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Hybridization of DNA Probes with Whole-Community Genome for Detection of Genes That Encode Microbial Responses to Pollutants: *mer* Genes and Hg^{2+} Resistance†

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

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

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Nucleic acids extracted from microbial biomass without prior culturing were hybridized with probes representing four *mer* operons to detect genes encoding adaptation to Hg^{2+} in whole-community genomes. A 29-fold enrichment in sequences similar to the *mer* genes of transposon Tn*501* occurred during adaptation in a freshwater community. In an estuarine community, all four *mer* genes were only slightly enriched (by three- to fivefold), suggesting that additional, yet uncharacterized, *mer* genes encoded adaptation to Hg^{2+}.

Aquatic microbial communities adapt to the presence of Hg^{2+} in their environment by increased Hg^{2+} tolerance (1, 7) and accelerated rates of Hg^{2+} reduction to Hg^0 (1, 3). Subsequent removal of Hg^0 from solution is due to low solubility and high vapor pressure (15). Because Hg^{2+} resistance and volatilization in gram-negative bacteria are encoded by genes that bear DNA sequence similarity to the *mer* operon of transposon Tn*21* [*mer*(Tn*21*)] (2, 12), it was hypothesized that adaptation of aquatic microbial communities, where gram-negative bacteria predominate (10), would be associated with an enrichment of *mer*(Tn*21*)-carrying bacteria. However, experiments to test this hypothesis indicated that *mer*(Tn*21*) was not enriched in Hg^{2+}-adapted microbial communities (1) and that alternative *mer* genes coded for resistance among strains of studied communities (3).

This conclusion was based on hybridization of bacterial colonies with a single probe, *mer*(Tn*21*) (2). Because only 0.1 to 22.2% of aquatic bacteria can grow on common laboratory growth media (4), the colony hybridization method probably missed the majority of organisms in the communities that volatilized Hg^{2+}. Thus, a method was needed to determine the abundance of specific genes in the community as a whole, so that the relationship between specific genes and the activities they encode could be studied.

Five *mer* operons, three of gram-negative and two of gram-positive origins, have been isolated and sequenced to date (12). Although the majority of Hg^{2+}-resistant isolates from adapted communities were gram negative (3), resistant gram-positive bacteria may contribute significantly to adaptation by aquatic microbial communities. In addition, the three gram-negative *mer* operons are of two types; the operons of transposon Tn*501* [*mer*(Tn*501*)] and the plasmid pDU1358 are composed of four structural genes and a regulatory gene, *merR*. The operon of Tn*21* contains an additional gene, *merC*, whose function is unknown (12). The abundance of the four types of *mer* genes (two of gram-positive and two of gram-negative origins) in adapted communities could indicate their relative significance in the bacterial response to Hg^{2+}.

This report describes the use of DNA probes to determine the abundance of sequences similar to the four characterized *mer* genes in nucleic acid fractions obtained from Hg^{2+}-adapted and -unadapted (control) microbial communities. A role in adaptation is suggested by an increase in sequences similar to *mer* in the adapted community compared with the control community.

MATERIALS AND METHODS

Preparation of adapted and control microbial communities. Estuarine water samples were collected on 30 November 1987 from Santa Rosa Sound, Fla. (water pH, 7.88; salinity, 25‰ temperature, 16.5°C), and freshwater samples were taken from Thompson's Bayou, Fla., on 5 April 1988 (water pH, 6.45; salinity, below detection; temperature, 21°C). Sampling sites, sampling procedures and measurements of water characteristics were described previously (1). Adapted communities were obtained by incubating 45 liters of sample supplemented with 250 μg of Hg^{2+} per liter for 2 days at 29°C in a Pyrex bottle (Corning Glass Works, Corning, N.Y.). Control communities, in 50-liter samples, were incubated under the same conditions in a Nalgene polypropylene carboy (Nalgene Labware Div., Nalge/Sybron Corp., Rochester, N.Y.) without Hg^{2+}. Detailed procedures used during exposure were as described previously (1).

Determination of Hg^{2+} resistance levels. Direct viable counts of Hg^{2+}-resistant bacteria and statistical analysis of the results were performed as described previously (7).

