Distribution of DNA Sequences Encoding Narrow- and Broad-Spectrum Mercury Resistance

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The distribution of DNA sequences homologous with three *mer* genes was determined in unselected and mercury-resistant water and sediment isolates. The maximum proportions of unselected bacterial isolates containing DNA hybridizing with the 358*merA*, 358*merB*, and 501*merR* probes, derived from gram-negative organisms, were 93.8, 21, and 100%, respectively. Up to 53.3% of mercury chloride-resistant isolates and 54% of methylmercury hydroxide-resistant isolates did not contain DNA homologous with 358*merA* or 358*merB*, respectively. Hybridizations performed at high and low stringencies demonstrated that divergence of the *merA* gene accounted for many of the mercury-resistant but probe-negative isolates. Sixteen mercury-resistant *Bacillus* spp. isolated from the least contaminated site all contained DNA homologous with 258*merA*, originally from a gram-positive organism, but only four hybridized weakly with 358*merA*. The results demonstrate the wide distribution of mercury resistance genes but, because of the diversity of genetic determinants, highlight the importance of using multiple detection techniques and gene probes derived from a variety of origins for such studies.

Mercury-containing compounds are abundant in many aquatic and terrestrial ecosystems as a result of either anthropogenic pollution or natural geological processes (15). Bacteria have, however, evolved mechanisms of mercury detoxification, and mercury resistance is a common phenotype among bacteria isolated from soil and water environments (16–18, 27). The majority of work carried out to date has been on the narrow-spectrum form of mercury resistance which confers resistance to inorganic mercury [HgCl_2 , $\text{Hg}(\text{NO}_3)_2$]. Studies have shown that the proportion of bacteria, isolated from a wide variety of mercury-contaminated soil and aquatic environments, resistant to inorganic mercury ranges from 0.007 to 49% (6, 18, 27). Resistance to the more toxic organic mercurial compounds such as methylmercury and phenyl mercuric acetate (broad-spectrum resistance) is less common. Radford et al. (26) reported transposable phenyl mercuric acetate resistance in a soil isolate of *Pseudomonas fluorescens*, and Nakamura et al. (23) found that 4.5% of bacteria isolated from mercury-contaminated sediment exhibited broad-spectrum resistance compared with 1.1% from a control site. Of 1,300 gram-negative bacteria isolated from hospitals, rural humans, and farm animals, 14.3% had narrow-spectrum mercury resistance compared with 1.4% which exhibited broad-spectrum mercury resistance (11). In a study of bacteria isolated from a river draining an area of cinnabar deposits, 35% were resistant to HgCl_2 and 6.6% degraded methylmercury (1).

With the development of DNA hybridization technology, research has turned towards assessing the occurrence of specific genetic determinants in the environment rather than just monitoring phenotypes. Sayler et al. (30) used DNA-DNA colony hybridization to detect toluene and naphthalene degradative genes in sediment samples, and DNA probes have been used to assess the occurrence of mercury resistance genotypes in environmental isolates (3, 4, 6, 11,

25). DNA probes have also been used to detect genes encoding polychlorinated biphenyl degradation in soil bacteria (10, 35), *Bradyrhizobium japonicum* (14) and *mer* genes (5) in whole-community genome preparations, and, in conjunction with the polymerase chain reaction, genetically engineered bacteria in environmental samples (8, 32). Barkay and Olson (6) used a 2.6-kb probe spanning most of the mercury resistance operon of Tn21 and found a positive correlation between sediment mercury concentration and the frequency of mercury resistance genes. Gilbert and Summers (11) used a combination of seven probes to distinguish between subpopulations of bacteria containing *mer* sequences related to either Tn21 or Tn501. More recently, a study was carried out with probes specific for the *merR* and *merC* genes, and it was concluded that, as sediment mercury concentrations increased, Tn21-like resistance systems became the predominant mercury resistance genotype (25).

In this study we describe the use of four probes from *mer* operons (three of gram-negative origin and one from a gram-positive organism) to determine the occurrence and distribution of mercury resistance genotypes in aquatic environments. The probes used were derived from the *merR*, *merA* (gram positive and negative), and *merB* genes. *merR* encodes a regulatory protein, *merA* encodes the structural mercuric reductase, and *merB* encodes organomercurial lyase, which cleaves organic groups from the mercury ion and is present in organisms encoding broad-spectrum mercury resistance (33).

