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Cadmium- and Mercury-Resistant *Bacillus* Strains from a Salt Marsh and from Boston Harbor

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Bacteria resistant to cadmium or mercury or both were isolated from the Great Sippewissett Marsh (Cape Cod, Mass.) and from Boston Harbor. Many of these metal-resistant isolates were gram-positive aerobic sporeformers, although not necessarily isolated as spores. Although several of the isolated strains bore plasmids, cadmium and mercury resistances appeared to be, for the most part, chromosomally encoded. DNA sequence homology of the gram-positive cadmium- and mercury-resistant isolates was not demonstrable with metal resistance genes from plasmids of either gram-positive (pI258) or gram-negative (pDB7) origin. Cadmium resistance of all the marsh isolates tested resulted from reduced Cd^{2+} transport. On the other hand, three cadmium-resistant harbor isolates displayed considerable influx but no efflux of Cd^{2+}. Hg-resistant strains detoxified mercury by transforming Hg^{2+} to volatile Hg^0 via mercuric reductase.

Cadmium (Cd) and mercury (Hg) salts induce specific human disease syndromes. Cd, in particular, has a long half-life and accumulates in tissues (23, 35).

Over the past decade, heavy-metal pollution of near-shore and fresh water has been increasingly recognized as a serious health problem. We are concerned with the isolation of Cd- and Hg-resistant (Cd^r and Hg^r) bacteria from polluted waters (Great Sippewissett Marsh, Cape Cod, Mass., an estuarine marsh, and Boston Harbor), the mechanisms of their resistance, the genetic determinants involved, the transfer of resistance to sensitive microorganisms, metal-resistant bacteria as an index of a polluted environment, and the possible use of such bacteria to clean up an environment.

Resistance to Hg and Cd has been reported for both gram-positive and gram-negative bacteria (10). The detoxification of inorganic Hg salts occurs via an enzymatic transformation of Hg^{2+} to Hg^0. The enzyme involved, mercuric reductase, is part of a complex *mer* operon which usually has been reported as being plasmid encoded (3, 28). Cadmium resistance determinants have been found both on plasmids (10) and chromosomally (18, 31). Bacterial resistance to this toxic metal appears to be due to several distinct mechanisms. Thus, plasmidborne cadmium resistance in *Staphylococcus aureus* results from an energy-dependent efflux of Cd^{2+} from resistant, but not from cadmium-sensitive (Cd^s), cells (34). In a Cd^r mutant of *Bacillus subtilis*, Cd transport is significantly reduced as compared with that in the Cd^s wild type (18). Another possible mechanism involves synthesis of novel proteins. Cd-binding proteins have been reported in gram-negative Cd^r isolates (13, 17, 22) and in a blue-green alga (cyanobacterium) (24), but Cd^s strains were not examined. Comparison of a Cd^r *Pseudomonas aeruginosa* strain and its Cd^s mutant gave no evidence for a binding protein unique to the Cd^r strain (14).

We report here the isolation of Cd^r and Hg^r gram-positive sporeformers from both the harbor and the marsh sites. Three lines of evidence (lack of plasmids in some strains, plasmid curing, transformation experiments) suggested that metal resistance in many of the strains was chromosomally encoded. None of the strains hybridized with two well-characterized plasmidborne genes that encode metal resistance. Inducible mercuric reductase activity was detected in all Hg^r strains. All the Cd^r marsh isolates which were tested showed reduced Cd transport as has been reported for a Cd-resistant mutant of *B. subtilis* (18). On the other hand, the tested Cd^r harbor isolates showed no evidence for reduced Cd transport or for efflux. Cd-binding polypeptides were detected in a Cd^r harbor isolate, but these were also apparent in its Cd^s mutant. Cd influx by both marsh and harbor isolates was, as previously reported (1, 18, 26), via the Mn^{2+} active transport system.

