Method for the Preferential Isolation of Actinomycetes from Soils

J. N. Porter, J. J. Wilhelm, and H. D. Tresner

Biochemical Research Section, Lederle Laboratories, American Cyanamid Company, Pearl River, New York

Received for publication October 21, 1959

Species of the genus Streptomyces and of related genera of the Actinomycetales, at one time considered to be microbiological curiosities of no great economic importance, have become the subjects of intensive searches for sources of new, biologically active compounds. The demonstrated ability of these microorganisms to produce useful antibiotics and to carry out other transformations of commercial interest has focused attention on factors bearing on their isolation from their natural habitat, the soil. It would be desirable, therefore, to be able to isolate aerobic soil-inhabiting actinomycetes with a minimum of interference from associated bacteria and higher fungi.

There have been a number of reports in the literature dealing with selective isolation of the higher fungi at the expense of bacteria or of bacteria at the expense of molds. Several investigators used antibiotics as the selective agents. For example, Beech and Carr (1955) found cycloheximide, gliotoxin, and frequentin to be effective selective yeast and mold inhibitors in apple juices and ciders. Phillips and Hanel (1950) found cycloheximide to be inactive against representatives of 27 species of bacteria and suggested that this antifungal antibiotic might be used to rid bacterial cultures of contaminating molds. On the other hand, Butler and Hine (1958) selectively isolated fungi from soil plated on potato dextrose agar adjusted to pH 5.6 to 6.1 and to which were added 100 μg per ml of the antibiotic novobiocin.

Turning to preferential actinomycete isolation, Crook et al. (1950) found sodium propionate to be an effective fungal inhibitor, useful for their Streptomyces program. Corke and Chase (1956), in their microbiological investigations of heavily mold-infested acid forest soils, studied the use of both sodium propionate and cycloheximide as medium additives. They reported cycloheximide to be the more effective compound of the two and recommended its addition to actinomycete isolation media at a level of 40 μg per ml of agar. In their hands, cycloheximide at 100 μg per ml showed no suppression of any of 85 actinomycete cultures. Dulaney et al. (1955) isolated Streptomyces selectively on a nutrient agar medium containing, per ml: cycloheximide, 20 units; polymyxin, 20 units; subtilin, 20 units; and penicillin, 20 units. They stated that the amounts of antibiotics in the medium were critical and that, whereas the selective inhibition of molds could be accomplished, the separation of streptomycetes and bacteria was a difficult achievement.

The fact that the development of Streptomyces colonies on agar plates could be favored over bacteria by selection of the nitrogen source in the medium was reported by Benedict et al. (1955). They noted that L-arginine is readily attacked by most streptomycetes and they recommended the use of this amino acid as a replacement for the glycine of the glycerol-glycine medium of Lindenbein (1952).

We have achieved a set of conditions very favorable to the preferential isolation of actinomycetes from soils by combining the principle of the selective inhibition of molds by means of an antifungal antibiotic with that of the use of Benedict’s modification of the Lindenbein medium to limit bacterial development. A surface-layering technique enhances the effectiveness of the isolation procedure.

METHODS AND RESULTS

Plating procedure. A plating method described by Kelner (1948) was employed in carrying out most of these studies. The technique is one which ensures the development of most microorganisms as surface colonies, thus greatly expediting their tentative identification and comparison. Fifteen-ml aliquots of an appropriate medium were poured into Petri dishes and allowed to harden to form a basal layer. Soil samples were suspended in sterile water, appropriately diluted and 1 ml of each desired dilution pipetted into 14-ml portions of agar medium maintained at 48°C in a water bath. Five-ml quantities were then pipetted onto the hardened surfaces of two basal layers, thus providing duplicate plates for each dilution. The composition of the agar, including additives, of both basal and surface layers was identical. An exception to this procedure was the plating out by the conventional nonlayering method of cultures contaminated with molds by design.

Nutrient media. A number of nutrient media, for example, asparagine dextrose, Emerson, Czapek, yeast extract, and Bennett agars, are commonly used to investigate the growth and development of aerobic actinomycetes. Where bacteria are numerous in soils,
none of these media are particularly satisfactory for streptomycete isolation and some, such as yeast extract agar, actually potentiate spreading bacterial colonies. The writers, however, have obtained consistently good results with Benedict's modification of the Lindenbein medium. That it is possible to obtain plates with this medium on which streptomycetes are growing almost exclusively is shown in figures 1 and 2. These figures also demonstrate the virtual impossibility of isolating actinomycetes from the same soil sample on some other media.

Antibiotic additives to culture media. Antibacterial antibiotics such as the tetracyclines, polymyxin, neomycin, and streptomycin alone or in combination were added to several agar media at levels ranging from 0.5 to 100 μg per ml. Selective inhibition of bacteria was not satisfactorily achieved. However, since the Benedict medium modification achieved the purpose of favoring the actinomycete to bacterium colony ratio, attention was turned to the use of antifungal agents in the medium.

