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Isolation of bioactive actinomycetes from marine sediments using rifampicin

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Summary. Bioactive actinomycetes were isolated from marine sediments using rifampicin. Plating the sediments on starch-casein agar, supplemented with rifampicin, eliminated the occurrence of contaminating microorganisms. Total counts, however, were reduced in the presence of rifampicin. Most of the isolates contained LL-2,6-diaminopimelic acid (DAP), whereas 37% contained *meso*-DAP. The use of increasing concentrations of rifampicin tended to yield a higher proportion of strains with cell extracts positive for *meso*-DAP. *Streptomyces* and *Micromonospora* represented the major genera identified. Antimicrobial activity was exhibited by 46% of the isolates, primarily against Gram-positive bacteria. Inhibition of Gram-negative bacteria was minimal, but antimycotic activity was displayed by 28% of the actinomycetes. Most of the latter activity was attributable to polyenes, particularly hexaenes. The results obtained indicate that rifampicin, added to starch-casein agar, is effective for the isolation of bioactive actinomycetes from marine sediments.

Introduction

It is well established that marine microorganisms have been largely unexplored as a source of bioactive agents for industrial production (Okami 1984). Of all available marine forms, the actinomycetes merit special consideration in view of the proven biosynthetic capabilities of numerous isolates from soil. Marine actinomycetes, however, have not received the attention accorded their terrestrial counterparts. As has already been expressed (Goodfellow and Haynes 1984), the former may comprise a selected gene pool possibly containing organisms with the potential to yield useful metabolic substances. These investigators, in fact, further added that methods which contribute to the isolation of specific groups of microorganisms make the exploitation of marine forms a desirable goal. Cross (1982) has reviewed a variety of such methods. These have been employed primarily for the isolation of actinomycetes from soils. The promise held out by investigations of marine actinomycetes as sources of potentially novel metabolites has prompted the suggestion that marine forms will prove to be as fruitful as those isolated from terrestrial habitats (Okami 1986).

Work in our laboratory has focused on the application of pretreatments and selective media for the isolation of biologically active actinomycetes from marine sediments. Heat, phenol, filtration through cellulose membrane filters, and the use of chitin agar all contributed towards the isolation of desirable strains from sediments collected in Sandy Hook Bay, New Jersey (Pisano et al. 1986). Similarly, heat, phenol, as well as benzalkonium chloride and various agar substrates greatly assisted in the isolation of antimycotic actinomycetes from Hudson River sediments (Pisano et al. 1987).

The present work was undertaken to expand the choice of selective agents which may be employed for the isolation of active strains of actinomycetes from marine sediments. Accordingly, we investigated the possible utility of rifampicin for this purpose. We are not aware that this antimicrobial agent has been employed previously for the isolation of bioactive actinomycetes from such sediments.

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Materials and methods

Sample sites. Sediments were collected at 18 sampling sites which included the south shore of Brooklyn, the East River, and New Jersey. Water depths ranged from 20 to 37 m, and samples were obtained from locations up to 1 mile offshore. Total counts of actinomycetes in each sample were performed using starch-casein agar (Okazaki and Okami 1972) containing cycloheximide and nystatin as indicated below.

Isolation medium. All sediments collected were dried and weighed. One gram samples of dried sediments were diluted (10^{-2} to 10^{-4}) in sterile saline and plated on starch-casein agar supplemented with rifampicin. It was utilized in concentrations of 2.5, 5.0, and 10.0 μg/ml, respectively. All samples were plated in duplicate. Unsupplemented starch-casein agar served as the control. To minimize fungal contamination, all isolation agar plates were supplemented with 50 μg/ml each of cycloheximide and nystatin. Plates were incubated at 28° C for a minimum of 4 weeks.

