THE ISOLATION AND ANTIBIOTIC SCREENING OF TWENTY-EIGHT ACTINOMYCETES FROM AN ACID SOIL AND CULTURAL STUDIES ON SEVERAL SHOWING SPECIAL PROPERTIES

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by

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HISTORICAL BACKGROUND

The actinomycetes are a group of unicellular branching microorganisms found widely distributed in nature, and seeming to form a transition group between bacteria and fungi, possessing characteristics related to each. The term "actinomycete" refers to any member of the five genera Nocardia, Mycobacterium, Actinomyces, Streptomyces, or Micromonospora which comprise the order Actinomycetales. (1) Waksman (2, p. 22) gives the following characteristics for the order:

> Organisms forming elongated, usually filamentous cells, with definite tendency to branching; hyphae not exceeding 1.5 micron in diameter, mostly about 1 micron or less. Usually producing a characteristic branched mycelium. Multiply by means of special spores, as well as by oidiospores or by conidia. The special spores are formed by fragmentation of the plasma within the spore-bearing hyphae, the latter being straight or spiral-shaped. The oidiospores are formed by segmentation, or by simple division of hyphae by means of transverse walls, in a manner similar to the formation of oidia among the true fungi. The conidia are produced singly, at the end of special, simple or branching conidiophores. They grow readily on artificial media and form well developed colonies. The surface of the colony may become covered with aerial mycelium. Some of the organisms are colorless or white, whereas others form a variety of pigments. They are either saprophytic or parasitic. In relation to temperature, most are mesophilic, though some are thermophilic. Certain forms are capable of growing at low oxygen tension.

The latter description may also be found in Bergey's Manual of Determinative Bacteriology. (3)

The genera which are of interest antibiotically speak-

ing are Nocardia, Micromonospora, and Streptomyces. Of these the most interesting and extensively studied is the genus Streptomyces. (4) In this genus are placed the aerobic nonpathogenic actinomycetes producing aerial mycelium and multiplying by forming true conidia in chains. (3)

Cohn (5) in 1875, was the first person to study an actinomycete, under the generic name of Streptothrix. From that date through 1943 over 31 different generic names were used in designating all the actinomycetes or certain constituent groups. (2)

The antagonistic action of actinomycetes to the growth of other microorganisms was first demonstrated by Gasperini in 1890. The actinomycete, apparently the same as Cohn's was able to live on the surface of bacteria and fungi because of its ability to digest the membrane or cell wall of the lower fungi. (2)

One year earlier the word "antibiosis" was coined by Vuillemin in reference to antagonism between two living forms. Ward (6) ten years later, applied the word in respect to microbial antagonism and it has lasted to the present time.

The theory of antibiosis really obtained its start, however, in 1917, when Greig-Smith (1) observed the inhibition of certain spreading colonies of Bacillus mycoides and Bacillus vulgaris on nutritive agar by soil actinomycetes.

In 1921, Lieske (2) tested a large number of actinomycetes for their antibacterial effect and succeeded in showing that certain bacteria pathogenic to man may be inhibited by some excretion of the actinomycete which he believed to be a specific bacteriolytic enzyme.

Three years later, a "Streptothrix" was isolated from the air by Gratia and Dath (6) which lysed dead or living staphylococci.

From 1936 to 1941, Welsch (6) did extensive work on an organism very similar to the one described by Gratia and Dath. The bacteriolytic substance was named Actinomycetin and the organism, Streptomyces albus G.

Rosenthal (2) obtained an actinomycete culture from dust which inhibited living Clostridium diphtherium and lysed the dead cells. At the same time (1925) he introduced suitable methods for measuring the bacteriostatic and bacteriolytic properties of actinomycete cultures.

At this time other researchers became interested in the antagonistic actions of the actinomycetes as a group and so large numbers of actinomycetes were being worked with instead of only one or two cultures.

The first detailed study on the distribution of antagonistic actinomycetes was done in 1937 by Nakhimovskaia (4). She reported that 58.75% of 80 actinomycete cultures isolated from a variety of soils possessed antagonistic properties.

A year later Krassilnikov and Koreniako (2) reported that species primarily in the genus Streptomyces were antagonistic to a variety of organisms particularly the genera Nocardia, Mycobacterium, and Micrococcus. The activity was believed to be due to a single substance possessed by all the species tested which were active. The substance studied by these and other Russian workers was thought to be similar to lysozyme, a lytic enzyme.

Kriss (1), in 1940, was one of the first workers to try to isolate the antagonistic substance using various solvents, but he had little success. By 1942 researchers throughout the world were beginning to organize the previous works. It was concluded that actinomycetes possessing antagonistic properties are widely distributed in nature. Waksman (7) found 43.4% of 244 cultures isolated at random from a variety of soils possessed antagonistic properties.

Alexopoulos (8) found that 56.25% of 80 cultures were antagonistic to a fungus Colletrotrichum gloesporioides.

Out of 660 cultures of actinomycetes isolated on a nonselective basis from soils from 5 locations in northern Canada, 61.2% showed antagonism against at least one test organism out of the eight that were tested (9).

Because of the widespread and developing interest in these antagonistic substances, it inevitably followed that a substance would be found which would be of practical clinical use. This occurred in 1944 when Waksman (10)

published his discovery of Streptomycin after examing some thousands of actinomycetes, hundreds of fungi, and many bacteria during the preceding five years.

With this discovery and the drug's subsequent use in medicine to kill pathogenic gram negative bacteria in vivo, the subject of microbial antagonism took on its greatest significance. It was at this time that the word antibiotic came into widespread existence. Waksman (11) defined the word "antibiotic" as a "substance produced by microorganisms which has the capacity of inhibiting the growth or even destroying other microorganisms."

A slightly different definition was suggested by Benedict and Langlyke (12). They defined it as a "chemical compound derived from or produced by living organisms which is capable in small concentration, of inhibiting the life processes of microorganisms."

Immediately following the discovery of Streptomycin, the pharmaceutical industries began their own research on the actinomycetes and in antibiotic screening. Large plants were constructed for the production of Streptomycin and for large scale soil investigations. Antibiotics were sought which were of low toxicity and yet had a wide spectrum of antibiotic activity, that is, they were actively antagonistic to a number of microorganisms composed of different physiological types such as gram positive and gram negative bacteria, fungi, etc., and yet were not poisonous to animals.

Streptomycin is a wide spectrum antibiotic primarily active against gram negative and gram positive bacteria. The work of isolating antibiotic producing actinomycetes from various soils gathered throughout the world was begun in earnest with the discovery of this antibiotic, and is still being carried on.

Industrial laboratories are organized for mass screening programs and search thousands of soil samples yearly. The number of actinomycetes studied is small in relation to the number isolated. The industries are interested only in the antibiotic which will compete favorably with those already on the market. Antibiotics that have entered the production stage are few compared with the large numbers which have been found.

The work reported in this paper, and other research like it, is justified by the very fact that the industries are organized for mass screening only. Their time spent on any particular soil sample and the organisms isolated from it is restricted. They are not interested in the number of active cultures found, or in the classification of the actinomycetes whose antibiotics will not compete favorably with those on the market today.

Although the work in this thesis is restricted to a single soil, it is spent on a more thorough investigation of the cultures isolated from it. The purpose of this work was to obtain from a soil as many actinomycetes as possible

showing either antagonism toward a wide spectrum of bacterial species or showing other special properties. The cultural and morphological characteristics of these were investigated and the cultures were identified if possible.

ANALYSIS OF THE PROBLEM

When a problem is selected in which the particular group of microorganisms to be investigated is a part of a mixed microbial population, the problem automatically enlarges itself to include the isolation of that particular group.

In this work, the actinomycetes existing in a particular soil sample were wanted for subsequent checking of their antibiotic properties, and the classification of those showing special and/or unusual properties.

Several methods have been used for isolating antibiotic organisms from soils and other sources where mixed microbial populations are found. All the methods, however, are variations of one called the "crowded plate technique" which in turn is only a special case of the "plate dilution culture method". Instead of isolating all colonies exhibiting similar morphological characteristics as in the latter method, only colonies exhibiting zones of inhibition are selected in the "crowded plate technique".

The major disadvantage to this method is the uncertainty as to whether an appropriate test organism exists in the soil which will show up the antibiotic activity of all the organisms in the group being investigated.

A summary of the techniques for isolating antibiotic

organisms from mixed cultures is given by Flory, Chain, and coworkers in Antibiotics. (v.1, pp. 81-88)

For the evaluation of the actinomycete population of a soil and the isolation of cultures for later antibiotics work, the plate dilution culture method is used most extensively. (1),(2).

In its simplest form, this method consists of plating out a suspension of soil with a suitable medium, incubating, and then selecting the particular type of microorganisms sought for future investigation.