MATERIALS AND METHODS

Isolation of bacterial strains. Bacterial strains were isolated from cores of bottom mud of two sites in Great Sippewissett Marsh and from a Boston Harbor site near the mouth of the Neponset River. The marsh sites were an unfertilized control area (site 7) and an area which had been highly fertilized with sewage sludge for nearly a decade (site 8) under an experimental program jointly conducted by the Boston University Marine Program and the Woods Hole Oceanographic Institute. Strains of gram-positive sporeforming bacteria from unheated or from heated (75°C, 10 min) mud samples, isolated on ZoBell agar (25) containing 0.1 mM $MnCl_2$ or on LB agar, were tested for ability to grow on LB agar supplemented with 250 μM $CdCl_2$ (Cd^r) or 125 μM $HgCl_2$ (Hg^r). Strains producing colonies on ZoBell agar or on LB agar after heating of the mud inoculum were considered to have been present as spores. Harbor and marsh samples from which bacterial strains were isolated were analyzed for metal content by the Soil and Environmental Chemistry Laboratory, The Pennsylvania State University, University Park, Pa.

Isolated bacterial strains and their mutants were given three-digit Rosenstiel Center (RC) designations. Several known *Bacillus* strains were used, and these are referred to in text by their American Type Culture Collection (ATCC) or U.S. Army Quartermaster (QM B) accession numbers.

Manganese sparing of growth inhibition by Cd^{2+}. Cd^s marsh and harbor *Bacillus* strains were incubated at 30°C (marsh isolates) or at 37°C (harbor isolates) in LB broth or in LB supplemented with $CdCl_2$, with or without $MnCl_2$. Growth was measured as the increase in A_{595} (Bausch & Lomb Spectronic 20 spectrophotometer).

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Plasmids and DNA isolation. *Escherichia coli* carrying pDB7 (*mer* operon) was kindly sent by A. Summers. *S. aureus* RN453 carrying plasmid pI258 was received from the laboratory of R. Novick, and *Bacillus thuringiensis* var. *kurstaki* HD244 was obtained from the *Bacillus* Genetic Stock Center (Columbus, Ohio).

For large-scale plasmid DNA preparation, the method of Tanaka and Sakaguchi (32) was used. Small-scale plasmid DNA extractions were carried out as described by Birnboim and Doly (5) and by Kado and Liu (16). Chromosomal DNA was prepared by the method of Marmur (21).

Probe DNA. Plasmid DNA or restriction enzyme fragments were excised from low-melting-temperature agarose and were extracted and purified as described by Maniatis et al. (20).

Mutagenesis and plasmid curing. Cd^s and Hg^s mutants of the cadmium- and mercury-resistant harbor strain RC607 were obtained by exposing a washed, lysozyme-treated spore sample (10^8 spores per ml) to the mutagen ethyl methanesulfonate (20 μl/ml). The mutagen-treated sample was shaken at 30°C for 19 h, during which time spores had germinated and outgrown. The cells, collected by centrifugation (6,800 × *g*), were washed with KH_2HPO_4-K_2HPO_4 (0.1 M, pH 7.1) and plated on LB agar. After overnight incubation at 42°C, the survivors (0.2%) were replicated on LB agar containing $HgCl_2$ (125 μM) or $CdCl_2$ (250 μM).

Strains RC413 and RC421 were each cured of their plasmid with acridine orange (29). Plasmid-free survivors were identified by colony hybridization techniques (12) with ^{32}P-labeled plasmid DNA as a probe.

Transformation. *B. subtilis* JAS9 (19) was rendered competent for uptake of chromosomal and plasmid DNA (6, 9). Drug- or metal-resistant transformants were selected on LB agar containing antibiotic, $HgCl_2$, or $CdCl_2$.

Protoplasts of the marsh isolates were prepared for transformation by the procedure of Chang and Cohen (8).

Labeling of DNA and Southern analysis. Chromosomal and plasmid DNAs to be used as probes were labeled in vitro (27) with [α-^{32}P]dCTP. Southern (30) blot transfers were carried out with Zeta-Probe membranes. The filters were prehybridized for 4 h at 45°C in a solution containing 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50% formamide, 2× Denhardt solution (1× Denhardt solution is 0.2 g of Ficoll, 0.2 g of polyvinylpyrrolidone, and 0.2 g of bovine serum albumin in 1 liter of 3× SSC), 0.1% sodium dodecyl sulfate (SDS), and 100 μg of calf thymus DNA. Hybridization was carried out under stringent conditions at 45°C for 16 to 20 h in the same solution containing 10^6 cpm of probe DNA per ml.