Concentration of microorganisms and nucleic acid extraction. Following prefiltration (through a 3-μm-pore-size filter), cells of adapted and control communities were concentrated by tangential flow ultrafiltration by using a Pellicon Cassette System (Millipore Corp., Bedford, Mass.) equipped with a 5-ft^2 (0.65 m^2) coarse screen membrane (100K NMWL, excluding molecules with molecular weights <100,000). Following ultrafiltration, the membranes and tubing of the ultrafiltration unit were rinsed by cycling 400 ml of saline (0.85% NaCl) for 5 min. The wash saline was combined with the concentrated samples to yield a final volume of 500 to 1,000 ml. Cells were concentrated by centrifugation (20,000 × *g* at 5°C for 60 min) and rinsed with 10 ml of cold saline, and pellets were stored at −20°C.

Nucleic acids were extracted and purified from cell pastes by generally following the method of Ogram et al. (9).

* Corresponding author.

† Contribution no. 664 of the U.S. Environmental Research Laboratory, Gulf Breeze, Fla.

TABLE 1. *mer* probes used in hybridization with community genomes and enrichment of sequences homologous to the probes in Hg^{2+}-adapted microbial communities

mer gene detected	Restriction fragment used as a probe	Control DNA		Enrichment factor in[a]:		Reference
		Positive	Negative	Freshwater comm.	Estuarine comm.	
Gram negative:						
Tn*21* and Tn*501*	2.6-kb[b] *Eco*RI from pACNR25	pACNR25	*E. coli* HMS174	29.05 (35)	3.19 (26)	2
Tn*21*	0.35-kb *Bss*HII-*Ava*II from pPG102[c]	pPG102	pME440[d]	4.17 (47)	3.57 (23)	5
Gram positive:						
Bacillus sp. strain RC607	3.8-kb *Eco*RI-*Sph*I from pYW46	pWY46	*E. coli* HMS174	0.71 (13)	5.02 (38)	16
S. aureus pI258	6.4-kb *Sst*I-*Xba*I from pRAL2A	pRAL2A	*E. coli* HMS174	1.75 (29)	4.36 (11)	17

[a] Ratio of *mer* abundance in the Hg^{2+}-adapted community (comm.) to *mer* abundance in the control community (see text). Ratios are between the means of two or three hybridization signals for Hg^{2+}-adapted and control communities. Numbers in parentheses are maximal percent errors for the ratios, calculated as suggested by Sokal and Rohlf (13).

[b] kb, Kilobase.

[c] Fragment is internal to *merC* of *mer*(Tn*21*).

[d] pME440 caries an intact Tn*501* (6). A strain containing pME440 was the gift of D. Haas (Mikrobiologische Institut, Zurich, Switzerland).

Frozen pellets were thawed and suspended in 5 ml of 0.12 M sodium phosphate buffer (pH 8.0), sodium dodecyl sulfate was added to a final concentration of 1.2%, and suspensions were incubated at 70°C for 1 h. Suspensions were placed in the jar of a Bead-Beater (Biospec Products, Bartlesville, Okla.), 2.5 g of prebaked glass beads (0.1 to 0.15 mm in diameter) were added, the jar was immersed in an ice-water bath, and cells were beaten for 1 min. Glass beads were separated by centrifugation for 10 min at 11,950 × *g* at 5°C and washed twice with 2.5 ml of 0.12 M sodium phosphate (pH 8.0). Supernatants were combined and extracted with phenol-chloroform (8). Aqueous phases were dialyzed against three changes of TE buffer (10 mM Tris hydrochloride, 1 mM EDTA [pH 8.0]), and nucleic acids were concentrated by cold precipitation (8). The precipitation step was repeated once more, and resulting nucleic acids were dissolved in 500 μl of TE buffer. The chemical purity of samples was determined by spectrophotometric scans with a DU-40 Beckman spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). Pure nucleic acid samples, with optical density ratios at 260 to 280 nm of 1.8 to 2.0, were stored at −20°C.

Preparation of *mer* probes. Four probes were used to detect *mer* homologous DNA in the genomes of Hg^{2+}-exposed and control communities. Restriction fragments used as probes, probe specificity, and target DNA used as positive and negative controls are listed in Table 1. *Escherichia coli* SK2267(pPG102) was a gift from A. Summers (University of Georgia). *E. coli* JM83(pYW46) was provided by I. Mahler (Brandeis University), and R. Laddaga (Bowling Green State University) sent strain JM83 (pRAL2A). These cultures were grown in LB broth containing either 100 μg of chloramphenicol (Sigma Chemical Co., St. Louis, Mo.) per ml or 12.5 μg of tetracycline (Sigma) per ml.

