APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1990, p. 1695–1701
0099-2240/90/061695-07$02.00/0

Vol. 56, No. 6

Genes Encoding Mercuric Reductases from Selected Gram-Negative Aquatic Bacteria Have a Low Degree of Homology with *merA* of Transposon Tn*501*†

TAMAR BARKAY,[1]* MARK GILLMAN,[2] AND CYNTHIA LIEBERT[2]

Microbial Ecology and Biotechnology Branch[1] and Technical Resources Inc.,[2] Environmental Research Laboratory, U.S. Environmental Protection Agency, Gulf Breeze, Florida 32561

Received 25 October 1989/Accepted 3 April 1990

An investigation of the Hg^{2+} resistance mechanism of four freshwater and four coastal marine bacteria that did not hybridize with a *mer* operonic probe was conducted (T. Barkay, C. Liebert, and M. Gillman, Appl. Environ. Microbiol. 55:1196–1202, 1989). Hybridization with a *merA* probe, the gene encoding the mercuric reductase polypeptide, at a stringency of hybridization permitting hybrid formation between evolutionarily distant *merA* genes (as exists between gram-positive and -negative bacteria), detected *merA* sequences in the genomes of all tested strains. Inducible Hg^{2+} volatilization was demonstrated for all eight organisms, and NADPH-dependent mercuric reductase activities were detected in crude cell extracts of six of the strains. Because these strains represented random selections of bacteria from three aquatic environments, it is concluded that *merA* encodes a common molecular mechanism for Hg^{2+} resistance and volatilization in aerobic heterotrophic aquatic communities.

A widely employed mechanism of bacterial resistance to mercurial compounds is the reduction of Hg^{2+} to the volatile form, Hg^0. This biotransformation is mediated by an inducible NADPH-dependent (and in some cases, NADH-dependent) flavin-containing disulfide oxidoreductase enzyme, mercuric reductase (36). The gene encoding mercuric reductase (*merA*), together with genes coding for Hg^{2+} transport and for regulatory functions, comprises the *mer* operon (24). In a wide variety of gram-negative and -positive organisms, *mer* operons are located on plasmids and transposons (36), but chromosomally located *mer* operons have been found in *Staphylococcus aureus* (41), a *Bacillus* sp. (38), and *Thiobacillus ferrooxidans* (31). A variable degree of homology among *mer* genes and polypeptides of different bacteria exists (32), but reductases of even the most evolutionarily distant strains share 40% of their amino acid residues (39). The following other mechanisms for resistance to mercurial compounds have been reported but not as thoroughly studied: methylation of Hg^{2+} by an anaerobe with a high tolerance to CH_3Hg^+ (25), removal of Hg^{2+} by its precipitation as HgS by an H_2S-producing anaerobe (27), and alteration of outer membrane permeability in an aerobic organism (26). In addition, morphological abnormalities have been observed in bacteria adapted to growth in the presence of Hg^{2+}, but the relation of these phenomena to resistance is not clear (37).

Reductive mercury detoxification has served as a model system for the study of the role of a specific molecular mechanism in the response of natural microbial communities to toxic pollutants, because of its ubiquity and the detailed knowledge of its mechanism (3, 6, 7). It has been shown that aquatic microbial communities eliminated Hg^{2+} by a process that required an acclimation period (3), that the volatile mercurial was Hg^0 (6), and that reduction of Hg^{2+} was mediated by bacteria rather than by photosynthetic microbes or heterotrophic microflagellates (6). However, in acclimated communities, the number of organisms having DNA sequences homologous to characterized *mer* operons was 2 to 3 orders of magnitude lower than the number of Hg^{2+}-resistant bacteria (3), many resistant strains failed to hybridize with a *mer* probe (6), and an increase in the abundance of *mer* genes in the genomes of acclimated communities could only partially account for increases in the level of tolerance to Hg^{2+} (7). Thus, genes that were not detected by the employed *mer* probe must have coded for Hg^{2+} resistance and reduction in aquatic microbial communities. These genes either mediated alternative resistance mechanisms or their nucleotide sequences have diverged sufficiently from those of the characterized *mer* operons so that cross-hybridization could not occur.

Here we report that randomly selected Hg^{2+}-resistant strains that did not hybridize with an operonic *mer* probe had genes homologous to *merA* of transposon Tn*501* [*merA* (Tn*501*)] when tested under a low stringency of hybridization, that these strains volatilized Hg^{2+} when grown under inducing conditions, and that volatilization was mediated by NADPH-dependent mercuric reductases in crude cell extracts.

MATERIALS AND METHODS

Bacterial strains. Previously isolated gram-negative Hg^{2+}-resistant strains (6) were grouped according to colony morphology (34). One or two isolates representing groups that were exclusively comprised of strains that did not hybridize with a *mer*(Tn*21*) probe (6) were selected for further study (Table 1). These strains were characterized by using API Rapid NFT strips (Analytab Products, Plainview, N.Y.).

Coastal marine isolates (a total of 50 strains) were divided into five groups, two of which did not include *mer*(Tn*21*)-positive strains. The first group, comprised of six isolates, was represented by *Pseudomonas* sp. strain Z1031 and *Pseudomonas vesicularis* Z1028. *P. vesicularis* Z1042 and Z1033 represented the second group, which contained a total of three isolates. Among the 63 resistant strains that were

* Corresponding author.

