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Isolation and Diversity of Actinomycetes in the Chesapeake Bay†

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Chesapeake Bay was investigated as a source of actinomycetes to screen for production of novel bioactive compounds. The presence of relatively large populations of actinoplanetes (chemotype II/D actinomycetes) in Chesapeake Bay sediment samples indicates that it is an eminently suitable ecosystem from which to isolate actinomycetes for screening programs. Actinomycetes were isolated from sediment samples collected in Chesapeake Bay with an isolation medium containing nalidixic acid, which proved to be more effective than heat pretreatment of samples. Actinomycete counts ranged from a high of 1.4×10^5 to a low of 1.8×10^2 CFU/ml of sediment. Actinomycetes constituted 0.15 to 8.63% of the culturable microbial community. The majority of isolates from the eight stations studied were actinoplanetes (i.e., chemotype II/D), and 249 of these isolates were obtained in a total of 298 actinomycete isolates. Antimicrobial activity profiles indicated that diverse populations of actinoplanetes were present at each station. DNA hybridization studies showed considerable diversity among isolates between stations, but indicated that actinoplanete strains making up populations at nearby stations were more similar to each other than to populations sampled at distant stations. The diversity of actinoplanetes and the ease with which these organisms were isolated from Chesapeake Bay sediments make this a useful source of these actinomycetes.

Actinomycetes have provided many important bioactive compounds of high commercial value and continue to be routinely screened for new bioactive substances. These searches have been remarkably successful, and approximately two-thirds of naturally occurring antibiotics, including many of medical importance, have been isolated from actinomycetes (15). Actinomycetes are abundant in terrestrial soils, a source of the majority of isolates shown to produce bioactive compounds. One result of intensive screening programs carried out over the past several decades is that there is a growing problem of "rediscovery" of already known bioactive compounds (13). One approach to address this problem is to expand the source of actinomycetes by carrying out ecological assessments of environments other than terrestrial soils. There is also growing interest in the nonstreptomycete actinomycetes as sources of novel compounds (13). We isolated actinomycetes from Chesapeake Bay sediments and found that the majority of isolates were actinoplanetes, indicating that the Chesapeake Bay is a suitable source of actinomycetes to screen for production of novel bioactive compounds.

Goodfellow and Haynes (7) reviewed the literature on isolation of actinomycetes from marine sediments and suggested that this source may be valuable for the isolation of novel actinomycetes with the potential to yield useful new products. However, it has not yet been resolved whether actinomycetes form part of the autochthonous marine microbial community of sediments or whether actinomycetes isolated from marine sediment samples originated from terrestrial habitats and were simply carried out to sea in the form of resistant spores. Jensen et al. (9) recently reported a bimodal distribution of actinomycetes in near-shore tropical marine environments, with streptomycetes predominating at shallow depths and an increase in actinoplanetes with increasing depth. This distribution and the requirement for seawater for growth of the actinoplanete isolates indicate that autochthonous actinomycete populations exist (9). This is also suggested by the isolation of actinomycetes from deep oceanic sediments distant to land (23–25). However, actinomycetes from marine samples have rarely undergone screening for novel metabolites, and there is evidence that actinomycetes usually make up only a small proportion of the bacterial flora of marine habitats, with absolute numbers of actinomycetes much lower than in terrestrial habitats (5, 7). The consequence is that it should be more difficult to obtain large numbers of isolates from marine samples for screening purposes. In view of the potential importance of marine actinomycetes as a source of novel bioactive compounds, we investigated methods to improve the isolation of actinomycetes from marine and estuarine environments and applied these methods to isolation of actinomycetes from Chesapeake Bay sediments.

MATERIALS AND METHODS

Sampling. Sediment samples were collected from the Chesapeake Bay on 10 August 1991, 7 to 8 October 1991, and 14 March 1992 by using a Smith-MacIntyre sediment grab sampler. Sampling stations were located along the midaxis of the Chesapeake Bay at the following latitudes: 1, 39°08′N; 2, 38°45′N; 3, 38°18′N; 4, 37°56′N; 5, 37°30′N; 6, 37°10′N. In addition, stations were located at the mouth of the bay (station 7: 36°59′N 75°58′W) and ca. 10 km offshore (station 8: 37°00′N 75°40′W) (Fig. 1). The surface of each grab sample was aseptically collected with a sterile syringe, and all samples were processed on board ship within 30 min of collection.