In our antibiotic screening program a polyene antibiotic, designated A-5283, with a broad spectrum of activity against fungi was isolated. This antibiotic, subsequently identified as pimaricin, was found to be eminently suitable for eliminating molds from soil dilution plates at a level of 50 μg per ml. Moreover, at 100 μg per ml, A-5283 was without observable effect on Streptomyces populations.

1 Composition (per L): glycerol, 20 g; L-arginine, 2.5 g; NaCl, 1 g; CaCO₃, 0.1 g; FeSO₄·7H₂O, 0.1 g; MgSO₄·7H₂O, 0.1 g; agar, 20 g.

---

Figure 1. Isolation plates from a New Jersey soil, 3 X 10⁻⁴ dilution. Benedict agar (upper left), yeast agar (upper right), asparagine dextrose agar (lower left), Bennett agar (lower right).
Figure 2. Isolation plates from an Indiana soil, $3 \times 10^{-4}$ dilution. Benedict agar (upper left), yeast agar (upper right), asparagine dextrose agar (lower left), Bennett agar (lower right).

Figure 3. Isolation plates from a Mississippi soil. Top row, left to right: Benedict agar plates containing 0, 5, 25 and 50 µg of antibiotic A-5283 per ml of agar, respectively. Bottom row, left to right: Plates containing asparagine dextrose, Emerson, Czapec, yeast, and Pennett agar, respectively. No A-5283.
Antibiotic A-5283 is water-insoluble and can withstand autoclaving at 15 lb pressure for 15 min with less than 10 per cent loss of activity. A stock solution of A-5283 at 1 mg per ml is prepared in N,N-dimethylformamide and dispensed in agar media at desired levels before autoclaving. It has been our experience, however, that agar media containing A-5283 cannot be kept for more than 3 days, even under refrigeration conditions, without further loss of antibiotic activity. Figures 3 and 4 illustrate dilution plates made from a Mississippi soil and from a local greenhouse soil. Complete mold suppression is shown on Benedict agar plates containing 50 \( \mu \text{g} \) per ml of A-5283. Less complete inhibition is indicated at lower levels. Heavy mold growth and complete suppression of actinomycetes from the same soils occurred on asparagine dextrose, Emerson, Czapek, yeast, and Bennett agars lacking A-5283.

The facility with which streptomycetes may be selectively isolated from soil on the Benedict agar-A-
5283 combination has led us to incorporate the technique in our routine isolation program. In 521 soil samples collected on a world-wide basis and studied by this technique, the numbers of streptomycetes per g were highly variable, normally ranging from $10^4$ to $10^5$. Bacteria were a serious handicap to isolation in the case of 49 soils, and yeasts or molds were a problem in 11 soils. In only one soil, however, were contaminants present in such numbers that the isolation of Streptomyces by our method was impossible. For practical reasons, actual comparisons with other isolation media on the same soils was not feasible. However, in an earlier phase of the program, unamended asparagine dextrose agar was used to isolate Streptomyces from 332 soils of various origins. In the case of 92, contaminants were a serious problem and from 32 no streptomycete isolates could be obtained.

**Culture decontamination.** Since A-5283 is such an efficacious antifungal agent for soil isolation work, the use of this antibiotic in decontaminating mold-infested Streptomyces cultures also suggested itself. To test the idea, a spore suspension of a strain of Streptomyces lavendulae was prepared in sterile saline and portions mixed separately with each of the following fungi: Rhizopus nigricans, Penicillium chrysogenum, Saccharomyces cerevisiae, and Neurospora crassa. Approximately 0.5-ml amounts of each mixture and of S. lavendulae spores alone were incorporated in yeast-malt agar containing 50 μg per ml of A-5283 in poured Petri plates. Plates without A-5283 were included. The antibiotic A-5283 had no visible effect on the development of the Streptomyces cultures but the growth of the fungal contaminant was completely inhibited in all cases (figure 5). Several mold-contaminated Streptomyces cultures in our collection have been successfully freed of the fungus contaminant by the use of this antibiotic.

At appropriate concentrations, other antifungal antibiotics can be employed effectively both in soil isolation plates and in decontamination work. We have found both nystatin and cycloheximide at 50 μg per ml to be satisfactory in these respects.

**SUMMARY**

A method is described of combining an antifungal antibiotic with a glycerol-arginine medium which selectively encourages the development of aerobic, soil-inhabiting actinomycetes on isolation plates. Surface layering of the inoculated agar ensures the growth of most organisms as surface colonies. Such antifungal antibiotics as pimaricin (A-5283 from our antibiotic program) nystatin, and cycloheximide were used successfully to suppress molds in isolation plates. These antibiotics were also found to be useful in decontaminating mold-infested Streptomyces cultures.

**REFERENCES**