For DNA-DNA homologies, samples were loaded in alternate wells of 0.7% agarose gels. After transfer to a blotting membrane and exposure to the probe, strips representing each sample were cut out, transferred to scintillation fluid, and counted in a Beckman LS 1801 scintillation counter. The percentage of homology was estimated relative to hybridization with homologous DNA taken as 100% homology (nonspecific binding to heterologous bacteriophage λ DNA was subtracted).

Colony hybridization. Colonies were transferred to Whatman 541 filters and lysed, and their DNA was denatured in situ (12). Colonies were probed with nick-translated ^{32}P-labeled plasmid DNA.

Transport experiments. Accumulation of Cd^{2+} by Cd^r and Cd^s isolates was assayed by the method of Laddaga et al. (18). Cells (0.5 mg [dry weight]/ml) harvested from tryptone broth (tryptone, 8 g; NaCl, 5 g/liter) were incubated at 37°C with 0.1 μM $CdCl_2$ and $^{109}Cd^{2+}$. At intervals, the radioactivity of filtered (0.45-μm-pore-size filters; Millipore Corp., Bedford, Mass.) and washed samples was measured. Efflux of $^{109}Cd^{2+}$ was induced by 10:1 dilution of the culture with Cd-free tryptone broth as described by Tynecka et al. (34).

Polyacrylamide gel analysis of $^{109}Cd^{2+}$-labeled cells. Cells were grown in LB broth to an optical density at 595 nm of 0.24. The cells were centrifuged (6,800 × *g*), suspended in tryptone broth, and exposed for 10 min to $^{109}Cd^{2+}$. The labeled cells were washed twice with Tris (10 mM, pH 8.0), resuspended in 1.0 ml of Tris (10 mM, pH 8.0) containing 25% sucrose and 4 mg of lysozyme, and incubated at 37°C for 30 min. A 10-μl portion of 10% SDS was added, and the solution was sonicated for 20 s to reduce viscosity. Protein concentration was determined, and 10-μl samples were counted to measure total radioactivity. Duplicate samples were loaded on 15% SDS-polyacrylamide gels (29). After completion, half the gel was silver stained, and the other half was dried, placed on X-ray film (XAR-5; Eastman Kodak Co., Rochester, N.Y.), and exposed at −70°C.

Mercuric reductase assay. The $HgCl_2$-dependent oxidation of NADPH was measured by following the decrease in A_{340} with crude cell extracts from Hg^r and Hg^s strains (15).

Chemicals. SDS, antibiotics, ethyl methanesulfonate, acridine orange, and NADPH (tetrasodium salt) were purchased from Sigma Chemical Co. (St. Louis, Mo.). The Zeta-Probe, protein assay kit, and silver staining kit were obtained from Bio-Rad Laboratories (Rockville, N.Y.). Restriction enzymes were purchased from New England BioLabs, Inc. (Beverly, Mass.) and used as directed. Protein markers were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), and $^{109}Cd^{2+}$ and [α-^{32}P]dCTP were from New England Nuclear Corp. (Boston, Mass.). Low melting-temperature agarose (SeaPlaque) was purchased from FMC Corp., Marine Colloids Div. (Rockland, Maine).

RESULTS

Marsh and harbor flora. From 45 to 70% of the bacteria in marsh core samples were Cd resistant; the Hg-resistant population was much smaller, ranging from 0 to 0.2% of the population recoverable on ZoBell agar or on LB agar (Table 1). In contrast, harbor samples yielded approximately equal numbers of Cd- and Hg-resistant organisms (1.9 and 2.6%, respectively). Very few of the strains were antibiotic resistant.

Sporeformers were widely prevalent. Indeed, ca. 15% of the isolates, both from the marsh and from the harbor, were present as spores, i.e., they formed colonies from a heated inoculum. From 7 to 22% of the marsh isolates present as spores were Cd resistant; none was Hg resistant. In contrast, none of the harbor isolates present as spores was Cd resistant, while 4% were Hg resistant (Table 1).

