Collected Papers on **Antibiotics**

Section XI

«Description, Classification & Identification of Streptomycetes Producing Antibiotics»

April 1976

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METHODS FOR CHARACTEROZATION OF *STREPTOMYCES* SPECIES

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METHODS FOR CHARACTERIZATION OF STREPTOMYCES SPECIES[1]

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ABSTRACT. The methods used by collaborators in the International Streptomyces Project (ISP) for emendation of descriptions of type and neotype strains of the genus Streptomyces (Actinomycetales) are presented.

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An international cooperative effort, now in progress, is directed toward collection of type cultures of the Streptomyces species for deposition with the Centraalbureau voor Schimmelcultures (CBS), Baarn. From this center the reference cultures will be supplied to other culture collections so that they are available throughout the world.

An essential adjunct to this activity is the redescription of each type culture in terms of currently acceptable criteria and methods. The urgent need for an authentic reference collection, accompanied by standardized characterizations for each species, has been pointed out by spokesmen for the several meetings and conferences which culminated in this project. (See, for example, Gottlieb, 1959, 1961; Küster, 1959; Krasil'nikov, 1961.) More than 40 investigators[2] representing 17 countries are participating in this research. Each culture is described independently by three of these cooperating specialists in different laboratories before it is deposited in the reference collection.

[1] This project is supported in part by a research grant from the National Science Foundation, U.S.A. The Subcommittee on Actinomycetes of the Committee on Taxonomy, A.S.M. and the Subcommittee on Taxonomy of Actinomycetes of the International Committee on Bacteriological Nomenclature are co-sponsoring advisors.

[2] Participants in the 1954-1965 studies are listed on p. 338.

This manual contains the criteria and methods adopted for the project. It reflects the results of two extensive cooperative studies directed toward selection of stable properties and reproducible procedures for characterization of streptomycetes. One study conducted under the direction of the Subcommittee on Actinomycetes of the Committee on Taxonomy, American Society for Microbiology was reported by the Chairman, Dr. D. Gottlieb (1961). A similar preliminary study on an international basis was reported for the Subcommittee on Taxonomy of Actinomycetes of the International Committee on Bacteriological Nomenclature by the Secretary, Dr. E. Küster (1961, 1964). The descriptive criteria are essentially the same as those included in the recommendations of this international subcommittee for descriptions of Actinomycetales appearing in patent applications (Gottlieb, 1963). The methods in mimeographed form have been used successfully for the description of type and neotype strains of more than 200 named species submitted to ISP collaborators during 1964 and 1965, and are now in use for a continuation of the project. Only minor editorial changes have been made except that the test for nitrate reduction (including medium 8, Bacto-nitrate broth) has been omitted. This characteristic proved unstable and has been dropped from the study.

It is hoped that the characterizations used in this manual will be included in future descriptions of Streptomyces species so that direct comparison can be made with descriptions for type cultures in the reference collections.

MATERIALS AND GENERAL METHODS

CULTURE MEDIA

Prepare Difco[3], [4] dehydrated culture media as instructed on labels of containers. If the dehydrated media are not used, use formulas and instructions in this manual as a guide to preparation of the media.

[3] All culture media described in this manual have been prepared especially for the I.S.P. by Difco Laboratories as preformulated dehydrated media. This important contribution by Difco Laboratories is gratefully acknowledged. When Difco dehydrated media are used, instructions on labels supersede instructions in the manual.

[4] Difco Laboratories, Detroit, Michigan, U.S.A. 48201

Sterilize culture media in the autoclave at 121°C. Sterilize loosely packed tubes or flasks containing less than 500 ml for 15 minutes; sterilize larger quantities for 20 minutes. (Do not autoclave carbon compounds to be used in the carbon utilization tests. Special instructions for sterilizing these compounds are given with medium 9.) Adjust pH of media with NaOH or HCl before addition of agar and before sterilization.

Trace salts solution (Use as directed in media 3, 4, 5 and 7 if prepared from formulas. Do not add to the corresponding Difco dehydrated media.)