Before the method can be used satisfactorily, however, certain factors such as the type of nutritive medium and its reaction, the temperature and time of incubation, and the treatment of the soil prior to incubation must be decided upon. To date, there has been published no data comparing the different factors and conditions used by past investigators. Personal choice has been the governing factor of such a selection. (4)

For this reason, it was thought advisable to include the isolation as the first part of the experimental work of this problem. Results were obtained by varying the different conditions and attempting to select finally a particular medium, reaction, incubation temperature, and soil treatment which would give results comparable to those obtained by using several variations of each factor used in the plate dilution culture method.

After isolation, the actinomycete cultures were screened for antibiotic activity using the cross streak method.

Since the beginning of the search for antibiotics, many methods and special techniques have been developed for determining not only whether an antibiotic is produced by a given organism, but also the quantity produced. A very good summary of these methods may be found in Antibiotics. (v.1, pp. 75-81)

There is one method which has been important from the start and is still the most commonly used for testing not only for an antibiotic, but also for the number and types of organisms upon which the antibiotic acts. This method is the " cross streak method" developed by Garré (1) in 1887, and is now widely used in industry as a primary screening method where many actinomycetes are to be checked for antibiotic properties against many other microbes.

The procedure is as follows: The antagonist, i.e., the actinomycete is streaked across a nutritive agar plate which is then incubated at a suitable temperature until the organism has grown out abundantly. Then secondary streaks of the test organisms are made at right angles to the actinomycete streak. The plate is again incubated, this time at a temperature optimal for growth of the test organisms. If the actinomycete has produced an antibiotic antagonistic to a test organism, then the latter will be inhibited in the neighborhood of the actinomycete growth at

a distance proportional to the sensitivity of the test organism to the antibiotic, and the amount and diffusibility of the antibiotic produced.

The type of medium used to produce antibiotic activity and the types of test organisms used to detect this activity are discussed later in relation to the procedure of screening the isolated cultures.

After all the isolated actinomycete cultures were screened for antibiotic activity, several showed certain properties that made them worthy of further investigation.

Classification of these cultures was therefore attempted. There was the possibility that either a previously unknown organism, or a known organism with uncharacterized properties would be found. The work of classifying actinomycete cultures, however, is complicated by the fact that there has been found no morphological or physiological characteristics which may be used to divide the species of a particular genus into smaller definite groups. (4) There are 73 species of the genus Streptomyces listed in Bergey's Manual of Determinative Bacteriology. (3) There has been found to date no natural breakdown of this number of species into smaller groups. A grouping based on the color of pigments on certain types of media, and morphology of the species has been attempted, but due to the variability of composition of these media, the variability of pigment production, and the use of no specified medium

upon which the morphology was studied, the grouping is slightly better than none at all.

It can be seen then, that when the classification of an actinomycete culture is attempted, all the known species of actinomycetes must be checked individually against the unknown culture.

Waksman (5) published a compilation of the characteristics of 41 different species of soil actinomycetes in 1919. The descriptions included the morphological, cultural, and biochemical characteristics of each. These descriptions may also be found in Bergey's Manual.

Due to the extent of Waksman's work, some of the media which he used were prepared for this work so that the cultures worked on in this paper could be compared with those of his. Other media used in Bergey's Manual were also prepared.

The results of isolation, antibiotic screening, and classification are summarized on page 67.

ISOLATION OF ACTINOMYCETES FROM SOIL

Experimental

The plate dilution culture method was decided upon for isolating the actinomycetes from the soil. All other methods used for isolation of these organisms are special variations of this method and limit the worker to the isolation of only the actinomycetes which show antibiotic activity in the isolation medium.

As stated on page 9, the plate dilution culture method consists of plating out a suspension of soil with a suitable medium, incubating at a given temperature, and selecting the particular type of microorganisms sought for future work. To date, there is no set of conditions universally accepted for use in isolating actinomycetes from soils. The reason for this may be that the different soils investigated vary broadly in their organic contents and reactions, thus also varying their microbial population.

The pH of the soil sample used in this work checked at 5.1 to 5.3. This reaction is very favorable to the growth of fungi but not to actinomycetes. It would be difficult merely to select a particular medium and the various other factors which would allow the actinomycetes to produce good growth while restricting the fungal growth.

The first step then was to find a nutritive medium which

would not allow fungi or bacteria to grow in such profusion as to make the isolation of the slower growing actinomycetes impossible.

If possible, no surface reductants or fungal inhibitors were wanted in the procedure. There is a possibility that they inhibit actinomycetes as well.

Many media have been used in the past for isolating actinomycetes from soils. The following media were tested here:

1. actinomyces agar (13)
2. Czapek's agar (14)
3. Jensen's agar (15)
4. nitrate sucrose agar (2)(16)
5. streptomyces agar (1)
6. brain-heart infusion agar (17)
7. malt agar
8. North geletin agar (17)
9. nutrient agar (2)
10. potato dextrose agar
11. S-1 agar

The composition of each medium and its reaction can be found in table I.

Twelve plates of each medium were inoculated with soil diluted from 10^2 to 10^4, four plates per dilution. The plates were incubated at room temperature and observed for actinomycete growth every day until they became overgrown with fungi. On the third day, 8 cultures were isolated. One was from actinomyces agar, 2 were from streptomyces agar, and 5 were from nitrate sucrose agar. The colonies on the nitrate sucrose agar were all between 2 and 3 mm. in diameter, while the other 3 colonies were only about 1 mm. in diameter. All were isolated from plates of the lowest dilution.

After only four days incubation, it became evident that most of the media tested were inadequate for use with this particular soil. All three dilutions of media numbers 3, 5, 9, and 10 were covered by spreading growths of both bacteria and fungi. Media numbers 2, 6, 8, and 11 had spreading type bacteria dominating the plates. All the others except number 4, nitrate sucrose agar, had fungi covering the plates. On the plates of nitrate sucrose agar, the bacteria and fungi grew out in well-defined colony formations which allowed time and room for actinomycete growth to take place. However, only three more cultures were obtained.

The three cultures obtained from the actinomyces agar and streptomyces agar were cultivated in sterile plates of the same medium from which they were isolated, then transferred onto nitrate sucrose agar plates to check for growth on that medium. All three were found to grow out abundantly. For this reason, plus the fact that nitrate sucrose agar had the least interfering growth, it alone was chosen for subsequent isolation work.

At this time, the method of inoculation was changed. The soil which had been diluted was now added directly to the surface of the hardened agar plates and spread by means of a sterile needle. In this way it was hoped that more actinomycete growth would be evidenced.

TABLE I

Formulae of the Eleven Media Tested in the Plate Dilution Culture Method for Isolating Soil Actinomycetes

Ingredients grams/liter	Culture Media										
	Actinomyces Agar	Brain heart infusion Agar	Czapek's Agar	Jensen's Agar	Malt Agar	Nitrate sucrose Agar	North Gelatin Agar	Potato dextrose Agar	S-1 Agar	Streptomyces Agar	Nutrient Agar
Peptone		10					20		10		5
Tryptone										5	
Casein				.2			2				
Veal (infusion)							500				
Brain heart (infusion)		200									
Potato (infusion)								200			
Beef extract											3
Egg albumen									.5		
Malt extract					30						
Sucrose						30					
Dextrose	1		40	2				20		10	
Gelatin							20				
Glycerine	10										
Sodium asparaginate	1										
$CaCl_2$	.1										
KH_2PO_4		2.5	1	.5		1					
NaCl		5					5			2	
Sodium citrate									1	1	
$FeSO_4$	trc		.01	trc		trc			.2	trc	
$MgSO_4$	.2			.2		.5			.2		
KCl	.1		.5			.5					
$NaNO_3$			3			2					
$NH_4H_2PO_4$	1.5									2	
Agar	17	15	15	15	15	15	15	15	17	17	15
pH	6.0	7.0	6.0	6.5	4.5	6.8	7.3	6.0	6.8	6.5	7.0

It has been noted (18) that the spores of the actinomycetes have waxy coatings which are not readily wet. They will therefore float on the surface of a liquid. Thus many of the spores will be missed when obtaining a dilution sample using a pipette placed beneath the surface of the dilution liquid.

After the selection of nitrate sucrose agar as the isolation medium, its pH was adjusted from 6.0 to 8.0 in steps of 0.5. Colonies of actinomycetes were found only on the plates containing the medium at a pH of 6.5 and 7.0. Allen set the pH of nitrate sucrose agar at 7.0. The medium after autoclaving and without adjusting was found to be 6.8 $\pm$ .2. No noticeable results were found between the unadjusted agar medium and that adjusted at 6.5 or 7.0, and so the unadjusted nitrate sucrose agar was used. It should be noted that cultures were isolated whenever possible.