† Contribution no. 689 of the U.S. Environmental Research Laboratory, Gulf Breeze, FL 32561.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid[a]	Relevant characteristic or marker	Source (reference)
Environmental strains		
P. stutzeri VS1063 (97.8)	Hg^r	This study
Pseudomonas sp. strain VS1069 (100)	Hg^r	This study
Gram-negative motile rod PCA5030	Hg^r	This study
P. cepacia PCA109 (99)	Hg^r	This study
Gram-negative motile rod Z1031	Hg^r	This study
P. vesicularis Z1028 (93.8)	Hg^r	This study
P. vesicularis Z1042 (99.5)	Hg^r	This study
P. vesicularis Z1033 (99.5)	Hg^r	This study
Reference strains		
P. aeruginosa PAO1	Prototroph	R. Olsen, University of Michigan, Ann Arbor
P. aeruginosa PAO1(pEPA81)[b]	Hg^r, Cb^r	S. Cuskey, Environmental Protection Agency, Gulf Breeze, Fla.
P. aeruginosa PAO25(pVS1)	Hg^r	D. Haas, Mikrobiologische Institut, Zurich, Switzerland (35)
Plasmids		
pMERA	Subclone of *merA* from pVSI	This study
pACNR25	Subclone of *mer* from R100	(7)
pYW46	Subclone of *mer* from *Bacillus* sp. strain RC607	I. Mahler, Brandeis University, Waltham, Mass. (38)
pRAL2A	Subclone of *mer* from pI258	R. Laddage, Bowling Green State University, Bowling Green, Ohio (41)

[a] Identifications are given for strains that could be clearly characterized by using the API Rapid NFT strips (>90% confidence level, as reported by the manufacturer of the test kits). When test results could not be clearly interpreted by using the available API data base, only a simple description of the strain is given. Numbers in parentheses indicate the confidence level of identification (percent).

[b] Plasmid pEPA81 is a derivative of pRO2317 (G. J. Zylstra, S. M. Cuskey, and R. H. Olsen, *EPA Manual for Methods in Biotechnology*, in press) into which a restriction fragment carrying the *mer* operon from plasmid pVS1 (i.e., Tn*501*) was cloned (Cuskey and Barkay, unpublished data).

isolated from Vortex Spring, Fla., 7 formed a unique group that was *mer*(Tn*21*) negative and 2 (*Pseudomonas* sp. strain VS1069 and *Pseudomonas stutzeri* VS1063) were selected for further study. A second freshwater community from Thompson's Bayou, Fla., consisted of 104 isolates that were divided to 14 groups, 6 of which were exclusively comprised of *mer*(Tn*21*)-negative strains. Two such groups (of 10 isolates each) were represented by *Pseudomonas cepacia* PCA109 and by strain PCA5030.

Reference pseudomonads (Table 1) were obtained from the culture collection at the U.S. Environmental Protection Agency laboratory in Gulf Breeze, Fla. *Pseudomonas aeruginosa* PAO1(pEPA81) and PAO25(pVS1) were both employed as positive controls in volatilization and mercuric reductase assays (see below). Strain PAO25(pVS1) was a gift from D. Haas (Mikrobiologisches Institut, Zürich, Switzerland).

Growth conditions. Growth conditions were selected to optimize the growth of the various strains included in this study. Coastal marine organisms were routinely grown in marine medium (5 g of peptone [Difco Laboratories, Detroit, Mich.], 1 g of yeast extract [Difco], 500 ml of aged seawater, 500 ml of distilled water). Freshwater isolates were cultured in half-strength plate count broth (Difco). Bacto-Agar (Difco) was added (1.5%) to these media for solidification. Unless otherwise specified, Hg^{2+} (as $HgCl_2$) was included at 10 μg/ml. All environmental strains were grown at 30°C. Reference pseudomonads were grown in Luria broth (LB) at 37°C, and, where indicated, media were supplemented with 600 μg of carbenicillin per ml or 10 μg of Hg^{2+} (as $HgCl_2$) per ml.

DNA:DNA hybridization with a *merA* probe. A 1,195-base-pair *Sph*I-*Nar*I restriction fragment spanning three quarters of the *merA* gene of Tn*501* (12) was subcloned (21) into pUC18 to give the recombinant plasmid pMERA. Radiolabeled preparations of the 1,195-base-pair *merA* fragment were obtained as described previously (7). Digestions with *Nar*I (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and *Sph*I (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) were performed as recommended by the manufacturers. Membranes for hybridization were prepared as described elsewhere (7) by applying 1 μg of purified denatured genomic DNA from environmental strains prepared as described by Maniatis et al. (21), by using a slot blot apparatus (Schleicher & Schuell, Inc., Keene, N.H.). Purified plasmids carrying *mer* genes used as control target DNA (Table 1) were applied to the membrane to yield a final amount of 1.0 ng (as nanograms of target DNA per slot).

Hybridizations were carried out at high and low stringencies. For high-stringency hybridization, prehybridization, hybridization, and preparation of membranes for autoradiography were as described previously (5). For low-stringency hybridization, membranes were prehybridized in 4× SET buffer (0.6 M NaCl, 0.12 M Tris hydrochloride [pH 8], 4 mM EDTA)–10× Denhardt solution– 0.2% sodium dodecyl sulfate–100 μg of calf thymus DNA per ml for 5 h at 30°C. Hybridizations were carried out in fresh buffer (as for prehybridization) containing 50% deionized formamide (Sigma Chemical Co., St. Louis, Mo.) and labeled probe (10^7 dpm/reaction) at 30°C for 12 h. To allow hybridization with *merA* genes of gram-positive organisms, the temperature of hybridization was adjusted as suggested by Beltz et al. (9), by using DNA sequence information for *merA* of Tn*501* (12), *Bacillus* sp. strain RC607 (39), and *S. aureus* (19). The alignment of the corresponding MerA polypeptides as suggested by Wang et al. (39) was used for all DNA sequence comparisons. Hybridization reactions, under both stringencies, contained 25 μg of denatured pUC18 DNA per ml; the

DNA was previously digested with *Eco*RI (Boehringer Mannheim Biochemicals). Posthybridization washes of membranes that were hybridized at a low stringency were as suggested by Beltz et al. (9). Autoradiograms of dried membranes were obtained as described previously (5).