$FeSO_4 \cdot 7H_2O$	0.1 g
$MnCl_2 \cdot 4H_2O$	0.1 g
$ZnSO_4 \cdot 7H_2O$	0.1 g
Distilled water	100.0 ml

Medium 1: Tryptone-yeast extract broth (Pridham and Gottlieb, 1948)

Bacto-Tryptone (Difco)	5.0 g
Bacto-Yeast Extract (Difco)	3.0 g
Distilled water	1.0 liter

pH 7.0 to 7.2 before autoclaving

Dispense 5 ml of broth into test tubes with a diameter of 20 mm or more. Use these test tubes for initiating growth from lyophile pellet. One tube will be needed for each culture studied.

Dispense 50 ml of the broth into each 250 ml Erlenmeyer flask (or 25 ml into 125 ml flask). These flasks will be used for preparation of washed inoculum (p. 322). One flask will be needed for each culture studied.

Medium 2: Yeast extract—malt extract agar (Pridham et al., 1956-57)

Bacto-Yeast Extract (Difco)	4.0 g
Bacto-Malt Extract (Difco)	10.0 g
Bacto-Dextrose (Difco)	4.0 g
Distilled water	1.0 liter

Adjust to pH 7.3, then add --

Bacto agar	20.0 g

Liquefy agar by steaming at 100° C for 15-20 minutes.

Dispense appropriate amount for a slanting into at least 6 tubes for each culture. Sterilize by autoclaving; cool tubes as slants. Use the agar slants for preparation of stock cultures (page 321).

Also sterilize medium 2 in flasks for pouring the sterilized medium into Petri dishes. Seven standard 90 mm dishes containing 25 ml per plate will be needed for each culture. (Pages 325 and 330).

Medium 3: Oatmeal agar (Küster, 1959a).

Oatmeal. . . . . 20.0 g

Agar. . . . . 18.0 g

Cook or steam 20 g oatmeal in 1000 ml distilled water for 20 minutes.

Filter through cheese cloth.

Add distilled water to restore volume of filtrate to 1000 ml.

Add trace salts solution (page 315). . . 1.0 ml

Adjust to pH 7.2 with NaOH.

Add 18 g agar; liquefy by steaming at 100°C for 15-20 minutes.

Sterlize in flasks for pouring into Petri dishes. Seven standard 90 mm dishes containing 25 ml per dish will be needed for each culture. (Pages 325 and 330)

Swirl medium before pouring to assure even distribution of the oatmeal.

Medium 4: Inorganic salts-starch agar (Küster, 1959a.)

Solution I: Difco soluble starch 10.0 g. Make a paste of the starch with a small amount of cold distilled water and bring to a volume of 500 ml.

Solution II:

K_2HPO_4 (anhydrous basis)	1.0 g
$MgSO_4 \cdot 7H_2O$	1.0 g
NaCl	1.0 g
$(NH_4)_2SO_4$	2.0 g
$CaCO_3$	2.0 g
Distilled water	500 ml
Trace salts solution (p. 315)	1.0 ml

pH should be between 7.0 and 7.4. Do not adjust if it is within this range.

Mix starch suspension and salts solution.

Add agar (Difco). . . . . 20.0 g

Liquefy agar by steaming at 100°C for 15-20 minutes.

Sterilize in flasks for pouring into Petri dishes. Seven standard 90 mm dishes containing 25 ml per dish will be needed for each culture. (Pages 325 and 330)

Medium 5: Glycerol-asparagine agar (Pridham and Lyons, 1961)

L-asparagine (anhydrous basis)	1.0 g
Glycerol.	10.0 g
K_2HPO_4 (anhydrous basis)	1.0 g
Distilled water	1.0 liter
Trace salts solution (page 315).	1.0 ml

The pH of this solution is about 7.0-7.4. Do not adjust if it is within this range.

Agar . . . . . 20.0 g

Liquefy agar by steaming at 100°C for 15-20 minutes.

Sterlize in flasks for pouring into Petri dishes. Seven standard 90 mm dishes containing 25 ml per dish

will be needed for each culture. (Pages 325 and 330)

The final pH of the medium after sterilization with agar and solidification is about 7.4.

