IDENTIFICATION OF STREPTOMYCES SPECIES USING
FLUORESCENT ANTIBODY-MEMBRANE FILTER
TECHNIQUES

APPROVED:

[Signatures]

Major Professor

Minor Professor

Minor Professor

Director of the Department of Biology

Dean of the Graduate School
IDENTIFICATION OF STREPTOMYCES SPECIES USING
FLUORESCENT ANTIBODY-MEMBRANE FILTER
TECHNIQUES

THESIS

Presented to the Graduate Council of the
North Texas State University in Partial
Fulfillment of the Requirements

For the Degree of

MASTER OF ARTS

By

Jackson David Singleton Jr., B. A.
Denton, Texas
August, 1967
TABLE OF CONTENTS

LIST OF TABLES ........................................ iv

Chapter

I. INTRODUCTION ........................................ 1
   History
   Statement of Problem

II. MATERIALS AND METHODS ............................. 10
   Organisms
   Cytoplasmic Antigen Preparation
   Antisera Production
   Conjugation Procedures
   Membrane Filter Methods
   Quantitative Determinations

III. RESULTS AND DISCUSSION ............................ 22
   Organisms
   Antisera Production
   Membrane Filter Methods
   Quantitative Determinations
   Conclusion

IV. SUMMARY .............................................. 35

BIBLIOGRAPHY ............................................ 37
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Heterologous Cross-Reactions Between Antisera and Cytoplasmic Antigens of <em>Streptomyces</em> Organisms</td>
<td>23</td>
</tr>
<tr>
<td>II. Comparison of Homologous Reactions Among Antibody Preparations</td>
<td>24</td>
</tr>
<tr>
<td>III. Antibody Titer of Antibody-Preparations Against Six <em>Streptomyces</em> Species</td>
<td>25</td>
</tr>
<tr>
<td>IV. Fluorescent Antibody Tests on <em>Streptomyces</em> Grown on Membranes for Various Incubation Periods</td>
<td>28</td>
</tr>
<tr>
<td>V. Quantitative Determination of <em>Streptomyces</em> Cells from Dilution Samples</td>
<td>29</td>
</tr>
<tr>
<td>VI. Quantitative Determination of <em>Streptomyces</em> Cells from Dilution Samples</td>
<td>31</td>
</tr>
</tbody>
</table>
CHAPTER I

INTRODUCTION

Serum antibodies may be labeled by chemical combination with fluorescent dyes without detectable change in the immunological properties of the protein. The resulting fluorescent antibody solution is employed as a specific cyto-chemical stain on bacterial cells. Wherever antigen-antibody conjugates form, the fluorescent-tagged antibody is fixed; unreacted fluorescent protein can be washed away. The section is then examined under a fluorescence microscope assembly; the brilliant yellow-green light from the deposited fluorescein-antibody reveals the presence and location of the homologous antigen.

History

These methods of fluoresceine labeling of antibody for microscopic tracing were introduced by Coons et al. (3). A fluorescein derivative, B-anthryl isocyanate, was used as a conjugate to label Type III antipneumococcus rabbit serum. The conjugated antiserum rendered Type III pneumococci specifically fluorescent under ultraviolet light, but Type II pneumococci did not fluoresce when exposed to the same procedure. This method developed by Coons for labeling
antibody is known as direct immunofluorescent staining.

Weller and Coons (12) developed a modified staining procedure in which indirect staining with fluorescent antibody is carried out. The specific antigen-antibody complex is rendered visible by treating it with a fluorescein-labeled antibody prepared against the specific (antibody) globulin. This procedure is generally referred to as the indirect fluorescent-antibody test.

The isocyanate compound used by Coons in these experiments was difficult to prepare and remained stable for only a short period of time. Thus, many workers were unwilling to utilize fluorescent-antibody methods until Riggs et al. (7) synthesized another fluorescent agent, fluorescein isothiocyanate, which remained stable for long periods of time. It was prepared by treating aminofluorescein with thiophosgene. When pure, it was a bright yellow powder which could be stored in an ordinary stoppered container at room temperature for at least one year without loss of activity.

Since the initial use of fluorescein isothiocyanate, a great amount of work has been done in the application of these fluorescent-antibody methods to bacteriological problems. Recently, the diagnostic possibilities of these methods have been utilized in regard to the order Actinomycetales, more specifically the genus Streptomyces.
Slack and Moore (10) used the fluorescent antibody technique to separate *Actinomyces* into three serological groups (A, B, and C). They used the methods and procedures which were explained in detail in a later experiment by Slack et al. (11). In the latter work a fourth serological group, group D, was reported. One hundred and thirty-eight cultures of *Actinomyces* species (mostly *Actinomyces bovis*, *israelii*, and *naeslundii*) were placed into one of four serological groups. A number of species of *Corynebacterium*, *Propionibacterium*, *Lactobacillus*, *Streptomyces* and *Nocardia* were found to be unreactive, even though these genera are morphologically or culturally similar to the genus *Actinomyces*. The fluorescent-antibody technique was considered more advantageous than other serological methods because it was rapidly performed and only a small amount of culture was needed.