Determination of Hg^{2+} resistance levels. Resistance was quantitated by using the disk inhibition test described previously (4). Cell suspensions were prepared as follows: turbidities of late-log-phase cultures of freshwater strains were adjusted (by using a spectrophotometer [Spectronic 21; Milton Roy Co., Rochester, N.Y.]) to an A_{500} of 0.25. Cells were centrifuged (12,000 × *g* for 10 min at 5°C) and resuspended in 1/10 of the original culture volume. Coastal marine cultures were prepared by adjusting turbidities (measured with a colorimeter [Klett-Summerson Photoelectric colorimeter; A. H. Thomas Co., Philadelphia, Pa.] equipped with a red [no. 66] filter) to correspond to a dry weight of 0.1155 mg/ml [the dry weight of strain PAO1(pEPA81) giving a turbidity of 30 Klett units]. The appropriate growth media (see above) were inoculated by spreading 0.1 ml of cell suspension prior to application of disks impregnated with Hg^{2+}. Disks (10 mm in diameter) were cut from chromatography paper (3MMChr; Whatman Ltd., Maidstone, England).

Whole-cell Hg^{2+} volatilization assays. Resting cell volatilization assays were performed by the method of Weiss et al. (40). Cultures were induced by overnight growth in media supplemented with 10 μM Hg^{2+}, except for strains Z1042 and Z1033, which were grown with 2.5 μM Hg^{2+}. Cells were washed with assay buffer and resuspended to a turbidity of 30 Klett units. Strains Z1042 and Z1033 grew as tight aggregates that were dispersed by the addition of 25 mM citrate to the rinse buffer (dispersion was also achieved with 50 mM phosphate or 50 mM nitrilotriacetic acid [Sigma]). Assay buffer for freshwater and reference strains was as described previously (40), except that chloramphenicol (Sigma) was present at 100 μg/ml. For coastal marine organisms, the assay buffer was modified by replacing phosphate with HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanelsulfonic acid; Sigma) at 50 mM (pH 7.4) containing 0.1 M NaCl. Assay buffers used for strains Z1033 and Z1042 also contained 0.01% yeast extract, with Hg^{2+} concentration reduced to 2.5 μM. All assays were carried out in 10-ml reaction volumes in a shaking water bath at 30°C for 45 min. Samples were removed periodically, and remaining Hg^{2+} was analyzed as described by Barkay et al. (6).

Mercuric reductase assays. Crude cell extracts were prepared by the method of Fox and Walsh (15). Mercury-dependent NADPH (or NADH) oxidation rates were determined as described previously (15) at 30°C, by using a spectrophotometer (model 8452A Diode Array Spectrophotometer; Hewlett-Packard Co., Palo Alto, Calif.). For Hg^{2+} reduction rate determinations, reactions (15) were performed in 3-ml reaction mixtures at 30°C with shaking, 0.3-ml samples were periodically removed, and remaining Hg^{2+} was determined as described above. Qualitative assays of mercuric reductase activities, needed to adjust assay conditions (see Results), were carried out by detecting Hg^0 evolved from 5-ml reaction mixtures placed in 30-ml graduated glass impingers (Wheaton Industries, Millville, N.J.) attached to a cold-vapor mercury analyzer (6). Protein concentrations were measured by using a BCA* bicinchoninic acid Protein Assay Reagent (Pierce Chemical Co., Rockford, Ill.) as recommended by the manufacturer.

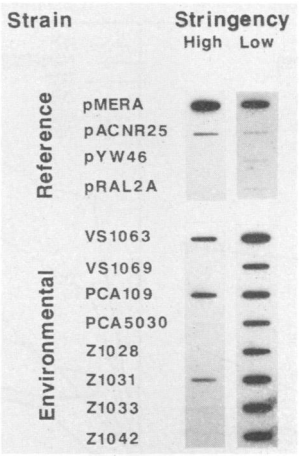

FIG. 1. Detection of *merA* in the genomes of the studied environmental strains. Purified genomic DNA samples (1 μg) of environmental strains were hybridized with a *merA* probe at high and low stringencies. Purified plasmid DNA carrying *merA* genes of different bacterial origins (see Materials and Methods) was used as a reference.