MATERIALS AND METHODS

Site descriptions and isolation of bacteria. Water and sediment samples were collected from three locations: East Fork Poplar Creek, Tennessee (EFPC); Davis Creek Reservoir, California (DCR); Little Rock Lake, Wisconsin (LRL). The study locations were chosen because information on the distribution of mercury species within the water column and sediment was available. EFPC was contaminated in the

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TABLE 1. Bacterial densities and phenotypic mercury resistance at the three research sites^a

Sample location	Bacterial densities (CFU/ml in water; CFU/g in sediment)	HgCl ₂ -resistant bacteria (%)	Methyl-HgOH-resistant bacteria (%)
East Fork Poplar Creek			
Water	5.7×10^3 – 1.5×10^6	0.20–83.3	0.0–0.0015
Sediment	5.9×10^6 – 6.9×10^8	1.40–15.4	0.003–0.06
Davis Creek Reservoir			
Water	1.4×10^2 – 2.7×10^3	0.0–5.3	0.0
Sediment	6.8×10^6 – 1.1×10^7	0.33–1.32	0.11–0.16
Little Rock Lake			
Water	1.6×10^1 – 2.4×10^4	0.0–49.2	0.0
Sediment	1.4×10^5 – 2.0×10^5	0.02	0.0

^a Values are expressed as a range from the lowest to the highest recorded.

1960s by spills from an ammunitions plant, close to the stream's source. DCR was originally impounded in 1984 and has been contaminated by feeder streams which run through old mining areas. LRL is a small (18-ha) seepage lake which has been used for lake acidification studies (37) with no known source of mercury contamination.

EFPC was sampled at seven locations in 1988 (six locations within 1.5 miles [ca. 2.4 km] of the source of contamination and a seventh about 8 miles [ca. 12.9 km] downstream) and five locations in 1989 (four within 1.5 miles of the source and one downstream). At each location a mixed water sample (maximum depth, 0.5 m) and a sediment sample (top 15 cm) were taken. The DCR water column was sampled at three depths (surface, 10 m, and 20 m) at one location, and sediment was collected at three locations. Sediment was collected from two locations at LRL, and three depths of the water column (surface, 5 m, and 8 m) were sampled at both locations. The densities of viable bacteria at the sites are given in Table 1, in which are represented samples taken from the DCR water column in the summer and fall of 1988 and in the spring, summer, fall, and winter of 1989. Sediment samples from DCR were collected in the summer of 1988 and the spring, summer, and winter of 1989. The water column of LRL was sampled in fall 1988 and the spring and fall of 1989. LRL sediment was sampled once in the fall of 1989. Water and sediment samples from EFPC were collected in summer 1988 as well as in the summer and winter of 1989.

A 10-g portion of sediment was resuspended in 90 ml of dilution buffer (0.3 mM KH₂PO₄, 2.0 mM MgCl₂ · 6H₂O, pH 7.2) by shaking at 100 rpm for 30 min. Dilutions of the sediment suspensions and water samples were plated in triplicate onto plate count agar (PCA; Difco Laboratories, Detroit, Mich.), PCA plus 25 µg of Hg per ml as HgCl₂ (Mallinckrodt, Paris, Ky.), and PCA plus 3 µg of Hg per ml as methyl-Hg hydroxide (Alfa Products, Danvers, Mass.). Plates were incubated for 2 to 5 days at 22°C. Gram-negative isolates were identified by using API Rapid NFT kits (Analytab Products, Plainview, N.Y.). Gram-positive isolates were identified to the genus level on the basis of Gram reaction, microscopic examination of cell morphology, spore stain, oxidase and catalase reactions, and colony morphology.

Preparation of filters for probing. Plates containing 20 to

300 colonies were chosen for probing, and replica plates were made from these. Replica plates were made with either RepliPlate colony transfer pads (FMC, Rockland, Maine) or sterile toothpicks. Colony lifts were performed by using Biotrans nylon membranes (ICN Biomedicals, Irvine, Calif.), and the cells were lysed in 1.5 M NaCl–0.5 M NaOH, followed by neutralization in 3 M sodium acetate, pH 5.5. After drying at room temperature, the filters were baked under vacuum at 80°C for 1 h. A slot blot manifold (Schleicher & Schuell, Keene, N.H.) was also used to apply DNA samples to GeneScreen Plus membranes (DuPont, NEN Research Products, Boston, Mass.). In this case, 1 ml of an overnight L-broth culture, incubated at 25°C, was pelleted and the cell pellet was resuspended in 100 µl of a solution containing 10 mg of lysozyme per ml, 10 mM Tris, and 1 mM EDTA (Sigma Chemical Co., St. Louis, Mo.) and incubated on ice for 30 min. Following addition of 250 µl of 1% sodium dodecyl sulfate (SDS) in 0.2 M NaOH, the tubes were incubated on ice for a further 5 min and then centrifuged at 14,000 rpm (Eppendorf 5415 centrifuge) for 10 min. The supernatant was removed to a fresh tube, and the DNA was denatured by addition of NaOH to a final concentration of 0.25 M. After chilling on ice for 10 min, the DNA was diluted in 0.125 M NaOH–0.02 M NaCl–0.002 M sodium citrate, as recommended by the membrane supplier, and then applied to the membrane in 150-µl volumes. The DNA solution remained on the membrane for 30 min without suction, and the liquid was then drawn through by applying a vacuum for 30 s.