Fermentations. Actinomycetes isolated from different sediments were cultivated in submerged culture in test tubes (150 × 25 mm) containing 12 ml medium. The latter corresponded to the seed medium described by Tunac et al. (1985) except that Proflo (Traders Protein, Ft. Worth, Tex, USA) was substituted for cotton seed meal. The fermentation tubes were inoculated with one loopful of growth of a specific actinomycete maintained on starch-casein agar. Inoculated tubes were incubated for 72–96 h on a rotary shaker (Psycrotherm, New Brunswick Scientific, NJ, USA) operated at 250 rpm at 28° C. After an appropriate period of incubation, the tubes were removed from the shaker and the contents centrifuged at 2000 *g* for 20 min. The resulting supernatant broths and mycelial pellets were retained for analysis.

Determination of bioactivity. Antimicrobial activity was determined by assaying fermentation broths against a battery of five bacteria, two yeasts, and three filamentous fungi. The test bacteria included *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis,* and *Mycobacterium smegmatis.* The fungi tested were comprised of *Candida albicans, C. krusei, Aspergillus niger* ATCC 12845, *Fusarium moniliforme* ATCC 10052, and *Trichoderma viride* ATCC 8678. Fungal assay plates were prepared as described previously (Pisano et al. 1987). All microoganisms were plated on glucose-peptone agar (Difco, Detroit, Mo) to which paper discs (12.5 mm Schleicher and Schuell, Keene, NH, USA), impregnated with 0.1 ml of a specific fermentation broth, were added. The plates were incubated for a total of 48 h at 28° C for the detection of antifungal activity and at 37° C for antibacterial activity.

Nature of antifungal activity. The nature of the antimycotic activity detected was determined spectrophotometrically (Pisano et al. 1987).

Identification of actinomycetes. Criteria employed for the indentification of the actinomycetes isolated were identical to those described peviously (Pisano et al. 1986). These included the determination of cell wall chemotypes (Becker et al. 1964).

Data analysis. Statistical comparisons of colony counts were based on Student's *t* test. Values of $P < 0.05$ were considered significant.

Results and discussion

Determinations of the number of actinomycetes present in the various sediments varied between a maximum of 1.1×10^6 colony-forming units (cfu)/g sample to a minimum of 1.0×10^2/g. Overall, highest counts occurred on starch-casein agar plates which did not contain rifampicin. Contaminating microorganisms of a mucoid nature were commonly found on such plates. These tended to stain Gram-negative. On agar plates supplemented with rifampicin, however, the presence of undesirable colonies was eliminated, but total counts were reduced. A similar observation was reported with a rifampicin-supplemented, starch-casein agar utilized for the selective isolation of streptomycetes from soil (Vickers et al. 1984). In the present study, the decrease in counts obtained at all concentrations of rifampicin, as compared to counts on control plates, were found to be statistically significant (Fig. 1). All plates supplemented with cycloheximide and nystatin were devoid of fungal contamination.

Cell wall analyses performed on the actinomycete isolates revealed that a majority yielded extracts which contained LL-2,6-diaminopimelic acid (DAP). Out of a total of 147 isolates, however, 55 (37%) contained the *meso*-isomer. In contrast, the incidence of *meso*-DAP-containing actinomycetes was only 4.5% in an earlier investigation of bioactive strains obtained from Hudson River sediments (Pisano et al. 1987). In the latter case rifampicin was not utilized for strain isolation. In the present investigation, the proportion of *meso*-DAP-containing actinomycetes obtained at each level of rifampicin employed was deter-

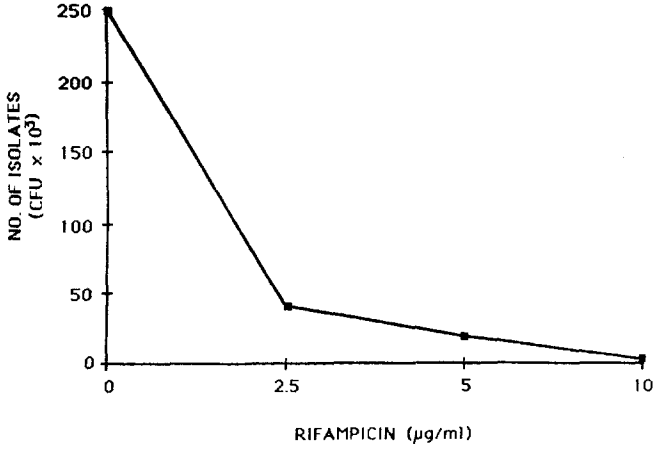

Fig. 1. Effect of various concentrations of rifampicin on colony counts (cfu) obtained using starch-casein agar. All plotted points represent average values