Many of the Cd- and Hg-resistant isolates were sporeformers, although not necessarily present as spores. Generally the sporeformers from the marsh formed pigmented (yellow, pink) colonies, grew better at 25 to 30°C than at 37°C, and preferred NaCl-containing media such as ZoBell agar to media with less NaCl. The three harbor isolates appeared not to require NaCl.

Although more metal-resistant isolates were recovered from a highly fertilized marsh site than from a control area, no direct relationship was established between Cd or Hg concentration and either the presence or the size of the bacterial population resistant to these metals. None of three

TABLE 1. Bacterial isolates and metals from Great Sippewissett Marsh and Boston Harbor samples

Sites	Organisms[a]						Metals[b]	
	CFU/ml	Cd^r (%)	Hg^r (%)	Present as spores (%)	Cd^r spores (%)	Hg^r spores (%)	Cd	Hg
Marsh[c]								
7	ca. 9×10^5	44.0	0	14.0	7.0	0	0.2	0.0
8	ca. 3.3×10^6	67.0	0.2	11.0	22.0	0	9.7	0.0
Harbor	ca. 1.4×10^6	1.9	2.6	17.0	0	4.0	4.3	0.0

[a] Resistance was assayed as growth on LB agar plates containing 250 μM $CdCl_2$ (Cd^r) or 125 μM $HgCl_2$ (Hg^r).
[b] Metal analyses (parts per million in a dried core sample) by the Soil and Environmental Chemistry Laboratory of The Pennsylvania State University.
[c] Site 7, Control area; site 8, highly fertilized area.

sites (two from the marsh, one from the harbor) was demonstrably mercury polluted (Table 1) at the time of sampling although previous analyses (7; A. Giblin, personal communication) did show ca. 0.2 ppm (0.2 μg/ml) of Hg^{2+} in a control, unfertilized area and ca. 0.65 ppm (0.65 μg/ml) of Hg^{2+} in a heavily fertilized area of Great Sippewissett Marsh. Mercury-resistant bacteria were isolated from the fertilized marsh site (site 8) and from the harbor. No Hg^r or Cd^r bacteria were found among bacteria (10^5 CFU/ml) isolated from the relatively unpolluted waters of Biscayne Bay, Fla., adjacent to the Rosenstiel School of Marine and Atmospheric Science, University of Miami (data not shown).

Metal resistance of many of the isolates is chromosomally encoded. Several lines of evidence support the idea that Cd and Hg resistance was, in many cases, not encoded by plasmids.

(i) Many of the metal-resistant strains lacked plasmids (Table 2). Of seven Hg^r isolates, five were plasmid-free. Of 15 Cd^r isolates, 8 were plasmid-free. Since very large plasmids are sometimes difficult to visualize, *B. thuringiensis* var. *kurstaki* HD244 which contains at least eight plasmids ranging in size up to 250 kilobases (kb) was used as a control strain. None of the metal-resistant strains contained very large plasmids (Fig. 1).

(ii) Two Cd^r strains, RC413 and RC421, each contained one plasmid. The plasmids were cured with acridine orange (29). Plasmid-free survivors were identified by colony hybridization with ^{32}P-labeled plasmid DNAs as probes and were replicated on Cd-containing plates. The cured colonies obtained from both strains RC413 and RC421 were still Cd resistant.

(iii) CsCl-purified plasmid DNA from all the plasmid-containing Cd^r and Hg^r strains was used to transform competent cells of *B. subtilis* JAS9. No metal-resistant transformants were obtained under conditions which allowed transfer and expression of a *Bacillus cereus* plasmid, pBC16 (4).

We were unable to render marsh or harbor isolates competent for transformation by the competence regimens of Bott and Wilson (6) and Thorne and Stull (33). However, some of the isolates could be transformed as protoplasts. We attempted to transfer plasmids from the Hg^r marsh isolate RC416 to the plasmid-cured, Hg^s marsh isolate RC413. Strain RC416 carried two plasmids, approximately 4 and 12.5 kb (Fig. 1), which were separated on an agarose gel, purified, and used to transform RC413 protoplasted as described by Chang and Cohen (8). Transformed RC413 protoplasts were allowed to regenerate on nonselective medium (8) and were then transferred to filters for colony hybridization. The 4- and 12.5-kb plasmid DNAs were ^{32}P labeled and used as probes. Of ca. 10^4 colonies, 5 carried the 4-kb plasmid, and 3 of 10^4 colonies were transformed with the 12.5-kb plasmid. None of the transformed colonies became Hg^r.