Arai et al. (1) used the indirect fluorescent antibody test to study the serological relationships among groups of antagonistic *Streptomyces* that are related in their ability to produce antibiotics or in their morphological and cultural characteristics. These results indicated that this method can be successfully applied to the serological investigation of these organisms. It was also demonstrated that spores and sporophores were as satisfactorily stained as were shake-cultured vegetative mycelium.
Recently the fluorescent-antibody technique has been used in conjunction with membrane filter methods as a diagnostic tool in identification of various bacterial species. Carter and Leise (2) attempted to collect bacteria on membrane filters and stain them with specific fluorescein-tagged antiserum. They were unsuccessful, however, because of the natural fluorescence of the membrane filter and the difficulty of passing the ultraviolet light through the filter. A short time later they demonstrated that twelve-hour old colonies of *Bacillus anthracis* could be stained with specific fluorescent antiserum on black Millipore membrane filters. The colonies were observed with a low-power dissecting microscope using top-lighting techniques.

Ryschenkow and Wertlake (8) developed a technique applicable to microorganisms in fluid that yields specific fluorescence of organisms against a black Millipore filter background. Both the direct and indirect fluorescent-antibody techniques were used. These procedures were recommended for rapid identification of small numbers of microorganisms in liquid culture.

Danielsson (4) demonstrated that *Escherichia coli* and *Shigella guanabara*, when suspended in tap water (or cultured in nutrient broth) and then trapped on non-fluorescent membrane filters, could be identified within one hour by means of the fluorescent-antibody method. The number of bacteria suspended in test samples was estimated with a Burker
counting chamber and by plating on Endo agar. His results indicated the recovery of the test organisms by direct count of fluorescing cells on membrane filters ranged from 61-100 percent of the total number of added test bacteria counted with a Burker chamber. The range was 63-101 percent when compared with viable cell counts on Endo agar. Thus, the technique also allowed a quantitative determination of the bacteria identified serologically in concentrations of 100,000 cells per liter or more.

In continuation of this work, Danielsson and Laurell (5) increased the sensitivity for demonstration of small quantities of bacteria in water by means of the fluorescent-antibody technique in combination with the membrane filter method. By direct staining of bacteria on non-fluorescent membrane filters, the lower limit could be reduced to 1000 bacteria per liter. Another procedure, involving a one to two hour growth period in liquid enrichment media, demonstrated bacteria present in a concentration of two to fifty cells per liter. These methods also functioned well for mixed cultures in which an unidentified bacteria was dominant in relation to the bacteria diagnosed. Thus, this experiment offers new possibilities in reducing the time required for diagnostic identification of organisms trapped on a membrane filter as well as providing a sensitive method of quantitative determination of cells in pure or mixed culture on the filter.
The fluorescent-antibody technique and the membrane filter method together can be applied to provide a means of rapid identification of members of the genus **Streptomyces**. This application would become useful in light of recent discoveries concerning taste and odor compounds present in streams and reservoirs; many of these reservoirs provide a major water supply for urban areas. Silvey et al. (9) found that a number of taste and odor compounds may be attributed to members of the genus **Streptomyces**. These odors are sometimes described as "marshy," "woody," "fishy," "earthy," and "musty". Field and laboratory data (6) now show that the taste and odor compounds can serve as a source of nutrients for the gram-positive heterotrophic bacilli, including **Bacillus cereus**. If this organism is added in large volumes to a reservoir before the taste and odor compounds reach too high a threshold odor level, it has been theorized that destruction of these compounds will occur as they are produced by the **Streptomyces** (6). An indicator of the threshold level is the **Streptomyces** population density, which is most likely determined from colony counts of water samples taken at or near the surface away from the shore. A rapid identification is necessary in order to perceive any rise in population density before the concentration of taste and odor compounds reaches a level which cannot be controlled by the addition of **Bacillus cereus**. The one to two week period now necessary for culture and identification of
Streptomyces is too long for applications of the above procedure.

Statement of Problem

It is the purpose of this investigation to modify existing methods in an attempt to provide a rapid identification of members of the genus Streptomyces.
CHAPTER BIBLIOGRAPHY


CHAPTER II

MATERIALS AND METHODS

Organisms

The organisms used in this study were obtained from the American Type Culture Collection (ATCC), Washington, D. C., and the International Streptomyces Project (ISP), Ohio Wesleyan University, Dr. E. B. Shirling. The following organisms were used: Streptomyces parvus, ATCC 12433, Streptomyces purpureochromogenes, ATCC 3343, Streptomyces griseus, ATCC 10137, Streptomyces odorifer, ISP 5347, Streptomyces antibioticus, ISP 5234, and Streptomyces parvus, ISP 5348. All stock cultures were maintained in tubes of tomato-juice oatmeal agar slants. To prepare the medium, twenty grams of oatmeal and fifty-one grams of tomato-juice agar (Difco Laboratories, Detroit, Michigan) were dissolved in one liter of distilled water. The final pH was adjusted to 6.3 with sodium hydroxide. The organisms were grown and maintained at room temperature.