Medium 6: Peptone-yeast extract iron agar (Tresner and Danga, 1958)

Bacto-Peptone Iron Agar, dehydrated (Difco)	36.0 g
Bacto-Yeast Extract (Difco)	1.0 g
Distilled water	1.0 liter

pH should b 7.0-7.2 before autoclaving; adjust if necessary.

Liquefy agar by steaming at 100°C for 15-20 minutes.

Dispense appropriate amount for slanting into 2 tubes for each culture. Sterilize and solidify as slants. (Page 334). (Note that less than 1 liter of this medium is easily prepared by using a proportionately smaller amount of the dehydrated peptone iron agar and adding yeast extract in proportion of 0.1% of water used.)

Bacto-Peptone Iron Agar, dehydrated, contains the following ingredients when reconstituted as 36.58 grams per liter of water: Bacto-Peptone, 15 g; Proteose Peptone, Difco, 5 g; Ferric Ammonium Citrate, 0.5 g; Dipotassium Phosphate, 1 g; Sodium Thiosulfate, 0.08 g: Bacto-Agar, 15 g.

Medium 7: Tyrosine agar (Shinobu, 1958)

Glycerol.	15.0 g
L-tyrosine (Difco)	0.5 g
L-asparagine (Difco)	1.0 g
K_2HPO_4 (anhydrous basis).	0.5 g
$MgSO_4 \cdot 7H_2O$	0.5 g
NaCl	0.5 g
$FeSO_4 \cdot 7H_2O$	0.01 g
Distilled water	1.0 liter
Trace salts solution (page 315)	1.0 ml

Adjust to pH 7.2-7.4

Bacto-Agar	20.0 g

Liquefy by steaming at 100°C for 15-20 minutes.

Dispense appropriate amount for slanting into 2 tubes for each culture; sterilize and solidify as slants. (Page 333).

Medium 8: Nitrate broth (Deleted because of unreliability of the nitrate-reduction test).

Medium 9: Carbon utilization medium (Modified from Pridham and Gottlieb, 1948)

A. Sterile carbon sources

Use chemically pure carbon sources certified to be free of admixture with other carbohydrates or contaminating materials. Carbon sources for this test are:

No carbon source (negative control)
D-glucose (positive control)
L-arabinose

Sucrose	D-fructose
D-xylose	Rhamnose
I-inositol	Raffinose
D-mannitol	Cellulose

Sterilize without heat by one of the following methods:

1. Filtration. Filter sterilize 10% solution through bacteriological filter. (i-Inositol and cellulose are not sufficiently soluble for sterilization by this method—use one of the methods described below.)

2. Ether sterilization. Weigh an appropriate amount of the dry carbon source and spread as a shallow layer in a pre-sterilized Erlenmeyer flask fitted with a loose cotton plug. Add sufficient acetone-free ethyl ether $(C_2H_5)_2O$ to cover the carbohydrate. (OBSERVE PRECAUTIONS AGAINST FIRE! Allow ether to evaporate at room temperature under a ventilated fume hood overnight or longer. When all ether has evaporated add sterile distilled water aseptically to make a 10% w/v solution of the carbon source.

3. Ethylene oxide sterilization (Judge and Pelczar, 1955). Make a 10% w/v solution of the carbon source. Cool the liquid in an ice bath to 3-5° C and add 1 volume per cent liquid (cold) ethylene oxide with a chilled pipette or syringe. Agitate the solution. Leave it in the cold ice bath under a ventilated fume hood for

1 hour. Transfer to a warm water bath (about 45°C) UNDER FUME HOOD to permit complete volitilization of the ethylene oxide. The vapors are toxic and explosive.

Carbon sources sterilized by one of these three methods will be added to the basal mineral salts agar to give a final concentration of 1%. For example, add 10 ml of 10% solution to 100 ml basal medium, or 100 ml of a 10% solution to 1000 ml basal medium.

