Actinomycetes & their Antibiotics

GUIDE TO THE CLASSIFICATION

AND IDENTIFICATION OF THE

Actinomycetes and their Antibiotics

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Preface

Barely four years ago, an effort was made to summarize the existing knowledge of the nature and physiology of the actinomycetes in a book entitled "The Actinomycetes." No attempt was made to include in that volume any detailed system of classification or species characterization of these organisms, since it was felt that Bergey's Manual was quite sufficient for that purpose. Nor was any attempt made to characterize in detail the antibiotics produced by actinomycetes, since the available information was rather limited at that time.

The rapid progress made during the last few years in the screening of actinomycetes, especially of species belonging to the genus *Streptomyces*, for their ability to produce antibiotics, the use of many of these antibiotics for chemotherapeutic purposes, and the addition of numerous descriptions of new species of actinomycetes necessitate a more detailed presentation of the systematic position and identification of these organisms and of the antibiotics produced by them. In describing these new organisms, many investigators have been unable to identify their newly isolated cultures with those described in the last edition of Bergey's Manual, with the result that they preferred to give their cultures new names, often without regard to the established types. These new descriptions are found in various journals and even in patents. This guide has been prepared for the primary purpose of making this information available to workers in this field. Those species which have been insufficiently described or which appear to be synonyms are not listed in the guide, but will be found in the new edition of Bergey's Manual, now in the process of preparation.

In the preparation of this guide, advantage has been taken of progress made in the classification of the actinomycetes during the last 30 years, thanks largely to the work of Orskov, Jensen, Waksman and Henrici, Erikson, and Krassilnikov. Much, however, still remains to be done, especially on the physiology and biochemistry of actinomycetes. This is true particularly of one group of metabolic products, namely, the antibiotics. Because these substances have become so vitally important, an effort has been made to list all the antibiotics so far isolated, either in crude or in pure form, and to give their salient properties and uses.

This guide has been prepared with the primary purpose of facilitating the work of the student of antibiotics produced by actinomycetes and to help him in the identification of the organisms producing these antibiotics, as well as of the antibiotics themselves.

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PART I

The Actinomycetes

By Selman A. Waksman

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Classification of the Actinomycetes

Introduction

Two important factors must be recognized in any attempt to classify the actinomycetes, especially for the purpose of establishing specific differences. These are, first, the ability of the organisms to produce vegetative and aerial mycelium; and second, the great variability of the cultures. To facilitate recognition of these organisms and to establish differences for classification purposes, well-defined media and standard conditions of cultivation must be used.

The filamentous growth and the true branching of the actinomycetes differentiate these organisms from the true bacteria. It is often difficult if not impossible to distinguish between the profuse branching of certain mycobacteria and the short-lived mycelium of the nocardias, except for the fact that the nocardias produce mycelium consistently in the early stages of their development. There is thus a gradual transition between the mycobacteria and the nocardias. There is also a definite transition between the nocardias and the streptomycetes, due to the constant and marked nature of the aerial mycelium of the latter and the transitory and undifferentiated aerial mycelium of the former.

In differentiating a nocardia from a streptomyces, one must consider the following differences: 1. A nocardia usually does not produce aerial mycelium, and if it does, such mycelium is no different from the vegetative mycelium produced by the organism and never forms spirals; a streptomyces, on the other hand, produces a characteristic aerial mycelium, a property which may be lost, however, on continued cultivation or under special conditions, and such mycelium frequently forms characteristic spirals. 2. A nocardia multiplies by concentration and segmentation of the protoplasm within a filamentous cell, followed by dissolution of the cell membrane, the fragmented portions of the mycelium usually developing into fresh mycelium under favorable conditions, either by germ tubes or by lateral budding; a streptomyces produces true spores or conidia, the vegetative mycelium not segmenting spontaneously into bacillary or coccoid forms, but remaining nonseptate and coherent even in old cultures, thus producing the characteristic tough-textured, leathery growth. 3. In a nocardia, the aerial mycelium represents an extension upward of the vegetative mycelium; it does not exhibit any differentiated protoplasm and

is sterile and abortive; on the other hand, when a streptomyces has lost the capacity of producing aerial mycelium, a form analogous to that of a nocardia may result, except for the structure of the mycelium and the capacity of the degenerated streptomyces to regain the lost capacity. 4. Another difference between the nocardias and the streptomycetes is the acid-fastness or partial acid-fastness of some of the former when grown in certain media.