Purified plasmid DNA was isolated by the method of Sadhu and Gedamu (11) and digested with restriction enzymes (purchased from Bethesda Research Laboratories, Gaithersburg, Md., except *Bss*HII, which was a product of New England BioLabs, Inc., Beverly, Mass.) as recommended by Maniatis et al. (8). Restriction fragments were separated by electrophoresis through 1.0% low-melting-point agarose gels (FMC BioProducts Corp., Rockland, Maine) in a Tris-borate buffer (8). Fragments were removed from agarose gel slices, as recommended by the manufacturer of the low-melting-point agarose. Probes were labeled with 5′-[α-^{32}P]dCTP (specific activity, 3,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) by using a nick translation kit (Boehringer GmbH, Mannheim, Federal Republic of Germany) as recommended by the manufacturer.

Hybridization of *mer* probes with nucleic acid fractions isolated from microbial communities. Purified nucleic acids were blotted onto S&S NC membranes (BA85; Schleicher & Schuell, Inc., Keene, N.H.) as recommended by the manufacturer, by using a Minifold II Slot Blotter (Schleicher & Schuell). Nucleic acids were diluted in twofold increments to give a range of 1.0 to 0.0625 μg per slot when 100-μl samples were applied to the membrane. Amounts of control target

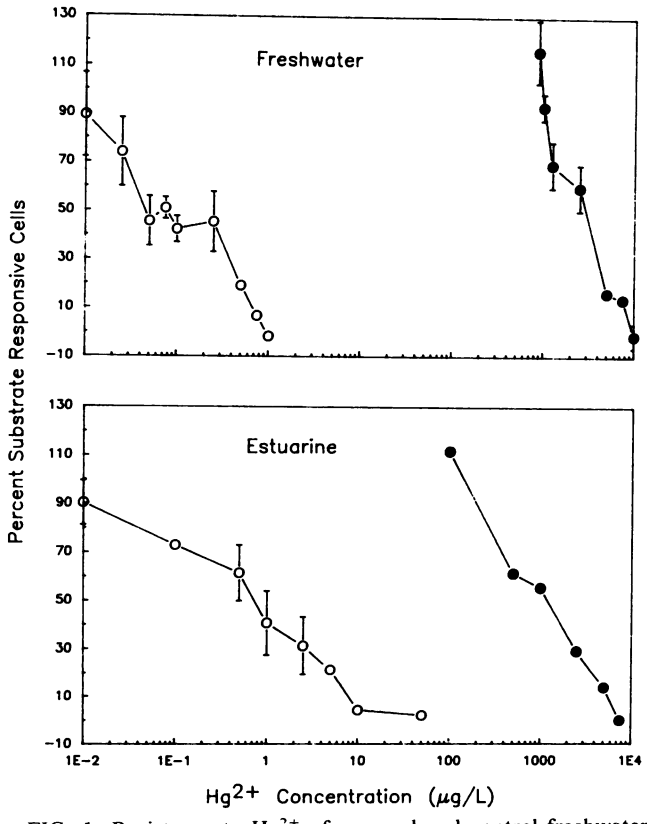

FIG. 1. Resistance to Hg^{2+} of exposed and control freshwater and estuarine microbial communities. Hg^{2+}-resistant direct viable counts are from communities exposed to 250 μg of Hg^{2+} per liter (●) and control communities (○).