Soil inoculated plates of nitrate sucrose agar were incubated at three temperatures - room temperature (approximately 25°C), 35° $\pm$ 1°C, and 54° $\pm$ 2°C.

Waksman (5) found that most of the actinomycetes will grow readily at temperatures ranging from 15°C to 37°C, the higher temperature favoring a more rapid growth. The temperature of 54° was included to grow out any thermophilic species from the soil.

A few cultures were isolated at room temperature which would not originally grow at 35°C. On subsequent attempts the cultures began to produce slight growth at the raised temperature, but they would have been missed if only the higher temperature had been used for isolation. Only one species was isolated at 54° ± 2°C. The majority of the cultures were obtained at 35° ± 1°C.

Thereafter, the following conditions were set forth in the procedure for the cultivation and isolation of the soil actinomycetes.

Plates of nitrate sucrose agar were poured approximately 20 ml. of agar medium per plate, and allowed to harden. The soil which was kept refrigerated at 4°C was inoculated onto the surface of the agar in a quantity of .15 - .2 grams per plate. Twigs and other debris within the soil were included with the soil. The plates were then incubated at three temperatures - room temperature, 35° ± 1°C, and 54° ± 2°C. The plates were checked after 3 days incubation and every day thereafter for about a week. Suspected actinomycete colonies were picked from the plates by means of a sterile inoculating needle and transferred into a sterile nitrate sucrose plate for primary pure cultivation.

Cultural characteristics which identify actinomyces colonies are the following. The agar colonies are usually small and tough, adhering to the medium. They are either flat or semicircular and growing deep into the medium. If

they are semicircular, they may exhibit radial folds on the surface, or they may be smooth and shiny. If they are smooth and shiny, they are distinguished from bacteria in that the colony is leathery and can not be broken up. It is removable all at one time. Almost all actinomycete growth gives off a typical earthy odor when cultured which readily identifies them. Microscopically, actinomycetes may be distinguished from other organisms primarily by the production of branching mycelium not exceeding 1.5 microns in diameter and usually only about 1 micron in diameter. All actinomycetes are gram positive.

Cultures were isolated and kept on the basis of their diverse appearance. Presumably, one could isolate cultures as long as the sampling material held out. However, there is, to date, no published information dealing with the isolation of actinomycetes on any basis other than random sampling or diversity of appearance. Cultural characteristics of the isolated cultures were checked against each other on nitrate sucrose agar and S-1 agar. Differences in the rate of growth, contour of the surfaces of the colonies, edge of the colonies, color of the vegetative mycelium, color of the aerial mycelium, color of the spores, or color of any soluble pigment were searched for.

If any of the isolated cultures differed from the others in any way mentioned it was placed on an agar slant of nitrate sucrose agar and one of S-1 agar, incubated for

three days, and then refrigerated until it was to be tested with the rest for antibiotic activity.

Results and Discussion

Twenty-eight cultures were selected for screening of their antibiotic activities. Records were not kept of the actual number of colonies isolated from the soil inoculated plates. The isolations were continued until no further cultures were isolated which differed from those already obtained. This method seemed justified in two respects. First, during the final isolations, no different appearing cultures were found. Second, in random sampling it would be possible to pick many colonies of the more profusely growing strains of actinomycetes and yet miss cultures which appeared rarely on the plates. This would later involve the screening of many identical cultures. If the non-antibiotic strains of actinomycetes were the commonest in the soil, then the work would be of no value.

It is interesting to note that on the soil inoculated plates which were incubated at 54°C, only one actinomycete was found. This actinomycete showed rather peculiar properties. It produced abundantly a deep, bluish-purple water soluble pigment that when treated with hydrochloric acid turned bright red. The culture also gave off a pleasantly smelling fruity odor instead of the usual musty or earthy odor so often associated with actinomycete growths.

Of the other 27 cultures, only 3 showed any water soluble pigments. However, most of them produced pigments within their mycelia or spores.

When checked morphologically, some cultures produced spiral formation in their aerial mycelia while others produced no aerial mycelia. Some cultures produced conidia readily while others produced none at all.

Because of the large number of variations in characteristics shown in the 28 cultures, it was expected that there would also be shown many characteristic spectra of antibiotic activity.

SCREENING ACTINOMYCETES FOR ANTIBIOTIC ACTIVITY

A major difficulty which confronts any worker in the field of antibiotics is that of selecting conditions which will give favorable results. Failure to use the proper medium when testing for antibiotic activity can give negative results with organisms which actually are good antibiotic producers. This fact is illustrated by several industries which have included in their patents the formulae of the culture media used in producing their antibiotics (2)(19).

The selection of the temperature and time of incubation is also just as important as the selection of the medium. Organisms vary considerably in these respects. The optimal temperature for antibiotic production is not necessarily the optimal temperature for growth. And antibiotic production by a particular organism may reach its maximum after a few days incubation, or after a few weeks.

Last, but not less important, is the critical selection of the test organisms. Unless an antibiotic is sought for its action against a specific organism, a wide variety are used in testing for antibiotic activity. Streptomycin would have been missed if tested against only yeasts or fungi. Penicillin, which is very active against gram positive bacteria would not have been discovered if tested against gram negative bacteria.

The preceding paragraphs were included in order that the reader could understand more clearly the problems which had to be met before selecting the conditions used in this work for screening the isolated cultures.

Experimental

Nutritive agar plates were poured two days before using to check for sterility and to allow the surface of the agar to dry. The antagonist, i.e. the actinomycete, was streaked across one side of the agar plates by means of a loop in a path 5 mm. in width and about 25 mm. from the edge of the agar at the middle of the streak's length. The plates were then incubated at 35° ± 1°C for about 60 hours. At that time, secondary streaks of the test bacteria were made at right angles to the actinomycete streak. The secondary streaks were made by means of a loop, streaking away from the actinomycete. Twenty-four hour broth cultures of the bacteria were used.

After the secondary streaks were made, the plates were again incubated at 35°C. This temperature was close to the optimum temperature for all the test organisms except Sarcina lutea. Its optimum temperature is given in Bergey's Manual as 25°C. However, an excellent growth was obtained at 35°C.

After another 12 hours, the plates were removed from the incubator and readings were made in millimeters of the

zone between the actinomycete streak and the bacterial growth.

Readings were made by placing a celluloid ruler on the bottom of the Petri dish with the scale measurement superimposed over the streak of the test organism. A small microscope light held under the inverted culture plate gave a well defined end point to the test streak. Readings were made only to the nearest millimeter. Greater accuracy was not needed. The measurements were taken for purposes of comparing the cultures with one another and for comparing their inhibitive effects on the different physiological types of test organisms such as the gram positive and gram negative bacteria. The results are recorded in table IV.

The media which have been used for the production of antibiotics run the gamut of nutritional substances. Many of them contain tryptone plus some particular carbohydrate. Others contain meat extracts and peptones, with or without sugar added. With this in mind, four media were prepared for this work which contained some of the common sources of nitrogen and carbon used in the past. The media were also selected so that they would supplement each other to a certain extent. It was thought that perhaps in this way, the production of an antibiotic may be found to be connected with the use of a certain sugar or other substance. The formulae of the four media used for screening the actinomycetes are given in table II.

TABLE II

Formulae of the Four Media Used in Screening the Actinomycete Cultures for Antibiotic Activity

Penassay base Agar	Glucose Agar	Tryptose Phosphate Agar	Tryptose Sucrose Agar
	Glucose - - -10 g	Glucose - - 2 g	Sucrose - - 2 g
Beef extract - 1.5g	Beef extract- 5 g	Tryptose- -20 g	Tryptose- -20 g
Yeast extract - 3 g	NaCl- - - - - 5 g	NaCl - - - 5 g	NaCl- - - - 5 g
Peptone- - - - 6 g	Peptone - - -10 g	K_2HPO_4 - - 2.5g	K_2HPO_4- - - 2.5g
Agar - - - -17 g	Agar- - - - -17 g	Agar - - - 17 g	Agar - - -17 g

The screening of each culture was carried out on four media because it was known that an antibiotic may be produced in one medium and not in others. Possibly it would be worth while to run the tests on several media of varying composition. Whatever the medium used, however, it must not only allow the actinomycete cultures to grow out, but, just as important, the medium must allow the test organisms also to produce growth. These four media met the conditions very favorably.

The reactions of the media were unadjusted. The major reason for this was the fact that after checking the reactions used for 16 media employed in the industrial production of actinomycete antibiotics such as Chloromycetin, Streptomycin, Aureomycin, and Tyrothricin, it was found that they varied from 6.0 to 7.2 before inoculation. After incubation the variance was from 4.5 to 9.0. These variations occurred not only between different cultures, but also for the same culture using different media. Variations could also be found in the reaction of a single medium depending upon the number of days incubation, and seemingly unaffected within certain limits by the original reaction of the medium. (2)(19) There appeared to be no evidence which would support the adjustment of the reactions already shown by the four media used in this work.