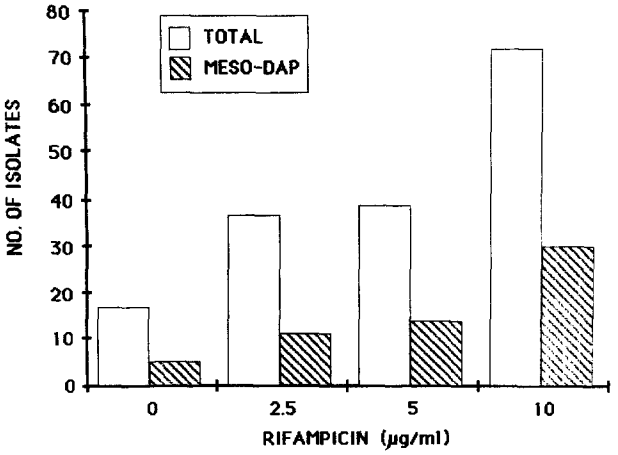

Fig. 2. Relationship between rifampicin concentrations employed and the proportion of strains isolated which contained *meso*-diaminopimelic acid (*meso*-DAP)

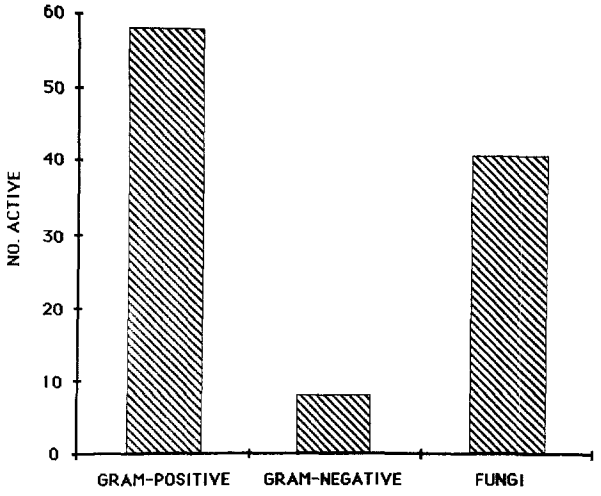

Fig. 3. Groups of microorganisms inhibited by bioactive actinomycetes isolated from marine sediments

mined. Figure 2 illustrates the results obtained. At a level of 2.5 µg/ml of rifampicin, 11 of 37 isolates (30%) contained *meso*-DAP which was slightly above the ratio of *meso*-DAP to LL-DAP-containing cultures found with control plates. At rifampicin concentrations of 5 µg/ml and 10 µg/ml the proportions of *meso*-DAP-containing isolates were 36% and 42%, respectively. Thus, from the foregoing it appears that there was a tendency to isolate a higher proportion of *meso*-DAP-containing strains as rifampicin levels were increased. Other investigations have demonstrated that rifampicin is useful for the isolation of *Actinomadura* strains from soils (Chormonova 1978; Athalye et al. 1981). In the present study we were unable to identify any of the isolates as being *Actinomadura* species. In fact, the majority of our strains yielded extracts containing LL-DAP and most were identified as streptomycetes. Those having the *meso*-isomer, for the most part, were identified as strains of *Micromonospora*.