The differentiation between species of *Streptomyces*, and those of *Micromonospora* is not very difficult, since both formation of aerial mycelium and sporulation are markedly distinct for both genera. Some difficulty may be experienced in differentiating species of these two genera from those of *Thermoactinomyces*, since the latter also produce aerial mycelium, similar to species of *Streptomyces*, and they produce single spores, similar to *Micromonospora*, but such spores are produced in the aerial mycelium rather than in the vegetative mycelium as in the case of the *Micromonospora*.

Classification of Actinomycetes According to Bergey

According to the latest edition of Bergey's Manual, the order ACTINOMYCETALES is divided into three families, *Mycobacteriaceae*, *Actinomycetaceae*, and *Streptomycetaceae*. The first family comprises the genus *Mycobacterium*, which is beyond the scope of this treatise. The second and third families comprise the true actinomycetes, with which this treatise is primarily concerned. The genera *Nocardia* and *Actinomyces* fall into the second family, and the genera *Streptomyces* and *Micromonospora* belong to the third. Another genus has recently been added, *Thermoactinomyces*, comprising largely certain thermophilic forms.

Of the various species belonging to the five genera of true actinomycetes, those of the genus *Actinomyces* have not, so far, been found capable of producing antibiotics. Among the species belonging to the other four genera, those of the genus *Streptomyces* are by far the most important; hence a detailed consideration of the nature of this genus is justified.

The Genus Streptomyces

Species of *Streptomyces* produce a well-developed mycelium. The diameter of the hyphae seldom exceeds 1.0 μ and is usually only 0.7–0.8 μ. The hyphae vary greatly in length. Some are long with limited branching; others are short and much branched. The vegetative mycelium does not form cross walls; it does not break up into rod-shaped and coccus-like bodies. The streptomycetes reproduce by means of conidia or by bits of mycelium. The spores or conidia are formed in special spore-bearing hyphae or sporophores which arise from the aerial mycelium, either monopodially or in the form of tufts or whorls. The sporulating hyphae are straight or

curved. The curvatures range from mere waviness to the formation of perfect spirals, which may be compact, in the form of fists, or long and loose.

The growth of streptomyces "colonies" on artificial media is smooth or lichnoid, hard and densely textured, raised, and adhering to the medium. The colony is usually covered completely or partially, in the form of spots or concentric rings, by aerial mycelium, which may be variously pigmented, depending on the species and on the composition of medium. In liquid media, especially in shaken cultures, growth of a streptomyces is usually in the form of flakes, which gradually fill the container, or in the form of spherical growths or puffballs. It is the former type of growth that is most desirable from the point of view of antibiotic production.

Many of the cultures, either in the form of colonies on the surface of solid media or as flaky growth in submerged culture, may undergo rapid autolysis. The production of antibiotics usually corresponds with the lysis of the cultures. Frequently the lysis is brought about by a phage, actinophage, which is injurious because of the premature destruction of the mycelium.

The analogy between the aerial mycelium and sporulation of species of *Streptomyces* and those of certain fungi has frequently led to confusion in the classification of actinomycetes as a whole. More recent evidence, notably the sensitivity of actinomycetes to antibiotics and to specific phages, as well as the nature of their circumscribed growth, tends to place the actinomycetes as a group, including the genus *Streptomyces*, closer to the true bacteria than to the true fungi.

Finally a word must be said about the "physiological races" of the streptomycetes. These organisms undergo in culture certain qualitative and quantitative variations. The streptomycin-producing strain of *S. griseus*, for example, has yielded two types of variants, one of which produced no aerial mycelium and formed no streptomycin, and the other of which produced a red pigmented vegetative mycelium aud formed another antibiotic (rhodomycetin) but not streptomycin. The qualitative differences of the variants are best expressed in the amount of antibiotic produced, which may vary as much as a hundredfold for the same species. From the point of view of antibiotic production, the following five subgroups of *S. griseus* may now be recognized: (a) those that produce streptomycin (as well as certain other antibiotics, such as cycloheximide and streptocin), (b) those that produce grisein, (c) those that produce candicidin, (d) those that produce still unidentified antibiotics, and (e) those that do not yield any antibiotic, at least under the particular condition of culture. Whether all of these variants have originated from a single original species of *S. griseus* is a matter of speculation. The limited evidence seems to point to the affirmative.