Cytoplasmic Antigen Preparation

A loopful of stock culture was inoculated into 250 milliliter Erlenmeyer flasks containing fifty milliliters of Difco Emerson's Broth. This was incubated for six days on a rotary shaker (Eberbach Corp., Ann Arbor, Michigan) at room
temperature, after which time a five milliliter inoculum of this suspended culture was transferred to a one liter Erlenmeyer flask containing 250-300 milliliters of Emerson's Broth. Each new culture was placed on a rotary shaker for six days at room temperature. At the end of this period, the flasks were removed and allowed to remain stationary for eight days; thus, a total culture period of two weeks elapsed during incubation.

At the end of the incubation period, immediately before harvesting the organisms, samples were taken from the flask and inoculated in triplicate onto tomato-juice oatmeal agar. At the end of three days these plates were macroscopically observed for possible contamination of the flask culture.

To collect the Streptomyces cultures, which consisted of suspended mycelial clumps as well as a mycelial mat extending over the surface of the medium, the flask contents were filtered through Munktell (number six) filter paper discs. The mass of mycelium retained was collected and placed in a small beaker.

Each beaker was then partially submerged in an ice bath and sonicated for fifteen minutes with a Branson Model S-75 sonifier (Branson Instruments, Inc., 37 Brown House Road, Stamford, Connecticut), disrupting the cell walls and releasing cell cytoplasm. A small sample was examined microscopically to verify the breakage of cell walls. Subsequently, this mixture was centrifuged in an International
PR-2 centrifuge (5°C) for twenty minutes at 12,000 rpm. The resulting supernatant, containing the cytoplasmic antigenic material, was collected and stored at approximately -65°C. The antigenic material obtained in this way was used for both antiserum production and immunodiffusion testing. These methods of cell growth and cytoplasmic extraction followed closely those presented by Guthrie et al. (4) and modified by Hutcheson (5) and Brunson (1).

Antisera Production

The cytoplasmic antigenic fraction was emulsified in Freund's incomplete adjuvant at a 1:1 ratio and the subcutaneous injection schedule established by Guthrie, Roach, and Ferguson (3) was followed. Rabbits weighing approximately one and one-half kilograms were injected with two milliliters of the emulsified material every other day for a series of three injections. One week following the last injection, another series of three injections was made; thus, each animal received a total of six milliliters of the cytoplasmic fraction.

The rabbits were bled from the ear artery once before the injection schedule began and each succeeding week thereafter until the antibody titer reached 1:64. Then, during the next two week period, forty to sixty milliliters of blood were taken intracardially to be used in subsequent conjugation procedures. The antisera were stored at -65°C until further use.
To determine the antibody titer of antisera, gamma-globulin, and conjugates used in this study, methods patterned after the agar-gel double diffusion technique of Ouchterlony (8) were used. Petri plates were prepared which were filled with twenty-five milliliters of one percent Ionagar No. 2 (Consolidated Laboratories, Inc., Chicago Heights, Illinois) containing phenol in a final concentration of five-tenth percent. Wells were cut in the agar using a "Feinburg Agar Gel Cutter" (Consolidated Laboratories, Inc., Chicago Heights, Illinois). The well pattern consisted of six smaller wells symmetrically surrounding a larger center well. Antiserum was added to the large well and dilutions of specific antigen solution were added to the smaller wells. An eleven centimeter Munktell No. 3 filter paper disc saturated with distilled water was placed over the petri plate bottom to provide a moist atmosphere. A positive antigen-antibody reaction consisted of the presence in the agar of a band of precipitation between the center well and any of the smaller wells after an incubation period of four days.

Conjugation Procedures

The conjugation procedures employed required extraction of gamma-globulin from serum, conjugation of fluorescein isothiocyanate to the serum fraction, and removal of unreacted fluorescein dye from the conjugated solution. Initially whole serum was conjugated, but weak staining of
antigens resulted. Thus, the gamma-globulin fraction was extracted from the serum to reduce non-specific reactions as well as provide a more concentrated antibody source. Two fractionation procedures were used. The first procedure was that set forth by Campbell et al. (2), which involved the addition of saturated ammonium sulfate solution to the serum. A method yielding more gamma-globulin was later used in which the serum solution was made to a final concentration of thirty-five percent ammonium sulfate by slow addition of the solid crystals (209 grams ammonium sulfate per liter of serum). Thirty minutes were allowed for equilibration. The solution was centrifuged for twenty minutes at 5000 rpm, the supernatant decanted, and the precipitated gamma-globulin dissolved in phosphate-buffered saline (one-fourth original volume). This solution was then dialyzed for three days against phosphate buffered saline (pH 7.2). A volume of one liter was changed twice daily until the sulfate ions were removed.