All actinomycete cultures produced luxurious growth after 60 hours incubation at 35°C. Sporulation also began at this time.

If an antibiotic were produced by any culture, it should at least have been produced in a colony containing all stages in the actinomycete's life cycle.

It was therefore concluded that the test organisms could be streaked at this time and any antibiotic cultures would be demonstrated.

Twelve hours of incubation at 35°C was sufficient to produce heavy growth streaks of the test bacteria and so readings were taken at that time. The total time elapsing between streaking the actinomycetes and reading the plates for antibiotic activity was 72 hours.

The streaks of the test organisms were made from brain heart infusion broth cultures incubated at 35°C.

Twelve bacterial species were selected for testing the antibiotic activity of the actinomycete cultures. It is necessary during the isolation of antagonistic organisms and the study of the antibiotic substances to use more than one test organism including one or more gram positive and one or more gram negative bacteria. Antibiotics vary greatly in their action on different organisms possessing different physiological properties. The antagonistic action of a given antibiotic may be highly selective, affecting detrimentally only a single strain of a bacterial species, or it may act generally, not only on a given species but also on gram positive and gram negative bacteria and fungi as well.

In most methods of testing for antibiotics, the choice

of the test organisms rests with the experimenter. There is no standard procedure developed through rational planning for selecting them. Most of the general groups of organisms found in any bacteriology laboratory have been used for this work. In general, the only factor to be considered in selecting the test organisms is that they are picked from a variety of physiological groups.

For this reason, although the work was limited to bacteria, both gram positive and gram negative bacteria were used. In these two groups, both pathogenic and soil types can be found. In the gram positive group, both rods and cocci are present. See table III.

Results and Discussion

Table IV shows the antagonistic properties of 28 actinomycete cultures tested on each of four media against 12 bacterial species.

The actinomycete cultures producing wide spectra of antibiotic activity are seen to be organisms 241, 58, 201, 216, 415, 200, and 202. Organisms 58 and 415 produced their antibiotics on all four media, although decreased activity was shown on penassay base agar by number 58. Apparently, they are able to utilize a number of carbon and nitrogen sources for synthesizing their antibiotics. Organisms 200 and 202 were antagonistic to the test organisms only on penassay base agar. This may mean that a more complex

TABLE III

General Characteristics of the Twelve Test Bacteria
Used in the Cross Streak Method for Demonstrating Antibiotic Activity
of the Actinomycete Cultures

Test Organisms	Gram Reaction	Characteristics	
Micrococcus pyogenes var. aureus 6538-P	+	coccus	pathogenic
Salmonella typhosa	-	motile rods	pathogenic
Bacillus subtilis NRRL B543	+	motile rods	spore producers, soil organisms
Escherichia coli	-	short rods, var. motility	enteric form, isolated from intestinal disease
Klebsiella pneumoniae	-	non-motile rods	encapsulated, pathogenic
Pseudomonas aeruginosa	-	motile rods	pathogenic, freshly isolated
Salmonella pullorum	-	non-motile rods	pathogenic, freshly isolated
Streptococcus sp.	+	coccus	pathogenic
Shigella sonnei	-	non-motile rods	pathogenic
Sarcina lutea	+	coccus	soil organism
Proteus vulgaris	-	motile rod	saprophytic
Pseudomonas aeruginosa 16-3	-	motile rod	pathogenic, old culture

TABLE IV

Inhibition of Growth on Four Media of Twelve Bacterial Species by Twenty-eight Actinomycete Cultures

Media and Actinomycetes	Test Organism: Micrococcus pyogenes	Salmonella typhosa	Bacillus subtilis	Escherichia coli	Klebsiella pneumoniae	Pseudomonas aeruginosa	Salmonella pullorum	Streptococcus sp.	Shigella sonnei	Sarcina lutea	Proteus vulgaris	Pseudomonas aeruginosa 16-3
ORGANISM 58												
glucose agar	17	19	22	14	12	0	17	10	8	18	13	5
tryptose phosphate	15	16	17	13	14	0	19	6	12	23	16	10
tryptose sucrose	23	22	27	20	19	0	21	14	18	24	15	9
penassay base	6	7	8	5	7	0	6	5	0	9	0	0
ORGANISM 201												
glucose agar	0	0	0	0	0	0	0	0	0	0	0	0
tryptose phosphate	0	0	4	0	0	0	0	0	0	0	0	0
tryptose sucrose	0	0	0	0	0	0	0	0	0	0	0	0
penassay base	10	15	12	8	0	0	10	7	0	8	0	0
ORGANISM 203												
glucose agar	0	0	7	0	0	0	0	0	0	0	0	0
tryptose phosphate	0	0	10	0	0	0	0	0	0	0	0	0
tryptose sucrose	0	0	6	0	0	0	0	0	0	0	0	0
penassay base	0	0	15	0	0	0	0	0	0	0	0	0
ORGANISM 216												
glucose agar	0	0	0	0	0	0	0	0	0	0	0	0
tryptose phosphate	0	0	6	0	0	0	0	0	0	0	0	0
tryptose sucrose	0	0	0	0	0	0	0	0	0	0	0	0
penassay base	10	17	14	10	6	0	15	5	6	13	5	0

Inhibition in millimeters demonstrated by cross streak method

TABLE IV continued

Media and Actinomycetes	Test Organism											
	Micrococcus pyogenes	Salmonella typhosa	Bacillus subtilis	Escherichia coli	Klebsiella pneumoniae	Pseudomonas aeruginosa	Salmonella pullorum	Streptococcus sp.	Shigella sonnei	Sarcina lutea	Proteus vulgaris	Pseudomonas aeruginosa 16-3
ORGANISM 254												
glucose agar	0	0	0	0	0	0	0	0	0	5	0	0
tryptose phosphate	0	0	0	0	0	0	0	6	0	6	0	0
tryptose sucrose	0	0	0	0	0	0	0	5	0	0	0	0
penassay base	0	0	8	0	0	0	0	5	0	9	0	0
ORGANISM 408												
glucose agar	15	0	11	0	0	0	0	5	0	15	0	0
tryptose phosphate	13	0	8	0	0	0	0	0	0	10	0	0
tryptose sucrose	16	0	9	0	0	0	0	0	0	12	0	0
penassay base	19	0	13	0	0	0	0	7	0	15	0	0
ORGANISM 413												
glucose agar	0	0	0	0	0	0	0	0	0	4	0	0
tryptose phosphate	0	0	0	0	0	0	0	0	0	0	0	0
tryptose sucrose	0	0	0	0	0	0	0	0	0	0	0	0
penassay base	0	0	0	0	0	0	0	0	0	6	0	0
ORGANISM 415												
glucose agar	30	19	34	7	6	6	17	35	12	35	15	8
tryptose phosphate	24	8	30	0	4	0	10	32	7	38	15	8
tryptose sucrose	29	15	32	7	4	0	13	52	7	37	10	11
penassay base	31	20	33	4	5	0	15	38	8	41	7	10
ORGANISM 419												
glucose agar	0	0	0	0	0	0	0	0	0	0	0	0
tryptose phosphate	0	0	0	0	0	0	0	0	0	5	0	0
tryptose sucrose	0	0	0	0	0	0	0	0	0	0	0	0
penassay base	0	0	0	0	0	0	0	0	0	0	0	0

Inhibition in millimeters demonstrated by cross streak method

TABLE IV continued

Media and Actinomycetes	Test Organism											
	Micrococcus pyogenes	Salmonella typhosa	Bacillus subtilis	Escherichia coli	Klebsiella pneumoniae	Pseudomonas aeruginosa	Salmonella pullorum	Streptococcus sp.	Shigella sonnei	Sarcina lutea	Proteus vulgaris	Pseudomonas aeruginosa 16-3
ORGANISM 425												
glucose agar	16	0	12	0	0	0	0	0	0	11	0	0
tryptose phosphate	14	0	9	0	0	0	0	4	0	15	0	0
tryptose sucrose	14	0	11	0	0	0	0	0	0	15	0	0
penassay base	20	0	15	0	0	0	0	4	0	14	0	0
ORGANISM 37												
glucose agar	0	0	0	0	0	0	0	0	0	0	0	0
tryptose phosphate	0	0	0	0	0	0	0	0	0	0	0	0
tryptose sucrose	0	0	0	0	0	0	0	0	0	0	0	0
penassay base	0	0	4	0	0	0	0	0	0	0	0	0
ORGANISM 200												
glucose agar	0	0	0	0	0	0	0	0	0	0	0	0
tryptose phosphate	0	0	0	0	0	0	0	0	0	0	0	0
tryptose sucrose	0	0	0	0	0	0	0	0	0	0	0	0
penassay base	8	18	13	9	6	0	17	15	12	18	11	0
ORGANISM 202												
glucose agar	0	0	0	0	0	0	0	0	0	0	0	0
tryptose phosphate	0	0	0	0	0	0	0	0	0	0	0	0
tryptose sucrose	0	0	0	0	0	0	0	0	0	0	0	0
penassay base	3	7	6	0	0	0	10	10	0	0	0	0
ORGANISM 204												
glucose agar	0	0	0	0	0	0	0	0	0	0	0	0
tryptose phosphate	0	0	0	0	0	0	0	0	0	0	0	0
tryptose sucrose	0	0	0	0	0	0	0	0	0	0	0	0
penassay base	0	0	8	0	0	0	0	0	0	5	0	0

