3. **Isolation of organisms.** Seed soil samples on definite media, certainly on the three following, simultaneously: synthetic Czapek's, starch-ammonium, and meat-peptone agars. Other media can be used depending on the interests of the investigator.

In addition to counts of actinomyces encountered on these media, it is desirable to know the number of colonies of other bacteria (mycobacteria, micrococi), fungi, etc. Calculation of the total microflora enables one to determine the degree of cultivation of the soil and secondly to establish the biological background of antagonistic actinomyces. The antagonists are usually revealed by means of 1 or 2 test organisms (Escherichia coli and Staphylococcus aureus). The more test organisms, the more antagonists are detected.

4. **Investigation of the biological properties of the antagonistic actinomyces under strictly defined conditions.** Nutritional media must be rigidly standardized, not complex in their composition, available to any investigator, and without coded [trade-name] components or coded total media. Certain media for characterization of actinomyces must be obligatory, others can be used for additional information according to the investigator's option.

As obligatory media the same ones should be used as were recommended for primary analysis of soil samples, namely, synthetic Czapek's, starch-ammonium, and meat-peptone agars. In addition, it is desirable to use certain defined media with different sources of carbon and nitrogen.

For general characterization, rather good criteria appear on media with corn extract, on media with fish extract, on potato plugs, etc. (Krasil'nikov, 1938, 1950).

The composition of obligatory media: (a) Czapek's medium; (b) starch-ammonium medium; (c) meat-peptone agar: Meat infusion (1 kg meat boiled in 2 L of water), 1000 ml; peptone, 10 g; NaCl, 0.5 g; agar, 2 per cent; pH 7.2. Sterilization 10 min at 120 C.

5. **Growing conditions.** Mesophils should be incubated at 25 to 27 C with aeration such that growth occurs with free access of oxygen. Accurate information on anaerobiosis should be given in regard to anaerobes. Culture should be grown stationary or on shakers (surface and submerged growth). For rotary or reciprocal shakers,
revolutions, or strokes per min should be stated. All this must be mentioned in descriptions of performed experiments.

6. Antagonistic or antimicrobial properties. Antimicrobial spectrum of actinomycetes should be studied. General and special action against a set of bacteria, mycobacteria, yeasts, fungi, and phages should be noted. The group of test organisms must be strictly constant and standardized for all investigators. For this purpose museums or centers for standard cultures of test organisms should be established in different countries. Test organisms should be controlled from time to time and compared with those in other countries.

7. Conditions for the production of antibiotics. Follow definite rules of standardization in regard to composition of nutritional media, temperature, aeration, etc.

8. Strictly specified information should be given in culture descriptions. (a) Morphological: structure and development of fruit-bearing organs, etc.; (b) cultural: pigmentation, etc.; (c) physiological and biochemical data; and (d) antagonistic properties and their specificity.

(A) Morphological criteria necessary for the establishment and recognition of actinomycete species, are as follows: (1) Sporulation—For spiralled sporophores the following should be noted: number of convolutions, open or tight spirals, long or short, does the shape of sporophores resemble a corkscrew or a tuber (club). It should be stated whether nonspiralled sporophores are short, brushlike, or long and whether the arrangement is monopodial or whorled.

(2) Shape of spores.—It should be noted whether the spores are spherical, oval, elongate, rodlike with rounded ends or cylindrical with cut ends, and whether spore formation occurs by fragmentation or by segmentation. The media favorable for the formation of sporophores and spores should be designated. Since spirals do not always appear under all growing conditions, they should be looked for on other media if they do not appear on typical media.

(3) Other morphological characteristics.—The manner of branching, formation of coerulea and sclerotia on the surface of colonies, formation of spherical cells of the chlamydospore type, etc., should be described. The media and conditions under which they are formed should be indicated.

(B) Cultural criteria. (1) Coloring of colonies and of media—Synthetic (Čapek’s, CP-I, CP-III, starch-ammonium, etc.) and complex protein (meat-peptone, etc.) should be used. Fundamental properties of pigments are solubility in water and in organic solvents, their relation to the acidity of medium, diffusion into media (liquid and agar), etc. Conditions of pigment formation in the same culture depends on temperature, aeration, composition of medium, pH, etc. The following should be determined: purity of pigment (single or several pigments); their characteristics; variability of pigmentation in the same culture under different growing conditions and on different media; and boundaries of variability, impurities of nonpigment character (gray-brown substances, products of tyrosin oxidation, etc.).

(2) Coloring of aerial mycelium—On defined (Čapek’s) and nondefined or protein media, the degrees of variability in coloring of aerial hyphae should be recorded.

In all cases of pigmentation descriptions, it is necessary to give colored illustrations of the culture or refer to number in the color chart. Of course, the corresponding color chart should be mentioned.

(C) Physiological properties. Although the ordinary criteria of physiological activity of actinomycetes do not possess great taxonomic significance, they should be indicated in culture descriptions. These criteria are as follows: gelatin liquefaction, coagulation and peptonization of milk, starch hydrolysis, growth on cellulose, nitrate reduction, etc.