To determine the protein concentration of the serum, gamma-globulin fractions, and conjugated solutions, two methods were utilized. The biuret method described by Campbell et al. (2) was first used, but later discontinued in favor of the more sensitive Folin-Ciocalteau method of Lowry et al. (6).

The actual conjugation procedure was carried out with fluorescein isothiocyanate (Colorado Serum Co., Denver,
Colorado) conjugated to either a serum solution of three percent protein or a gamma-globulin solution of five percent protein. The former was patterned after the procedure developed by Marshall et al. (7). The solution was adjusted to pH 9.0 using carbonate-bicarbonate solution and cooled to 4°C in a refrigerator. The fluorescein isothiocyanate (FITC) powder was added with constant stirring to a final concentration of 0.05 milligram dye per 1.0 milligram protein. The conjugation process was allowed to continue for eighteen hours. The conjugate was placed in a dialysis bag and dialyzed at 5°C against phosphate-buffered saline (pH 7.2) until as much unconjugated dye as possible was removed. This usually took two one-liter volume changes each day for three days. Using this procedure, antisera against Streptomyces parvus (ATCC 12433), Streptomyces griseus (ATCC 10137), and Streptomyces purpureochromogenes (ATCC 3343) were conjugated separately and also as an equally pooled mixture. Normal rabbit serum (i.e., without detectable antibody against Streptomyces) was conjugated to be used in control tests.

To conjugate the extracted gamma-globulin fractions, the conjugation procedure developed by Spendlove (9) was followed. A two percent FITC to protein ratio was used and the final conjugate was diluted to one milligram protein per milliliter. Using this method, antisera against Streptomyces parvus (ISP 5348), Streptomyces antibioticus (ISP 5234), and
Streptomyces odorifer (ISP 5347) were conjugated, as well as normal rabbit gamma-globulin.

To remove the smaller unconjugated FITC molecules from the larger protein-FITC conjugated molecules, a Pharmacia eighteen inch Sephadex column (Pharmacia Ltd., Uppsala, Sweden) was used. The conjugate sample was eluted through G-25 Sephadex with phosphate-buffered saline. This procedure was employed with all conjugated gamma-globulin fractions.

Membrane Filter Methods

A loopful of stock culture was aseptically placed in a 250 milliliter Erlenmeyer flask containing fifty milliliters of Emerson's Broth and cultured for five to eight days on a rotary shaker at room temperature. When heavy growth of mycelial clusters were visibly suspended in the medium, five milliliters of this suspension were transferred to a centrifuge tube, centrifuged, and washed twice with distilled water. The cells were then diluted with ten milliliters of distilled water, the larger mycelial clumps allowed to settle, and from the suspension containing smaller mycelial strands, a sample was taken and diluted 1:10,000. This final dilution was used to provide cell suspensions to be filtered onto membrane filters.

The following five types of membrane filters were used: Gelman GA-6 and type black-6-grid (Gelman Instrument Co.,
Ann Arbor, Michigan), and Millipore HAWP, HABP, and HABG (Millipore Filter Corp., Bedford, Massachusetts). Each of these types had a pore size of 0.45 microns and were forty-seven millimeters in diameter.

The membrane filters were sterilized by tightly packing them between several thicknesses of filter paper in a closed petri plate and autoclaving at fifteen pounds pressure for fifteen minutes.

A pyrex filter holder with a 250 milliliter funnel, on which a Millipore black membrane filter was applied, was used to filter the 1:10,000 dilution samples of Streptomyces organisms. One milliliter of the sample was added to fifty milliliters of water present in the funnel and filtered by application of negative pressure. The membrane was immediately removed and placed on a tomato-juice oatmeal agar plate. The membranes were incubated for eighteen, twenty-four, forty-eight, and eighty-one hours before they were stained with specific conjugate to test for fluorescence.

To stain the organisms trapped on the membrane filter, the FITC-gamma-globulin conjugate was flooded over the surface of the membrane. The membrane was then incubated for thirty minutes in a humid chamber. Fresh conjugate was added if necessary. The unreacted conjugate was removed by immersing the membrane in phosphate-buffered saline (PBS), pH 7.2, for ten minutes. This was repeated two more times in PBS for additional ten minute periods. The membranes
were dried in a drying oven for three minutes at 90°C. Each of the organisms from the ISP collection (S. parvus, S. odorifer, and S. antibioticus) were cultured and stained on the membrane filters by this method.

The following controls were also concurrently performed: (a) normal conjugated rabbit gamma-globulin was flooded on a membrane containing the above filtered organisms, and (b) a membrane with Streptomyces colonies was pretreated with specific unlabeled antisera, washed, and then flooded with the specific conjugated gamma-globulin.