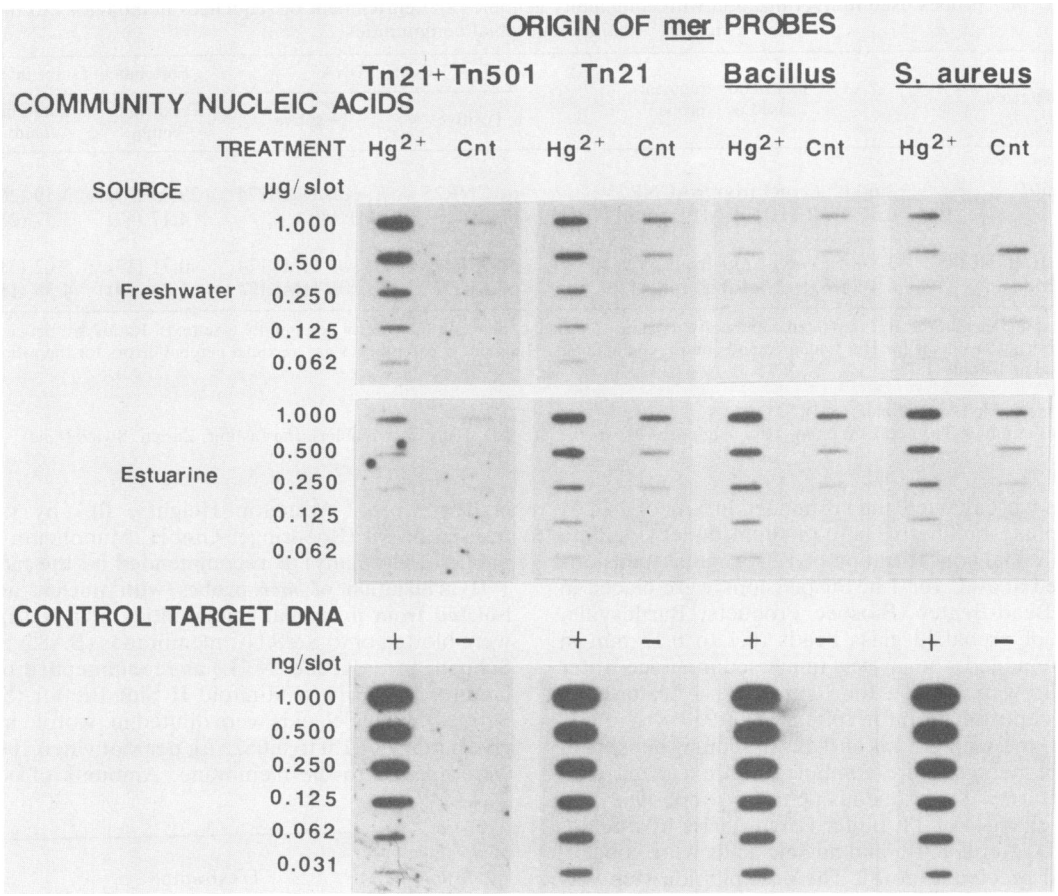

FIG. 2. Abundance of *mer* sequences in the community genomes of Hg^{2+} and control microbial communities. The autoradiogram is of radioactive signals resulting from hybridization of the four *mer* probes to the indicated amounts of nucleic acids extracted from Hg^{2+}-adapted (Hg^{2+}) and control (Cnt) freshwater and estuarine microbial communities.

DNA ranged from 1.0 to 0.031 ng per slot in twofold increments. Hybridization of the *mer*(Tn*21*) probe was carried out as described previously (2), and hybridization reactions containing 10% formamide (2) were used with the remaining three probes. Stringency conditions were selected by preliminary experiments to result in clear signals with positive controls and in no hybridization of negative controls. After hybridization, membranes were prepared for autoradiography by the method of Maniatis et al. (8) and autoradiograms were obtained after exposure of membranes to X-Omat X-ray film (Eastman Kodak Co., Rochester, N.Y.) for 10 days at −70°C.

Determination of *mer* abundance in whole-community genomes. The intensity of hybridization signals was quantified by integrating the area of peaks generated by light transmitted through autoradiograms by using a model 620 Video Densitometer equipped with 1-D Analyst software (Bio-Rad Laboratories, Richmond, Calif.). Absolute absorbance values, obtained from linear readings between amount of nucleic acids per slot and signal intensity, were normalized to 1 µg of community nucleic acids and used to determine enrichment factors. Standard deviations for absorbance values ranged between 32 and 0.5%.