Preparation of DNA probes. Sequences of gram-negative *mer* operons spanning the *merR* gene in Tn501 (20, 22) and the *merA* and *merB* genes in pDU1358 (13, 24) were subcloned into the pT7/T3-18 vector (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) and maintained in *Escherichia coli* JM83 (*merR*) and DH5α (*merA* and *merB*). A fragment of the gram-positive *merA* gene from pI258 (19) was subcloned into the Bluescript KS11+ vector (Stratagene, La Jolla, Calif.) and maintained in *E. coli* DH5α. The original clones were supplied by N. Brown, S. Silver, and R. A. Laddaga. The recombinant plasmid DNA was prepared by cesium chloride-ethidium bromide density gradient centrifugation (29), and the DNA was digested with appropriate enzymes, using the manufacturer's recommendations (Bethesda Research Laboratories; Boehringer Mannheim, Indianapolis, Ind.). Fragments were separated electrophoretically, and the DNA fragments of interest were electroeluted (29) and purified by passage through Elutip-D columns (Schleicher & Schuell). The DNA for the 501*merR* probe was 675 bp in length, was cut out with *EcoRI*, and contained all of the *merR* gene as well as a short sequence of DNA accounting for 20 bp of the *merT* gene. The DNA for the 358*merA* probe consisted of 1 kb of DNA from the *merA* gene of pDU1358 and was cut out with *EcoRI* and *HindIII*, and the DNA fragment for the 358*merB* probe was 457 bp of the *merB* gene, cut out with *KpnI* and *SalI*. The DNA for the 258*merA* probe was a 943-bp *HindIII* fragment from the *merA* gene of pI258. None of the probes contained vector DNA.

Hybridizations. Probe DNA was labeled with [³²P]dCTP (ICN) by random priming (Bethesda Research Laboratories). Hybridizations of DNA from colony lifts were carried out in 50% formamide at 42°C and washed at 50°C to achieve 93, 88, and 92% stringency with the 501*merR*, 358*merA*, and 358*merB* probes, respectively. To achieve lower stringencies, hybridizations were also performed with 358*merA*, using 35 and 20% formamide (77.5 and 67% stringency,

TABLE 2. Occurrence of *mer* genes in, summer 1988^a

Sample	% Probe-positive bacteria isolated nonselectively (PCA) and selectively (PCA + HgCl ₂) ^b		
	501 <i>merR</i>	358 <i>merA</i>	358 <i>merB</i>
Isolated on PCA			
Water column	54.8 ± 28.8 (<i>n</i> = 1,587) ^c	80.5 ± 21.5 (<i>n</i> = 1,595)	8.7 ± 13.1 (<i>n</i> = 5,078)
Sediment	29.9 ± 29.6 (<i>n</i> = 831)	33.6 ± 36.0 (<i>n</i> = 1,648)	7.2 ± 14.9 (<i>n</i> = 1,636)
Isolated on PCA + HgCl ₂			
Water column	57.2 ± 10.9 (<i>n</i> = 220)	78.6 ± 14.8 (<i>n</i> = 216)	24.3 ± 62.5 (<i>n</i> = 435)
Sediment	67.2 ± 28.7 (<i>n</i> = 982)	57.3 ± 33.1 (<i>n</i> = 1,717)	14.3 ± 14.0 (<i>n</i> = 1,584)

^a Mean ± standard deviation of gene frequencies over seven water and sediment locations.^b 25 µg of Hg per ml.^c *n* = total number of isolates probed.

respectively) at 42°C followed by washing at 50°C. To conserve reagents and supplies, 10 colony lift membranes were hybridized together in a single hybridization bag (Bethesda Research Laboratories). Following autoradiography, filters were stained in 0.25% methylene green to confirm cell lysis (25). Hybridizations of DNA applied to membranes by the slot blot manifold were performed in 50% formamide–1% dextran sulfate at 42°C followed by washing at 65°C as described by the filter supplier.

