

Novel Method for Selective Isolation of Actinomycetes

C. F. HIRSCH* AND D. L. CHRISTENSEN

*Merck Sharp & Dohme Research Laboratories, Merck & Co., Inc., P.O. Box 2000, Rahway,
New Jersey 07065*

Received 17 March 1983/Accepted 12 July 1983

A new technique for the selective isolation of actinomycetes from natural mixed microbial populations is described. A nutrient agar medium was overlaid with a 0.22- to 0.45- μ m-pore cellulose ester membrane filter, and the surface of the filter was inoculated. During incubation, the branched mycelia of the actinomycetes penetrated the filter pores to the underlying agar medium, whereas growth of non-actinomycete bacteria was restricted to the filter surface. The membrane filter was removed, and the agar medium was reincubated to allow the development of the isolated actinomycete colonies. This procedure selects actinomycetes on the basis of their characteristic mycelial mode of growth, offers a general method for their selective isolation, and does not rely on the use of special nutrient media or of antibacterial antibiotics.

The actinomycetes are a group of bacteria which possess many important and interesting features. They are of considerable value as producers of antibiotics and of other therapeutically useful compounds, they exhibit a range of life cycles which are unique among the prokaryotes, and they appear to play a major role in the cycling of organic matter in the soil ecosystem (7, 15).

The isolation of actinomycetes from the mixed microflora present in nature is complicated by their characteristic slow growth relative to that of other soil bacteria. This has resulted in the development of selective isolation procedures based primarily on one or both of the following approaches: (i) nutritional selection, in which media are formulated with nutrients which are preferentially utilized by actinomycetes (4-6, 8-10), and (ii) selective inhibition, in which compounds such as antibiotics are incorporated into media to selectively inhibit non-actinomycete bacteria (3, 6, 11, 16). The major difficulties with each of these approaches are that neither is strictly selective for actinomycetes and that each has the inherent potential to actually inhibit the growth of some actinomycetes. We report here a method for the isolation of actinomycetes which is simple, selective, and based on an entirely new approach.

(A preliminary account of this work has been published [Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, I12, p. 96].)

MATERIALS AND METHODS

Membrane filters. The membrane filters used were obtained from Millipore Corp. All filters were 90 mm

in diameter, were composed of mixed esters of cellulose, and were steam sterilized before use.

Growth conditions. A synthetic agar medium (CMS) used in most experiments had the following composition: citric acid, 2 mM; $MgCl_2 \cdot 6H_2O$, 1.25 mM; KCl, 10 mM; Na_2SO_4 , 2 mM; $NaH_2PO_4 \cdot H_2O$, 10 mM; NH_4Cl , 100 mM; glucose, 200 mM; 3-(*N*-morpholino) propanesulfonic acid-tetramethyl ammonium hydroxide buffer, pH 7.2, 200 mM; trace elements solution (TS), 10 ml/liter; agar (Difco Laboratories), 20 g; and distilled water, 1,000 ml. The composition of TS was as follows: $FeCl_3 \cdot 6H_2O$, 1 mM; $MnSO_4 \cdot H_2O$, 2.5 mM; $CuSO_4 \cdot 5H_2O$, 0.2 mM; $CaCl_2 \cdot 2H_2O$, 10 mM; H_3BO_3 , 1 mM; $CoCl_2 \cdot 6H_2O$, 0.1 mM; $ZnCl_2$, 0.5 mM; and $Na_2MoO_4 \cdot 2H_2O$, 0.1 mM. TS was prepared in 0.1 N HCl. Glucose, magnesium chloride, and TS were sterilized and added separately to autoclaved and cooled CMS before the plates were poured. A modification of Bennett medium was also used in some experiments and contained, per liter of distilled water, the following: yeast extract, 1.0 g; beef extract, 1.0 g; casein hydrolysate (vitamin and salt free; ICN Pharmaceuticals, Inc.), 2.0 g; 1 M potassium phosphate buffer, pH 7.0, 30 ml; 50% (wt/vol) glucose, 20 ml; TS, 10 ml; and agar (Difco), 20 g. Glucose and TS were sterilized and added separately to the autoclaved and cooled modified Bennett medium before the plates were poured. All media contained cycloheximide and candicidin, each at 100 μ g/ml, to suppress the growth of fungi. Cycloheximide stock solution (1.25% [wt/vol]) was prepared in water and sterilized by filtration. Candicidin stock solution (2.5% [wt/vol]) was prepared in *N,N*-dimethylformamide. Each antibiotic was added to sterilized molten agar media before the plates were poured. All incubations were carried out at 28°C.

