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Application of pretreatments for the isolation of bioactive actinomycetes from marine sediments

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Summary. Actinomycetes were isolated from marine sediments collected in Sandy Hook Bay, New Jersey. A variety of pretreatments, including heat and phenol, were employed to improve the recovery of these microorganisms. In addition, plating of sediment samples on chitin agar, and filtration through cellulose membrane filters were also utilized. These pretreatments eliminated or severely curtailed the growth of contaminating microorganisms thereby facilitating the isolation of actinomycetes. A total of 120 isolates was obtained, of which 19 displayed significant antimicrobial activity. Most of the activity was directed against gram-positive bacteria, but inhibition of gram-negative species and a yeast were also evident.

Introduction

Goodfellow and Haynes (1984) have suggested that actinomycetes found in the marine ecosystem have been relatively neglected, and that they may be viewed as a selected gene pool possibly containing organisms capable of producing useful metabolic substances. They further suggested that the availability of methods which permit the isolation of specific groups of microorganisms make the exploitation of marine microorganisms a desirable goal. Cross (1982) has reviewed a variety of pretreatment techniques and selective media which are utilized to favor the isolation of specific genera of actinomycetes. The pretreatments discussed included enrichment, physical, and chemical methods. Cross indicated that pretreatments and selective media may be used to study the ecology of actinomycetes in natural habitats such as soil or water samples. Their primary utility appears to have been with soils.

Very little is known concerning the application of pretreatments to marine sediments to enhance the isolation of actinomycetes that synthesize antimicrobial substances. Since marine sediments represent an environment which is markedly different from that associated with soil samples, it is not clear how effective the pretreatment of such sediments would be for the recovery of bioactive actinomycetes.

This work was therefore undertaken to determine if the application of various pretreatment methods and the use of selective media would enhance the isolation of bioactive actinomycetes from marine sediments.

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Materials and methods

Sample collection. Marine sediments were collected at eight sampling sites in Sandy Hook Bay, New Jersey in May, 1985. Water depths at these locations varied from 0.33 to 7 m. Sediments were brought to the surface by means of a Van Veen sampler. Total counts of actinomycetes, in each sample, were performed using M3 agar (Rowbotham and Cross 1977).

Pretreatments. Pretreatments used included dry heat (Nonomura and Ohara 1969) followed by plating on starch-casein agar (Okazaki and Okami 1972), and exposure to 1% phenol with subcultures being made on glucose-asparagine agar (Lawrence 1956). Some of the sediments were plated directly on to chitin agar (Hsu and Lockwood 1975), or subjected to filtration through cellulose ester membrane filters followed by isolation on modified Bennett agar (Hirsch and Christensen 1983). Cycloheximide (100 μg/ml) as incorporated in the latter agar substrate to prevent fungal growth. All isolation agar media employed in this study were incubated at 28° C for at least four weeks. Sample dilutions of up to 10^{-4}, in sterile saline, were usually sufficient to obtain isolated colonies of actinomycetes. Controls, which did not receive a specific pretreatment, were included in all experiments.

Determination of bioactivity. Antimicrobial activity was readily determined by streaking individual actinomycete isolates on one side of a Petri dish covering approximately one-third of the surface of the glucose-peptone agar (Difco) layer. After incubation for 72–120 h at 28° C, a variety of test microorganisms were cross-streaked at right angles to the border of the actinomycete. The test species were comprised of *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis, Mycobacterium smegmatis,* and *Candida krusei.* The cross streaked plates were reincubated at 28° C for 48 h and examined for inhibition of the test strains.

Identification of actinomycetes. Criteria for the identification of actinomycetes included characteristic growth on starch-casein agar, chitin hydrolysis, selective filtration (Hirsch and Christensen 1983), and microscopic examination. In addition, analyses were made of whole cell hydrolysates (Becker et al. 1964) of the active strains to determine cell wall chemotypes.