Inhibition in millimeters demonstrated by cross streak method

TABLE IV continued

Media and Actinomycetes	Test Organism											
	Micrococcus pyogenes	Salmonella typhosa	Bacillus subtilis	Escherichia coli	Klebsiella pneumoniae	Pseudomonas aeruginosa	Salmonella pullorum	Strepto-coccus sp.	Shigella sonnei	Sarcina lutea	Proteus vulgaris	Pseudomonas aeruginosa 16-3
ORGANISM 214												
glucose agar	0	0	0	0	0	0	0	0	0	0	0	0
tryptose phosphate	0	0	0	0	0	0	0	0	0	0	11	0
tryptose sucrose	0	0	0	0	0	0	0	0	0	0	10	0
penassay base	0	0	0	0	0	0	0	0	0	0	0	0
ORGANISM 218												
glucose agar	0	0	0	0	0	0	0	0	0	0	0	0
tryptose phosphate	0	0	0	0	0	0	0	0	0	0	0	0
tryptose sucrose	0	0	0	0	0	0	0	0	0	0	0	0
penassay base	0	0	0	0	0	0	0	0	0	0	0	0
ORGANISM 217												
glucose agar	0	0	0	0	0	0	0	0	0	0	0	0
tryptose phosphate	0	0	0	0	0	0	0	0	0	0	0	0
tryptose sucrose	0	0	0	0	0	0	0	0	0	0	0	0
penassay base	0	0	0	0	0	0	0	0	0	0	0	0
ORGANISM 223												
glucose agar	8	0	5	0	0	0	0	6	0	5	0	0
tryptose phosphate	0	0	0	0	0	0	0	0	0	0	19	0
tryptose sucrose	0	0	0	0	0	0	0	0	7	0	17	0
penassay base	6	0	5	0	0	0	0	6	0	7	0	0
ORGANISM 233												
glucose agar	0	0	0	0	0	0	0	0	0	0	0	0
tryptose phosphate	0	0	0	0	0	0	0	0	0	0	0	0
tryptose sucrose	0	0	0	0	0	0	0	0	0	0	0	0
penassay base	0	0	0	11	0	0	0	0	6	19	0	11

Inhibition in millimeters demonstrated by cross streak method

TABLE IV continued

Media and Actinomycetes	Test Organism: Micrococcus pyogenes	Salmonella typhosa	Bacillus subtilis	Escherichia coli	Klebsiella pneumoniae	Pseudomonas aeruginosa	Salmonella pullorum	Streptococcus sp.	Shigella sonnei	Sarcina lutea	Proteus vulgaris	Pseudomonas aeruginosa 16-3
ORGANISM 241												
glucose agar	17	0	15	0	0	0	0	25	6	13	5	0
tryptose phosphate	14	0	14	0	0	0	0	10	0	8	0	0
tryptose sucrose	0	0	0	0	0	0	0	7	0	0	0	0
penassay base	11	0	11	0	0	0	0	6	0	8	0	0
ORGANISM 273												
glucose agar	0	0	0	0	0	0	0	5	0	0	0	0
tryptose phosphate	0	0	0	0	0	0	0	0	0	0	0	0
tryptose sucrose	0	0	0	0	0	0	0	0	0	0	14	0
penassay base	0	0	7	0	0	0	0	5	0	0	0	0
ORGANISM 305												
glucose agar	0	0	0	0	0	0	0	0	0	0	0	0
tryptose phosphate	0	0	0	0	0	0	0	0	0	0	0	0
tryptose sucrose	0	0	0	0	0	0	0	0	0	0	0	0
penassay base	0	0	0	0	0	0	0	0	0	0	0	0
ORGANISM 414												
glucose agar	10	0	8	0	0	0	0	6	0	11	0	0
tryptose phosphate	14	0	15	0	0	0	0	4	0	13	0	0
tryptose sucrose	14	0	10	0	0	0	0	5	0	15	0	0
penassay base	20	0	14	0	0	0	0	7	0	16	0	0
ORGANISM 215												
glucose agar	14	0	12	0	0	0	0	6	0	12	0	0
tryptose phosphate	18	0	16	0	0	0	0	7	0	14	0	0
tryptose sucrose	17	0	14	0	0	0	0	7	0	15	0	0
penassay base	15	0	15	0	0	0	0	6	0	14	0	0

Inhibition in millimeters demonstrated by cross streak method

TABLE IV concluded

Media and Actinomycetes	Test Organisms											
	Micrococcus pyogenes	Salmonella typhosa	Bacillus subtilis	Escherichia coli	Klebsiella pneumoniae	Pseudomonas aeruginosa	Salmonella pullorum	Streptococcus sp.	Shigella sonnei	Sarcina lutea	Proteus vulgaris	Pseudomonas aeruginosa 16-3
ORGANISM 417												
glucose agar	8	0	16	0	0	0	0	17	0	10	0	0
tryptose phosphate	5	0	4	0	0	0	0	0	0	0	0	0
tryptose sucrose	0	0	4	0	0	0	0	0	0	0	0	0
penassay base	10	0	12	0	0	0	0	14	0	10	0	0
ORGANISM 421												
glucose agar	0	0	0	0	0	0	0	0	0	0	0	0
tryptose phosphate	0	0	0	0	0	0	0	0	0	0	0	0
tryptose sucrose	0	0	0	0	0	0	0	0	0	0	0	0
penassay base	0	0	0	0	0	0	0	0	0	0	0	0
ORGANISM 431												
glucose agar	0	0	0	0	0	0	0	0	0	0	0	0
tryptose phosphate	0	0	0	0	0	0	0	0	0	0	0	0
tryptose sucrose	0	0	0	0	0	0	0	0	0	0	0	0
penassay base	0	0	0	0	0	0	0	0	0	0	0	0
ORGANISM 274												
glucose agar	0	0	4	0	0	0	0	0	0	5	0	0
tryptose phosphate	0	0	0	0	0	0	0	0	0	0	0	0
tryptose sucrose	0	0	0	0	0	0	0	0	0	0	0	0
penassay base	0	0	5	0	0	0	0	5	0	8	0	0

Inhibition in millimeters demonstrated by cross streak method

substance is needed such as the yeast extract before the antibiotic substances can be produced. A slight inhibition of Bacillus subtilis on tryptose phosphate agar was produced by both 201 and 216, however, because the activity is slight it is only mentioned in passing. Other than this slight inhibition, organisms 201 and 216 resemble organisms 200 and 202 in producing their antibiotics only on penassay base agar.

Another organism which produces its antibiotic only on penassay base agar is organism 233. Its spectrum of action seems to be against specific gram positive and gram negative bacteria. In the group acted upon, however, are found both soil organisms and pathogens.

There is some doubt whether organism 241 produces one antibiotic or two. This question arises upon noting that only gram positive bacteria are inhibited upon three of the four media, while on the fourth, glucose agar, increased activity against the gram positive organisms is found and at the same time inhibition of two gram negative organisms occurs. There are two possible explanations for this phenomenon. First, the greater activity shown on the glucose agar may signify that the actinomycete has a faster growth rate on this medium than on any of the others. It may be that a second antibiotic is produced later and has activity against gram negative bacteria only. Second, it may be that only one antibiotic is produced by organism

241 and due to greater production in glucose agar it exhibits inhibition against organisms which are antagonized only by larger amounts of the antibiotic.

In comparing the four media, penassay base agar gave the best results allowing 75% of the actinomycetes to produce inhibition against at least one of the test bacteria. Tryptose phosphate agar, glucose agar, and tryptose sucrose agar allowed activity to be shown by 53.6%, 50%, and 46.4% of the actinomycetes in the order stated.

Not only would a reduced number of active cultures have been observed by using a single medium, but also certain activities exhibited on different media by specific cultures would have been missed. Organisms 223 and 274, for instance, inhibit just gram positive bacteria on penassay base agar and glucose agar. On tryptose sucrose agar, number 274 inhibits a gram negative organism and number 223 inhibits two gram negative organisms. This would seem to indicate the production by each of them of two different antibiotics.