RESULTS

Homology of *merA* genes of environmental strains with *merA*(Tn*501*). The hypothesis that *mer* genes had not been previously detected in the studied strains because of low DNA sequence homology was tested by hybridization of genomic DNA with a *merA*(Tn*501*) probe at a high stringency (permitting cross-hybridization between *merA* genes of gram-negative organisms) and at a low stringency (permitting cross-hybridization between all currently characterized *merA* genes) (Fig. 1). At a high stringency, the *merA* probe hybridized with control plasmid DNA of pACNR25 [of *mer*(Tn*21*) origin] and pMERA [of *mer*(Tn*501*) origin] but not with pYW46 [containing *mer* of *Bacillus* sp. strain RC607; *merA(Bacillus)*] or pRAL2A [*mer* originating in the *S. aureus* plasmid pI258; *merA(Staph)*]. Although the overall DNA sequence homology of the *merA* probe with *merA(Bacillus)* and *merA(Staph)* is low (38 and 47%, respectively), the regions encoding the reductase-active sites have a higher degree of homology. A region of 132 base pairs in *merA(Bacillus)* (39) has 61% homology with the *merA* probe, and a region of 45 base pairs in *merA(Staph)* (19) has 78% homology with the *merA* probe. Because *merA(Staph)* did not hybridize at a high stringency, sequence homology greater than 78% was required for hybrid formation under the selected conditions. (Although *merA* of Tn*501* is only 70% homologous with *merA* of Tn*21*, specific regions bear 96% homology [8] and cross-hybridization was demonstrated [Fig. 1].) Low-stringency conditions were set up to permit cross-hybridization between *merA*(Tn*501*) and *merA(Bacillus)*, because they possess the lowest degree of homology of all sequenced *merA* genes (61% in the regions encoding the active sites). Because all control plasmids hybridized at a low stringency, genes having regions with at least 61% DNA sequence homology should be detected under these conditions. Negative controls consisting of equal amounts (i.e., 1 μg per slot) of *Escherichia coli*, *P. aeruginosa*, *Bacillus cereus*, and calf thymus DNA did not hybridize with the *merA* probe at either stringency.

Three of the environmental strains, VS1063, PCA109, and Z1031, hybridized with the *merA* probe at a high stringency (Fig. 1), indicating that they carry *merA* genes homologous

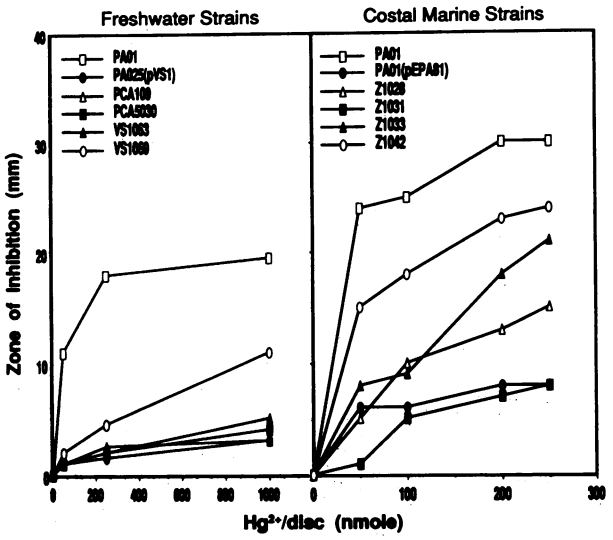

FIG. 2. Resistance level of environmental strains to Hg^{2+}. The results show the diameters of the zones of inhibition (less the diameter of disks) observed around disks impregnated with the indicated amounts of Hg^{2+}. Marine strains were tested on marine medium. Freshwater strains were tested on half-strength plate count agar.

to the previously characterized gram-negative genes. The remaining five strains carried *merA* genes with a lower degree of homology, because they only hybridized at a low stringency. Therefore, genes encoding mercuric reductases in these gram-negative strains seem to be more evolutionarily distant from *merA*(Tn*501*) than the previously defined *merA* genes of gram-negative bacteria (32). However, determination of the exact evolutionary relationships of *merA* in the environmental strains awaits DNA sequence analysis.

Resistance level to Hg^{2+}. The disk inhibition test was used to compare levels of Hg^{2+} resistance of environmental isolates with those of *P. aeruginosa* strains containing *mer*(Tn*501*) (Fig. 2). Three of the freshwater and one of the coastal marine isolates (Z1031) were as resistant as the control strains, PAO25(pVS1) and PAO1(pEPA81), but strain VS1069 and the remaining three coastal marine strains exhibited an intermediate level of resistance. On marine medium, the control strains PAO1 and PAO1(pEPA81) had zones of inhibition of 27 and 8 mm, respectively, around disks impregnated with 250 nmol of Hg^{2+}, whereas the corresponding zones of inhibition for strains Z1033, Z1042, and Z1028 were 13 to 23 mm. Strains Z1033 and Z1042 were inhibited by 10 μM Hg^{2+} in marine broth and, therefore, were cultured in medium supplemented with 2.5 μM Hg^{2+}. Experiments with reference strains of *Pseudomonas fluorescens*, *Pseudomonas putida*, *P. stutzeri*, and *Pseudomonas perfectomarina* (the last two strains were a gift from G. Stewart, University of South Florida, Tampa) showed zones of inhibition from 24 to 40 mm around disks containing 250 nmol of Hg^{2+} (data not shown). Thus, Hg^{2+} resistance was above the intrinsic tolerance of some pseudomonads, even for the coastal marine organisms.

Inducible Hg^{2+} volatilization activities. All eight environmental strains had inducible Hg^{2+} volatilization activities. Induced cultures rapidly removed Hg^{2+}, whereas little or no Hg^{2+} was lost from assays of uninduced cells (Fig. 3). Freshwater isolates were active under conditions defined for *merA*-carrying strains [such as the positive control PAO1 (pEPA81)]. However, assay conditions for coastal marine bacteria were modified as follows. (i) The osmotic potential of assay buffers was increased by the addition of 0.1 M NaCl to prevent cell lysis. (ii) Phosphate buffer was replaced by HEPES, at the same strength and the same pH (see Materials and Methods), because volatilization by induced strain Z1031 was inhibited by 50 mM sodium phosphate buffer (Fig. 3E). Volatilization was not affected by 0.3 M NaCl (data not shown), suggesting that inhibition was due to phosphate. (iii) Yeast extract (0.01%) was included in the assay buffer for strains Z1033 and Z1042, resulting in an increase in initial rates from 0.263 ± 0.035 to 0.528 ± 0.020 nmol/min per mg for induced Z1042 and from 0.219 to 0.605 nmol/min per mg for induced Z1033. Thus, supplementing assays with yeast extract allowed a clear demonstration of the differences between induced and uninduced activities (Fig. 3F).

