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Heavy-Metal and Antibiotic Resistance in the Bacterial Flora of Sediments of New York Bight

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The New York Bight extends seaward some 80 to 100 miles (ca. 129 to 161 km) from the Long Island and New Jersey shorelines to the edge of the continental shelf. Over 14×10^6 m^3 of sewage sludge, dredge spoils, acid wastes, and cellar dirt are discharged into this area each year. Large populations of *Bacillus* sp. resistant to 20 μg of mercury per ml were observed in Bight sediments contaminated by these wastes. Resistant *Bacillus* populations were much greater in sediments containing high concentrations of Hg and other heavy metals than in sediments from areas further offshore where dumping has never been practiced and where heavy-metal concentrations were found to be low. Ampicillin resistance due mainly to β-lactamase production was significantly ($P < 0.001$) more frequent in *Bacillus* strains from sediments near the sewage sludge dump site than in similar *Bacillus* populations from control sediments. *Bacillus* strains with combined ampicillin and Hg resistances were almost six times as frequent at the sludge dump site as in control sediments. This observation suggests that genes for Hg resistance and β-lactamase production are simultaneously selected for in *Bacillus* and that heavy-metal contamination of an ecosystem can result in a selection pressure for antibiotic resistance in bacteria in that system. Also, Hg resistance was frequently linked with other heavy-metal resistances and, in a substantial proportion of *Bacillus* strains, involved reduction to volatile metallic Hg (Hg^0).

Interactions of bacteria with heavy-metal ions have aroused considerable interest in recent years. Studies of these interactions have focused especially on conversions of mercuric ions in soils (15) and in marine (11) and freshwater sediments (18). These processes may result in loss of volatile inorganic Hg (Hg^0) from sediments that contain the element in other forms including toxic methyl-Hg. Therefore, loss of Hg in this form may be of considerable ecological benefit in depuration of these contaminated sediments.

Sediments of areas in the New York Bight, the area of ocean off the Long Island and New Jersey shorelines, have been heavily polluted for over 50 years by a variety of domestic and industrial wastes and dredge spoils that contain high concentrations of heavy metals. Levels of metals in sediments near the sewage sludge disposal area have ranged from 10 to 100 times greater than in comparable unpolluted sediments further offshore in the Bight (1). Preliminary bacteriological studies have indicated that *Escherichia coli* with multiple-antibiotic and heavy-metal resistances are common at one site in sediment of the sewage sludge dump area (7), and it was suggested that heavy-metal contamination of the sediment was the selective pressure for the plasmids encoding the resistances. Heavy-metal resistance in a number of different bacterial genera has been shown to be plasmid mediated (17, 20) and in some cases is present together with antibiotic resistances (9, 10, 13). In instances where these genes are grouped on the same plasmid, it is reasonable to assume that either heavy metals or antibiotics could serve as selection pressures for populations of bacteria hosting these plasmids.

The work described in this paper was performed to compare the heavy-metal and antibiotic resistance of aerobic sediment bacterial flora of the sewage sludge dump area with unpolluted areas of the New York Bight to test the hypothesis that heavy-metal contamination may influence the occurrence of antibiotic resistance in the flora of an ecosystem contaminated by heavy metals.

MATERIALS AND METHODS

Sampling. Sediment samples were collected during September 1975 and February 1976 at Marine Ecosystems Analysis stations and from a series of other stations near and on proposed north and south alternate dump sites in the New York Bight (Fig. 1 and 2).