Results and discussion

Total counts of actinomycetes in the Sandy Hook Bay samples ranged from a maximum of 2.4×10^6 cfu/g sample to a minimum of 10^3 cfu/g. In all, approximately 120 isolates, identified as actinomycetes, were recovered. The use of heat reduced the number of undesirable bacteria occurring on the isolation plates. Occasionally, however, mucoid colonies were evident. Microscopic examination revealed the absence of hyphae and typically, the cells stained gram-negative. In contrast, controls which were not heated, were usually overgrown with contaminants. Nonomura and Ohara (1969) reported that treating soil with heat at 120° C drastically reduced the number of undesirable bacteria and in addition, the isolation of streptomycetes was also considerably curtailed. They reported heat to be selective for the isolation of *Microbispora* and *Streptosporangium.* Heat has also been employed for the isolation of salt marsh actinomycetes (Hunter et al. 1981). In the present study, phenol pretreatment eliminated the outgrowth of all but a few bacterial colonies. In addition, the occurrence of fungal growth was sharply reduced. Control plates, prepared from untreated sediments, exhibited an abundance of contaminating fungi. A similar observation was reported previously (Lawrence 1956). Figure 1 illustrates the appearance of a variety of predominantly actinomycete colonies obtained from marine sediments, pretreated with phenol, in the work being reported. Chitin agar, as a selective medium, proved to be effective for the isolation of actinomycetes (Fig. 2). Contaminating microorganisms did not occur in significant numbers and hence, did not interfere with the isolation of preferred strains. The selectivity of chitin-containing media

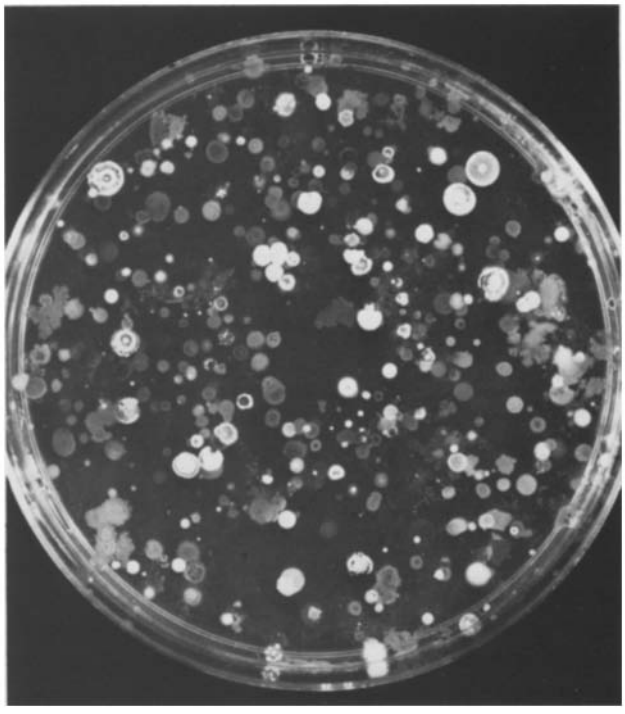

Fig. 1. Appearance of actinomycete colonies isolated from a sample treated with 1% phenol. Colonies were plated on glucose-asparagine agar