Positive and negative controls were included in all hybridization analyses. The positive control was *E. coli* JM83 (for 501*merR*) or *E. coli* DH5α (358*merA* and 358*merB*) containing the pT7/T3-18 vector with unlabeled probe DNA inserted. The negative control was *E. coli* DH5α containing the cloning vector alone. These strains were spread onto PCA or PCA-plus-HgCl₂ plates and allowed to grow. Colony lifts were then made as described above; once the DNA was fixed, the membranes were cut into nine sections. One section of both positive and negative control membranes was included in each hybridization bag containing 10 test membranes. When probing DNA applied to membranes via the slot blot manifold with 258*merA*, the positive control was unlabeled 258*merA* DNA and the negative control was 358*merA* DNA.

In addition to autoradiography, hybridization and relative signal intensities were also detected by using a scanning beta-detector and image analysis software (Ambis Systems, San Diego, Calif.).

Mercury determinations. The concentration of mercury in the water column and sediment of the research sites was determined by previously described methods (12, 34).

RESULTS AND DISCUSSION

Isolation of bacteria. Nonselective (PCA) and selective (PCA plus HgCl₂ or PCA plus methyl-HgOH) plates were used for the initial isolation of bacteria. The densities of viable bacteria in the water column of the three sites ranged from 1.6×10^1 CFU/ml in LRL to 1.6×10^6 CFU/ml in EFPC; in the sediment, densities ranged from 1.4×10^5 CFU/g at LRL to 6.9×10^8 CFU/g at EFPC (Table 1). Up to 83.3% of bacteria from the water column and 15.4% from the sediment of EFPC were resistant to inorganic mercury. The highest level of methylmercury resistance was 0.16%, in DCR sediment (Table 1).

From these isolation plates, replica plates were prepared for probe analysis. Initially, the replica plates were made by using colony transfer pads to obtain about 100 colonies. However, because of the heterogeneity of colony morphology, lysis was found to be inconsistent and $73\% \pm 19\%$ (*n* =

316 plates) of the colonies were lost in the transfer process. As an alternative, sterile toothpicks were used to transfer 50 randomly picked colonies per plate, which were then probed. All gene occurrence data are presented as the number of probe-positive colonies calculated as a percentage of the number of colonies with confirmed lysis. Using toothpicks to generate filters containing 50 colonies each, we obtained a lysis efficiency of $86\% \pm 12\%$ (*n* = 95 filters).

Distribution of *mer* sequences in mercury-contaminated sites. The EFPC site was sampled at seven locations in the summer of 1988 and at five locations in the summer and winter of 1989, with total mercury concentrations ranging from 8 to 183 µg/g in the sediment and from 0.4 to 5.9 µg/liter in the water column. There were no significant differences (*P* > 0.05) in the occurrence of *mer* genes among the locations of the EFPC site, so the results have been condensed. Tables 2 and 3 give the occurrence of sequences homologous to the three gram-negative probes in bacteria from EFPC isolated nonselectively on PCA and selectively on PCA plus HgCl₂ or PCA plus methyl-HgOH. The results are presented as means of gene frequency over the seven (1988) or five (1989) locations. Up to 93.8% of unselected water column isolates contained sequences homologous to the 358*merA* gene and up to 100% contained DNA hybridizing with the 501*merR* probe. The maximum proportions of unselected sediment isolates containing DNA homologous to 501*merR* and 358*merA* were 59.1 and 72.1%, respectively.

These results indicate that genes encoding mercury resistance are far more common than phenotypic data suggest (Table 1). For example, based on phenotypic plate counts, up to 15.4% of the bacterial population in EFPC sediment were resistant to mercury chloride (25 µg/ml), but up to 72.1% of the same population carried DNA sequences homologous with the 358*merA* gene. A similar finding was reported for genes encoding polychlorinated biphenyl degradation in soil bacteria (35). Possible explanations for this difference follow. (i) Some of the bacteria probing positive with the 358*merA* probe did not contain all of the genes of the operon necessary for mercury resistance. The mercury resistance operons of Tn501 and pDU1358 each contain at least five genes (7), and we did not determine the occurrence of the *merP*, *merT*, or *merD* genes. (ii) Some of the organisms may carry the entire mercury resistance operon but blocks in transcription or translation or both prevent expression of resistance. Mercury resistance is an inducible phenotype (9, 33), so some of our bacteria may be resistant but fail to grow on selective plates without prior exposure to inducing concentrations of mercury. (iii) Some of the bacteria probing positive were mercury resistant but at a level of