DNA-DNA homologies. There was some homology between the DNA of marsh *Bacillus* isolate RC418 and several other marsh isolates (Table 3), but no significant hybridization was detected with several known *Bacillus* species nor with three harbor isolates. On the other hand, there was

TABLE 2. Plasmids in metal-resistant bacilli

Strain[a]	Growth on LB agar with:		No. of plasmids[b]
	Cd^{2+} (250 μM)	Hg^{2+} (100 μM)	
RC413	+	−	1
RC414	+	−	2
RC415	+	−	3
RC416	+	+	2
RC417	+	+	0
RC418	+	+	0
RC419	+	−	0
RC420	+	−	0
RC421	+	−	1
RC422	+	+	0
RC423	+	+	0
RC424	+	+	0
RC601	+	−	3
RC606	+	−	2
RC607	+	+	3

[a] Strains RC4XX from marsh; RC6XX from harbor.
[b] Plasmid DNA extraction (5, 16).

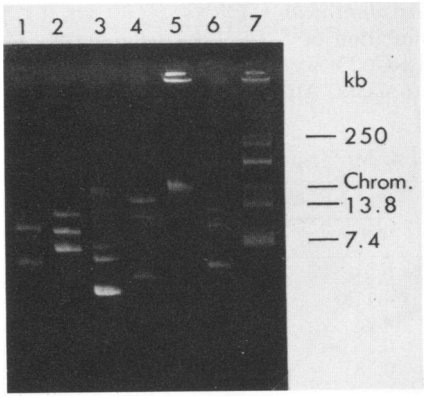

FIG. 1. Plasmid profile of metal-resistant strains and of *B. thuringiensis* var. *kurstaki* HD244. Lanes: 1, RC413; 2, RC414; 3, RC415; 4, RC416; 5, RC417; 6, RC607; 7, HD244. Sizes (in kilobases) of some of the HD244 plasmids are indicated for reference. Chrom., Chromosomal.

TABLE 3. DNA-DNA homologies

Bacillus strain	Cd resistance	% Homology[a] with DNA from:	
		RC418	*B. cereus*
B. subtilis RC350	s	<3	NT[b]
B. licheniformis RC351	s	<3	NT
B. megaterium RC352	s	<3	NT
B. cereus RC701	r	<3	100
Marsh *Bacillus* strains			
RC418	r	100	<3
RC301	s	5	NT
RC414	r	5	NT
RC416	r	15	NT
RC417	r	23	NT
Harbor *Bacillus* strains			
RC601	r	<3	94
RC606	r	<3	89
RC607	r	<3	91

[a] *Hin*dIII-cut chromosomal DNA from RC418 and from *B. cereus* ATCC 14579 was ^{32}P labeled and used as hybridization probes.
[b] NT, Not tested.

hybridization between *B. cereus* DNA and the DNA of the three harbor isolates (Table 3).

Diversity of Cd and Hg resistance genes. Colony blot hybridization was used to determine whether the marsh and harbor isolates contained DNA sequences homologous to the well-studied Hg^r genes organized in the *mer* operon of plasmid pDB7 or to Hg^r and Cd^r genes from *S. aureus* plasmid pI258. With *E. coli*(pDB7) as a positive control, no hybridization was detectable between the Hg resistance probe prepared from pDB7 and any of our Hg^r *Bacillus* isolates. Similar results have been reported by Barkay et al. (2). Further, there was no hybridization between pI258 (of gram-positive origin) and our Cd-resistant isolates.