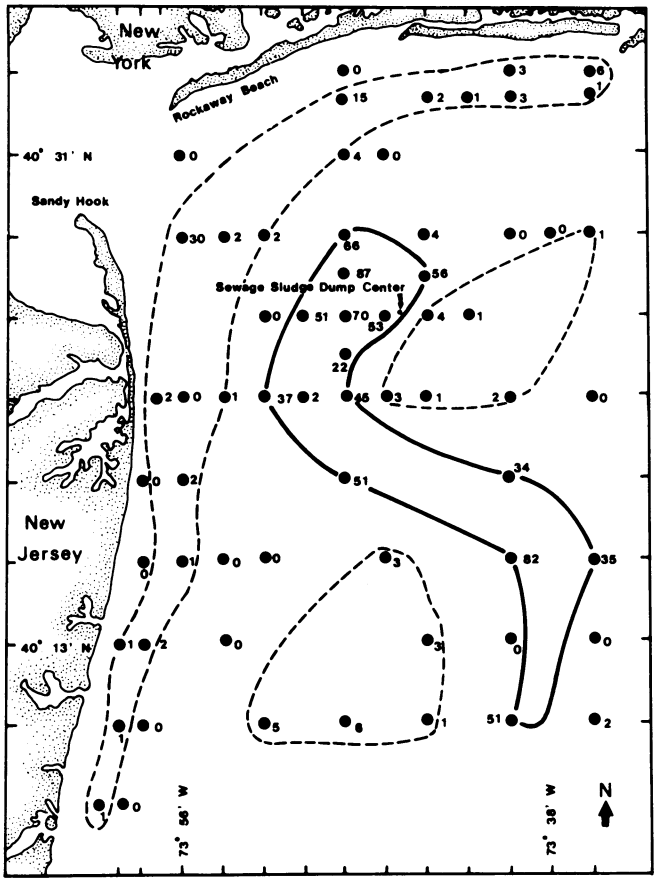

FIG. 1. *Sampling stations in inner New York Bight. Percentages of sediment bacteria resistant to 20 μg of Hg per ml are shown as figures beside each station. Approximate isopleths are drawn at 1 and 30%.*

Ship positions at sampling were located by Loran. Stations within 4 miles (ca. 6.4 km) of the center (40°25'N, 73°45'W) of the sewage sludge dump site were arbitrarily classed as dump-site stations. Stations near proposed alternate dump sites were designated as control stations. Samples from all other stations were classified as intermediate. A number of the intermediate stations lay in the upper Hudson Shelf Valley—a topographic depression which has accumulated silts and clays from the Hudson/Raritan estuary and is known to have exceptionally high metal concentrations (1). Sediments were collected with either a Smith McIntyre or Shipek bottom grab. After each collection the grab was hosed with clean seawater and then disinfected with 0.1% sodium hypochlorite solution. The top 1 inch (ca. 2.54 cm) of sediment was sampled by means of sterile disposable tongue depressors, the sediment being transferred to sterile, capped 50-ml plastic centrifuge tubes. Samples were stored at 10°C until returned to the laboratory, where portions of each were transferred to sterile vials for storage at −70°C.

Enumeration and isolation of bacteria. Tryptose-glucose-yeast (TGY) agar containing 3.5% NaCl and used alone or supplemented with either 2 or 20 μg of Hg ($HgCl_2$) per ml was used for pour-plate counts of total and Hg-resistant bacteria and for propagation of isolates. Weighed fractions of sediment (0.3 to 2.5 g, depending on sampling station) were added to flasks containing 65 ml of 3.5% NaCl solution in water. The flask contents were mixed, and then 10-ml portions were removed and added to 10-ml double-strength TGY agar with 3.5% NaCl at 46°C as well as to this medium supplemented with 4 and 40 μg of Hg per ml. After addition of sediment suspensions, the contents of all tubes were thoroughly mixed on a Vortex mixer and then poured into sterile petri dishes. All plates were run in duplicate and incubated at room temperature for 48 h. After counting, between 10 and 20 randomly chosen colonies were harvested with a sterile Pasteur pipette from each pour plate and placed on plain or TGY agar supplemented with either 2 or 20 μg of Hg per ml. Percentages of bacteria resistant to 20 μg of Hg per ml on initial isolation were calculated for each sediment using the formula: (no. of colonies on TGY + 20 μg of Hg per ml/no. of colonies on TGY) × 100.