TABLE 3. Occurrence of *mer* genes in East Fork Poplar Creek, winter 1989^a

Sample	% Probe-positive bacteria isolated nonselectively (PCA) and selectively (PCA + HgCl ₂ ^b or PCA + methyl-HgOH ^c)		
	501 <i>merR</i>	358 <i>merA</i>	358 <i>merB</i>
Isolated on PCA			
Water column	57.7 ± 22.9 (<i>n</i> = 235) ^d	15.6 ± 19.4 (<i>n</i> = 158)	5.6 ± 5.5 (<i>n</i> = 237)
Sediment	42.6 ± 8.4 (<i>n</i> = 205)	52.8 ± 14.3 (<i>n</i> = 205)	6.2 ± 5.1 (<i>n</i> = 204)
Isolated on PCA + HgCl ₂			
Water column	95.2 ± 6.1 (<i>n</i> = 191)	56.0 ± 11.1 (<i>n</i> = 197)	7.4 ± 4.4 (<i>n</i> = 190)
Sediment	72.4 ± 18.9 (<i>n</i> = 241)	84.6 ± 11.2 (<i>n</i> = 242)	11.5 ± 5.1 (<i>n</i> = 242)
Isolated on methyl-HgOH			
Sediment	66.1 ± 10.1 (<i>n</i> = 226)	73.9 ± 8.9 (<i>n</i> = 226)	58.8 ± 9.1 (<i>n</i> = 229)

^a Mean ± standard deviation of gene frequencies over five water column and sediment locations.

^b 25 µg of Hg per ml.

^c 3 µg of Hg per ml.

^d *n* = total number of isolates probed.

mercury below 25 µg/ml. (iv) Bacterial cells freshly isolated from natural environments are often starved or injured (28), and these stressed cells may not be able to express traits on selective media.

To test the third hypothesis, one sediment sample was plated on PCA plates containing a lower concentration of mercury. As expected, the proportion of the population resistant to mercury chloride at 10 µg/ml was 31.5% compared with 2.2% at 25 µg/ml.

The proportion of the EFPC bacterial population resistant to methylmercury (up to 0.06%) was much lower than for inorganic mercury. This was also reflected by the probing data, with a maximum of 17.2% of unselected isolates hybridizing with the 358*merB* probe. However, the occur-

rence of the gene was still far higher than would be expected from phenotypic data.

Bacteria were also isolated on selective media containing inorganic mercury or methylmercury and, as would be expected, there was a higher occurrence of DNA hybridizing with the three probes than in unselected isolates (Tables 2 and 3; Fig. 1). However, there was a surprisingly low occurrence of *merA* and *merB* genes among mercury chloride- and methylmercury-resistant isolates, respectively. Up to 96% of mercury-resistant EFPC isolates hybridized with the 358*merA* probe, but at some locations the occurrence was as low as 42.7%. Likewise, only 46 to 70% of methylmercury-resistant isolates contained DNA homologous with 358*merB*. Therefore, up to 57.3% of mercury chloride-

FIG. 1. Hybridization of 501*merR*, 358*merA*, and 358*merB* probes to DNA from 50 HgCl₂-resistant (25 µg of Hg per ml) bacteria isolated from EFPC sediment. Positive and negative controls were included for each probe. The positive control in each case was *E. coli* JM83 (501*merR*) or *E. coli* DH5α (358*merA* and 358*merB*) containing the cloning vector with unlabeled probe DNA inserted. The negative control was *E. coli* DH5α containing just the cloning vector. Details of the cloning vector are given in Materials and Methods. DNA was applied to membranes by colony lifts followed by cell lysis and neutralization as described in the text. Hybridizations were performed at high stringency in 50% formamide followed by washing at 50°C. Also shown is hybridization of the 358*merA* probe to unlabeled 358*merA* DNA (0.3 µg of DNA) and sheared salmon sperm DNA (21 µg of DNA) applied to a nylon membrane via a slot blot manifold. Hybridizations for these two control DNAs were also performed at high stringency in the presence of 50% formamide followed by washing at 65°C.

FIG. 2. Image produced by scanning beta-detector (AMBIS) following hybridization of the 358*merA* probe to the DNA of 24 mercury-resistant isolates from EFPC. Hybridization was carried out under conditions of high (panel a; 50% formamide) or low (panel b; 35% formamide) stringency.

resistant isolates did not contain DNA homologous with the 358*merA* probe, and up to 54% of methylmercury-resistant isolates did not contain the *merB* gene.