B. Pridham and Gottlieb trace salts (only 1 ml of this solution is used per liter of final medium)

$CuSO_4 \cdot 5H_2O$	0.64 g
$FeSO_4 \cdot 7H_2O$	0.11 g
$MnCl_2 \cdot 4H_2O$	0.79 g
$ZnSO_4 \cdot 7H_2O$	0.15 g
Distilled water	100.0 ml

Store at 3-5°C until required for use. Bring to room temperature before using. Prepare fresh solution each month. Disregard any precipitate or scale (probably iron salts) that forms during storage. (Only 1 ml of this solution will be used in the medium.)

C. Basal mineral salts agar (use analytical reagent grade chemicals)

$(NH_4)_2SO_4$	2.64 g
KH_2PO_4 anhydrous	2.38 g
$K_2HPO_4 \cdot 3H_2O$	5.65 g
$MgSO_4 \cdot 7H_2O$	1.00 g
Pridham and Gottlieb trace salts (B)	1.00 ml
Distilled water	1.00 liter

Dissolve ingredients and check pH. Adjust, if necessary, to 6.8-7.0 with 1 N NaOH or 1 N HCl.

Add agar (Difco) . . . . . 15.0 g

D. Complete medium

Sterilize basal agar medium (C); cool it to 60°C and add sterile carbon source (A) aseptically to give a concentration of approximately 1%. Agitate the mixture and pour 25 ml of medium per dish into 9 cm Petri dishes. Each organism will require 2 Petri dishes with no carbon (as a negative control) plus duplicate plates for each carbon source tested. (Page 335)

METHODS FOR INITIATING GROWTH AND PREPARING STOCK CULTURES FROM LYOPHILE PELLET

1. Make a file scratch on ampoule at a location near upper part of looped cord (see Fig. 1).

2. Immerse the unbroken ampoule into 70% ethyl alcohol.

3. Enclose the alcohol-moistened ampoule with a piece of sterile cotton or cotton and gauze; then snap or break it at the file scratch (Fig. 2).

4. Use sterile forceps or a sterile stiff wire hook to transfer the looped string and pellet to the labeled tube containing 5 ml of sterile tryptone—yeast extract broth (Fig. 3). If a pellet fails to come out attached to the string, the cells or spores on the string will usually be adequate to start a good culture.

5. Shake the tube by hand until the pellet dissolves.

6. Incubate the tubes in a slanted position or on a mechanical shaker to give good aeration. Use of a shaker is the preferred method. Incubate at 25-28° C for 24-28 hours (or until there is evidence of spore germination or growth).

7. Look for possible contaminants with the microscope. Also streak one loopful of the broth culture onto the agar surface of a Petri dish containing medium 2 (yeast extract—malt extract agar). This plate can be examined after a few days to confirm absence of contaminants.

8. Inoculate 6 or more test tube slants of medium 2 (yeast extract—malt extract agar) and of medium 3 (oatmeal agar) with 0.1-0.2 ml of the 24-48 hour growth. Streak material over entire surface of agar slant.

9. Incubate the slants at 25-28° C for 14 days to get mature stock cultures for use in preparation of inoculum (see section which follows). Then store stock slants in refrigerator (6-10° C) until ready for use. Generally stock cultures for preparation of inoculum for characterization tests should be used within one month.

PREPARATION OF INOCULUM

Use stock culture slants (prepared as described in the preceding section) for preparation of (A) general inoculum for all inoculations except carbon utilization test, and (B) a special washed inoculum for determining carbon utilization patterns.

A. Preparation of general inoculum

1. Prepare a supply of stoppered test tubes containing 3-5 ml of sterile distilled water.

2. Use a wire loop and standard aseptic technique to transfer spores, or mycelial growth, from a stock culture slant to one of the tubes of sterile distilled water.

   a. If sporulation on the stock slant is good, transfer sufficient spore material to make a very turbid suspension in the distilled water. Normally most of the spore surface from a stock slant will be required. If necessary, use more than one slant to get a turbid suspension.

   b. If spores are not formed, use the wire loop to transfer mycelial material to the tube of sterile distilled water. Triturate the mycelium in the distilled water with a sterile glass rod or the tip of a sterile pipette. Produce a very turbid suspension of mycelial fragments. Do not use a mycelial suspension if a good spore suspension (a) can be obtained.