Antibiotic susceptibility testing. Antibiotics

were added to TGY agar as follows: 50 μg of ampicillin, streptomycin, and kanamycin per ml; 30 μg of chloramphenicol and tetracycline per ml. Antibiotic-supplemented plates were replica plated with isolates from pour plates, and susceptibility patterns were read after 48 h of incubation at room temperature.

MIC studies. The minimal inhibitory concentrations (MICs) of ampicillin and heavy metals for selected isolates were determined by replica plating on TGY agar containing (in micrograms per milliliter): ampicillin—10, 50, 250, 1,250, 6,250; Hg ($HgCl_2$)—1.5, 2.0, 5.0, 10.0, 15.0, 20.0, 50.0; Cd ($CdCl_2$)—8.0, 11.0, 28.0, 56.0, 84.0, 112.0; Zn ($ZnCl_2$)—32, 49.0, 65.0, 162.0, 324.0, 488.0, 650.0. The MIC of a particular reagent was taken as the next concentration beyond the last concentration at which growth was observed after incubation at 25°C for 48 h.

Identification procedures. Gram-negative organisms were identified to genus level according to the scheme of Shewan et al. (14). Gram-positive organisms were identified according to Cowan and Steel (3).

Test for β-lactamase production. The Gots Test (5) modified by addition of 3.5% NaCl to brain heart infusion agar was used for the detection of β-lactamase production.

Test for bacteriocin production. Bacteriocin production was detected in Gots medium without penicillin.

Assay for Hg reduction. The dithizone assay as described by Summers et al. (19) was used to demonstrate Hg reduction by representative Hg-resistant strains.

Metals analysis. Representative sediments for metals analysis were dried at 25°C and ground to a fine powder in a Spex 5100 mill (Spex Industries, Inc., Metuchen, N.J.). The analyses for Hg, Cd, Cu, and Zn were performed by atomic absorption spectrophotometry at the Northeast Fisheries Center Laboratory of the U. S. Department of Commerce, Millford, Conn.

RESULTS

Sediments from 42 stations sampled during the September 1975 cruise and from 79 stations sampled during the February 1976 cruise were included in the study. Bacterial counts for sediments from both of these cruises are shown in Table 1. Substantial numbers of bacteria capable of growing on medium containing 20 μg of Hg per ml were found only in sediments from the dump site and in sediments from intermediate stations along the upper Hudson Shelf Valley. Bacteria growing on medium containing 20 μg of Hg per ml were regarded as Hg resistant, although some of these bacteria exhibited a rapid decrease in their level of Hg resistance after initial isolation.

Identification data are summarized in Table

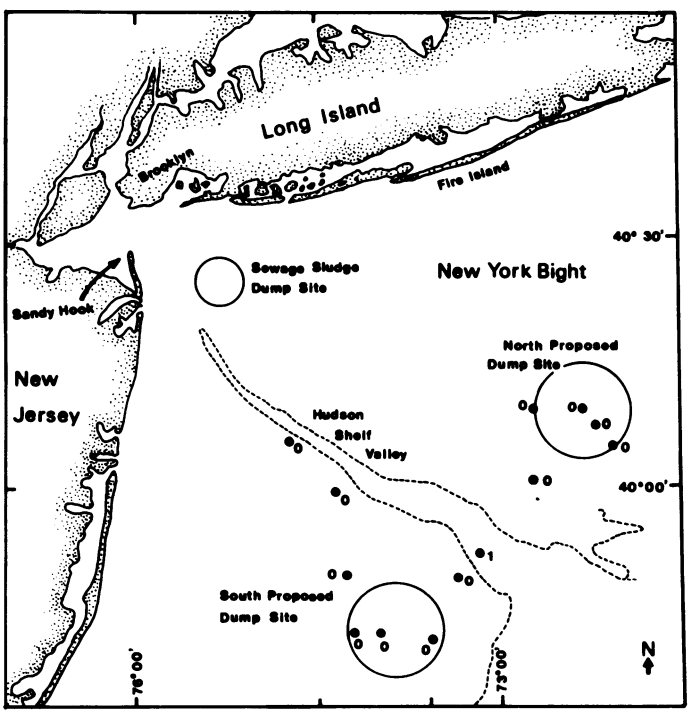

FIG. 2. *Sampling stations in outer New York Bight. Ocean dumping of urban wastes has never been practiced in these areas. Percentages of sediment bacteria resistant to 20 μg of Hg per ml are shown as figures beside each station.*