In addition to the group of organisms showing activity against a wide spectrum of the test bacteria, three other active groups of cultures may be found. See table V.

First, there is the group of 14 cultures which inhibited just gram positive bacteria. Second, there is a small group of only three cultures which produced activity on certain media against just gram negative bacteria.

Third, there is the group of cultures which produced antagonistic affects only upon specific bacterial species. Organism 203 inhibited only Bacillus subtilis on all four media.

Organism 413 inhibited Sarcina lutea on penassay base agar and glucose agar. Organism 214 inhibited just Proteus vulgaris on tryptose phosphate agar. Some of the first two groups of organisms are also placed here.

TABLE V

The Twenty-eight Actinomycete Cultures
Grouped According to the Range of Their Antagonistic Action

Group 0
Inhibition against neither gram positive nor gram negative bacteria

Penassay base agar	419, 214, 218, 421, 37, 217, 305
Glucose agar	201, 216, 419, 37, 200, 202, 204, 214 218, 217, 233, 305, 421, 431.
Tryptose phosphate	413, 37, 200, 202, 204, 218, 217, 273, 274, 305, 421, 431, 233.
Tryptose sucrose	201, 216, 413, 419, 37, 200, 202, 431, 204, 214, 218, 217, 233, 274, 305, 421.

Group I
Inhibition against gram positive bacteria only

Penassay base agar	203, 254, 408, 413, 425, 37, 204, 223, 241, 273, 414, 215, 417, 274.
Glucose agar	203, 254, 413, 408, 223, 414, 215, 417, 425, 273, 274.
Tryptose phosphate	201, 203, 216, 254, 408, 419, 425, 241, 414, 215, 417.
Tryptose sucrose	203, 417, 254, 241, 408, 425, 414, 215.

Group II
Inhibition against gram negative bacteria only

Penassay base agar	none
Glucose agar	none
Tryptose phosphate	223, 214.
Tryptose sucrose	223, 273.

Group III
Inhibition of only 1 or 2 bacterial species

Penassay base agar	203, 413, 37, 204, 273.
Glucose agar	203, 254, 413, 273, 274.
Tryptose phosphate	203, 216. 254, 419, 214, 223, 417
Tryptose sucrose	203, 254, 223, 241, 273, 417

Group IV
Inhibition of both gram positive and gram negative bacteria

Penassay base agar	58, 201, 200, 202, 216, 415, 233.
Glucose agar	58, 415, 241.
Tyyptose phosphate	58, 415.
Tryptose sucrose	58, 415.

MORPHOLOGICAL AND CULTURAL STUDIES

Experimental

Nine cultures were selected for further study on the basis of antibiotic activity or other properties shown by them in the course of this work.

Organisms 58, 200, 201, 202, 216, and 415 each produced inhibition of a wide spectrum of bacteria. Organism 233 showed activity primarily against gram negative bacteria although it also inhibited Sarcina lutea, a gram positive coccus. It was also peculiar in its antibiotic production on only one of the four media upon which it was tested. Organism 241 was unusual not only in its production of principally a gram positive antibiotic, but also in its production of a litmus-like pigment and its optimal growth temperature of 54°C. It was also the only organism isolated which gave a sweet, fruity odor instead of the usual earthy odor associated with actinomycetes. Organism 218 was the only culture isolated that produced a brown, water soluble pigment which was observed in almost every protein containing medium in which it was grown. It was also one of the five cultures which produced no activity whatsoever. Classification of it was attempted merely because it was distinct from the other 27 cultures by its pigment production.

In the past, identification of antibiotic producing actinomycetes has been attempted on the basis of morphology, pigment production, and/or physiological characteristics such as nitrate reduction, starch hydrolysis, and ability to utilize carbohydrates. All have been found to be rather unsatisfactory procedures.

Henrici (20) stated that it was not possible to identify most of the species of the genus Streptomyces which he had isolated. He believed that as much could be said for abandoning the species concept for this group as for any other group of microorganisms.

Routein and Finley (4) believed that any taxonomical work on this genus could be only provisional with reference to given cultures deposited in culture collections.

Benedict (21) said that he was forced to conclude "that the present system of classification of the actinomycetes, which is based on morphology and relatively few physiological tests, especially for the genus Streptomyces, made it extremely difficult for competent investigators to 'key down' an unknown culture."

Although it's quite possible that no natural relationships have been found in the group as yet, there has been a good deal of work done on the individual species. These species are listed in Bergey's Manual and are separated from each other mainly by cultural differences, i.e., their growth on various media.

In this thesis, classification was undertaken by preparing the various media mentioned in Waksman's classification work of 1916 (17) and 1919 (5) which accounts for over half of the species descriptions found in Bergey's Manual for the genus Streptomyces. The other media prepared were selected from Bergey's Manual.

On the solid media prepared, the cultures were observed for type of vegetative growth, presence and color of aerial mycelia, and the presence and color of soluble pigments. The cultures grown in liquid media were observed for type of growth, presence of soluble pigments, and special reactions due to the particular type of medium.

Morphologically, the growths on certain solid media were observed microscopically for straight or spiraling conidiophores and the presence of spores and their shape if present.

Two procedures were followed in checking the characteristics of the unknown cultures against the descriptions of the species in the literature. First, the characteristics of each isolated culture were checked individually against each species listed in Bergey's Manual. When a culture agreed generally with the description of a species listed in Bergey's Manual, the original reference to the species was found. This usually gave a more complete description.

The second procedure followed in attempting to identify the unknown cultures, was based on the fact that strains of a particular species may vary markedly in their

antibiotic production and at the same time in their morphological characteristics. With this in mind it was thought wise to check the unknown cultures of this work against all species listed in the literature as active antibiotic producers. There are several good publications in which attempts were made to compile such a list. Of these, the largest number of antibiotics described and the species listed can be found in the article by Benedict (21) which lists 115 antibiotics. Approximately 89 species account for all the antibiotics except those which are produced industrially by chemical manipulation.

The results of the cultural and morphological work are given in tables VI through X. At the end of each culture's description are listed any known species or species group to which the culture appeared to be related.

Results and Discussion

No individual charts are found for organisms 58 and 415 or organisms 200, 201, 202, and 216. In the course of the work it became evident that organisms 218, 233, and 241 were three different species. Organisms 58 and 415 comprised a fourth species, and organisms 200, 201, 202 and 216 composed a fifth species.

Identification of the five species was attempted. However, after comparing their descriptions with the literature descriptions of no less than 150 species and strains, the

author found that none of the unknown organisms could be identified with a known species in more than a general way.

This was due largely to the incomplete descriptions given for many organisms. In many cases reported in the literature, descriptions on three or four media were the basis for classifying a species (22).

Three of the unknown species were placed into specific groups with little or no indecision. There was little doubt that organism 218 belonged in the Streptomyces albus group. It agreed morphologically and culturally with the group characteristics. However, it resembled several of the species in this group without being unmistakably one or a variant of any of these species. For this reason the status of this organism could not be decided upon.

Organism 200 belongs quite definitely to the Streptomyces flavus group. Its golden yellow pigmentation alone would seem to warrent this. It resembles Streptomyces flaveolus and Streptomyces thermophilus in several respects. Its identification is complicated not only by its entirely different set of characteristics depending upon the temperature, but also by the fact that within the literature several species of the Streptomyces flavus group resemble each other so closely that with the slight natural variations they would be indistinguishable. An example of this may be found by comparing the descriptions of Strep-

TABLE VI

DESCRIPTION OF ORGANISM 218

General Morphological Characteristics

Spiral formation in aerial mycelium; long open spirals, spores are spherical or slightly oval observed on calcium malate agar.