Mechanisms of resistance to Cd and Hg. (i) Manganese sparing of inhibition by Cd. Growth of several Cd^s marsh *Bacillus* strains and of a Cd^s mutant of the Cd^r harbor *Bacillus* isolate RC607 was inhibited by the addition of Cd^{2+}; the cells were spared from the inhibitory effect of Cd^{2+} by the addition of Mn^{2+} (Table 4). Furthermore, Cd^{2+} transport by strain RC607 (Cd^r) was reduced in the presence of $MnCl_2$ (Fig. 2). These data support involvement of the Mn^{2+} active transport system for Cd^{2+} accumulation by the marsh and harbor bacilli, as previously reported for other gram-positive bacteria, e.g., *B. subtilis* (18), *S. aureus* (26), and *Lactobacillus plantarum* (1).

(ii) Accumulation of $^{109}Cd^{2+}$ by protoplasts. Two strains (RC305 and RC607) were labeled with $^{109}Cd^{2+}$ and allowed to form protoplasts. After centrifugation, the radioactivities of the supernatant fluid and of the protoplast-containing pellet were counted. For both strains, 82 to 88% of the total radioactivity was found in the protoplast fraction. Approximately 40% of the protoplast radioactivity was bound to protein.

(iii) Cd resistance mechanisms. Two major Cd resistance mechanisms have been postulated for gram-positive bacteria: the *S. aureus* model, involving a Cd^{2+} efflux system which transports Cd^{2+} out of Cd^r cells (34), and the *B. subtilis* model, involving reduced transport of Cd^{2+} by Cd^r cells (18, 31).

A Cd^s marsh isolate (RC305) and its spontaneous Cd^r mutant (RC505) were examined for cadmium transport (Fig. 3). The reduced transport, *B. subtilis* model appeared to be operative, the Cd^r mutant showing considerably less $^{109}Cd^{2+}$ accumulation that the Cd^s wild type; no efflux was detected. Eight Cd^r marsh isolates showed similar reduced Cd^{2+} transport (Fig. 3). As previously shown for *B. subtilis* (18) in studies involving the use of inhibitors and of low temperature, Cd^{2+} influx and efflux (when it occurs) are energy-dependent processes. In all cases which we examined, $^{109}Cd^{2+}$ transport by the marsh strains was markedly reduced at low temperature (data not shown).

Similar experiments carried out with the harbor isolate RC607 and its Cd^s mutant RC707 revealed considerable transport of $^{109}Cd^{2+}$ by both organisms (Fig. 2). No loss of radioactivity via efflux was demonstrable in the Cd^r strain. Harbor isolates RC601 and RC606 (both Cd^r) also displayed considerable influx and no efflux of $^{109}Cd^{2+}$ (data not shown).

The possible existence of metallothioneinlike, metal-binding proteins in gram-negative Cd^r strains exposed to very high levels of Cd has been reported (13, 17, 22). We

TABLE 4. Mn^{2+} sparing of growth inhibition by Cd^{2+}

Strain	Increase in optical density at 595 nm[a]		
	LB	LB + Cd^{2+}	LB + Cd^{2+} + Mn^{2+}
RC301[b]	0.91	0.053	0.65
RC303[b]	0.17	0.04	0.12
RC304[b]	0.28	0.10	0.28
RC707[c]	0.16	0.006	0.08
RC607[c]	0.18	0.14	0.20

[a] $OD_t - OD_i$, where OD is optical density, in LB or in LB supplemented with $CdCl_2$ with or without $MnCl_2$, after incubation for t hours.
[b] Cd^s marsh *Bacillus* strains. Cd^{2+}, 10 μM; Mn^{2+}, 50 μM; incubation time, 6 h.
[c] RC707 is the Cd^s mutant of Cd^r harbor *Bacillus* strain RC607. Cd^{2+}, 75 μM; Mn^{2+}, 20 μM; incubation time, 4 h.