A total of 147 strains of actinomycetes was isolated from the various sediments collected. Of these, 68, or 46%, displayed antimicrobial activity. By far, most of the inhibitory activity was directed against Gram-positive bacteria. As shown in Fig. 3, 58 strains of actinomycetes, representing 40% of the total number isolated, proved to be active. In this group, *B. subtilis* was the most susceptible, followed closely by *S. aureus; M. smegmatis* was the least susceptible of the Gram-positive strains. Activity against Gram-negative bacteria was minimal with only eight, or 5%, of the isolates proving effective. All eight isolates inhibited *E. coli*, whereas only one (strain SG-944) displayed anti-*Pseudomonas* activity. The eight strains were also active against Gram-positive bacteria. Antimycotic activity was evident against both yeasts and filamentous forms. A total of 41 (28%) isolates was inhibitory to one or more of the fungal test species. In general, the yeasts and filamentous fungi displayed equivalent susceptibilities to the antifungal substances elaborated by the actinomycestes. All but eight of the actinomycetes active against fungi were also inhibitory to Gram-positive and/or Gram-negative bacteria. Twelve of the isolates, which exhibited a broad spectrum of antifungal activity, were analysed to determine the nature of the active principle. The results obtained are shown in Table 1. All but three of the strains examined gave evidence that they synthesized polyenic substances. Hexaenes represented the major chromophore group detected, followed by heptaenes which were produced by two actinomycetes. No polyenes were found in association with strains SG-934, SG-935, and SG-936. It would be of interest to establish what kinds of antifungal substances are produced by the latter strains.

The results reported in the present investigation demonstrate that using rifampicin, as indicated above, may be successfully applied to ma-

Table 1. Nature of antifungal activity of strains of actinomycetes isolated from marine sediments

Chromophore class	Strain
Hexaenes	SG-896, SG-897, SG-947, SG-952, SG-954, SG-958, SG-971
Heptaenes	SG-900, SG-946
Unknowns	SG-934, SG-935, SG-936

rine sediments for the isolation of actinomycetes. Our findings suggest that an increase in the concentration of rifampicin is accompanied by a decrease in the total number of isolates obtained. Of interest, however, was the lack of contaminating bacteria. In addition, there was a steady increase in the proportion of *meso*-DAP-containing actinomycetes with increasing levels of the antibiotic. Thus, the greatest proportion of *meso*-DAP-containing actinomycetes occurred with rifampicin levels of 10 μg/ml.

In our hands, treatment of marine sediments with rifampicin proved to be selective for the isolation of strains of *Micromonospora*. Althalye et al. (1981) reported that addition of rifampicin (5 μg/ml) to several media allowed the selective isolation of *Actinomadura* strains from soil. In addition, the antibiotic contributed to the isolation of *Thermomonospora chromogena* and *Streptomyces albus* from hay and straw. Thus, it appears likely that the combination of rifampicin and a specific natural source may lead to the isolation of particular genera or even particular species of actinomycetes.

The antimicrobial activity exhibited by the bioactive actinomycetes isolated in the present investigation was similar to that obtained in a previous report. Specifically, it was demonstrated that actinomycetes isolated from marine sediments were primarily active against Gram-positive bacteria (Pisano et al. 1986). No *meso*-DAP-containing actinomycetes, however, were found among the bioactive isolates. In an investigation of the isolation of actinomycetes displaying antimycotic activity from Hudson River sediments, only one of the bioactive strains contained *meso*-DAP. In the present investigation, 55 of 147 isolates contained this isomer. Of these, 13% inhibited Gram-positive bacteria and several were active against fungi.

From the results reported here, rifampicin may be viewed as a useful agent for studies involving marine sediments. Its incorporation into media designed for the isolation of actinomycetes provides a selctivity which may not be attained using other procedures. The elimination of contaminating microorganisms from isolation media must be weighed against the reduction in total counts experienced with this antibiotic. If the latter is not perceived to be a major obstacle, then rifampicin should receive serious consideration for its inclusion in a program designed to isolate bioactive actinomycetes from marine sediments or other ecosystems.

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