Mercuric reductase activities. As expected, crude extracts of induced environmental strains demonstrated NADPH-dependent mercuric reductase activities (Table 2). The reductase activities of four strains (Z1031, PCA109, PCA5030, and VS1063) were quantitated by the standard Hg^{2+}-dependent NADPH oxidation assay (15), but the remaining four strains were inactive under these assay conditions. For two of these strains (Z1028 and VS1069), Hg^{2+} could have been inhibitory at the high concentration required for the NADPH oxidation assay (100 μM), since Hg^{2+} was volatilized when lower concentrations (10 μM) were employed in the Hg^{2+} reduction assays. Evolution of Hg^0 was noted in qualitative assays (see Materials and Methods) of cell extracts of strain VS1069, but activity was too unstable to be reproducibly quantitated. Cell extracts from induced VS1069 had both NADPH- and NADH-dependent Hg^0 evolution activities. Extracts from an uninduced culture had activities that were only slightly above those of the background levels (i.e., rate of Hg^0 evolution by a blank not containing cell extract). Mercuric reductase activities could not be detected in cell extracts of strains Z1042 and Z1033 by any of the employed assays. Repeated attempts to obtain assay conditions giving detectable activities (by using the qualitative Hg^0 evolution test) were unsuccessful. Modifications included the use of alternative assay buffers (22, 30), among them the buffer employed by Ji et al. (17) to measure reductase activities of marine *Caulobacter* strains; replacing phosphate with HEPES (in both assay and cell extract preparation buffers); and replacing β-mercaptoethanol with thioglycolate (87 mM), L-cysteine (87 mM), or dithiothreitol (2.3 mM). Thus, despite the demonstrated ability of intact cells of strains Z1033 and Z1042 to volatilize (i.e., reduce) Hg^{2+} (Fig. 3) and the presence of *merA* in their genomes (Fig. 1), conditions optimal for the demonstration of mercuric reductase activity could not be established. The mercuric reductase of strain Z1031 was equally as active in phosphate buffer (29.17 ± 1.17 mU/mg) as in HEPES buffer (29.29 ± 0.59 mU/mg) (Table 2). Measurable reductase activities in reactions in which NADH replaced NADPH (data not shown) were below the level of detection, except for strain Z1028, with activity (0.0184 mU/mg) bordering on the detection limit of the assay (0.016 mU/mg).

DISCUSSION

The Hg^{2+} resistance mechanism employed by eight bacterial strains selected to represent Hg^{2+}-resistant organisms that did not hybridize with a *mer*(Tn*21*) operonic gene probe (6) was shown to be inducible NADPH-dependent mercuric reductase-mediated Hg^{2+} volatilization (Fig. 3) (Table 2).