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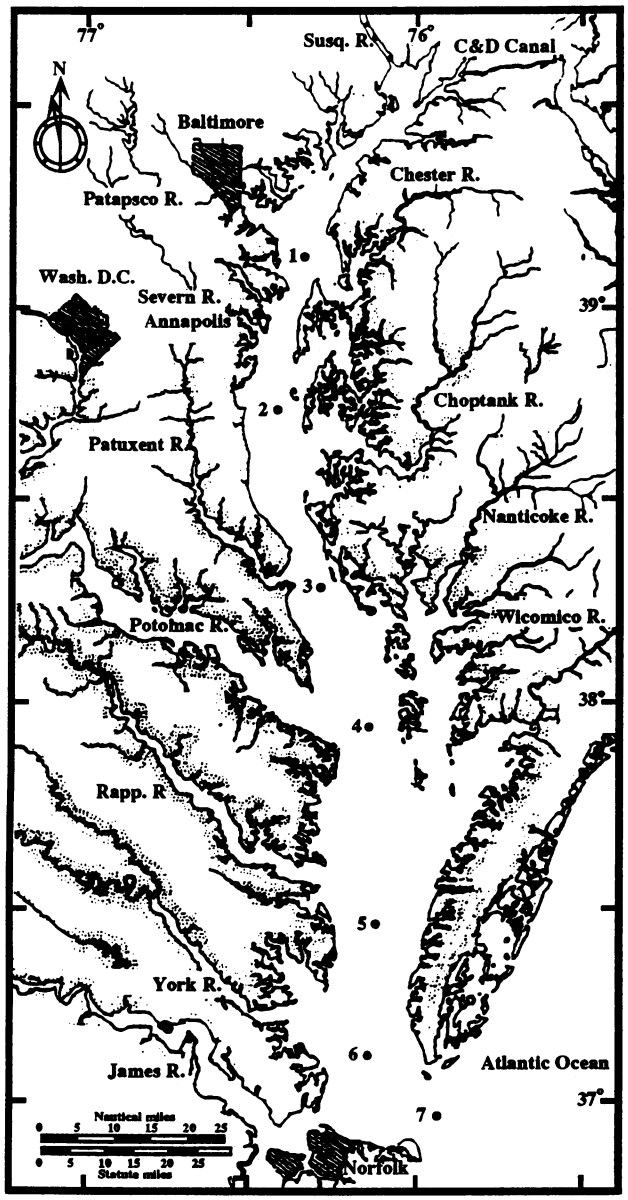

FIG. 1. Location of sampling stations in the Chesapeake Bay. Stations 1 to 6 were located on the midaxis of the bay at the following latitudes: 1, 39°08′N; 2, 38°45′N; 3, 38°18′N; 4, 37°56′N; 5, 37°30′N; 6, 37°10′N. Station 7 was at 36°59′N 75°58′W, and station 8 (not depicted) was at 37°00′N 75°40′W.

Sample treatment. Heat treatments were performed by holding sediment samples in a water bath at 50°C for 60 min. All samples were diluted with sterile 0.5% saline prior to inoculation in triplicate onto isolation plates.