Two possible reasons for this apparent absence of the *merA* and *merB* genes in mercury- and methylmercury-resistant isolates are as follows. (i) It is recognized that there is divergence among *mer* genes encoding proteins with the same function (9), and so it is likely that many of the mercury-resistant isolates had *merA* genes sufficiently divergent from the 358*merA* probe to give no hybridization signal at high stringency. (ii) Mechanisms of mercury resistance other than volatilization by mercuric reductase have been described (2, 33), and it is possible that some of our probe-negative isolates had such novel mechanisms of mercury resistance. To test the first of these explanations, we performed hybridizations on 72 mercury-resistant isolates from EFPC under conditions of varying stringency, to detect *merA* genes divergent from 358*merA*. Our standard conditions, using 50% formamide, resulted in hybridizations and washes being performed at 20 and 12°C below the T_m of 358*merA*, respectively. This achieved high stringency (88%). Hybridizations were also performed with 35 and 20% formamide, which resulted in hybridizations at 31 and 42°C below T_m and washes at 23 and 34°C below the T_m of 358*merA* (T_m , $69.3^\circ\text{C} + 0.41 \times [\text{guanine plus cytosine ratio}]$) (31). Using 50, 35, and 20% formamide, the proportions of mercury-resistant isolates ($n = 72$) probing positive with 358*merA* were 55.5, 79.1, and 90.2%, respectively. Thus, divergence of *merA* genes does account for a large proportion of our mercury-resistant/probe-negative isolates. However, it should be stated that probing at low stringency can be misleading since the greater base pair mismatching allowed could result in similar sequences, which do not encode mercuric reductase, probing positive. Figure 2 shows the images obtained with a scanning beta-detector of membranes containing DNA from mercury-resistant isolates hybridized in the presence of 50% (Fig. 2a) and 35% (Fig. 2b) formamide. There was no apparent correlation between bacterial species and hybridization signals at different stringencies since isolates that probed positive at high stringency represented four bacterial genera (Table 4). Of the 24 identified isolates, the only one which did not hybridize with the

358*merA* probe, even at low stringency, was a strain of *Pseudomonas fluorescens*.

The specificity of the probes was established by always including positive and negative controls in the hybridizations. The specificity of the 358*merA* probe was confirmed further by hybridization with salmon sperm DNA (Fig. 1). Although a faint signal was observed with this complex DNA control, the relative intensity of the signal per microgram of DNA was 1.8×10^{-4} -fold lower than the signal obtained when unlabeled 358*merA* was the target DNA (as determined by scanning beta detection).

TABLE 4. Identification of 24 mercury-resistant East Fork Poplar Creek isolates probed at three hybridization stringencies

Isolate	Hybridization with 358 <i>merA</i> at given formamide concentration		
	50%	35%	20%
1A <i>Pasteurella</i> sp.	+	+	+
1B <i>Pseudomonas alcaligenes</i>	+	+	+
1C Unidentified gram-negative rod	+	+	+
1D <i>Alcaligenes faecalis</i>	+	+	+
2A <i>Alcaligenes faecalis</i>	—	—	+
2B <i>Pseudomonas vesicularis</i>	+	+	+
2C <i>Alcaligenes faecalis</i>	—	—	+
2D <i>Pseudomonas vesicularis</i>	+	+	+
3A <i>Pseudomonas fluorescens</i>	—	+	+
3B <i>Pseudomonas fluorescens</i>	+	+	+
3C <i>Pseudomonas putida</i>	—	+	+
3D <i>Pseudomonas alcaligenes</i>	—	+	+
4A <i>Pseudomonas fluorescens</i>	—	—	—
4B <i>Pseudomonas putida</i>	—	—	+
4C <i>Pseudomonas putida</i>	—	—	+
4D <i>Agrobacterium radiobacter</i>	—	—	+
5A <i>Pseudomonas fluorescens</i>	—	+	+
5B <i>Pseudomonas fluorescens</i>	—	+	+
5C Unidentified gram-negative rod	—	+	+
5D <i>Aeromonas hydrophila</i>	+	+	+
6A <i>Alcaligenes</i> sp.	—	+	+
6B <i>Pseudomonas putida</i>	—	+	+
6C <i>Pseudomonas fluorescens</i>	—	+	+
6D <i>Pseudomonas putida</i>	+	+	+

FIG. 3. Occurrence of *mer* genes in EFPC (1989) sediment isolates, expressed as percentages of isolates containing DNA homologous with (a) the 501*merR* probe, (b) the 358*merA* probe, and (c) the 358*merB* probe. Symbols: , unselected isolates; , HgCl₂-selected isolates; , methyl-HgOH-selected isolates.

Previous studies on the distribution of mercury resistance determinants have demonstrated DNA sequences homologous to *mer*(Tn21) in 50% of mercury-resistant freshwater bacteria but in only 12% of bacteria from saline communities (4) and that *merC*-positive operons occurred almost exclusively in *Escherichia* spp. among a collection of mercury-resistant gram-negative bacteria (11). It was also reported that most *Pseudomonas* strains from a collection of hospital isolates did not hybridize detectably with probes derived from Tn21 or Tn501 (11). This contrasts with our results because the majority of our gram-negative isolates were *Pseudomonas* spp., and many of these probed positive with the 501*merR* probe (Tables 2, 3, 4, and 5). It has also been reported previously that *Pseudomonas* spp. and *Alcaligenes* spp. were predominant among mercury-resistant Tn501-like and Tn21-like sediment isolates (25). These differences in bacterial genera associated with particular *mer* operons may be due to the different isolation sources used in the studies.