Inocula. Samples of soil, water, and various vegetable materials used in experiments were collected locally and from different locations around the world.

Electron microscopy. Filters for scanning electron microscopy were fixed in 3% glutaraldehyde, rinsed in

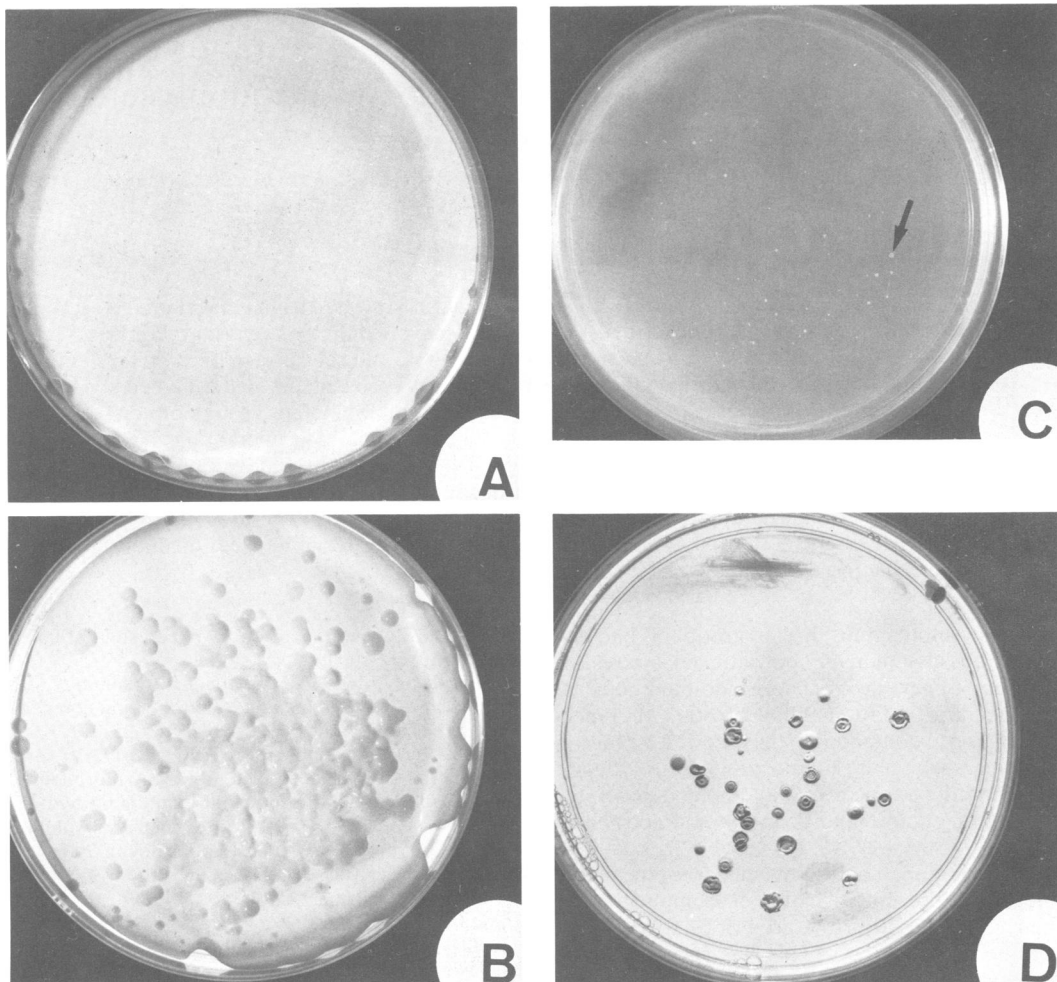


FIG. 1. Membrane filter isolation procedure for actinomycetes. Agar medium was overlaid with a sterile cellulose ester membrane filter (A), the filter surface was inoculated and the petri plate was incubated until colonies developed (B), the filter was removed to expose the actinomycete colonies (arrow) in the underlying agar (C), and the petri plate was reincubated to allow development of the colonies (D).

phosphate buffer, postfixed in 1% osmium tetroxide, and dehydrated with increasing concentrations of ethanol. The filters were examined by light microscopy, and intact actinomycete colonies were located, excised, and critically point dried. Specimens were affixed to aluminium stubs and gold coated, and representative areas were photographed by scanning electron microscopy.