Media and culture conditions. The medium used for actinomycete isolation and enumeration was starch-casein agar supplemented with 0.5% (wt/vol) NaCl and 10 μg of nalidixic acid (NA) per ml. Starch-casein agar supplemented with 0.5% (wt/vol) NaCl, but without NA, served as a control to assess the effect of NA treatment. All isolation media also contained nystatin and cycloheximide at final concentrations of 25 and 10 μg/ml, respectively, to minimize fungal contamination. All colonies with the tough, leathery characteristics of actinomycete vegetative mycelia (and in some cases with aerial mycelia and spore formation) were counted as actinomycetes. One-tenth-strength tryptic soy agar (Difco Laboratories, Detroit, Mich.) containing 0.5% (wt/vol) NaCl was used to determine aerobic heterotrophic counts. Microorganisms were cultured by spread plating sediment sample dilutions onto solid media. Plates were incubated at 24°C, and colonies were counted after incubation for 28 days. Tryptone yeast extract broth (ISP-1) (Difco) supplemented with 0.5% (wt/vol) NaCl and 0.2% (wt/vol) glucose was used to culture actinomycetes for taxonomic studies and DNA isolation. These cultures were incubated at 24°C, with shaking. To assess antimicrobial activities of isolates, actinomycetes were cultured at 24°C for 7 days on yeast malt extract agar (ISP-2) (Difco) supplemented with 0.5% (wt/vol) NaCl.

Detection of antimicrobial activities. Antimicrobial activities were determined by streak plating, using the following test microorganisms obtained from the American Type Culture Collection (ATCC): *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Streptococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Candida albicans* ATCC 14053.

Cell wall analysis. After cultivation in liquid medium, actinomycete cells were harvested. The cells were hydrolyzed and analyzed chromatographically for isomeric diaminopimelic acid configurations and for whole-cell sugar composition, as described by Hasegawa et al. (8). Chromatography was performed on cellulose-coated thin-layer chromatography plates (Baker-flex; J. T. Baker Inc., Phillipsburg, N.J.). Actinomycetes were designated by wall chemotype and whole-cell sugar pattern as outlined by Goodfellow (6).

DNA isolation. Actinomycete cells were harvested by centrifugation, and cell pellets were suspended in TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0) containing 2 mg of lysozyme per ml in a total volume of 5 ml. Samples were incubated at 30°C for 30 min, which was followed by addition of 1.2 ml of 0.5 M EDTA and protease (final concentration, 0.2 mg/ml) and an additional 30 min of incubation. After addition of 0.7 ml of 10% (wt/vol) sodium dodecyl sulfate (SDS), 1.8 ml of 5 M NaCl, and 1.5 ml of 10% CTAB–NaCl solution (10% [wt/vol] hexadecyltrimethyl ammonium bromide in 0.7 M NaCl), the mixtures were incubated at 65°C for 20 min. Samples were extracted with chloroform-isoamyl alcohol (24:1, by volume), and the supernatants were transferred to clean tubes. DNA was precipitated by addition of 0.6 volume of isopropanol and pelleted by centrifugation (48,000 × *g*, 15 min). DNA pellets were rinsed with 70% (wt/vol) ethanol, dried, dissolved in TE buffer, and quantified by absorption spectrophotometry at 260 nm.

DNA hybridization experiments. To obtain an indication of overall diversity among strains making up the actinomycete populations, between stations, DNA-DNA hybridization experiments were performed. DNA was extracted from 16 randomly selected actinoplanetes (i.e., chemotype II/D actinomycetes by cell wall analysis) from each station, and equal amounts of DNA from each isolate were mixed to obtain a pool of DNA representative of that station. These pools were compared by hybridization studies, in which each pool was radiolabelled and hybridized to dot blots containing DNA samples from all pools. Probe DNA was labeled with [α-^{32}P]dATP by nick translation, using a kit supplied by Boehringer Mannheim Corp., Indianapolis, Ind., and was purified by passage through a Sephadex G-50 column (12).

Target DNA was denatured by alkaline conditions and vacuum blotted onto MagnaGraph nylon membrane (Micron Separations Inc., Westboro, Mass.) in concentrations ranging from 50 to 1,000 ng per dot, using the procedure recommended by the manufacturer. DNA was bound to membranes by baking at 80°C for 30 min.