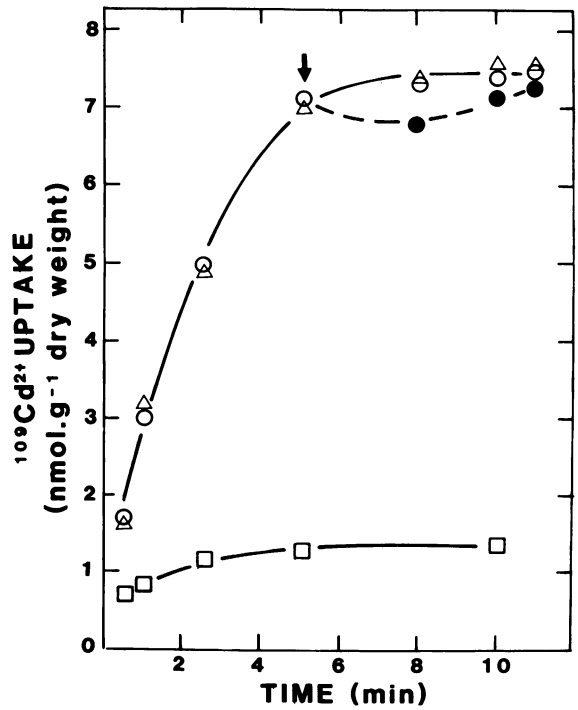

FIG. 2. $^{109}Cd^{2+}$ transport in the absence of Mn^{2+} by Cd^r harbor *Bacillus* strain RC607 (○) and its Cd^s mutant RC707 (△) and by RC607 in the presence of 20 μM Mn^{2+} (□). Arrow shows time of establishment of conditions which induce efflux (●).

attempted to find specific Cd-binding proteins in strain RC607. Cells were labeled with $^{109}Cd^{2+}$ and lysed, and duplicate samples were run in a 15% SDS-polyacrylamide gel. The Cd^r strain RC607, its Cd^s mutant (RC707), and a spontaneous Cd^r revertant all showed identical polypeptide patterns (Fig. 4A). Several of the polypeptides bound $^{109}Cd^{2+}$ with various affinities, but the pattern of the three strains was identical (Fig. 4B). Since the major labeled polypeptide comigrated with lysozyme (14,300 daltons), present in the cell lysate, $^{109}Cd^{2+}$ was added to the marker proteins (which included lysozyme). No radioactive band appeared (Fig. 4B, lane 4).

(iv) Hg resistance mechanism. Three strains of Hg^r marsh bacilli and of a Hg^r harbor isolate (RC607) were resistant by virtue of possessing mercuric reductase activity and thus were capable of reducing Hg^{2+} to volatile Hg^0. Enzyme activity was undetectable in an Hg^s mutant (data not shown).

DISCUSSION

This study concentrated on the gram-positive sporeformers isolated from two metal-contaminated saltwater sites, an estuarine marsh experimentally fertilized with heavy-metal-containing sludge (11) and the industrially polluted Boston harbor.

We wanted to determine whether the isolation of bacteria resistant to toxic metals could be used as an index of pollution. It is clear that substantial numbers of Hg^r bacteria existed in areas in which, according to spectrographic analysis (Table 1), the metal was either absent or present at concentrations below detection limits; many Cd^r strains were found in a plot which contained only background levels of Cd^{2+}. Since no Cd^r or Hg^r bacteria were recovered from waters with a pollution-free history, the selection and maintenance of a metal-resistant microbial community is likely to depend on the metal's having, at some time, been present. Indeed, sediment of the fertilized marsh (site 8, Table 1) was reported (7) as having 0.65 ppm of Hg^{2+}. However, once metal-resistant bacteria are present, it is not at all apparent

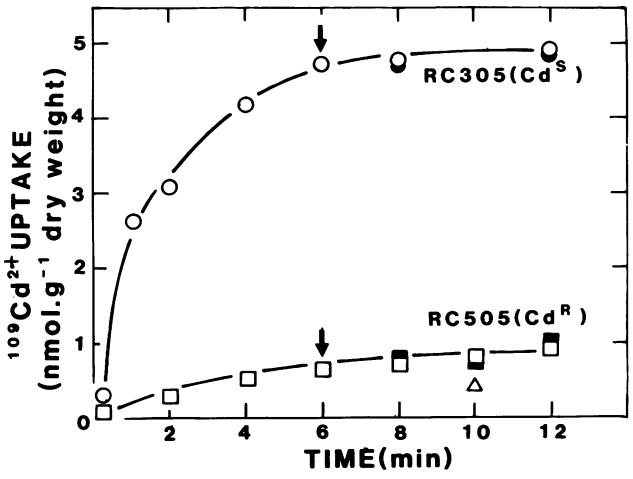

FIG. 3. $^{109}Cd^{2+}$ transport by Cd^s marsh *Bacillus* strain RC305 and its Cd^r mutant RC505. $^{109}Cd^{2+}$ accumulation (○, □), $^{109}Cd^{2+}$ in samples after establishment (arrows) of conditions which induce efflux (●, ■), and average 10-min $^{109}Cd^{2+}$ accumulation by eight Cd^r marsh *Bacillus* strains (△; range, 0.21 to 0.64 nmol g^{-1}) are indicated.