The stained colonies were observed under a low power dissecting scope with a Bausch and Lomb Mercury Power Supply SP 200 and a lamphouse containing a 200 watt Mercury arc lamp. A 5-58 exciter filter and a Y-8 barrier filter were utilized for light filtration. A top-lighting technique was used in which the light rays were projected obliquely on the exposed membrane surface at a thirty degree angle.

Quantitative Determinations

A culture of Streptomyces antibioticus was cultured in Emerson's Broth, centrifuged, and collected using the procedure previously presented. A stock dilution of 1:1000 was prepared with sterile tap water. From this stock dilution, a two-fold dilution was carried out to 1:8000. Thus, final dilutions of 1:1000, 1:2000, 1:4000, and 1:8000 were obtained. From another cell sample, dilutions of 1:300,
1:600, 1:1200, and 1:2400 were collected. For each dilution
the following three procedures were performed in triplicate:
(a) two-tenths milliliter was pipetted onto a tomato-juice
oatmeal agar plate and spread, (b) one milliliter was pi-
petted into a twenty-five milliliter sterile water aliquot
and filtered through a Millipore HABG membrane filter. The
filter was then placed on a tomato-juice oatmeal agar plate,
and (c) the second procedure, (b), was repeated.

The agar plates from procedures (a) and (b) were
incubated for seventy-two hours and the characteristic
Streptomyces antibioticus colonies were macroscopically
counted. The plates from procedure (c) were incubated for
only forty-eight hours and then stained in the described
manner. To remove the excess stain, the membranes were
placed on a filter holder with an attached funnel. Dis-
tilled water was washed through the membrane filter (using
negative pressure) carrying the excess stain with it. The
fluorescing colonies were then counted under a dissecting
scope equipped with the same ultraviolet assembly pre-
viously described.
CHAPTER BIBLIOGRAPHY


CHAPTER III

RESULTS AND DISCUSSION

Organisms

These specific *Streptomyces* species were chosen because the rabbit antiserum against each has demonstrated serological cross-reactions with a number of *Streptomyces* species. The three antisera against the organisms selected from the International Streptomyces Project (ISP) were found in a recent study to produce positive reactions with eighty-seven to ninety-one percent of twenty-four different species tested (2). This high percentage of cross-reactions increased the possibility of getting a positive test between an unknown Streptomycete and at least one of the three antisera. This possibility was further enhanced when the fluorescent-antibody technique was used, which is considered serologically more sensitive than the agar-gel diffusion test.

The antiserum against each of the six *Streptomyces* species were tested with the cytoplasmic fraction of each species using the agar-gel diffusion test. Table I presents the heterologous reactions of these tests. The results are recorded as the number of bands present in the agar. Each band represents a specific antigen-antibody precipitate.
Table I

<table>
<thead>
<tr>
<th>Antisera</th>
<th>Cytoplasm of Streptomyces Organisms*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATCC 3343</td>
</tr>
<tr>
<td><strong>S. purpureo</strong></td>
<td></td>
</tr>
<tr>
<td>ATCC 3343</td>
<td>3</td>
</tr>
<tr>
<td><strong>S. parvus</strong></td>
<td></td>
</tr>
<tr>
<td>ATCC 12433</td>
<td>3</td>
</tr>
<tr>
<td><strong>S. griseus</strong></td>
<td></td>
</tr>
<tr>
<td>ATCC 10137</td>
<td>0</td>
</tr>
<tr>
<td><strong>S. antibioticus</strong></td>
<td></td>
</tr>
<tr>
<td>ISP 5234</td>
<td>1</td>
</tr>
<tr>
<td><strong>S. parvus</strong></td>
<td></td>
</tr>
<tr>
<td>ISP 5348</td>
<td>1</td>
</tr>
<tr>
<td><strong>S. odorifer</strong></td>
<td></td>
</tr>
<tr>
<td>ISP 5347</td>
<td>2</td>
</tr>
</tbody>
</table>

*Listed by American Type Culture Collection Number or International Streptomyces Project Number.

**Streptomyces purpureochromogenes.

Table I indicates that all of the antisera contain antibody specific for at least one antigen present in each cytoplasmic fraction (except between S. griseus and S. purpureochromogenes). Thus, any of these antisera should be useful in the fluorescent-antibody test to give a positive reaction with all Streptomyces species which have an antigen in common with these cytoplasmic fractions.
Antisera Production

Agar-gel diffusion tests were run on the antisera, gamma-globulin, and/or conjugate specific for each Streptomyces species used in this study. The purpose of these tests was to determine whether any antigens were lost in preparation of gamma-globulin from antiserum or in preparation of conjugate from gamma-globulin. Table II records the results of these tests as the number of visible bands.