2. *Bacillus* was by far the most common genus observed in all sediments, and time of year made little difference in the frequency at which this genus was detected. Since both the enumeration and identification data from both cruises were essentially similar, it was deemed reasonable to pool data from both cruises. Moreover, the similarity of the flora in dump, intermediate, and control sediments greatly enhanced the epidemiological validity of comparisons of flora from these different locations. The only marked seasonal variation in generic distribution observed was in sediments from intermediate stations where *Bacillus* represented 76% of isolates in September 1975 compared with 93% in February 1976. The difference was made up mostly of organisms of the *Vibrio-Aeromonas* genera. It is probable that the brief exposure of sediments to 46°C during preparation of the pour plates greatly reduced counts of *Vibrio-Aeromonas* and *Pseudomonas* sp. (2). *Enterobacteriaceae* represented less than 1% of the flora in all sediments for both cruises. Concentrations of Cd, Cu, Hg, and Zn in the three categories of sediments are shown in Table 3.

The antibiotic resistances of 1,422 *Bacillus* clones randomly harvested from pour plates are shown in Table 4. Ampicillin was by far the most common antibiotic resistance and was much more frequent in dump (26%) than in control (12%) or intermediate sediments (14%). This difference was highly significant ($P < 0.001$). Streptomycin resistance was common among isolates from the latter categories of sediments, which may indicate selection against streptomycin-resistant bacterial populations in dump sediments. Chloramphenicol resistance was not observed in any isolate.

Table 5 contains a comparison of antibiotic resistances of *Bacillus* isolates from dump sediments from pour plates containing 2 μg of Hg with those from plates containing 20 μg of Hg per ml. The rationale for this comparison was based on the premise that if Hg resistance was sometimes linked with antibiotic resistance, then there should be more antibiotic-resistant clones

TABLE 1. *Mean counts of total and Hg-resistant aerobic bacteria at sewage sludge dump and other stations in the New York Bight*

Cruise	Site[a]	Hg-resistant aerobic bacteria (CFU[b]/g of wet sediment) on:				
		Plain TGY agar	TGY agar + 2.0 μg of Hg/ml[c]	% of total[d]	TGY agar + 20.0 μg of Hg/ml	% of total
September 1975	Sewage sludge dump (15)	6.2×10^3	6.4×10^3	>100	1.9×10^3	23.2
	Intermediate (15)	1.9×10^3	1.8×10^3	95.0	3.5×10^1	0.9
	Control[e] (12)	6.3×10^1	6.8×10^1	>100	1	0.2
February 1976	Dump (13)	4.6×10^3	5.4×10^3	>100	2.7×10^3	42.5
	Intermediate (47)	1.2×10^3	1.2×10^3	100	6.2×10^2	9.8
	Control[e] (19)	1.2×10^2	1.5×10^2	>100	1	0.6

[a] The numbers in parentheses are numbers of stations examined.
[b] CFU, Colony-forming units.
[c] Hg was present as $HgCl_2$.
[d] Mean percentages of Hg-resistant organisms were calculated from the separate counts on plain TGY agar for each station.
[e] Control stations were located at or near the projected alternate dump sites.