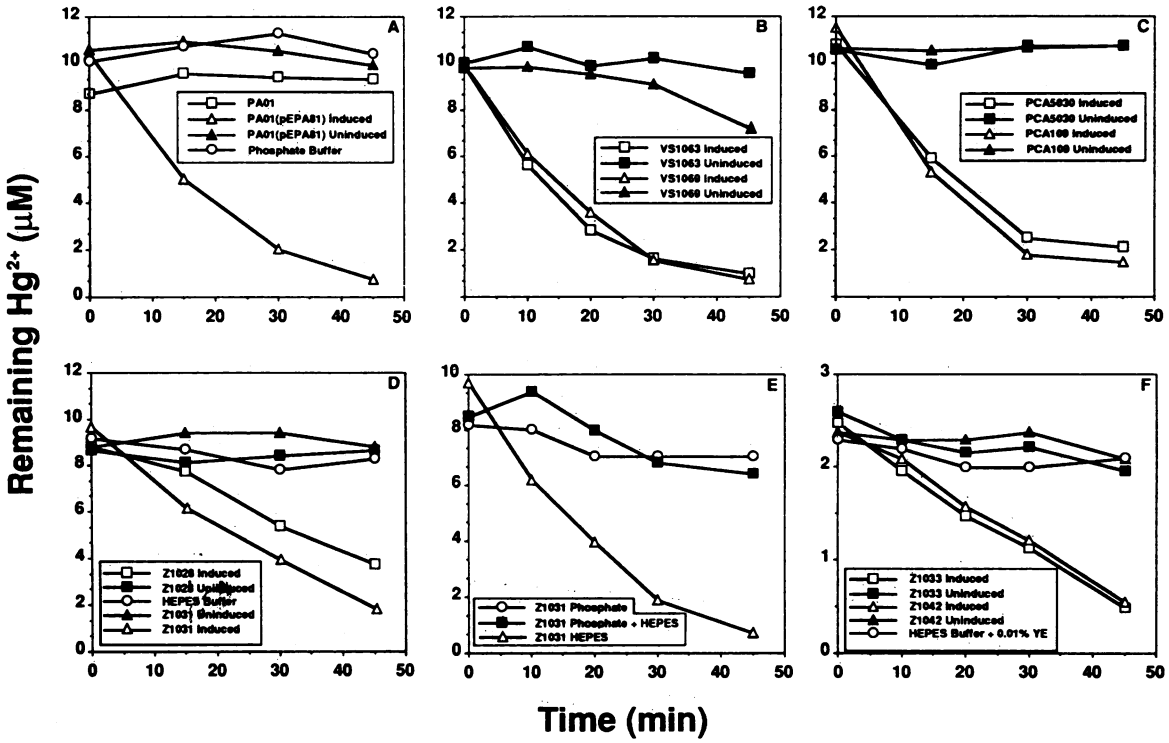

FIG. 3. Hg^{2+} volatilization by induced and uninduced strains. (A) Reference strains tested in phosphate-based assay buffer (see Materials and Methods). (B) Freshwater strains isolated from Vortex Spring, Fla., tested in phosphate-based buffer. (C) Freshwater strains isolated from Thompson's Bayou, Fla., tested in phosphate-based buffer. (D) Coastal marine strains tested in HEPES-based buffer. (E) Phosphate inhibition of Hg^{2+} volatilization by induced strain Z1031. (F) Coastal marine strains tested in HEPES-based buffer supplemented with 0.01% yeast extract (YE).

TABLE 2. Mercuric reductase activities in crude cell extracts of environmental strains

Strain	Sp act (mU/mg of protein)[a]		Induction ratio[b]
	Induced	Uninduced	
Hg^{2+}-dependent NADPH oxidation			
PAO1	ND[c]	<0.32[d]	
PAO1(pVS1)	117.0 ± 4.9	<0.32	>366
Z1031 (phosphate buffer)	29.2 ± 1.2	1.4 ± 0.5	21
Z1031 (HEPES buffer)	29.3 ± 0.6	1.8 ± 0.1	16
PCA109	36.8 ± 3.1	<0.32	>115
PCA5030	22.4 ± 2.7	<0.32	>70
VS1063	132.5 ± 16.5	0.465 ± 0.3	285
Hg^{2+} reduction			
PAO1	ND	<0.016[e]	
PAO1(pVS1)	258.5 ± 39.0	<0.016	>16,156
Z1028	0.526 ± 0.014	0.021	25
VS1069	+	–[f]	

[a] mU = nanomoles of NADPH (or Hg^{2+}) oxidized (reduced) per minute.
[b] Ratio between activities of induced and uninduced extracts.
[c] ND, Not determined.
[d] Limit of detection was 0.8 nmol of NADPH oxidized per min/100 μl of cell extract.
[e] Limit of detection was 0.125 nmol of Hg^{2+} reduced per min/100 μl of cell extract.
[f] No quantitative data available (see Results). Results of qualitative assay: +, active; –, inactive (see Materials and Methods).

This activity was encoded by *merA* genes that, in five of the strains (Fig. 1), had a lower degree of homology with *merA*(Tn*501*) than previously sequenced *merA* genes in gram-negative bacteria. The *merA* genes of the remaining three strains are evolutionarily as close to *merA*(Tn*501*) as is *mer*(Tn*21*). Thus, the hypothesis that alternative mechanisms endowed Hg^{2+} resistance in the studied strains is rejected. Because these strains represented random selections of Hg^{2+}-resistant bacteria from three aquatic environments (6), it can be concluded that the *merA*-mediated reduction is a common molecular mechanism for Hg^{2+} resistance and volatilization in aerobic heterotrophic aquatic communities.

Although resistance was similar in principle to the widely employed bacterial Hg^{2+} resistance mechanism, some differences emerged. These may be most significant among coastal marine strains, because (i) three of four strains had an intermediate resistance level (Fig. 2), (ii) the reductase of one of these strains was inhibited by high Hg^{2+} concentrations (Table 3) and the remaining two reductases could not be tested in standard assays, and (iii) the *merA* genes of these strains were detected only at a low stringency of hybridization (Fig. 1). Ji et al. (17) showed similar trends with marine *Caulobacter* strains; Hg^{2+} concentrations above 5 μM were inhibitory for bacterial growth and concentrations above 10 μM were inhibitory for reductases in crude cell extracts. Thus, mercuric reductases in marine bacteria may possess a greater diversity of properties than previously described. This, together with previous findings that highly Hg^{2+}-tolerant strains are absent in saline envi-

ronments (6) and that *mer*(Tn*21*)-like DNA sequences are infrequent in the community genome of an acclimated estuarine community (7), suggests that the molecular details of bacterial Hg^{2+} resistance in saline environments may have unique characteristics. Further studies are needed for a better understanding of the mercuric reductases of marine bacteria.

Such studies may be warranted in light of the importance of marine microbial activities in the geochemical cycling of mercury (14, 18). Elevated Hg^0 concentrations in the near surface atmosphere over the equatorial upwelling of the Pacific Ocean were related to increased biological productivity (14). It was calculated that this process could account for as much as 36% of the global flux of mercury into the atmosphere (18). Furthermore, microbial processes may be the major cause of mercury volatilization in marine environments (6), because of unfavorable conditions for nonbiological processes, such as reduction-disproportionation reactions (2) and activities mediated by components of humic acids (1, 33).

However, marine bacteria with typical reductases exist because the coastal marine strain Z1031 was as resistant to Hg^{2+} as was PAO1(pEPA81). Furthermore, its reductase was not inhibited by 100 μM Hg^{2+} and its *merA* gene had a high degree of homology with *merA*(Tn*501*). Yet the salt requirements for cell stability (see Results) and growth (data not shown) suggest that Z1031 is a real marine bacterium rather than a terrestrial organism isolated from a coastal marine environment (20, 42). Phosphate inhibition of whole-cell Hg^{2+} volatilization (Fig. 3E), but not of mercuric reductase activities (Table 2), suggests that phosphate interfered with the transport of Hg^{2+} through the cell wall in strain Z1031.