Hybridization. Membranes were prehybridized at 65°C for 2 h in prehybridization solution containing denatured salmon sperm DNA (6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 5× Denhardt solution [12], 1% [wt/vol] SDS, 50 μg of denatured salmon sperm DNA [Sigma Chemical Co., St. Louis, Mo.] per ml) and hybridized at the same temperature for 12 h in hybridization solution (6× SSC, 0.5% SDS [wt/vol], 50 μg of denatured salmon sperm DNA per ml). A 20× SSC stock solution was made as described in Maniatis et al. (12). Membranes were washed twice each in 5× SSC–0.5% (wt/vol) SDS (15 min at room temperature), 1× SSC–0.5% (wt/vol) SDS (15 min at 37°C), and 0.1× SSC–1% (wt/vol) SDS (5 min at 37°C). After drying at room temperature, disks of membranes containing each DNA dot were cut. Radioactivity of the DNA dots was determined by liquid scintillation counting. Hybridization of each probe to the target dot containing DNA from which that probe was derived was defined as 100% hybridization, and hybridization to dissimilar pools was expressed as a percentage of this self-hybridization level, i.e., observed hybridization of a particular pool, P_o (%), $= 100 \times [(R_p - R_b)/(R_s - R_b)]$, where R is the radioactivity from the target DNA dot and the subscripts p, b, and s denote, respectively, pool DNA, background (calf thymus DNA), and self-hybridization.

RESULTS AND DISCUSSION

The Chesapeake Bay was selected as an ecosystem in which to study the ecology of actinomycetes because it has a wide range of salinities, from freshwater at the head of the bay to seawater at the mouth. In addition, a large amount of soil carrying spores of terrestrial actinomycetes enters the head of the bay. This material is carried to the bay by the Susquehanna River, which accounts for approximately 50% of the fresh water flowing into the bay, with the bulk of the remainder flowing from the Potomac and James Rivers (20). Previously, this laboratory studied factors affecting isolation of actinomycetes from Chesapeake Bay (1, 23). The present study was designed specifically to evaluate Chesapeake Bay as a source of actinomycetes for screening programs. We therefore investigated the diversity of actinomycetes in Chesapeake Bay and assessed whether the range of strains isolated from this source differed from those typically isolated from terrestrial soil samples.

Isolation of actinomycetes from Chesapeake Bay sediments. Heat treatment is often used as a pretreatment of marine sediments, prior to actinomycete isolation, to reduce the numbers of gram-negative bacteria commonly found in marine samples and often overrunning isolation plates (2, 9, 19). The effectiveness of addition of NA to the isolation medium on actinomycete counts was compared with conventional heat pretreatment. Results are shown in Table 1. An improvement in actinomycete counts was found with heat pretreatment, compared with untreated samples, but counts from three stations sampled in October (stations 1, 2, and 3) were reduced by heat pretreatment. In general, counts obtained from an isolation medium supplemented with NA were similar, both with and without heat pretreatment, but were significantly higher than counts from heat pretreatment alone.

TABLE 1. Effects of NA and heat treatment on isolation of actinomycetes from marine sediments

Mo and station	Dilution	Avg CFU/100 μl of dilution (n = 3)			
		No heat treatment		Heat treatment	
		−NA	+NA	−NA	+NA
August					
1	1/1,000	6.3	68.3	32.3	77.0
2	1/100	0.0	27.0	1.3	21.7
3	1/100	1.0	3.3	1.3	5.0
4	1/100	3.3	39.7	22.3	40.3
October					
1	1/1,000	9.7	43.0	8.0	58.3
2	1/500	12.3	75.3	4.3	49.7
3	1/500	20.7	37.0	13.3	21.3
4	1/50	5.7	13.7	9.3	14.3
5	1/50	7.0	13.7	10.3	20.3
6	1/50	1.3	3.7	4.7	4.0
7	1/50	0.0	0.7	0.3	0.3
8	1/50	0.7	1.3	1.0	0.7

The effect of heat pretreatment and NA on the proportion of actinomycetes in the total number of bacteria on isolation plates was also investigated, and the proportion of actinomycetes increased with addition of NA to the isolation medium. Heat treatment was generally less effective than NA in increasing the proportion of actinomycetes, but both treatments, in combination, were most effective in maximizing the proportion of actinomycetes, which ranged between ca. 40 and 90% (Fig. 2).

From the literature, it is clear that a variety of selection methods have been used to facilitate isolation of actinomycetes from marine samples (5, 7, 18). These methods include pretreatment of samples with heat or phenol or both and plating on media containing rifampin to select for bioactive actinomycetes.