TABLE 2. *Bacterial genera isolated from sewage sludge dump and other sediments in the New York Bight*

Genus	Source of sediment					
	Dump stations		Intermediate stations		Control stations	
	Sept. 1975	Feb. 1976	Sept. 1975	Feb. 1976	Sept. 1975	Feb. 1976
Bacillus sp.	94[a] (186/198)	99 (192/194)	76 (110/145)	93 (133/143)	99 (90/91)	89 (144/162)
Aeromonas sp. *Vibrio* sp. *Staphylococcus epidermidis* *Streptococcus* sp.	6 (12/198)	1 (2/194)	24 (35/145)	7 (10/143)	1 (1/91)	11 (18/162)

[a] Percentage of strains isolated.

in the pour plates containing the higher concentration. As can be seen in Table 5, this hypothesis seems true with respect to ampicillin resistance. The difference in frequency of ampicillin-resistant clones between 2- and 20-μg/ml Hg pour plates was highly significant ($P < 0.001$).

The higher concentration of Hg also seemed to have an adverse effect on populations of streptomycin-resistant organisms and may be the explanation of the fewer streptomycin-resistant clones recorded in dump sediments in Table 5.

Percentages of *Bacillus* sp. with combined ampicillin-mercury resistances in dump, intermediate, and control sediments were 17.3 (172/987), 8.4 (19/227), and 3.0 (6/208), respectively. The difference between the dump- and control-site percentages was highly significant ($\chi^2 = 20.42$, $P < 0.001$).

A total of 74% (196/263) of randomly chosen ampicillin-resistant *Bacillus* strains from the dump site were β-lactamase producers in the Gots test. The remaining 26% did not produce detectable free β-lactamase. A bacteriocin-like activity was exhibited by 36% (13/36) of β-lactamase producers and by 85% (57/67) of ampicillin-resistant strains which were β-lactamase negative in the Gots test. MICs of ampicillin for 69 resistant *Bacillus* sp. varied considerably (Table 6).

MICs of Hg for *Bacillus* from sewage sludge sediments varied from 2 to 50 μg/ml with a tendency toward a bimodal distribution (Table 7). Cd and Zn MICs were less variable in these organisms. In the case of many of these organisms with resistance to two or more heavy metals, MICs of each heavy metal varied together. Of the remainder, most strains exhibited a relationship between Hg and Zn levels. In Table 8 it can be seen that Hg and Zn resistances occurred together in 48% of strains. Cd resistance occurred frequently in combination with both of the other metal resistances but seldom on its own or with Zn resistance. Strains with single Hg or Zn resistances were also common.

Hg reduction was observed in 33% (6/18) of *Bacillus* strains from dump sediments. In the case of the remainder, results were inconsistent for six and negative for six. The validity of the assay procedure was checked by placing X-ray (Kodak Safety Film RP) film over a culture plate supplemented with 20 μg of Hg per ml and containing an inoculum of a Hg-resistant *Bacillus* that was positive in the dithizone assay and

TABLE 3. *Heavy metals in sediments of the New York Bight*

Metal	Mean concn (μ/g, dry wt) of heavy metals from:		
	Control (7)[a]	Intermediate (9)	Dump (15)
Cd	<0.47	1.01 (<0.47-2.43)[b]	2.52 (<0.47-9.60)
Cu	<1.94	11.46 (<1.94-71.00)	50.00 (2.06-255.0)
Hg	0.19 (0.12-0.31)	0.75 (0.24-2.4)	1.51 (0.18-4.90)
Zn	11.21 (4.60-14.1)	42.48 (8.1-191.0)	135.80 (10.00-541.00)

[a] Numbers in parentheses indicate number of sites assayed.
[b] Ranges of concentrations are shown in parentheses.

TABLE 4. *Antibiotic resistances of 1,422 Bacillus clones from sediments in the New York Bight*

Antibiotic	Source of sediment		
	Dump stations	Intermediate stations	Control stations
Ampicillin	26.0[a]	14.0	12.0
Streptomycin	2.7	12.0	8.0
Kanamycin	0.2	2.6	0.5
Tetracycline	0.2	0.4	0.5
Ampicillin and kanamycin	3.2	6.2	2.0
Other resistance combinations	1.1	2.6	0.5
Sensitive	65.0	66.0	77.0

[a] Mean percent resistant strains.