Figure 3 shows the distribution of bacteria containing DNA hybridizing with the 501*merR*, 358*merA*, and 358*merB* probes at five locations in EFPC sediment. There was no apparent correlation between the occurrence of the mercury resistance genes and the concentration of total mercury at the sample locations within EFPC. However, EFPC had higher levels of mercury contamination than DCR (0.8 to 5.5 μ g of total mercury per g of sediment), and probe data demonstrated that isolates containing the *mer* genes were generally less common in DCR than in EFPC (Tables 5 and 6), as were bacteria phenotypically resistant to mercury (Table 1). The maximum proportion of nonselectively isolated bacteria in DCR sediment probing positive with 358*merA* was 15.6% compared with 72.1% in EFPC sediment. Therefore, when considering overall sites, *mer* genes were more prevalent in the most contaminated site. Strong positive correlations between sediment mercury concentration and the frequency of *mer* genes has been reported previously (6), and Tn21-like operons became the predominant genotype as sediment mercury concentrations increased (25). As with EFPC, DCR bacteria carrying *mer* genes were more prevalent than isolates expressing mercury resistance. The proportions of unselected bacteria containing DNA homologous with 358*merA* at three DCR sediment locations sampled in 1989 were 2.9, 2.2, and 15.6%. The proportions of the bacterial populations expressing mercury resistance at the same locations were 0.3, 0.4, and 0.9%, respectively.

Occurrence of DNA sequences homologous with a gram-

positive *merA*. The LRL site contained far less mercury than the other two sites, with 3 ng to 0.2 μ g of total mercury per g in the sediment and 0.08 to 2.1 ng/liter in the water column. Only 0.02% of the bacterial population in the sediment was resistant to inorganic mercury and no methylmercury resistance was detected in either the sediment or the water column, thus confirming the overall correlation between mercury concentration at the site and the occurrence of mercury-resistant bacteria.

Unlike EFPC and DCR sediment, which contained a diverse bacterial flora, LRL sediment contained bacteria with just two colony types on nonselective and selective

TABLE 5. Occurrence of *mer* genes in Davis Creek Reservoir, summer 1988

Sample	No. of probe-positive isolates/no. of isolates probed with confirmed lysis (%)		
	501 <i>merR</i>	358 <i>merA</i>	358 <i>merB</i>
Isolated on PCA			
Water column			
Surface	1/100 (1.0)	0/92 (<1.1)	6/72 (8.3)
10 m	8/91 (8.8)	0/81 (<1.2)	0/71 (<1.4)
20 m	0/99 (<1.0)	0/87 (<1.1)	0/92 (<1.1)
Total ^a	290	260	235
Sediment			
Station A	5/55 (9.1)	1/28 (3.6)	0/26 (<3.8)
Station B	0/32 (<3.1)	0/22 (<4.5)	0/26 (<1.1)
Station C	0/104 (<1.0)	0/90 (<1.1)	0/93 (<1.1)
Total	191	140	145
Isolated on PCA + HgCl ₂ ^b			
Water column			
Surface	56/97 (57.7)	41/62 (66.1)	42/85 (49.4)
10 m	3/86 (3.5)	0/49 (<2.0)	3/45 (6.7)
20 m	3/133 (2.3)	1/42 (2.4)	4/86 (4.6)
Total	316	153	216
Sediment			
Station A	11/41 (26.8)	3/37 (8.1)	0/35 (<2.9)
Station B	19/49 (38.8)	10/43 (23.3)	0/51 (<2.0)
Station C	14/42 (33.3)	3/29 (10.3)	0/30 (<3.3)
Total	132	109	116

^a Total number of water column or sediment isolates probed.

^b 25 μ g of Hg per ml.