Cultural Characteristics Observed on Various Media at 35°C and Room Temperature

	Incubation 35°C	Incubation Room Temperature
Czapek's agar modified (glycerin)		
Growth	colorless to cream	same as 35°C
Aerial mycelium	white all over surface of growth	same as 35°C
Soluble pigment	none	none
Calcium malate-glycerin agar		
Growth	grayish tan; raised, leathery type growth	colorless to white vegetative
Aerial mycelium	white	white
Soluble pigment	brown	yellowish brown
Glucose agar		
Growth	no growth	colorless changing to dark brown; raised, much wrinkled growth
Aerial mycelium		white changing to grayish black after 5 days
Soluble pigment		dark brown to black

TABLE VI continued

Nutrient agar		
Growth	colorless; flat & spreading	same as 35°C
Aerial mycelium	tan, powdery surface	same as 35°C
Soluble pigment	none	none
Starch agar		
Growth	tan, raised, spreading	light gray vegetative; raised, smooth & spreading
Aerial mycelium	white, turning gray after 6 days	white, powdery with droplets on surface turning gray after 6 days
Soluble pigments	none	none
Enzymatic zone	none	none
Potato dextrose agar		
Growth	buff colored vegetative, composing raised colonies; much wrinkled & leathery	smooth, round, raised colonies; light gray vegetative mycelium
Aerial mycelium	none	none
Soluble pigment	none	none
Gelatin		
Growth	submerged, colorless; no ring formation	white growth
Aerial mycelium	none	none
Soluble pigment	greenish brown	brown
Liquefaction	none	none

TABLE VI continued

Kligler's iron agar		
Growth	gray, raised, wrinkled growth; leathery & compact.	same as 35°C
Aerial mycelium	none	none
Soluble pigment	none	none
H_2S production	+	+
Skim milk		
Growth	yellowish brown ring formation; white aerial mycelium	reddish brown ring white aerial mycelium
Coagulation	none	none
Soluble pigment	greenish brown	slight brown
Litmus milk		
Growth	black ring formation; white aerial mycelium; brown soluble pigment	yellowish brown ring; aerial mycelium white; brown soluble pigment
Coagulation	none	none
Reaction	deeply alkaline	alkaline
Reduction	none	none
Peptonization	none	none
Czapek's agar with sucrose		
Growth	none	none

Temperature: optimum 35°C

TABLE VI continued

Generic Name of Unknown Species and
Probable Actinomyces Group to Which the Species Belongs

Genus: Streptomyces (3, p.929)

Actinomyces group: Streptomyces albus group (22, pp. 28-29)

TABLE VII

DESCRIPTION OF ORGANISM 233

General Morphological Characteristics

Spiral formation in aerial mycelium; long, open spirals; observed on calcium malate agar. Spores oval shaped.

Cultural Characteristics Observed on Various Media at 35°C and Room Temperature

	Incubation 35°C	Incubation Room Temperature
Czapek's agar modified (glycerin)		
Growth	colorless; scant, flat and spreading	no growth
Aerial mycelium	white, scant	
Soluble pigment	none	
Calcium malate-glycerin agar		
Growth	yellowish brown; flat spreading	white to dark yellow; flat & spreading
Aerial mycelium	tan to greenish tan	white to tan changing to olive green, powdery surface
Soluble pigment	green to greenish brown after 3 days	none
Glucose agar		
Growth	cream colored to dark brown; flat and spreading	cream colored; flat, spreading
Aerial mycelium	cream colored to greenish tan; powdery surface	white to tan with slight greenish tinge; powdery surface
Soluble pigment	none	none

TABLE VII continued

Nutrient agar		
Growth	flat, spreading; cream colored	same as 35°C
Aerial mycelium	cream colored turning olive green; powdery	same as 35°C
Soluble pigment	none	none
Starch agar		
Growth	colorless changing to yellow after 6 days; flat & spreading	scant, flat; colorless changing to greenish tan in 6 days
Aerial mycelium	white changing to tan after 6 days; powdery surface; concentric ring formation	white changing to greenish tan; powdery surface; concentric ring formation
Soluble pigment	green, produced only after 14 days	same as 35°C
Enzymatic zone	none to very slight (1-2 mm.)	none
Potato dextrose agar		
Growth	colorless; flat & spreading	buff colored; flat & spreading
Aerial mycelium	tan powdery surface with white powdery edges to the colony	white to pink with a slight greenish tinge to surface; powdery
Soluble pigment	none	none
Gelatin		
Growth	light yellow ring formation; light yellow growth produced through out liquified portion	same as 35°C

TABLE VII continued

Gelatin - continued		
Aerial mycelium	white to light yellow	same as 35°C
Soluble pigment	none	none
Liquefaction	2" (total) in 14 days	same as 35°C
Kligler's iron agar		
Growth	grayish brown; raised & much wrinkled	same as 35°C
Aerial mycelium	none	scant white
Soluble pigment	none	none
H_2S production	none	none
Skim milk		
Growth	light yellow ring formation	light yellow ring formation
Coagulation	none	none
Soluble pigment	none	none
Peptonization	complete in 6 days	3/8" in 6 days
Litmus milk		
Growth	produced on surface of liquid; white aerial mycelium; light yellow vegetative	same as 35°C
Coagulation	none	none
Reaction	Alkaline in 3 days	Alkaline in 5 days
Reduction	none	none
Peptonization	1 5/8" in 3 days	¼" in 4 days; total (2") in 8 days.

TABLE VII continued

Czapek's agar with sucrose		
Growth	none	none

Temperature: Optimum 35°C

Generic Name of Unknown Species and Probable Actinomyces Group to Which the Species Belongs

Genus: Streptomyces (3, p. 929)

Actinomyces group: Streptomyces griseus group (22, pp. 23-24; 25, pp. 14-16, 27-28; 26, pp. 259-269.)

TABLE VIII

DESCRIPTION OF ORGANISM 241

General Morphological Characteristics

Spiral formation in aerial mycelium; short compact spirals; observed on calcium malate agar. Spores spherical in shape. Generally, some oval spores observed.

Cultural Characteristics Observed on Various Media at 35°C and Room Temperature

	Incubation 35°C	Incubation Room Temperature
Czapek's agar modified (glycerin)		
Growth	flat; very slight growth	no growth
Aerial mycelium	white, powdery, scant	
Soluble pigment	bright blue	
Calcium malate-glycerin agar		
Growth	colorless to white; flat	no growth
Aerial mycelium	white turning gray in 5 days	
Soluble pigment	bright plum blue	
Glucose agar		
Growth	grayish black; leathery growth, wrinkled	gray growth; much wrinkled
Aerial mycelium	none	none
Soluble pigment	deep violet	none

TABLE VIII continued

Nutrient agar		
Growth	scant, flat; colorless	no growth
Aerial mycelium	white, powdery	
Soluble pigment	blue, rapidly spreading	
Starch agar		
Growth	light gray; flat and spreading	no growth
Aerial mycelium	buff colored; turning gray in 6 days	
Soluble pigment	bright blue	
Enzymatic zone	7 mm.	
Potato dextrose agar		
Growth	gray; smooth, raised, colonial growth; entire edge; leathery	no growth
Aerial mycelium	none	
Soluble pigment	none	
Gelatin		
Growth	submerged; very slight growth	no growth
Aerial mycelium	none	
Soluble pigment	none	
Liquefaction	complete (2") in 11 days	

TABLE VIII continued

Kligler's iron agar		
Growth	grayish in color; raised, much wrinkled	small, round, smooth slightly raised colonial growth; entire edge; gray in color
Aerial mycelium	white in color, scant	none
Soluble pigment	none	none
H_2S production	none	none
Skim milk		
Growth	colorless, slight	no growth
Coagulation	none	
Soluble pigment	reddish brown ring suggests perhaps slight reddish pigment	
Litmus milk		
Growth	reddish, brown ring formation with cream colored aerial mycelium	no growth
Coagulation	none	
Reaction	neutral	
Reduction	none	
Peptonization	3/8" in 8 days	
Czapek's agar with sucrose		
Growth	slight flat growth; scant, white aerial mycelium	no growth

Temperature: Optimum 55°C

Generic Name of Unknown Species

Genus: Streptomyces (3, p. 929)

TABLE IX

DESCRIPTION OF ORGANISMS 58 AND 415

General Morphological Characteristics

Straight sporulating hyphae, no spirals on calcium malate agar. Spores oval in shape.