Because of these variations among strains of a single species, it has often been difficult to identify freshly isolated cultures with already established types. This has been particularly difficult when the latter were known by published description only. But even when type cultures were available for comparison, they may have undergone such marked changes as a result of long cultivation in artificial media that they no longer represent the original isolate. This may be illustrated again with *S. griseus*. When the original strain of this culture, which was isolated in our laboratory in 1915, was tested in 1946 for antibiotic formation, it was found to be unable to produce any antibiotic. When the same culture, which was deposited in the Baarn (Holland) Collection in 1920, was tested, it was found to be able to form an antibiotic that was not streptomycin. When the original culture kept in our collection was irradiated by Kellner, a strain that was capable of producing streptomycin was obtained. One may, therefore, only speculate whether this proves that the original 1915 strain possessed the capacity of producing streptomycin and lost it, or that it did not possess it at all, but on irradiation produced a mutant that acquired such capacity.

Need for Standard Media and Standard Conditions of Growth

The use of standard media, comprising both synthetic and complex organic media, for the growth of actinomycetes is particularly important in any attempt to characterize and identify these organisms. The same is true of the need for a standard environment, notably aeration and temperature control. Synthetic media have found extensive application in the study of the morphology and physiology of these organisms. This is true especially for cultural characterization of these organisms, particularly pigmentation. Organic media, often quite specific, are used for obtaining supplementary evidence, especially for strains that do not grow at all or grow only very weakly on the common inorganic media.

The great majority of actinomycetes are aerobic, very few are anaerobic, many are microaerophilic. To supply proper aeration, the organisms are grown on the surface of solid media, or in shallow liquid media, or in a thoroughly aerated submerged condition.

Temperatures of 25°–30° are usually used for incubation of the great majority of streptomycetes, nocardias, and micromonospora. Frequently somewhat different temperatures are used for seed production and for antibiotic formation under factory conditions. Pathogenic organisms require 37°C, and thermophiles need 50°–60°C or even higher temperatures.

Among the stable morphological properties of actinomycetes essential for purposes of characterization and classification, one must list the structure and subsequent changes in the vegetative mycelium, the production and

nature of the aerial mycelium, the nature of the sporulating branches or sporophores, and the nature of the spores.

Among the physiological and cultural properties essential for characterization purposes, pigmentation of the vegetative and aerial mycelium is most important; the formation of soluble pigments, both in synthetic and in complex organic media, is also significant. Among the other characteristics, one may list utilization of starch (diastase formation), various proteins (protease), sucrose (invertase), cellulose (cellulase?), and a variety of other carbon compounds. Reduction of nitrate, production of odor, and coagulation of milk (acid production) are among the other characteristics. Still other activities include the utilization of fats, paraffin, and phenol.

The various fermentation properties, comprising the utilization of sugars and related compounds, with and without the formation of acids, can supply additional information for species differentiation. The antagonistic activities and the ability to produce antibiotics have recently come into popular use for the description of actinomycetes. Their sensitivity to phages and to known antibiotics is also of considerable importance in establishing specific differences.

Many species of actinomycetes have been described in the literature as pathogenic to plants or animals. The fact that such cultures were isolated from infectious diseases is no proof, however, that they are the causative agents of such diseases.

Many of the earlier and even some of the more recent descriptions of actinomycetes are superficial, and it is often difficult to compare the characteristics of a freshly isolated culture with those of the published description. No wonder, therefore, that it has been much easier to give a fresh culture a new specific name rather than to attempt its identification with a species already described.

In identifying cultures of actinomycetes, extensive use has been made, therefore, of their morphological and physiological properties. The cultural characteristics, notably growth characteristics on various organic and synthetic media, formation of soluble pigments, proteolytic and diastatic properties, utilization and transformation of specific organic and inorganic compounds, have received particular attention. Pridham and Gottlieb emphasized recently that the utilization of carbon compounds should receive greater consideration for species determination than hitherto. All the particular species tested were able to utilize *d*-glucose, *d*-mannose, dextrin, and glycerol, but not erythritol, phenol, cresols and the sodium salts of formic, oxalic, and tartaric acids. The utilization of such compounds, however, as rhamnose, raffinose, xylose, lactose, mannitol, dulcitol, inositol, and the sodium salts of acetic and succinic acids, was selective.

It has been suggested that rather than single species, group species should

be recognized, as will be shown later. Thus, one could recognize such well-established types as *S. albus*, *S. griseus*, *S. lavendulae*, *S. flavus*, *S. coelicolor*, and certain others. The various species within a group could be considered as related to a given type. This is possible where a large number of isolates are available.

Numerous systems for the classification of the various groups of actinomycetes have been proposed. Two of these are most comprehensive and are, therefore, presented here. One is that of Krassilnikov, and the other, that of Waksman and Henrici, as included in Bergey's Manual.