3. This distilled water suspension of spores or mycelial fragments may be used immediately as general inoculum or may be held at room temperature 3-4 hours. Prepare fresh inoculum suspensions for tests performed on different days.

B. Preparation of washed inoculum

1. Prepare 5 ml of turbid suspension of spores or mycelium in sterile water as described for general inoculum.

2. Transfer 4-5 ml of this suspension to 50 ml of medium 1 (tryptcne—yeast extract broth) in a 250 ml Erlenmeyer flask (or 25 ml in a 125 ml flask).

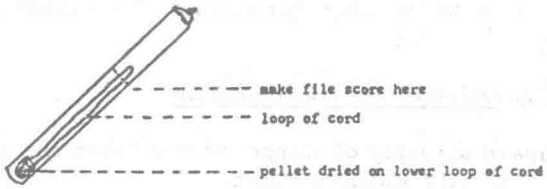

Figure 1: Sealed evacuated ampoule containing lyophile pellet and cord

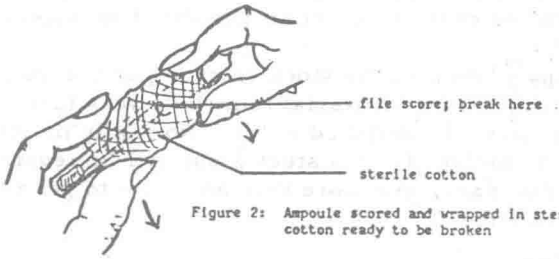

Figure 2: Ampoule scored and wrapped in sterile cotton ready to be broken

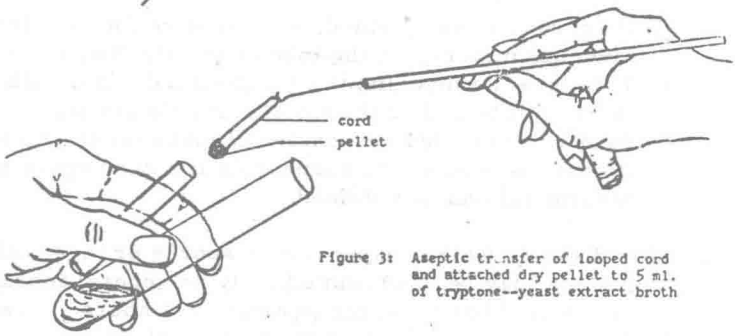

Figure 3: Aseptic transfer of looped cord and attached dry pellet to 5 ml. of tryptone--yeast extract broth

3. Incubate the flask for 48 hours at 25-28°C. If possible the flask should be aerated on a mechanical shaker during the incubation.

4. Use vigorous agitation with sterile glass beads, a mechanical blender such as the Waring Blendor, or other appropriate means to break up the 48-hour growth.

5. Transfer 5-10 ml of this fragmented broth culture into sterile centrifuge tubes equipped with sterile caps.

6. Centrifuge the suspension.

7. Decant the supernatant broth. Add sterile distilled water (or sterile 0.85% NaCl) to restore the original volume in the centrifuge tube. Mix and resuspend the washed sediment with a sterile rod or pipette.

8. Repeat steps 6 and 7. (Compare amount of sediment from different cultures. When amount of sediment is much less than normal amount, use proportionately less water for the final resuspension.)

9. Use the resuspended inoculum at once or within 3 hours to inoculate carbon utilization tests.

PROCEDURES FOR CHARACTERIZATION OF CULTURES

I. MORPHOLOGICAL CHARACTERIZATIONS

Accurate morphological characterization of the Actinomycetes producing catenulate spores is obviously dependent upon use of a culture medium giving good sporulation. Four media which gave good performance in this respect in previous cooperative studies are listed below as "standard" media. If these four media all fail to give good development of the sporulating aerial mycelium, then an additional medium promoting good spore formation should be used.[5] If it is necessary to use an additional medium, also include a record of observations of the growth on the standard media. Spore chain and sporophore morphology should be determined by observation of a fully matured culture with good

[5] Cooperators in the ISP will please communicate the formula of any additional medium used to Dr. Shirling, who will then forward the formula to the other two cooperating investigators studying the culture.