An objective of this study was to develop a protocol that reduced contamination of isolation plates by gram-negative bacteria common to marine and estuarine environments and allowed isolation of as broad a range of actinomycetes as possible. Starch-casein agar containing NA proved effective for this task. To our knowledge, this is the first report of the use of NA to enhance isolation of actinomycetes from marine and estuarine samples.

Heat pretreatment of samples was omitted since heat treatment selects against actinomycetes present in samples as actively growing mycelia, allowing subsequent outgrowth only of actinomycetes present as heat-resistant spores. Heat treatment did, in fact, result in a marked decrease in actinomycete counts of samples collected at stations 2 and 3 in October (Table 1).

Distribution of actinomycetes. Actinomycete counts ranged from a high of 1.4×10^5 to a low of 1.8×10^2 within the bay, although lower counts were recorded at the mouth of the bay and offshore. Results are presented in Table 2. Actinomycete counts, in relation to the total microbial population, ranged from 0.36 to 8.63% in samples collected within the bay. The highest actinomycete count was recorded at station 1 during all three cruises. Although variations in total microbial counts and actinomycete counts were observed between August, October, and March at the four stations sampled on the three cruises, the percentage of actino-

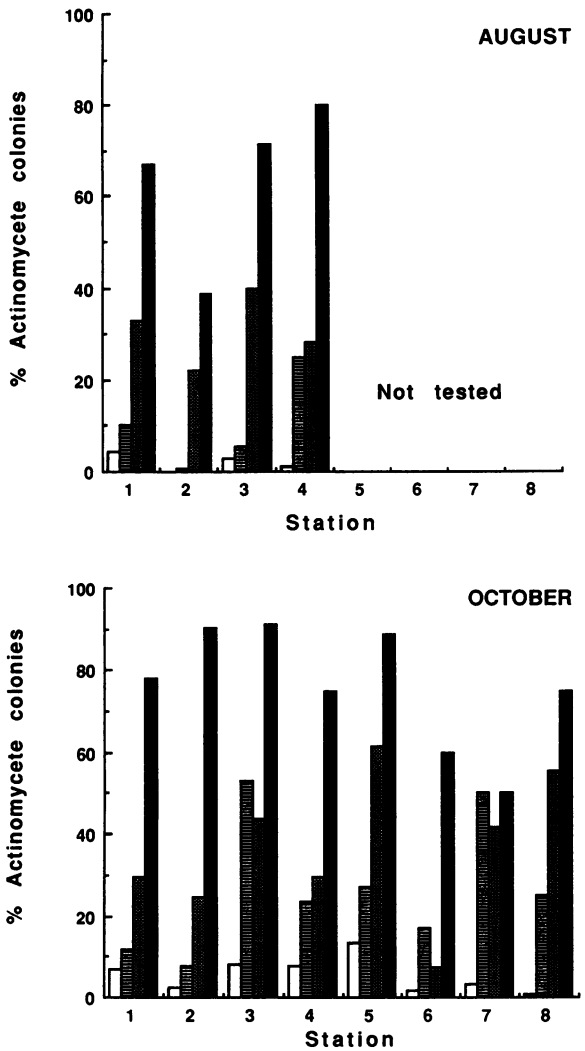

FIG. 2. Effect of NA and heat treatment on the percentage of actinomycetes in the total culturable microbial population. Actinomycete and total microbial counts were measured after 4 weeks of cultivation on starch-casein agar containing 10 μg of NA per ml, nystatin (25 μg/ml), and cycloheximide (10 μg/ml) and on one-tenth-strength tryptic soy agar, respectively. Both media contained 0.5% (wt/vol) NaCl. □, no treatment; ▤, heat treatment; ▩, NA treatment; ■, heat and NA treatment.

mycetes was found to be remarkably constant at these stations.