TABLE II

COMPARISON OF HOMOLOGOUS REACTIONS AMONG ANTIBODY PREPARATIONS

<table>
<thead>
<tr>
<th>Cytoplasmic Solutions</th>
<th>Homologous Antibody Preparations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Antiserum</td>
</tr>
<tr>
<td><em>S. odorifer ISP 5347</em></td>
<td>3</td>
</tr>
<tr>
<td><em>S. parvus ISP 5348</em></td>
<td>3</td>
</tr>
<tr>
<td><em>antibioticus</em> ISP 5234</td>
<td>4</td>
</tr>
<tr>
<td><em>S. parvus ATCC 12433</em></td>
<td>2</td>
</tr>
<tr>
<td><em>purpureochromogenes</em></td>
<td>3</td>
</tr>
<tr>
<td><em>griseus ATCC 10137</em></td>
<td>2</td>
</tr>
</tbody>
</table>

*This conjugate was a mixture of *S. griseus*, *S. parvus*, and *S. purpureochromogenes* conjugated antiserum.

The loss of bands, as indicated by comparing the gamma-globulin reactions with the conjugate reactions, suggested that there was a decrease of certain antigen-specific antibody in the conjugated gamma-globulin (conjugate). This could possibly reduce cross-reactivity between the conjugate and any Streptomyces species which contained antigen specific
for the antibody that had decreased in concentration. Because the fluorescent-antibody (FA) test is more sensitive than agar-gel diffusion, antibody which did not show up in the latter may still be present in enough concentration in the conjugate to give positive results in the FA test. Thus, Table II can only be used to show a decrease in antibody concentration, and does not show complete loss.

Another important consideration in a comparison of the three antibody preparations was the change in antibody titer that could occur in the extraction of gamma-globulin from antiserum and its conjugation to FITC. Table III presents the antibody titer (determined by agar-gel diffusion) for those antibody preparations tested against each of the six organisms used.

**TABLE III**

**ANTIBODY TITER OF ANTIBODY-PREPARATIONS AGAINST SIX STREPTOMYCES SPECIES**

<table>
<thead>
<tr>
<th>Cytoplasmic Solutions</th>
<th>Homologous Antibody Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Antiserum</td>
</tr>
<tr>
<td><strong>S. odorifer ISP 5347</strong></td>
<td>1:64</td>
</tr>
<tr>
<td><strong>S. parvus ISP 5348</strong></td>
<td>1:64</td>
</tr>
<tr>
<td><strong>S. antibioticus ISP 5234</strong></td>
<td>1:128</td>
</tr>
<tr>
<td><strong>S. parvus ATCC 12433</strong></td>
<td>1:64</td>
</tr>
<tr>
<td><strong>S. purpureochromogenes ATCC 3343</strong></td>
<td>1:64</td>
</tr>
<tr>
<td><strong>S. griseus ATCC 10137</strong></td>
<td>1:128</td>
</tr>
</tbody>
</table>

*This conjugate was a mixture of *S. griseus*, *S. parvus*, and *S. purpureochromogenes* conjugated antiserum.*
The results in this table indicated that there was a definite decrease in antibody concentration in the preparation of conjugate from gamma-globulin. This was to be expected due to the high dilution of the conjugate. It was necessary to dilute the conjugate in order to eliminate non-specific reactions which occur with a high concentration of conjugated dye. The reduction in antibody titer of the conjugate was not enough to reduce the positive reactions obtained by its use in the fluorescent-antibody test. By diluting the conjugate to one milligram protein per milliliter, strong positive reactions were still obtained, but non-specific reactions were eliminated.

Part of this dilution was accomplished by elution from the sephadex column. This procedure not only resulted in about fifty percent dilution, but also removed practically all unconjugated FITC, one of the causes of non-specific staining. The sephadex column proved much more effective than dialysis in removing unconjugated dye.

Membrane Filter Methods

A number of cell dilutions were filtered on membrane filters in an attempt to find a dilution which would yield a separation of Streptomyces colonies on the membrane surface. If the mycelial concentration was too large, a solid mycelial mat covered the membrane; if too dilute, no colonies were present.
Six different types of membrane filters were tested to determine which were best suited for the purpose of this study. Two problems were encountered. All of the white membrane filters autofluoresced when exposed to ultra-violet light. Fluorescent colonies were still observed, although not as distinctly as on the black membrane filters. The second problem was the brittleness of all the membrane filters after they were autoclaved, with the exception of Millipore black filters (HAB plain and HAB grid). The brittle membranes were easily cracked or broken when handled. Thus, the two types of Millipore black filters were selected for use in this study.