Classification System of Krassilnikov

A. Sporophores branching monopodially.
  I. Spiral-shaped sporophores, produced on hyphae of aerial mycelium.
    1. Spores spherical or oval.
      a. Cultures colorless, not producing any pigmentation.
        a^1. Aerial mycelium white.
          a^2. Saprophytes, living on dead material.
            Streptomyces albus
          b^2. Parasites.
            a^3. Living in bodies of men and animals.
              Streptomyces bovis
            b^3. Living on plants.
              Streptomyces totschidlowskii
        b^1. Aerial mycelium dark gray.
          Streptomyces griseus
        c^1. Aerial mycelium green.
          Streptomyces glaucus
      b. Cultures pigmented blue.
        a^1. Pigment of the anthocyanin type, similar to litmus.
          Streptomyces coelicolor
        b^1. Blue pigment not changing with acidity of medium.
          Streptomyces cyaneus
      c. Cultures violet, forming two basic pigments (red and blue), both dissolved into the substrate.
        a^1. Cultures not forming any fluorescent substance in liquid media. . . . *Streptomyces violaceus*
        b^1. Cultures producing in synthetic media a fluorescent substance of blue-green color similar to pyocyanin.
          Streptomyces pluricolor
      d. Cultures black-violet, forming red and blue pigments, as well

as a brown pigment of the type of melanin, which changes the violet color of the culture to violet-black.

Streptomyces violaceus-niger

e. Cultures red-colored, producing pigments insoluble in water, of the lipoactinochrome type; color of medium not changing with acidity.

a¹. Cultures not forming any brown or black pigments; they are always red, sometimes with a brownish tinge but not black.

a². Saprophytes.

Streptomyces ruber

b². Parasites.

Streptomyces madurae

b¹. Cultures producing on synthetic media, in addition to pigments, a black or dark brown substance which gives the culture a red-brown to black color.

Streptomyces melanocyclus

f. Cultures yellow, citron-yellow, or brownish yellow.

a¹. Saprophytes.

Streptomyces flavus

b¹. Parasites.

a². Living in bodies of men and warm-blooded animals.

Streptomyces hominis

b². Living in bodies of cold-blooded animals.

Streptomyces lacertae

c². Living on plants.

Streptomyces setonii

g. Cultures orange.

a¹. Saprophytes.

Streptomyces aurantiacus

b¹. Parasites. *Streptomyces phenotolerans*

h. Cultures green or brownish green.

Streptomyces viridochromogenes

i. Cultures black, producing a pigment of the melanin type.

Streptomyces niger

j. Cultures pigmented dark brown, but not black.

a¹. Saprophytes.

Streptomyces chromogenes

b¹. Parasites. *Streptomyces gracilis*

2. Spores cylindrical or elongated.

a. Cultures colorless.
Streptomyces longisporus
b. Cultures red, sporophores mostly straight.
a¹. Saprophytes.
Streptomyces longisporus-ruber
b¹. Parasites.
a². Living in bodies of men and animals.
Streptomyces spumalis
b². Living on plants.
Streptomyces salmonicolor
c. Cultures orange.
Streptomyces fradiae
d. Cultures yellow.
a¹. Saprophytes.
Streptomyces longisporus-flavus
b¹. Parasites living on plants.
Streptomyces scabies
e. Cultures citron-yellow.
Streptomyces virgatus
f. Cultures green.
Streptomyces viridans
g. Cultures brown or chocolate color.
Streptomyces halstedii
h. Cultures black.
Streptomyces nigrificans

II. Sporophores straight or wavy, but not spiral.
1. Spores produced by means of fragmentation of plasma within cells.
a. Spores spherical or oval.
a¹. Cultures colorless.
Streptomyces globisporus
b¹. Cultures green.
a². Saprophytes.
Streptomyces viridis
b². Parasites.
Streptomyces cretaceus
c¹. Cultures brown.
Streptomyces globosus
b. Spores cylindrical or elongated.
a¹. Cultures colorless.
Streptomyces candidus
b¹. Cultures pigmented.
Streptomyces cylindrosporus

2. Spores produced by means of segmentation of aerial hyphae.
   a. Cultures colorless
      Streptomyces farinosus
   b. Cultures pigmented red.
      Streptomyces oidiosporus
   c. Cultures yellow-orange.
      Streptomyces longissimus
   d. Cultures brown.
      Streptomyces fumosus

B. Sporophores produced in whorls.
   I. Sporophores straight.
      Streptomyces verticillatus
   II. Sporophores spiral-shaped.
      1. Spores spherical, oval.
         Streptomyces reticuli
      2. Spores cylindrical, elongated.
         Streptomyces circulatus