Taxonomic analysis. A total of 298 actinomycetes were isolated, and these were divided into four groups on the basis of chemotaxonomic evaluation. The major population was composed of actinomycetes of chemotype II/D cell wall structure (actinoplanetes). The proportion of actinoplanetes in the total actinomycete population increased towards the mouth of the bay. All isolates in samples collected from the station at the mouth of the bay and the offshore station (stations 7 and 8, respectively) were identified as actinoplanetes (Table 3). Conversely, the number of other actinomycetes (chemotype I, III/B, III/C, and IV/A cell walls) was highest at station 1, at the head of the bay, and decreased towards the mouth. A similar trend was observed in samples collected in August and October (Table 3).

TABLE 2. Actinomycete counts in sediment samples collected from the Chesapeake Bay[a]

Mo and station	Microbial counts (CFU/ml of sediment)		% Actinomycetes
	Total	Actinomycetes	
August			
1	4.60×10^6	6.83×10^4	1.48
2	4.37×10^4	2.70×10^3	6.18
3	8.50×10^3	3.33×10^2	3.92
4	1.30×10^5	3.97×10^3	3.05
October			
1	2.47×10^6	4.30×10^4	1.74
2	4.37×10^5	3.77×10^4	8.63
3	4.19×10^5	1.85×10^4	4.42
4	2.02×10^4	6.85×10^2	3.39
5	1.74×10^5	6.85×10^2	0.39
6	1.20×10^5	1.84×10^2	0.15
7	2.77×10^3	3.34×10^1	1.21
8	1.84×10^4	6.65×10^1	0.36
March			
1	7.50×10^6	1.44×10^5	1.92
2	3.73×10^5	2.49×10^4	6.68
3	9.50×10^5	2.94×10^4	3.09
4	2.38×10^5	8.50×10^3	3.57

[a] Actinomycete counts were determined on starch-casein medium containing NA, and total microbial counts were determined on one-tenth-strength tryptic soy agar medium.

Lechevalier (10) includes seven genera among the chemotype II/D actinomycetes. The great majority of isolates from the Chesapeake Bay appeared to belong to one of these genera, i.e., the genus *Micromonospora*, and were assigned to this genus on the basis of characteristic orange to brown colonies, often including black mucoid masses, but possessing neither sporangia nor motile spores (6, 10).

The predominance of chemotype II/D actinomycetes in Chesapeake Bay sediments was interesting, because the predominant actinomycetes in terrestrial soil are the streptomycetes (5), a group expected to be dominant in the Chesapeake Bay also, if actinomycetes in bay samples originated primarily from soil runoff. The most numerous

TABLE 3. Taxonomic diversity of actinomycetes in sediment samples at each station

Mo and station	Actinomycetes (% of total isolated)			
	I[a]	II/D	III/B,C	IV/A
August				
1	3.0	82.1	4.5	10.4
2	0.0	95.8	0.0	4.2
3	0.0	100.0	0.0	0.0
4	2.7	94.6	2.7	0.0
October				
1	12.1	60.6	18.2	9.1
2	11.4	80.0	5.7	2.9
3	17.6	82.4	0.0	0.0
4	10.5	84.2	5.3	0.0
5	11.7	76.6	11.7	0.0
6	10.0	90.0	0.0	0.0
7	0.0	100.0	0.0	0.0
8	0.0	100.0	0.0	0.0

[a] Chemotype.

TABLE 4. Relative percent hybridization of DNA from actinoplanetes from stations 1 to 5 and 8 sampled in October 1991[a]

Target DNA	% Hybridization with given probe					
	1	2	3	4	5	8
1	100.0	78.9	60.9	37.9	24.7	29.4
2	46.3	100.0	82.3	74.2	57.9	66.3
3	53.5	82.0	100.0	78.7	70.4	81.1
4	44.6	82.5	82.8	100.0	81.8	88.6
5	42.0	80.6	83.6	71.6	100.0	106.5
8	45.3	76.6	57.6	60.2	70.0	100.0

[a] Equal quantities of DNA from 16 chemotype II/D actinomycetes from each station were mixed and used as probe and target DNAs. The hybridization signal of each probe to target DNA from which that probe was derived was defined as 100% hybridization, and hybridization to other pools was expressed as a percentage of this self-hybridization signal.