TABLE 5. *Antibiotic resistances of Bacillus clones from dump sediments harvested from pour plates containing two different concentrations of Hg*

Antibiotic	% Resistant strains in supplemented medium (μg of Hg/ml)[a]	
	2	20
Ampicillin	8.1 (10/123)	29.0 (216/757)
Streptomycin	9.0	2.8
Kanamycin	2.8	1.1
Tetracycline	0	0.1
Ampicillin and kanamycin	3.3	3.3
Other resistance combinations	2.4	0.4
Sensitive	75.6	63.0

[a] The difference in frequency of ampicillin resistance between the two Hg levels was highly significant ($\chi^2 = 23.08$, $P < 0.001$).

TABLE 6. *MICs of ampicillin for 69 resistant Bacillus strains*

MIC (μg/ml)	% of strains
50	12
250	14
1,250	26
6,250	26
>6,250	22

TABLE 7. *MICs of Hg, Cd, and Zn for Bacillus strains from sewage sludge dump sediments of the New York Bight*

Metal (no. of strains examined)	MIC (μg/ml)	% of total
Hg[a] (376)	2	0.5
	5	36.2
	10	24.7
	15	17.3
	20	3.7
	50	7.4
	>50	10.4
Cd[b] (376)	8	72.3
	11	2.4
	28	12.2
	56	10.6
	84	2.4
	112	0
Zn[c] (375)	32	0
	49	22.0
	65	10.4
	162	25.3
	488	42.4
	650	0

[a] As $HgCl_2$.
[b] As $CdCl_2$.
[c] As $ZnCl_2$.

incubating in the dark at room temperature for 24 h. Fogging of the film produced by elemental Hg vapor occurred in the case of the positive culture plate. An agar plate containing 20 μg of Hg per ml but without inoculum produced no fogging of the film.

DISCUSSION

Hg-resistant *Bacillus* sp. were very common in sediments at the sewage sludge dump site and in the upper Hudson Shelf Valley in contrast to sediments at clean, unpolluted stations further out on the continental shelf where these resistant organisms were rarely observed. The distribution of resistant organisms conforms remarkably well with the known distribution of heavy metals in the New York Bight (1), and so it is reasonable to infer that heavy-metal contamination has been exerting a selective pressure for resistant *Bacillus* and other bacterial populations in this area. Sludge dump sediments had concentrations of Hg that were more than seven times as great as those in control sediments (Table 3). One sediment sample from the dump area contained 4.9 μg of Hg per g—a level which may be reflective of recent dumping since sewage sludge from metropolitan New York plants has been shown to contain up to 15 μg of Hg per g (5). It is likely that Hg concentrations in sediments become less after dumping because of dilution effects during passage through the water column, because of mixing with other sediments on the bottom, and finally because of dissipation of elemental Hg into the water column by resistant Hg-reducing bacteria.

Sediments from intermediate stations in the upper Hudson Shelf Valley also exhibited high counts of resistant *Bacillus* together with elevated concentrations of heavy metals. This may reflect inputs from dredge spoil and estuarine effluents since a number of these sampling stations were too far from the sewage sludge dump to have been substantially affected by drift from the latter. The existence of resistant bacterial populations at these stations supports the possibility of in situ selection of resistant clones. In the case of dump sediments, it is probable that resistant bacteria were already present in the sludge at the time of dumping. However, many resistant marine *Vibrio* and *Aeromonas* strains (unpublished observations) were found in these sediments, indicating that an in situ selection process was also taking place. The similarity in distribution of bacterial genera between dump and control sediments further supports this conclusion.