Little is known about Hg^{2+} transport in gram-negative bacteria and about its role in their resistance mechanism. An intact outer membrane is essential for expression of Hg^{2+} resistance (13). Ionic mercury interacts with bimolecular lipid membranes (10), but it is transported in an uncharged form (11). Resistant strains have an active Hg^{2+} transport system consisting of a periplasmic protein (MerP) and an inner membrane-spanning protein (MerT), but little is known about their function (32, 36). Phosphate may affect Hg^{2+} transport at any of these sites. Alternatively, inhibition of volatilization may result from a more general effect of phosphate on the metabolism of strain Z1031.

Detection of phenotypes of environmental bacteria by hybridization with function-specific DNA probes is a common procedure in microbial ecology (3, 28, 29; W. E. Holben, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, Q-72, p. 342), and it has been proposed as a tool in the management of polluted environments (16). The results presented here indicate that application of this approach requires that the genetic drift that occurs during evolution of DNA sequences be considered. While only three of the strains hybridized with the *merA* probe at a high stringency of hybridization, all eight hybridized when a lower stringency was employed (Fig. 1). Stringencies of hybridization were adjusted by considering available sequence data for *merA* genes (32). Unfortunately, data bases of similar quality do not yet exist for other genes encoding responses to environmental pollutants. The alternative (and complementary) approach of using rapid assays for substrates or products of biotransformations (23) could replace function-specific probing in surveys of environmental isolates.

ACKNOWLEDGMENTS

Gratitude is extended to Peter Chapman for his advice and support during the course of this study, to Jeff Freund for technical assistance, to Joe Capps for the drawings, and to Inga Mahler, Anne Summers, Dieter Haas, Richard Laddaga, and Greg Stewart for supplying bacterial strains.

LITERATURE CITED

1. **Alberts, J. J., J. E. Schindler, R. W. Miller, and D. E. Nutter, Jr.** 1974. Elemental mercury evolution mediated by humic acid. Science **184**:895–897.
2. **Baltisberger, R. J., D. A. Hildebrand, D. Grieble, and T. A. Ballintine.** 1979. A study of the disproportionation of mercury (I) induced by gas sparging in acidic aqueous solutions for cold-vapor atomic absorption spectrometry. Anal. Chim. Acta **111**:111–122.
3. **Barkay, T.** 1987. Adaptation of aquatic microbial communities to Hg^{2+} stress. Appl. Environ. Microbiol. **53**:2725–2732.
4. **Barkay, T., and R. R. Colwell.** 1983. Altered mercury transport in a mutant of *Pseudomonas fluorescens* B69. J. Gen. Microbiol. **129**:2945–2950.
5. **Barkay, T., D. L. Fouts, and B. H. Olson.** 1985. Preparation of a DNA gene probe for detection of mercury resistance genes in gram-negative bacterial communities. Appl. Environ. Microbiol. **49**:686–692.
6. **Barkay, T., C. Liebert, and M. Gillman.** 1989. Environmental significance of the potential for *mer*(Tn*21*)-mediated reduction of Hg^{2+} to Hg^0 in natural waters. Appl. Environ. Microbiol. **55**:1196–1202.
7. **Barkay, T., C. Liebert, and M. Gillman.** 1989. Hybridization of DNA probes with whole-community genome for detection of genes that encode microbial responses to pollutants: *mer* genes and Hg^{2+} resistance. Appl. Environ. Microbiol. **55**:1574–1577.
8. **Barrineau, P., P. Gilbert, W. J. Jackson, C. S. Jones, A. O. Summers, and S. Wisdom.** 1984. The DNA sequence of the mercury resistance operon of the IncFII plasmid NR1. J. Mol. Appl. Genet. **2**:601–619.
9. **Beltz, G. A., K. A. Jacobs, T. H. Eickbush, P. T. Cherbas, and F. C. Kafatos.** 1983. Isolation of multigene families and determination of homologies by filter hybridization methods. Methods Enzymol. **100**:266–285.
10. **Bevan, D. R., W. J. Worrell, and K. D. Barfield.** 1983. The interaction of Ca^{2+}, Mg^{2+}, Zn^{2+}, Cd^{2+} and Hg^{2+} with phospholipid bilayer vesicles. Colloids Surf. **6**:365–376.
11. **Bienvenue, E., A. Boudou, J. P. Desmazes, C. Gavach, D. Georgescauld, J. Sandeaux, R. Sandeaux, and P. Seta.** 1984. Transport of mercury compounds across bimolecular lipid membranes: effect of lipid composition, pH and chloride concentration. Chem. Biol. Interactions **48**:91–101.
12. **Brown, N. L., S. J. Ford, R. D. Pridmore, and D. C. Fritzinger.** 1983. Nucleotide sequence of a gene from the *Pseudomonas* transposon Tn501 encoding mercuric reductase. Biochemistry **22**:4089–4095.
13. **Calcott, P. H.** 1981. Transient loss of plasmid-mediated mercuric ion resistance after stress in *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. **41**:1348–1354.
14. **Fitzgerald, W. F., G. A. Gill, and J. P. Kim.** 1984. An equatorial Pacific Ocean source of atmospheric mercury. Science **224**: 597–599.
15. **Fox, B., and C. T. Walsh.** 1982. Mercuric reductase: purification and characterization of a transposon-encoded flavoprotein containing an oxidation-reduction-active disulfide. J. Biol. Chem. **257**:2498–2503.
16. **Goldstein, R. A., B. H. Olson, and D. B. Porcella.** 1988. Conceptual model of genetic regulation of mercury biogeochemical cycling. Environ. Technol. Lett. **9**:957–964.
17. **Ji, G., S. P. Salzberg, and S. Silver.** 1989. Cell-free mercury volatilization activity from three marine *Caulobacter* strains. Appl. Environ. Microbiol. **55**:523–525.
18. **Kim, J. P., and W. F. Fitzgerald.** 1986. Sea-water partitioning of mercury in the equatorial Pacific Ocean. Science **231**:1131–1133.
19. **Laddaga, R. A., L. Chu, T. K. Misra, and S. Silver.** 1987. Nucleotide sequence and expression of the mercurial-resistance