Chemicals. Cycloheximide and candicidin were purchased from Calbiochem Corp. All other chemicals were of reagent grade or of the highest purity available.

RESULTS

Isolation procedure. Actinomycete colonies growing on agar media characteristically extend onto and well beneath the agar surface (14). In contrast, colonies of non-actinomycete bacteria

generally limit their growth to the immediate agar surface. It was expected that selective isolation of actinomycetes could be achieved by placing a suitable barrier between the growing bacterial colonies and the agar surface which would allow only actinomycetes to penetrate to the underlying agar. A cellulose ester membrane filter of proper pore diameter was found to provide such a barrier. A procedure that uses membrane filters for the isolation of actinomycetes from mixed microbial populations was developed (Fig. 1). An agar medium was overlaid with a sterile membrane filter (Fig. 1A) without trapping air between the filter and the agar surface. During application to the agar, the filter must not be allowed to crack or have its integrity otherwise destroyed. Liquid or solid

TABLE 1. Recovery of actinomycetes by membrane filter isolation

Pore size (μm)	% Recovery ^a at:			
	1 day	2 days	3 days	4 days ^b
0.22	0	0	15	156
0.30	0	1.8	52	274
0.45	0	2.3	97	243

^a The percent recovery was calculated as the number of actinomycete colonies present on the agar divided by the number of actinomycete colonies present on the filter surface, multiplied by 100.

^b Recovery of >100% after 4 days reflects the difficulty of recognizing and counting actinomycete colonies which had been overgrown by other bacteria on the filter surface.

inoculum was then applied to the filter surface, and the inoculated plates were incubated until visible growth of bacteria occurred (Fig. 1B). The membrane filter was then removed aseptically from the agar medium (Fig. 1C), and the plate was reincubated until growth was observed. As shown in Fig. 1D, the colonies present on the plate were exclusively actinomycetes. Our experience has shown that two factors are particularly important in achieving success with the method. (i) Since penetration of the membrane filter by actinomycetes is necessary to achieve a physical separation of actinomycete and non-actinomycete colonies, it is important to choose culture conditions that will encourage growth and colony formation by the desired actinomycetes. (ii) The application of the inoculum must be limited to the central area of the filter, maintaining at least a 1.0-cm-wide sterile border along the filter edge. This precaution will prohibit growth of non-actinomycete bacteria over the edge of the filter and onto the underlying agar surface.

Effect of filter pore size on actinomycete selection. Membrane filters of different pore sizes were tested for their ability to select actinomycetes exclusively. Plates of CMS and modified Bennett medium were overlaid with filters of 3.0-, 2.0-, 1.0-, 0.65-, 0.45-, 0.30-, 0.22-, 0.10-, 0.05-, and 0.01- μm -pore diameters. The filters were inoculated with particles of soil and incubated at 28°C. After 4 days, the filters were removed, and the plates were reincubated for 4 days to allow growth of the colonies. The bacterial populations selected by the filters of different pore sizes were evaluated macroscopically and microscopically for the presence of non-actinomycete or actinomycete colonies or both. It was found that filters with a pore diameter of $\geq 0.65 \mu\text{m}$ did not discriminate between actinomycetes and other types of bacteria, whereas filters with a pore diameter of $\leq 0.10 \mu\text{m}$ blocked

penetration to the agar of all bacteria, even after incubation for up to 10 days. Only filters in the pore size range of 0.45 to 0.22 μm were observed to allow exclusive penetration of actinomycetes.

Effect of pore size on the efficiency of recovery from agar of actinomycetes on the filter surface. Replicate plates of CMS overlaid with 0.22-, 0.30-, and 0.45- μm -pore filters were inoculated, and at 1-day intervals, one set of plates was removed from incubation and the number of actinomycete colonies present on each of the three filters of different pore sizes was counted. The filters were then removed, and the plates were reincubated to allow development of actinomycete colonies on the agar. The number of colonies present on the agar was then counted, and the percent recovery was determined as described in footnote *a* of Table 1. The results show (Table 1) that after 3 days of incubation, the 0.45- μm -pore filters yielded the highest percent recovery of actinomycete colonies. Filters of 0.30- μm -pore diameter were only about 50% as efficient as 0.45- μm -pore filters after 3 days, whereas 0.22- μm -pore filters yielded the lowest percent recovery.