actinomycetes in Chesapeake Bay samples were *Micromonospora* spp., suggesting that actinoplanetes form part of the indigenous microflora in these sediments or that spores of these organisms are well adapted to survival under the conditions found in the Chesapeake Bay. It is interesting to note that Jensen et al. (9) observed an increase in actinoplanetes with increasing water depth off islands in the Bahamas and proposed that actinoplanetes were active members of the microbial community in that environment. Salt tolerances and requirements of actinomycetes isolated from the Chesapeake Bay were not determined and may have been useful in determining whether isolates were indigenous to the bay or originated from terrestrial runoff. Although the possibility that many of the organisms isolated in this study were of terrestrial origin cannot be excluded, nevertheless, the predominance of "unusual" actinomycetes such as actinoplanetes makes Chesapeake Bay sediments a useful source of actinomycetes for screening programs for isolates that produce novel bioactive compounds.

Diversity of actinomycetes in the Chesapeake Bay. Recently, marine actinomycetes have received increasing attention as a source of novel bioactive substances (16, 17, 21). To assess whether actinoplanetes from the Chesapeake Bay may be a useful population to screen for bioactive compounds, it was of interest to determine the diversity within this population. The diversity of actinoplanetes isolated from sediment samples collected at different stations was estimated by DNA hybridization, an approach increasingly employed to compare bacterial communities of different environmental samples (22) and which has proved to be effective for natural bacterioplankton assemblages (11).

Hybridization experiments were designed to give some indication of the variety of actinoplanetes isolated from sediment samples collected at different stations in the Chesapeake Bay without having to perform extensive biochemical characterization of large numbers of isolates. These isolates could not be distinguished from each other by morphological criteria. Results of hybridization experiments are shown in Table 4. In general, probes showed stronger hybridization with target DNA from nearby stations than with target DNA from more distant stations. These data suggest that chemotype II/D actinomycete populations were different at different stations in the Chesapeake Bay, but that populations of nearby stations resembled each other more closely than isolates of populations from more distantly located stations. This implies that several well-separated stations should be sampled to obtain the maximum diversity of actinoplanetes for screening for bioactive compounds.

TABLE 5. Diversity of actinoplanetes (chemotype II/D) at each station based on antimicrobial profiles of isolates, determined with six test organisms

Mo and station	Total isolates	Total antimicrobial profiles	Diversity index[a]
August			
1	55	12	0.22
2	23	10	0.43
3	6	2	0.33
4	35	12	0.34
October			
1	20	6	0.30
2	28	11	0.39
3	28	8	0.29
4	16	8	0.50
5	13	7	0.54
6	9	6	0.67
7	2	2	1.00
8	14	6	0.43

[a] Diversity index = total number of antimicrobial patterns/total number of isolates tested.

We also investigated the diversity of chemotype II/D actinomycete strains present in individual samples, distinguishing isolates on the basis of their antimicrobial profiles. This approach distinguishes between strains which may belong to a single species, which is useful since production of bioactive agents is strain specific, not species specific (14). Antimicrobial spectra of chemotype II/D actinomycetes were determined by using three gram-positive and two gram-negative bacteria and a yeast strain as the test organisms. The antimicrobial profile of each isolate (characteristic pattern of resistance or sensitivity of test organisms to that isolate) was determined. Antimicrobial profiles were used to compute a diversity index (diversity index = number of antimicrobial patterns/number of isolates tested) for each station. This index indicates the likelihood of a particular colony representing a novel isolate at that station. This value ranged from 0.22 to 1.0 (Table 5), and the results indicated considerable diversity of strains within the chemotype II/D actinomycete isolates from each station. Because the number of antimicrobial profiles increases as the number of test microorganisms used to determine these profiles increases, actual diversity may be higher than indicated, since only six test organisms were included. In any case, significant diversity was found among the actinoplanetes isolated from Chesapeake Bay sediments.

In summary, the Chesapeake Bay is concluded to be an eminently suitable ecosystem from which to isolate actinomycetes for screening programs designed to isolate strains producing bioactive compounds.

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