The optimum incubation period for colonies of \textit{S. parvus} (ISP 5348), \textit{S. antibioticus} (ISP 5234), and \textit{S. odorifer} (ISP 5347) on the membrane filter was determined. It was necessary to incubate the cells long enough to obtain positive results using the fluorescent-antibody technique, but not so long as to nullify the advantages of a rapid identification test. Concurrently, two controls were run on all three organisms, i.e., normal conjugated rabbit gamma-globulin was flooded on a membrane containing each of the filtered organisms, and a membrane with the \textit{Streptomyces} colonies was pretreated with specific unlabelled antisera, washed with phosphate-buffered saline, and then flooded with the specific conjugated gamma-globulin. The results of fluorescent-antibody tests on \textit{Streptomyces} colonies incubated on
membranes for eighteen, twenty-four, forty-eight, and eighty-one hours are recorded in Table IV.

**TABLE IV**

**FLUORESCENT ANTIBODY TESTS ON STREPTOMYCES GROWN ON MEMBRANES FOR VARIOUS INCUBATION PERIODS**

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Incubation Period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>18 Hour</td>
</tr>
<tr>
<td><strong>S. parvus</strong></td>
<td></td>
</tr>
<tr>
<td>ISP 5348</td>
<td>0</td>
</tr>
<tr>
<td><strong>S. antibioticus</strong></td>
<td></td>
</tr>
<tr>
<td>ISP 5234</td>
<td>0</td>
</tr>
<tr>
<td><strong>S. odorifer</strong></td>
<td></td>
</tr>
<tr>
<td>ISP 5347</td>
<td>0</td>
</tr>
<tr>
<td><strong>Controls</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

*Results are recorded as follows:

0 = no fluorescence  
+ = slight fluorescence  
++ = moderate fluorescence  
+++ = bright fluorescence

At eighteen and twenty-four hours, no positive fluorescence was observed, nor were any colonies macroscopically visible. After a forty-eight hour incubation period, distinct and specific fluorescence of colonies occurred. At this age the colonies were small with no definite diagnostic characteristics. The eighty-one hour incubated colonies demonstrated the same intensity of staining; the colonies were macroscopically large and well defined. None of the controls showed any fluorescence on the colonies.
Thus, the optimum incubation time was forty-eight hours. Colonies at this age produced consistent positive results when stained with specific conjugate. Also for this period of time, the test was relatively rapid compared to other diagnostic tests for *Streptomyces* organisms.

Quantitative Determinations

The tests to quantitate dilutions of *Streptomyces* were preliminary investigations that demonstrated the possible efficiency of such tests as well as some of the problems encountered. Table V contains data from a series of dilutions collected from a sample of suspended *Streptomyces antibioticus* mycelium.

**TABLE V**

**QUANTITATIVE DETERMINATION OF STREPTOMYCES CELLS FROM DILUTION SAMPLES**

<table>
<thead>
<tr>
<th>Colony Location</th>
<th>Dilution</th>
<th>Direct Colony Count*</th>
<th># of Cells/ml by Direct Colony Count**</th>
<th>Ultraviolet Colony Count*</th>
<th># of Cells/ml by U.V. Colony Count**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane Filter</td>
<td>1:1000</td>
<td>12</td>
<td>$1.2 \times 10^4$</td>
<td>13</td>
<td>$1.3 \times 10^4$</td>
</tr>
<tr>
<td></td>
<td>1:2000</td>
<td>5</td>
<td>$1.0 \times 10^4$</td>
<td>5</td>
<td>$1.0 \times 10^4$</td>
</tr>
<tr>
<td></td>
<td>1:4000</td>
<td>5</td>
<td>$2.0 \times 10^4$</td>
<td>6</td>
<td>$2.4 \times 10^4$</td>
</tr>
<tr>
<td></td>
<td>1:8000</td>
<td>1</td>
<td>$8.0 \times 10^3$</td>
<td>4</td>
<td>$3.2 \times 10^4$</td>
</tr>
<tr>
<td>Spread Plate</td>
<td>1:5000</td>
<td>2</td>
<td>$1.0 \times 10^4$</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td>1:10,000</td>
<td>4</td>
<td>$4.0 \times 10^4$</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td>1:20,000</td>
<td>3</td>
<td>$6.6 \times 10^3$</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td>1:40,000</td>
<td>3</td>
<td>$1.3 \times 10^4$</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

*These figures represent the average number of colonies per plate from three plates.

**These figures are to the nearest thousand.
Table V is divided into two sections representing Streptomyces dilutions on a membrane filter and dilutions on a spread plate. To record this data, the average number of colonies found on three plates of each dilution was determined. This was then multiplied by the dilution factor to give a number of cells per milliliter of cell suspension.

A comparison of the number of cells per milliliter in each dilution revealed that the cell quantities were relatively close considering some of the innate problems associated with dilutions of Streptomyces. First of all, these organisms clump when grown in a restricted environment (as in this experiment); and secondly, any dilution procedure produces a certain amount of error due to inaccuracy of measurement. There were only a few dilution samples in which there existed a relatively wide variance in cells per milliliter.

In a comparison of the average number of cells per milliliter counted on the spread plate with the average number of cells counted on the membrane filter by fluorescent staining, it was found that there were less than 2500 cells per milliliter difference.