Classification System of Waksman and Henrici

A. Saprophytes; psychrophilic to mesophilic.
   I. Soluble pigment on organic media absent, or faint brown, pink, purple, golden yellow, or blue.
      a. Pigment absent, or only faint brown pigment produced in protein media.
         a. Aerial mycelium abundant, white.
            a^1. Spirals formed; spores spherical to ellipsoidal.
               1. *Streptomyces albus*
            b^1. Long, open spirals; spores cylindrical.
               2. *Streptomyces longisporus*
            c^1. Straight sporophores, forming broom-shaped clusters; spores spherical to oval.
               3. *Streptomyces globisporus*
         b. Aerial mycelium whitish to light gray, in concentric zones.
            4. *Streptomyces annulatus*
         c. Aerial mycelium on synthetic sucrose agar sandy lavender to dark gray.
            5. *Streptomyces rochei*
      2. Aerial mycelium gray to blue-gray; soluble pigment blue.
         a. Strongly proteolytic.
            a^1. Spirals formed.
               a^2. Pigment at first red, changing to blue.
                  6. *Streptomyces coelicolor*.

b^2. Pigment at first yellow-red, changing to blue or bluish green.

7. *Streptomyces pluricolor.*

c^2. Pigment unchanged with acidity.

8. *Streptomyces cyaneus*

b^1. No spirals formed.

9. *Streptomyces vinaceus*

b. Weakly proteolytic; aerial mycelium poorly developed.

10. *Streptomyces violaceus*

3. Pigment at first green, becoming brown.

a. Aerial mycelium usually absent.

11. *Streptomyces verne*

b. Aerial mycelium dark gray, olive-colored, or gray-green.

12. *Streptomyces viridans*

4. Growth pink on synthetic media, yellowish on organic media; no soluble pigment.

13. *Streptomyces californicus*

5. Growth yellow to greenish or orange-colored; soluble pigment yellow to golden yellow.

a. Growth yellow to green; pigment insoluble.

14. *Streptomyces virgatus*

b. Growth sulfur-yellow; soluble pigment yellow.

a^1. Conidia oval to elliptical.

a^2. Aerial mycelium white.

15. *Streptomyces flaveolus*

b^2. Aerial mycelium light yellow.

16. *Streptomyces parvus*

c^2. Aerial mycelium white to gray to reddish gray.

17. *Streptomyces xanthophaeus*

b^1. Conidia spherical; cellulose decomposed.

18. *Streptomyces cellulosae*

c. Growth reddish brown to orange-colored to cinnamon-drab, covered with white to gray aerial mycelium; soluble pigment on synthetic media yellowish.

a^1. No soluble pigment on gelatin. No peptonization of milk.

19. *Streptomyces rimosus*

b^1. Faint yellowish coloration of liquefied gelatin. Milk rapidly peptonized.

20. *Streptomyces griseoflavus*

c^1. Soluble pigment golden yellow.

21. *Streptomyces aureofaciens*

d. Growth cream-colored to brown; aerial mycelium white to yellowish.

a^1. Soluble pigment yellow to yellow-orange.

22. *Streptomyces albidoflavus*

b^1. Soluble pigment yellowish to yellow-green.

a^2. Aerial mycelium white to yellow.

23. *Streptomyces lieskei*

b^2. Aerial mycelium gray.

a^3. Growth on synthetic agar yellowish green.

24. *Streptomyces flavovirens*

b^3. Growth on synthetic agar yellow; produces soluble yellow pigment on calcium-malate-glycerol agar.

24a. *Streptomyces celluloflavus*

c^3. Growth on glucose-asparagine agar yellow, becoming black.

25. *Streptomyces limosus*

6. Growth cream-colored; soluble pigment yellowish brown to reddish brown.

26. *Streptomyces griseoluteus*

7. Growth coral-red; aerial mycelium scant, white; soluble pigment brown.

a. Gelatin rapidly liquefied.

27. *Streptomyces bobiliae*

b. Gelatin slowly liquified.

28. *Streptomyces aurantiacus*

8. Growth on synthetic media mouse-gray; aerial mycelium white to gray.

a. Sporophores straight.

29. *Streptomyces griseolus*

b. Sporophores broom-shaped.

30. *Streptomyces fasciculus*

9. Growth cream-colored to yellowish to red; aerial mycelium white to gray.

a. Growth becoming red; aerial mycelium white.

31. *Streptomyces erythreus*

b. Growth yellow; aerial mycelium mouse-gray to drab.

32. *Streptomyces flavogriseus*

10. Soluble pigment on potato plug brown to brownish red to reddish purple.

a. Growth on potato greenish-colored; spirals formed.

33. *Streptomyces diastaticus*