More significant are data on assimilation of carbon and nitrogen compounds. These data are most informative in comparative studies of strains of different species belonging to the same group.

It is possible that data on phenol-oxidase reaction (according to Küster) may be significant in species differentiation.

(D) Among biochemical characteristics some metabolic products are significant, primarily, antibiotics. Their specificity of antimicrobial action and especially the specificity of mutual antagonism definitely make possible the differentiation of cultures in the same group. Therefore, criteria of this rank should be studied and disclosed with extreme care.

In culture descriptions it is necessary to give the following information. (1) Antimicrobial
spectrum, action against a set of bacterial cultures:—
Cocci: Staphylococcus aureus, Streptococcus pyogenes; mycobacteria: M. tuberculosis, Mycobacterium strain no. 5, M. citreus, M. rubrum; sporeformers: Bacillus subtilis, Bacillus mesentericus, Bacillus cereus var. mycoides, Bacillus antracoides; nonsporeformers: Escherichia coli, Serratia marcescens (B. prodigiosum), Proteus vulgaris (B. proteus), Pseudomonas aeruginosa (P. pyocyanea), Pseudomonas fluorescens. Further against yeasts and fungal cultures: Saccharomyces, Debaryomyces, Willia, Torulaspora, Candida, Aspergillus, Penicillium, Fusarium, Helminthosporium, Verticillium, etc. It is desirable to give information on antiphage action of cultures. The set of test organisms for the establishment of spectrum can be varied, keeping in mind the fact that in different groups of actinomycetes, different species of bacteria, yeasts, and fungi appear as significant test organisms. For instance, for the A. globisporus group, essential test organisms appear to be yeasts, but for some groups and subgroups of the gray actinomycetes these organisms do not have any important taxonomic significance, but more significant as test organisms are actinophages or bacteriophages and certain bacteria. For violet actinomycetes, most significant are cultures of nodule bacteria, etc.

(2) The specificity of antagonism, making possible the differentiation of cultures within the group, is an essential property of antagonists:—
By its means one can quickly and rather accurately determine the difference between cultures to be compared (Krasil’nikov, 1951). Therefore, the establishment of this specificity must be obligatory in culture studies; it must be accurately established whether the cultures to be compared inhibit each other or not. This comparison has a meaning only if the cultures studied do not differ from each other morphologically, culturally, and physiologically, but constitute a monolithic group in regard to these characteristics. It is self-evident that such comparison must be performed in conformity with strictly standardized experimental measures.

In addition, it is necessary to take into account the appearance of so-called self-inhibition, which, as our experiments have shown, is caused not by antibiotics, but by different agents, in some cases by phages, in other cases by “necrohormones” which bring about the lysis of cells (Krasil’nikov et al., 1958).

(E) Conditions for the production of antibiotic substances. The following should be indicated:

(1) The composition of nutritional media (defined and complex organic); use of carbon and nitrogen compounds:—Ordinary and special media should be tested, and the complete composition of media should be given.

(2) Aeration:—Growth of strains stationary (surface growth), on shakers or in fermentors (submerged growth) should be compared. The manner and intensity of aeration should be indicated, i. e., number of revolutions or strokes per min, and the amount of air supplied to the fermentor.

(3) Date of experimentation, temperature, etc., should be noted. It is desirable to mention the changes observed in media during growth as well as the process of antibiotic production. Biochemical changes, consumption of carbohydrates and nitrogen; pH of medium, etc., are of interest.

(F) Peculiarities of antibiotics. Antibiotic substances in a natural state (in beers, before isolation from beers) can be characterized by antimicrobial spectrum and by conditions of biosynthesis, as well as by physicochemical manifestations, by solubility in organic solvents, by the degree of adsorption, and by the manner of distribution on chromatographic paper. These methods make possible the detection of differences between antibiotic substances in different species assigned to the same group.

The taxonomic criteria offered here appear to be a preliminary schema for the recognition and differentiation of actinomycete species. This schema must be not only controlled, but also corrected and improved in conformity with new suggestions given by specialists. With the accumulation of new experimental data, this schema will change to some degree in one or another direction. Although not perfect, this schema, nevertheless, will unconditionally make possible the comparison and identification of the organisms under study and the determination of species relationships relatively accurately.

The existence of this schema does not exclude the necessity for a museum (or museums in different countries) of standard cultures with accurately established species characteristics as well as their synthesized antibiotics—standard
samples (chemically pure preparations). Only by comparing with these standards can one relatively closely establish the identity or discrepancy of the cultures studied.

When comparing new cultures with standard strains, the fact should be remembered that under longer laboratory storage, cultures change their characteristics and properties more or less. Some criteria are lost and others emerge, so that the culture acquires a different appearance. The comparison of a strain under investigation with such a changed standard culture can lead to mistaken conclusions and to false determination of species. Therefore, in establishment of species one should not only compare the new strain with standards, but also confront it with the original description, which is given as a result of studies of the fresh culture which has not been subjected to laboratory influences under storage.