In each dilution the number of colonies found on the membrane filter by direct count was very close to the number found on the filter by fluorescent staining. This demonstrates an acceptable degree of accuracy in a quantitative count of the colonies by fluorescent staining of Streptomyces.
Table VI presents results of another sample of suspended *Streptomyces antibioticus* mycelium, which was diluted and quantitated as before.

**TABLE VI**

**QUANTITATIVE DETERMINATION OF *STREPTOMYCES* CELLS FROM DILUTION SAMPLES**

<table>
<thead>
<tr>
<th>Colony Location</th>
<th>Dilution</th>
<th>Direct Colony Count*</th>
<th># of Cells/ml by Direct Colony Count**</th>
<th>Ultra-violet Colony Count*</th>
<th># of Cells/ml by U.V. Colony Count**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane</td>
<td>1:300</td>
<td>573</td>
<td>$1.7 \times 10^5$</td>
<td>455</td>
<td>$1.4 \times 10^5$</td>
</tr>
<tr>
<td></td>
<td>1:600</td>
<td>25</td>
<td>$1.5 \times 10^4$</td>
<td>56</td>
<td>$3.4 \times 10^4$</td>
</tr>
<tr>
<td></td>
<td>1:1200</td>
<td>18</td>
<td>$2.2 \times 10^4$</td>
<td>46</td>
<td>$5.5 \times 10^4$</td>
</tr>
<tr>
<td></td>
<td>1:2400</td>
<td>10</td>
<td>$2.4 \times 10^4$</td>
<td>14</td>
<td>$3.4 \times 10^4$</td>
</tr>
<tr>
<td>Spread</td>
<td>1:1500</td>
<td>193</td>
<td>$2.9 \times 10^5$</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>1:3000</td>
<td>20</td>
<td>$6.0 \times 10^4$</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>1:6000</td>
<td>15</td>
<td>$9.0 \times 10^4$</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>1:12,000</td>
<td>5</td>
<td>$6.0 \times 10^4$</td>
<td>***</td>
<td>***</td>
</tr>
</tbody>
</table>

*These figures represent the average number of colonies per plate from three plates.

**These figures are to the nearest thousand.

Table VI is set up as Table V. The cell dilutions were lower, thus a higher concentration of colonies appeared on the membrane filters and spread plates. The correlation of results between direct colony counts and counts using FA techniques was not as close in this table as in Table V, but again this is due to the clumping of mycelial strands. The higher counts in this table provide more accurate data than the counts in Table V.
Conclusion

Several methods presented in this study were either modifications of procedures previously published or were original procedures. Use of a top lighting technique with a low-power dissecting microscope was mentioned by Carter and Leise (1), but no explanation of procedure was given. In this study, in order to reflect as much ultra-violet light as possible into the lens of the dissecting scope, it was necessary to experimentally determine the optimum angle of incidence. Procedures developed in this study included the incubation of *Streptomyces* on membrane filters, and the staining technique applied to *Streptomyces* on membrane filters.

These methods provide a rapid means of diagnostic identification of some *Streptomyces* organisms. By combining various specific poly-valent anti-*Streptomyces* conjugates, it seems probable that a conjugate solution could be made that would provide positive FA reactions for the large number of *Streptomyces* species commonly found in streams and reservoirs.

More work is necessary before complete and effective quantitative methods are perfected, although preliminary investigations in this area were presented here. This problem may be simplified by using *Streptomyces* samples directly from natural water samples, which would not have the amount of mycelial clumping caused by growth in a restricted environment.
When these problems have been overcome, the techniques and methods found in this study may play a part in reducing the taste and odor compounds of lakes and reservoirs produced by *Streptomyces*.

CHAPTER IV

SUMMARY

By using the fluorescent-antibody technique in conjunction with membrane filter methods, a means of rapid identification and quantitation of *Streptomyces* organisms was developed. Broth cultures of *Streptomyces* were diluted, filtered onto membrane filters, and incubated on solid medium. The membranes were removed and the *Streptomyces* colonies stained with specific conjugate. Observation of fluorescent colonies was made using a low-power dissecting scope equipped with an ultraviolet light source. A top-lighting technique was employed in which the ultraviolet light was projected obliquely at a thirty degree angle onto the exposed membrane surface.

The optimum incubation period for *Streptomyces* colonies on the membrane filter was forty-eight hours. The colonies at this age displayed consistently positive fluorescence when stained with specific conjugate. Thus, the time necessary for identification of *Streptomyces* was reduced from one or more weeks to forty-eight hours.

Preliminary investigations to quantitate dilutions of *Streptomyces* were made using the techniques described. The results indicated possible usefulness of such tests. More
work is necessary before complete and effective quantitative methods are perfected.
BIBLIOGRAPHY

Books


Articles


Unpublished Materials