The data in Table 1 also indicate the time required for the penetration of actinomycete colonies through the filters. For example, on plates overlaid with 0.45- μm -pore filters, only a 2.3% recovery of actinomycetes was observed after 2 days of incubation. After 3 days of incubation, however, almost 100% of the actinomycetes were recovered on the agar. It appears that penetration of the filters by actinomycetes is a process closely related to growth of the colonies.

Penetration mechanism. The importance of the pore diameter in determining whether a membrane filter was penetrated by actinomycetes and the time required for actinomycetes to penetrate a filter suggested that the penetration mechanism involved growth of hyphae through the filter pores and not digestion of the filter matrix. This was investigated by scanning electron microscopy of a cross section of an actinomycete colony which penetrated a 0.22- μm -pore membrane filter (Fig. 2). It is clear that no obvious change in the filter matrix occurred in the vicinity of the actinomycete colony, but the colony penetrated the filter, as evidenced by mycelia on both the top and bottom surfaces of the filter. One can conclude that penetration of the membrane filter by actinomycetes occurs by growth of the branching mycelia through the openings of the filter matrix.

DISCUSSION

Membrane filters have been used by various investigators to isolate actinomycetes from wa-

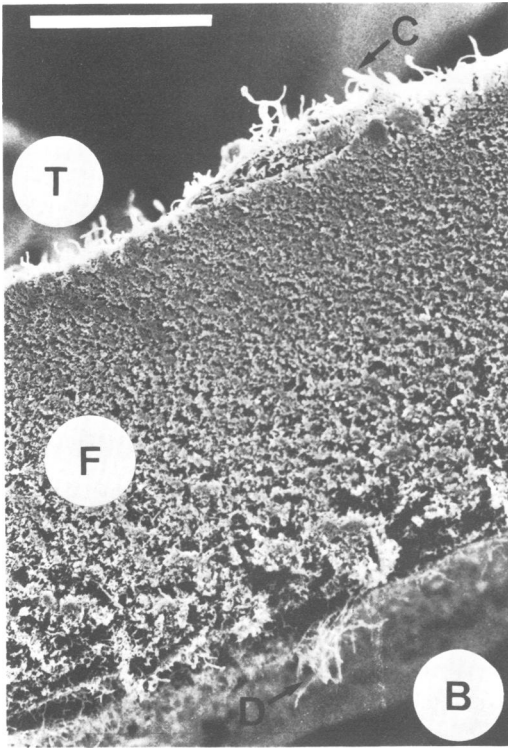


FIG. 2. Scanning electron micrograph of a cross section of an actinomycete colony which penetrated a 0.22- μm -pore membrane filter. (T) Top surface of the filter to which the inoculum was applied; (B) bottom surface of the filter which was in contact with the agar; (C) actinomycete colony present on the top surface of the filter; (D) tufts of mycelia from the colony in (C) which penetrated the filter matrix (F) and protrude from the bottom of the filter. Bar, 50 μm .

ter (1, 2). Trolldenier (12, 13) collected actinomycete spores from water on a membrane filter, incubated the spore side of the membrane in contact with nutrient agar, and observed the production of actinomycete colonies on the reverse side of the filter. We describe here a new procedure that uses membrane filters for the isolation of actinomycetes from mixed microbial populations. Actinomycetes are selectively separated from other bacteria by their ability to penetrate the pores of a membrane filter and to grow on an underlying agar medium, whereas the growth of other bacteria remains restricted to the filter surface. The ability of actinomycetes to penetrate the tortuous pores of the membrane filter apparently is due to their characteristic growth as highly branched mycelial networks. We have isolated many different actinomycete cultures from the environment by using this method. Based on colony morphology, culture behavior, and microscopic observation, it ap-

pears that our isolates include members of the *Streptomycetaceae*, *Micromonosporaceae* (mesophiles and thermophiles), *Nocardiaceae*, and *Actinoplanaceae*. Thus, this procedure is suitable for selective isolation of a taxonomic variety of actinomycetes.

Among the advantages offered by this technique are its simplicity and its ability to isolate actinomycetes from a variety of different samples without having to resort to the use of non-discriminating antibacterial antibiotics or restricting nutritional conditions. Moreover, the filter technique can complement existing procedures designed for the isolation of actinomycetes.