TABLE 6. Occurrence of *mer* genes in Davis Creek Reservoir, winter 1989

Sample	No. of probe-positive isolates/no. of isolates probed with confirmed lysis (%)		
	501 <i>merR</i>	358 <i>merA</i>	358 <i>merB</i>
Isolated on PCA			
Water column			
Surface	0/42 (<2.4)	1/42 (2.4)	0/41 (<2.4)
10 m	0/37 (<2.7)	0/40 (<2.5)	0/38 (<2.6)
20 m	0/31 (<3.2)	0/35 (<2.9)	0/29 (<3.4)
Total ^a	110	117	108
Sediment			
Station A	0/33 (<3.0)	1/35 (2.9)	0/35 (<2.9)
Station B	0/38 (<2.6)	1/45 (2.2)	0/38 (<2.6)
Station C	8/38 (21.1)	7/45 (15.6)	0/38 (<2.6)
Total	109	125	111
Isolated on PCA + HgCl ₂ ^b			
Sediment			
Station A	24/36 (66.7)	22/42 (52.4)	1/40 (2.5)
Station B	7/34 (20.6)	12/38 (31.6)	3/37 (8.1)
Station C	29/42 (69.0)	30/44 (68.2)	0/36 (<2.8)
Total	112	124	113

^a Total number of water column or sediment isolates.^b 25 µg of Hg per ml. No HgCl₂-resistant bacteria were isolated from the water column at this sampling time.

media. Of 18 mercury-resistant isolates, 16 were identified as *Bacillus* spp. and the remaining 2 were *Acinetobacter calcoaceticus*. Crude DNA samples were prepared from these 18 isolates by treatment with lysozyme and alkaline SDS, and the DNA was applied to membranes with a slot blot manifold. Figure 4 shows the results of probing these 18 isolates with the *mer* probes. Gram-positive organisms were predominant among mercury-resistant LRL sediment isolates, so a fourth probe, 258*merA*, derived from a plasmid (pI258)

encoding mercury resistance in *Staphylococcus aureus* (19), was used in addition to the three gram-negative-organism-derived probes. Of the 16 *Bacillus* strains, DNA from 12 produced strong hybridization signals with the 258*merA* probe (Fig. 4a); the remaining four strains produced weak to moderate signals. This finding contrasts with that of Mahler et al. (21), who reported no demonstrable homology between pI258 and *Bacillus* strains with resistance to mercury and cadmium. A 6.2-kb probe derived from *Bacillus* sp. strain RC607, exhibiting broad-spectrum mercury resistance, detected *mer* genes in 25% of mercury-resistant sporeforming isolates, but hybridized only weakly with the *mer* determinant of pI258 (36). Thus, there appears to be wide divergence among *mer* determinants in *Bacillus* spp. Four of the *Bacillus* strains (LRL3, LRL4, LRL9, and LRL14) hybridized weakly with the 358*merA* probe (Fig. 4b), but only two (LRL1 and LRL10) produced weak hybridization signals with the 501*merR* probe (data not shown).

The *A. calcoaceticus* strains (LRL11 and LRL18) hybridized strongly with the gram-negative-organism-derived probes, 501*merR* and 358*merA*, and produced a weak signal with the gram-positive-organism-derived 258*merA* probe. None of the LRL isolates had DNA homologous with the 358*merB* probe (data not shown), although all 16 *Bacillus* spp. were resistant to methylmercury hydroxide. Based on our results with *merA*, we would expect these methylmercury-resistant isolates to probe positive with a *merB* probe derived from a gram-positive organism.

Conclusion. Hybridization with DNA probes is now an accepted and extensively used technique for the detection of catabolic and resistance genes in bacteria isolated from the environment (4, 6, 10, 11, 25, 30, 35). However, because of the diversity of genetic systems encoding particular functions, it may be difficult to assess the catabolic or resistance potential of these environments with gene probes alone. Our results show that the distribution of mercury-resistant bacteria in aquatic systems cannot be accurately determined by either phenotypic expression or probe analysis alone. This is

FIG. 4. Detection of DNA sequences homologous with the *merA* gene in mercury-resistant sediment isolates from LRL. The DNA was applied to nylon membranes with a slot blot manifold and probed with (a) 258*merA* and (b) 358*merA*. Isolates LRL1 through LRL10 and LRL12 through LRL17 were identified as *Bacillus* spp.; LRL11 and LRL18 were *A. calcoaceticus*. Unlabeled 258*merA* and 358*merA* DNAs were applied to both membranes as controls.

similar to the conclusion drawn by Walia et al. (35) for the detection of polychlorinated biphenyl-degrading bacteria in soil. We also confirmed the general correlation between mercury concentration at contaminated sites and the occurrence of mercury-resistant bacteria and *mer* genes (6). Our results also demonstrate that limiting the number of probes used for the detection of a particular genotype can lead to an underestimation of the occurrence of that genotype, as can performing hybridization analyses at too high stringencies. No single probe or single hybridization analysis will detect all bacteria with a particular characteristic. Further research needs to be conducted to determine the optimum level of stringency to be used when assessing the distribution of a genetic determinant in the environment. Our findings suggest that the commonly used stringency of >90% will underestimate the actual occurrence of the determinant.

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