Location of sediment had a significant effect on occurrence of ampicillin resistance (Table 4). Clones resistant to this antibiotic were more than twice as numerous in sewage sludge dump as in clean sediments. This difference was highly significant. Furthermore, ampicillin resistance was three times as frequent in clones selected on medium containing 20 μg of Hg as on medium containing 2 μg of Hg per ml. Thus, a high level of Hg resistance significantly increased the probability of a selection of clones with ampicillin resistance and is suggestive of a linkage phenomenon. In Table 5 it can be seen that the Hg ampicillin resistance pattern was almost six times as common in *Bacillus* from sewage sludge

TABLE 8. *Heavy-metal resistance patterns of 366 Bacillus sp. from sewage sludge dump sediments of the New York Bight*

Resistance pattern[a]	% of total
Hg-Cd-Zn	24
Hg-Zn	24
Zn	20
Hg	14
Hg-Cd	3
Cd-Zn	2
Cd	1
Sensitive	12

[a] Criteria of resistance: Hg ≥ 5 μg/ml; Cd ≥ 11 μg/ml; Zn ≥ 65 μg/ml.

dump as in *Bacillus* from control, unpolluted sediments.

Although ampicillin and penicillin resistance due to β-lactamase is a frequent and well-studied phenomenon in some terrestrial *Bacillus* strains (12), its survival value to organisms in polluted marine sediments is not easily discerned. Its frequent occurrence in this situation is most easily explained by a selection pressure on a genetically linked characteristic. In some *Bacillus* sp. the genetic basis of β-lactamase production appears to be chromosomal (6). However Lovett and Bramucci (8) have recently shown the presence of a plasmid in a penicillinase-producing strain of *Bacillus* from soil, and unpublished work in our laboratory indicate that Hg resistance in *Bacillus* from Bight sediments is plasmid mediated, as detected by cesium chloride-ethidium bromide density gradient centrifugation and transformation studies. Genetic proof of linkage of Hg resistance and β-lactamase genes will depend on successful cotransformation with plasmid DNA, cotransduction of both determinants by bacteriophage, or simultaneous curing of both determinants.

Hg was chosen for inclusion in primary selection media because preliminary screening of different heavy metals indicated that it was the most toxic for sediment bacteria. Since multiple heavy-metal resistance patterns were much more common than single resistances (Table 8) in the *Bacillus* populations studied, it is probable that any of a number of different heavy metals may have been the selection pressure for a multiple-resistance pattern that included Hg resistance. Thus it was not critical which of these metals was included in the primary selection medium.

MIC data for ampicillin indicated considerable variability. Variability in level of Hg resistance was also evident (Table 7) but may, in part, be a reflection of innate instability of this resistance, which in many strains tended to decrease following initial isolation. The instability of Hg resistance was not associated with a similar instability in level of ampicillin resistance.

Hg-reducing bacteria in estuarine marine sediments of the Chesapeake Bay have previously been described (11). *Pseudomonas* was by far the most numerous Hg-resistant genus in that study. Populations of these organisms were highest in spring and were positively correlated with sediment Hg concentration and dissolved oxygen concentration. Our data (Table 1) indicate a similar rise (in spring) in Hg-resistant *Bacillus* populations in polluted areas of the New York Bight. The difference in bacterial populations between the Chesapeake Bay and the Bight may, in part, have been due to differences in isolation procedure, since Nelson and Colwell (11) used a surface-plating procedure for primary isolation, whereas we used pour plates which involved a brief exposure of the sediment to 46°C during preparation of pour plates. Also in the latter procedure we noted a marked tendency for colonies in Hg-supplemented media to be subsurface. A procedure which favored selection of surface colonies might, therefore, have discriminated against subsurface and generically different populations. It is clear, however, from both the Chesapeake Bay and New York Bight studies that Hg resistance can arise in generically quite different populations of marine sediment bacteria.

The selection of substantial populations of Hg-reducing *Bacillus* sp. in polluted marine sediments should have considerable ecological advantage by promoting mobilization and loss of elemental Hg from sediments. However, it seems that this ecological advantage carries with it the possibility of an increase in the gene pool for β-lactamase production, which, though not medically significant in the context of *Bacillus* sp., would have definite importance in the case of genera such as *Escherichia, Pseudomonas,* or *Vibrio*. Plasmid-mediated heavy-metal and antibiotic resistance in marine *Pseudomonas* and *Vibrio* sp. has, in fact, recently been described (16).

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