ACKNOWLEDGMENTS

We thank John Nichols and Solomon Scott for help with the scanning electron microscopy.

LITERATURE CITED

1. Al-Diwany, L. J., B. A. Unsworth, and T. Cross. 1978. A comparison of membrane filters for counting *Thermoactinomyces* endospores in spore suspensions and river water. *J. Appl. Bacteriol.* 45:249-258.
2. Burman, N. P., C. W. Oliver, and J. K. Stevens. 1969. Membrane filtration techniques for the isolation from water of coli-aerogenes, *Escherichia coli*, fecal streptococci, *Clostridium perfringens*, actinomycetes and microfungi, p. 127-134. In D. A. Shapton and G. W. Gould (ed.), *Isolation methods for microbiologists*. Academic Press, Inc., New York.
3. Dulaney, E. L., A. H. Larsen, and E. O. Stapley. 1955. A note on the isolation of microorganisms from natural sources. *Mycologia* 47:420-422.
4. El-Nakeeb, M. A., and H. A. Lechevalier. 1963. Selective isolation of aerobic actinomycetes. *Appl. Microbiol.* 11:75-77.
5. Kuster, E., and S. T. Williams. 1964. Selection of media for isolation of streptomycetes. *Nature (London)* 202:928-929.
6. Kutzner, H. J. 1981. The family *Streptomycetaceae*, p. 2028-2082. In M. P. Starr, H. Stolp, H. G. Truper, A. Balows, and H. G. Schlegel (ed.), *The prokaryotes. A handbook on habitat, isolation, and identification of bacteria*. Springer-Verlag, New York.
7. Lacey, J. 1978. Ecology of actinomycetes in fodder and related substrates, p. 161-168. In M. Mordarski, W. Kurylowicz, and J. Jeljaszewicz (ed.), *Nocardia and Streptomyces: proceedings of the international symposium on Nocardia and Streptomyces*. Warsaw, October 1976. Gustav Fischer Verlag, Stuttgart.
8. Lingappa, Y., and J. L. Lockwood. 1961. A chitin medium for isolation, growth, and maintenance of actinomycetes. *Nature (London)* 189:158-159.
9. Lingappa, Y., and J. L. Lockwood. 1962. Chitin media for selective isolation and culture of actinomycetes. *Phytopathology* 52:317-323.
10. Porter, J. N., J. J. Wilhelm, and H. D. Tresner. 1960. Method for the preferential isolation of actinomycetes from soil. *Appl. Microbiol.* 8:174-178.
11. Preobrazhenskaya, T. P., M. A. Sveshnikova, L. P. Terekhova, and N. T. Chormonova. 1978. Selective isolation of soil actinomycetes, p. 119-122. In M. Mordarski, W. Kurylowicz, and J. Jeljaszewicz (ed.), *Nocardia and Streptomyces: proceedings of the international symposium on Nocardia and Streptomyces*. Warsaw, October 1976. Gustav Fischer Verlag, Stuttgart.
12. Trolldenier, G. 1966. Über die Eignung Erde enthaltender

- Nährsubstrate zur Zählung und Isolierung von Bodenmikroorganismen auf Membranfiltern. Zentralbl. Bakteriolog. Parasitenkd. Infektionskr. Hyg. Abt. 2 **120**:496–508.
13. **Trolldenier, G.** 1967. Isolierung und Zählung von Bodenactinomyceten auf Erdplatten mit Membranfiltern. *Plant Soil* **27**:285–288.
 14. **Wildermuth, H.** 1970. Development and organization of the aerial mycelium in *Streptomyces coelicolor*. *J. Gen. Microbiol.* **60**:43–50.
 15. **Williams, S. T.** 1978. Streptomycetes in the soil ecosystem, p. 137–142. *In* M. Mordarski, W. Kurylowicz, and J. Jeljaszewicz (ed.), *Nocardia and Streptomyces: proceedings of the international symposium on Nocardia and Streptomyces*. Warsaw, October 1976. Gustav Fischer Verlag, Stuttgart.
 16. **Williams, S. T., and F. L. Davies.** 1965. Use of antibiotics for selective isolation and enumeration of actinomycetes in soil. *J. Gen. Microbiol.* **38**:251–261.