operon from *Staphylococcus aureus* plasmid pI258. Proc. Natl. Acad. Sci. USA **84**:5106–5110.
20. **MacLeod, R. A.** 1968. On the role of inorganic ions in the physiology of marine bacteria. Adv. Microbiol. Sea **1**:95–126.
21. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Nakahara, H., J. L. Schottel, T. Yamada, Y. Miyakawa, M. Asakawa, J. Harville, and S. Silver.** 1985. Mercuric reductase enzymes from *Streptomyces* species and group B *Streptococcus*. J. Gen. Microbiol. **131**:1053–1059.
23. **Nakamura, K., and H. Nakahara.** 1988. Simplified X-ray film method for detection of bacterial volatilization of mercury chloride by *Escherichia coli*. Appl. Environ. Microbiol. **54**: 2871–2873.
24. **Ni' Bhriain, N. N., S. Silver, and T. J. Foster.** 1983. Tn*5* insertion mutations in the mercuric ion resistance genes derived from plasmid R100. J. Bacteriol. **155**:690–703.
25. **Pan-Hou, H. S., and N. Imura.** 1982. Involvement of mercury methylation in microbial mercury detoxication. Arch. Microbiol. **131**:176–177.
26. **Pan-Hou, H. S., M. Nishimoto, and N. Imura.** 1981. Possible role of membrane proteins in mercury resistance of *Enterobacter aerogenes*. Arch. Microbiol. **130**:93–95.
27. **Pan-Hou, H. S. K., and N. Imura.** 1981. Role of hydrogen sulfide in mercury resistance determined by plasmid of *Clostridium cochlearium* T-2. Arch. Microbiol. **129**:49–52.
28. **Pettigrew, C. A., and G. S. Sayler.** 1986. The use of DNA:DNA colony hybridization in the rapid isolation of 4-chlorobiphenyl degradative bacterial phenotypes. J. Microbiol. Methods **5**: 205–213.
29. **Sayler, G. S., R. K. Jain, A. Ogram, C. A. Pettigrew, L. Houston, J. Blackburn, and W. S. Riggsby.** 1986. Applications for DNA probes in biodegradation research, p. 499–508. *In* F. Megusar and M. Gantar (ed.), Perspectives in microbial ecology. Slovene Society for Microbiology, Ljubljana, Yugoslavia.
30. **Schottel, J. L.** 1978. The mercuric and organomercurial detoxifying enzymes from a plasmid-bearing strain of *Escherichia coli*. J. Biol. Chem. **253**:4341–4349.
31. **Shiratori, T., C. Inoue, K. Sugawara, T. Kusano, and Y. Kitagawa.** 1989. Cloning and expression of *Thiobacillus ferrooxidans* mercury ion resistance genes in *Escherichia coli*. J. Bacteriol. **171**:3458–3464.
32. **Silver, S., and T. Misra.** 1988. Plasmid-mediated heavy metal resistances. Annu. Rev. Microbiol. **42**:717–743.
33. **Skogerboe, R. K., and S. A. Wilson.** 1981. Reduction of ionic species by fulvic acid. Anal. Chem. **53**:228–232.
34. **Smibert, R. M., and N. R. Krieg.** 1981. General characterization, p. 409–443. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
35. **Stanisich, V. A.** 1974. Interaction between an R factor and an element conferring resistance to mercuric ions in *Pseudomonas aeruginosa*. Mol. Gen. Genet. **128**:201–212.
36. **Summers, A. O., and T. Barkay.** 1989. Metal resistance genes in the environment, p. 287–308. *In* S. Levy and R. Miller (ed.), Gene transfer in the environment. McGraw-Hill Book Co., New York.
37. **Vaituzis, Z., J. D. Nelson, Jr., L. W. Wan, and R. R. Colwell.** 1975. Effects of mercuric chloride on growth and morphology of selected strains of mercury-resistant bacteria. Appl. Environ. Microbiol. **29**:275–286.
38. **Wang, Y., I. Mahler, H. S. Levinson, and H. O. Halvorson.** 1987. Cloning and expression in *Escherichia coli* of chromosomal mercury resistance genes from a *Bacillus* sp. J. Bacteriol. **169**:4848–4851.
39. **Wang, Y., M. Moore, H. S. Levinson, S. Silver, C. Walsh, and I. Mahler.** 1989. Nucleotide sequence of a chromosomal mercury resistance determinant from a *Bacillus* sp. with broad-spectrum mercury resistance. J. Bacteriol. **171**:83–92.
40. **Weiss, A. A., S. D. Murphy, and S. Silver.** 1977. Mercury and organomercurial resistances determined by plasmids in *Staphylococcus aureus*. J. Bacteriol. **132**:197–208.
41. **Witte, W., L. Green, T. K. Misra, and S. Silver.** 1986. Resistance to mercury and to cadmium in chromosomally resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **29**: 663–669.
42. **Zobell, C. E.** 1946. Marine microbiology. A monograph on hydrobacteriology. Chronica Botanica Co., Waltham, Mass.