Cultural Characteristics Observed on Various Media at 35°C and Room Temperature

	Incubation 35°C	Incubation Room Temperature
Czapek's agar modified (glycerin)		
Growth	light yellow vegetative, flat surface growth in 3 days	white growth turning slight yellow in 3 days; scant.
Aerial mycelium	grayish white in color; covering the vegetative growth completely in 3 days	white; scant
Soluble pigment	bright pink in 3 days	none
Calcium malate-glycerine agar		
Growth	yellow vegetative developing deep into medium	colorless vegetative; mycelium turning yellow in 5 days
Aerial mycelium	cream colored; thin	white, changing to greenish yellow in 5 days
Soluble pigment	rose or pink	pink; very slight
Glucose agar		
Growth	cream colored spreading vegetative	same as 35°C
Aerial mycelium	white, covering surface	same as 35°C
Soluble pigment	none	none

TABLE IX continued

Nutrient agar		
Growth	flat, scant growth	same as 35°C
Aerial mycelium	tan, powdery surface	same as 35°C
Soluble pigment	none	none
Starch agar		
Growth	light gray changing to brown after 6 days	same as 35°C
Aerial mycelium	white, changing to yellowish white in 8 days; powdery droplets on surface of medium	yellowish white later developing slight greenish droplets on surface of medium
Soluble pigment	none to very slight after 13 days; slight pink in color	none
Enzymatic zone	4 mm	none
Potato dextrose agar		
Growth	late in developing; grayish white vegetative; smooth, raised, & flat colonies with diffuse edge.	cream colored vegetative; flat growth
Aerial mycelium	cream colored later becoming white to yellow white; powdery surface	white going to light yellow; powdery surface
Soluble pigment	none	none
Gelatin		
Growth	yellow ring formation	yellowish white ring formation just below surface of liquified portion after 4 days

TABLE IX continued

Gelatin - continued		
Aerial mycelium	yellowish white	none
Soluble pigment	violet brown changing to rose after 14 days	light brown in liquified portion only
Liquifaction	2 inches in 15 days	total in 16 days
Kligler's iron agar		
Growth	much wrinkled, raised; gray in color	same as 35°C
Aerial mycelium	white covering entire surface	same as 35°C
Soluble pigment	none	same as 35°C
H_2S production	none	none
Skim milk		
Growth	yellowish white surface with white aerial mycelium	same as 35°C
Coagulation	none	none
Peptonization	slight; throughout tube in 5 days; no zone formed	¼" zone produced in 5 days
Soluble pigment	none	none
Litmus milk		
Growth	gray white surface	same as 35°C
Coagulation	none	none
Change of reaction	alkaline only after 12 days	same as 35°C
Reduction	none	none
Peptonization	slight after 16 days 1/16 inch.	same as 35°C

TABLE IX continued

Czapek's agar with sucrose

Growth none none

Temperature: Optimum 35°C

Generic Name of Unknown Species

Genus: Streptomyces (3, p. 929)

TABLE X

DESCRIPTION OF ORGANISMS 200, 201, 202, AND 216

General Morphological Characteristics

Straight hyphae, no spirals noted at 35°C on either Czapek's agar (modified) or calcium-malate agar. Straight sporulating hyphae with some open spiraling noted at room temperature on Czapek's agar (modified) and calcium-malate agar. Spores oval in shape.

Cultural Characteristics Observed on Various Media at 35°C and Room Temperature

	Incubation at 35°C	Incubation Room Temperature
Czapek's agar modified (glycerin)		
Growth	colorless to light yellow; flat & spreading	Grayish white; flat and spreading
Aerial mycelium	light yellow fluff with patches of white fluff	gray; powdery with patches of white
Soluble pigment	none	none
Calcium malate-glycerin agar		
Growth	yellow; flat, spreading growth	gray
Aerial mycelium	bright yellow powdery surface	gray aerial with patches of white and yellow
Soluble pigment	golden yellow	none
Glucose agar		
Growth	tan to yellow brown; raised, much wrinkled surface	deep tan to black vegetative; flat spreading, and abundant

TABLE X continued

Glucose agar - continued		
Aerial mycelium	white powdery surface	mouse gray, powdery surface with patches of white fluff
Soluble pigment	none	none
Nutrient agar		
Growth	colorless; flat, spreading	white growth; flat, spreading
Aerial mycelium	white powdery surface changing to tan with a pinkish tinge	ash white covering entire surface of growth
Soluble pigment	none	none
Starch agar*		
Growth	golden yellow; flat and spreading	gray yellow vegetative; flat & spreading growth
Aerial mycelium	sulfur yellow; powdery surface with white powdery patches	gray and powdery with patches of white fluff
Soluble pigment	none	none
Enzymatic zone	none	none
Potato dextrose agar*		
Growth	gray & golden vegetative growth mixed together; flat & spreading growth	same as 35°C
Aerial mycelium	mouse gray powdery patches mixed with golden yellow powdery patches	gray powdery patches mixed with white patches which turn golden yellow after 8 days
Soluble pigment	none	none

TABLE X continued

Gelatin		
Growth	yellow ring formation	yellow ring formation; yellow colonies throughout tube
Aerial mycelium	none	white
Soluble pigment	none	none
Liquefaction	total in 14 days (2")	total in 14 days
Kligler's iron agar		
Growth	tan; raised & much wrinkled	grayish white; raised & much wrinkled
Aerial mycelium	none	none
Soluble pigment	none	none
H_2S production	none	none
Skim milk		
Growth	yellow ring formation	grayish white ring formation
Coagulation	none	none
Soluble pigment	none	none
Peptonization	slight throughout tube after 6 days. No clear zoning	¼" zone formed at top of tube after 6 days
Litmus milk		
Growth	colorless ring formation; white aerial mycelium	same as 35°C
Coagulation	none	none

TABLE X continued

Litmus milk - continued		
Reaction	slightly alkaline after 7 days	alkaline after 7 days
Reduction	none	none
Peptonization	none	¼" after 8 days
Czapek's agar with sucrose		
Growth	scant white powdery surface growth; not penetrating into medium; no growth until after 14 days incubation	none

Temperature: Optimum 35°C

*The cultures of this species varied in the amount of patching shown on certain media such as starch agar. For instance, sections of the cultures would be yellow while adjacent sections would be gray, one color often predominating. By subculturing appropriate sections of any culture the predominating colors could be changed.

Generic Name of Unknown Species and Probable Actinomyces Group to Which the Species Belongs

Genus: Streptomyces (3, p. 929)

Actinomyces group: Streptomyces flavus group (22, pp. 24-26; 24, pp. 457-465)

tomyces parvus and Streptomyces cellulosae (22)(23). Furthermore, it may possibly be that many different species of this group would be identical with each other if they were all described on the same set of media under the same conditions of pH and temperature. For these reasons, no conclusions were drawn as to the identification of organism 200 with a particular species.

Organism 233 was tentatively classified as a member of the Streptomyces griseus group. The greenish pigmentation which is shown in the mycelium seems to justify this. This group is composed entirely of the variants of the species Streptomyces griseus which are separated on the basis of pigment and antibiotic production.

The other two species studied do not fit into any known groups. Although they each have certain properties which relate them to known species, it is the author's opinion that neither has been reported previously. After comparing organism 241 to over 150 species and strains, only one known species was found which had any major similarity to it. Organism 241 resembled Streptomyces coelicolor in its pigment production. Both pigments are litmus like in nature turning red in acid, and blue in alkali. Other than this characteristic, however, the two organisms are completely dissimilar both morphologically and culturally. Organism 241 is also definitely thermophilic, while Streptomyces coelicolor is mesophilic.

By this comparison, it was concluded that organism 241 is not just a new strain of Streptomyces coelicolor, but in most probability a new species.

Organism 58 (or 415) is characterized by its rose colored soluble pigment formed on several media. Along with this pigment there is a brown colored one produced in gelatin. Because of these pigments, the organism can be compared with Streptomyces purpurascens and Streptomyces erythrochromogenes, although it tends to resemble the former more than the latter. In its vegetative growth and aerial mycelia, however, it tends to resemble Streptomyces erythrochromogenes the closest and more so a specific strain described by Waksman in 1919. Organism 58 is weak in its diastatic action on starch, and in this it resembles only Streptomyces erythrochromogenes (5)(22). By exhibiting weak proteolytic properties, however, it resembles only Streptomyces purpurascens (22)(27) of the two species.

In spite of these close similarities to both species, there seems to be enough significant differences shown in the comparisons of each to warrant a separation of organism 58 from either. It was therefore concluded that organism 58 is a different species but belonging to a heretofore uncharacterized group of organisms in which Streptomyces purpurascens and Streptomyces erythrochromogenes are also members.

The results obtained from this work on these five species verifies the conclusions reached by Routein and Finley, Benedict, Henrici, (loc cit.) and other workers in the field by further showing that the classification of unknown species of actinomycetes can be only tentative until the work done in the past is better organized and a method of classification for the future is standardized.

SUMMARY

A favorable set of conditions for isolating actinomycetes from an acid soil first had to be determined. Isolation of actinomycete cultures was carried out until as many culturally and morphologically different appearing cultures as possible were obtained. Twenty-eight cultures were finally chosen as representative of all types of actinomycetes isolated.

These 28 cultures were then screened for antibiotic activity against 12 bacterial species using the cross streak method. Penassay base agar gave the best results out of the four media used for screening. Twenty-three or 82.2% of the cultures were active against at least one of the bacterial species. A large number of the cultures were active against gram positive bacteria. Little activity was shown toward the gram negative bacteria except by eight actinomycete cultures which were actively antagonistic to both gram positive and gram negative bacteria.

These eight highly active cultures, along with one inactive culture were characterized both morphologically and culturally in order to compare them with known actinomycete species. The nine cultures represented five different species. Of these, three organisms were identified as members of recognized groups. One unknown species belonged to the Streptomyces albus group, another to the

Streptomyces flavus group and the third to the *Streptomyces griseus* group. The other two cultures were tentatively classified as species unreported in the literature.

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