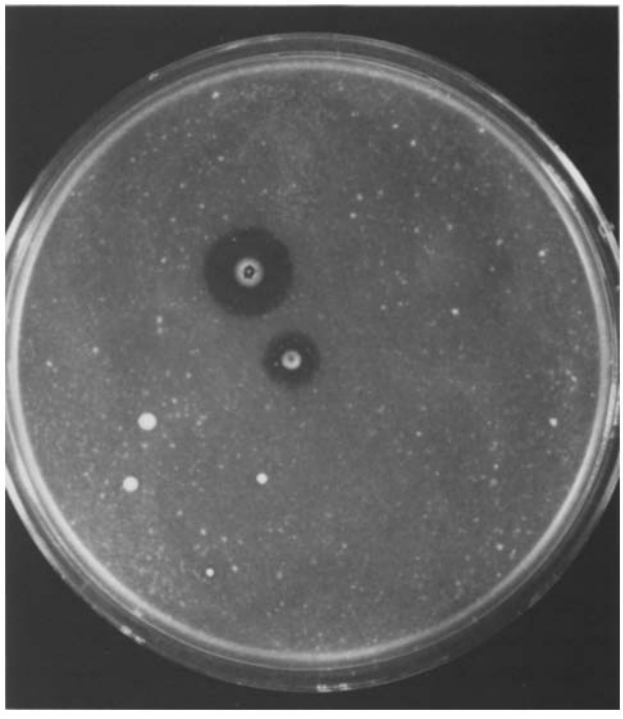

Fig. 2. Examples of chitinolytic colonies of actinomycetes plated on chitin agar

is attributed to what has been described as a near universal ability of actinomycetes to hydrolyze chitin (Hsu and Lockwood 1975), although this view is not entirely shared by others (Williams and Wellington 1982). The filtration method of Hirsch and Christensen (1983) was successfully applied, in the present study, to the isolation of bioactive actinomycetes from marine sediments. The presence of cycloheximide in the agar substrate suppressed fungal growth and allowed actinomycetes to pass through the filter, and to establish growth on the underlying agar surface without competition. Worthy of note is the fact that most of the active actinomycetes collected in this study resulted from filtration methodology. Analyses of whole cell hydrolysates disclosed that all 19 strains contained L-2,6-diaminopimelic acid as part of their cell wall composition. Based on cell wall chemistry, colonial characteristics, and microscopic examination, the active actinomycetes were identified as being streptomycetes.

A total of 19 strains of actinomycetes, which displayed antimicrobial activity, was recovered from the marine sediments collected in Sandy Hook Bay (Table 1). Only one active culture, SG-53, was obtained from heat treated sediments. Five of the active strains, Sg-102, Sg-113, Sg-125, Sg-127, and Sg-202, were collected from sediments pretreated with phenol. The greatest number of active strains were isolated from samples which were passed through cellulose ester membrane filters. Ten active strains were collected including SG-112, SG-117, SG-122, SG-123, SG-124, SG-130, SG-132, SG-135, SG-153, and SG-159. Three strains isolated from chitin agar also proved to be active. The ability to inhibit all test microorganisms was evident solely with strain SG-127. By far, the most prominent inhibitory activity noted was directed against gram-positive bacteria. All of the actinomycetes displayed significant inhibition of *B. subtilis* and 12 inhibited the growth of *S. aureus*. Activity against *E. coli* was attributed to three actinomycetes whereas only one inhibited *P. aeruginosa*. Almost all of the isolates were active against *M. smegmatis*. Interestingly, considerable antifungal activity was detected. More than one-half of the total number of active cultures inhibited *C. krusei*.

Table 1. Antimicrobial activity by strains of actinomycetes isolated from Sandy Hook Bay

Strain	*S. aureus*	*E. coli*	*P. aeruginosa*	*B. subtilis*	*M. smegmatis*	*C. krusei*
SG-53	−	−	−	+	+	+
SG-102	+	−	−	+	+	−
SG-112	−	−	−	+	−	+
SG-113	−	−	−	+	−	+
SG-117	−	−	−	+	+	−
SG-122	+	+	−	+	−	+
SG-123	+	−	−	+	+	+
SG-124	+	−	−	+	+	+
SG-125	+	−	−	+	+	+
SG-127	+	+	+	+	+	+
SG-130	−	−	−	+	+	+
SG-132	+	−	−	+	+	−
SG-135	−	−	−	+	+	+
SG-151	+	−	−	+	+	+
SG-152	−	−	−	+	+	+
SG-153	+	−	−	+	+	+
SG-159	+	−	−	+	+	−
SG-202	+	−	−	+	+	+
SG-207	+	+	−	+	+	−

+ = inhibition − = no inhibition

The results obtained in the present investigation demonstrate that the kinds of pretreatment methods reviewed by Cross (1982) and Williams and Wellington (1982), may be successfully applied to marine sediments to facilitate the isolation of actinomycetes. Both physical and chemical treatments were employed in the work being reported. In addition, the utility of selective media, such as chitin agar, for the recovery of bioactive strains of actinomycetes from these sediments was also underscored. Soil actinomycetes have long been a prime source of antimicrobial substances, but it is now apparent that strains from other locales, marine habitats in particular, may yield new and interesting antibiotics (Okami et al. 1979; Okazaki et al. 1975). Thus, it appears logical to extend our investigation of the marine habitat as a source of additional genera of actinomycetes in the search for novel metabolites. The applicability of special pretreatment techniques to marine sediments would be expected to facilitate the isolation of interesting species with special biosynthetic capabilities.

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