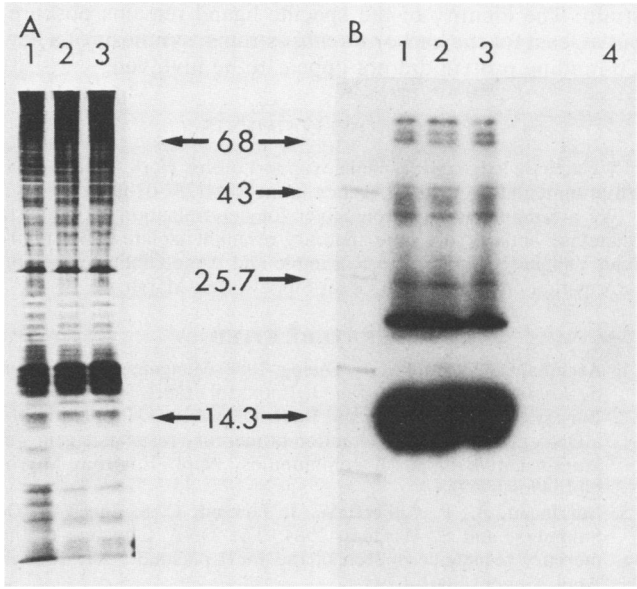

FIG. 4. 15% SDS-polyacrylamide gel electrophoresis of $^{109}Cd^{2+}$-labeled cell lysates. (A) Silver stain. (B) Autoradiogram. Lanes: 1, RC607; 2, Cd^s mutant; 3, Cd^r revertant; 4, marker proteins plus $^{109}Cd^{2+}$. Molecular weight standards: lysozyme (14,300), α-chymotrypsinogen (25,700), ovalbumin (43,000), bovine serum albumin (68,000).

that their numbers depend on metal concentration. Other, multiple environmental factors (7, 11) which affect the ability of organisms to take up metals also influence the ability of a metal to select and to maintain a metal-resistant population. A large Hg^r microbial population, actively transforming Hg^{2+} to volatile Hg^0, could help to account for the absence of detectable mercury, thus, in effect, cleaning up the environment.

Earlier investigations of metal resistance determinants have described both Cd^r and Hg^r genes as primarily plasmidborne. Recent findings have revealed the chromosomal existence of such determinants (31, 36). The chromosomal genes do not necessarily hybridize with plasmidborne resistance genes. Thus, a study of several chromosomally Hg- and Cd-resistant *S. aureus* strains, probed with the appropriate restriction fragments isolated from plasmid pI258, revealed homology for the Hg^r genes, but no hybridization was detected with the Cd^r determinants (36). The lack of demonstrable homology between our metal-resistant isolates and the absence of hybridization to pI258 DNA suggest considerable diversity among these resistance determinants (at least in gram-positive bacteria).

While the mechanism of resistance to inorganic Hg^{2+} via mercuric reductase appears to be operative in all the marsh and harbor strains examined and in all previously examined strains (28), Cd resistance seems to be based on diverse mechanisms. All the Cd^r marsh strains tested appeared to be resistant by virtue of reduced Cd transport as was previously described for *B. subtilis* (18). We were unable to determine the exact mechanism of Cd resistance of the harbor bacilli. Neither effluxing, nor reduced transport, nor specific binding proteins appeared to be involved in conferring resistance to Cd^{2+}. Yet large quantities of Cd^{2+} were transported into both sensitive and resistant cells. We feel that in view of the multiplicity of possible cell targets (35), the metal must be sequestered or chelated to ensure survival of the resistant

strain. The identity of the specific ligand remains obscure, but at least for the harbor *Bacillus* strains, synthesis of a new Cd-binding protein did not appear to be involved.

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