RESULTS AND DISCUSSION

Increased Hg^{2+} resistance in exposed communities. Exposure resulted in a dramatic increase in the tolerance level of the fresh and saline water communities to Hg^{2+} (Fig. 1). Regression analysis of the dose-response (percent substrate responsive cell versus log Hg^{2+} concentration) curves (Fig. 1) ($r^2 = -0.94$ and -0.92 for the control and exposed communities, respectively) indicated that the resistance level of the exposed estuarine community was increased by 3.5 orders of magnitude. The IC_{50}s (concentrations of Hg^{2+} that inhibited 50% of substrate-responsive cells) in the control and exposed estuarine communities were 0.567 ± 0.246 (mean of duplicate samples plus or minus standard deviation) and 1,194 µg of Hg^{2+} per liter, respectively. Duplicate samples for the exposed estuarine community were combined to obtain enough cells per counted microscopic field. An increase in Hg^{2+} resistance of over 4 orders of magnitude was observed after exposure of the freshwater community. The IC_{50} of the control community ($r^2 = -0.96$) was 0.117 ± 0.022 µg of Hg^{2+} per liter, and that of the exposed community ($r^2 = -0.97$) was 2,725 ± 159 µg of Hg^{2+} per liter.

Abundance of *mer* genes in the genomes of adapted and control microbial communities. The role of specific *mer* genes in adaptation to Hg^{2+} was suggested by their enrichment in the exposed community compared with their abundance in the control community (Fig. 2). The intensity of signals produced by hybridization with a constant amount of nucleic acids was considered to represent the abundance of the target sequence in the community genome. Enrichment

factors (Table 1) are the ratios between intensities of hybridization signals of exposed communities and those of control communities. These ratios express increases in abundance of specific *mer* genes that occurred during adaptation. For example, in the freshwater community, there was a large increase in sequences homologous to *mer*(Tn*21*) and *mer*(Tn*501*) (by a factor of 29.05) as a result of exposure to Hg^{2+}. This increase was mostly due to a Tn*501*-type *mer* operon because *merC* sequences were only 4.17 times more abundant in the exposed community than in the control community. The gram-positive *mer* genes did not contribute to adaptation in the freshwater community because their abundance was not enriched (Fig. 2 and Table 1). Thus, in the freshwater sample, an increase in IC_{50} of 3 orders of magnitude (Fig. 1) was associated with a 29-fold increase in abundance of *mer* genes similar to *mer*(Tn*501*). Recently, we have used a similar approach to study communities of a mercury-polluted freshwater stream. A 180-fold increase in abundance of *mer*(Tn*501*) but not of the other three *mer* genes was related to faster Hg^{2+} volatilization rates and higher Hg^{2+} resistance compared with those of communities from a control unimpacted stream (C. Liebert, M. Gillman, and T. Barkay, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, Q-187, p. 361).

Analysis of hybridization results of the estuarine sample suggested that the detected *mer* genes were weakly associated with adaptation to Hg^{2+}, with enrichment factors ranging from 3.19 [for *mer*(Tn*21*) and *mer*(Tn*501*)] to 5.02 (for *Bacillus* sp. strain RC607 *mer*). However, gram-positive *mer* genes played some role in adaptation to Hg^{2+} in the estuarine sample (Table 1). In light of the 3-order-of-magnitude increase in IC_{50} of Hg^{2+} (Fig. 1) and the small increase in abundance of sequences with similarity to any of the four *mer* probes (Fig. 2), other *mer* genes must have mediated adaptation to Hg^{2+} in the estuarine sample.

Thus, *mer*(Tn*501*)-like genes contributed to adaptation in a freshwater community, but in a saline community alternative *mer* genes played this role. Gilbert and Summers (5) used subgenomic *mer* probes to show that *mer*(Tn*21*) evolved in *E. coli*, whereas *mer*(Tn*501*)-like sequences were found in other enteric bacteria, including approximately 50% of the tested *Pseudomonas aeruginosa* strains. Thus, the predominant role of *mer*(Tn*501*) in Hg^{2+} adaptation of freshwater microbial communities, where the majority of culturable resistant aerobic heterotrophs were characterized by API NFT strips (Analytab Products, Mainview, N.Y.) as pseudomonads (T. Barkay and M. Gillman, manuscript in preparation), corresponds with evolutionary patterns of *mer* in enteric bacteria (5).

Stahl et al. (14) recently described a direct quantification of specific bacterial species in natural microbial assemblages by hybridization of 16S rRNA-directed oligonucleotide probes with nucleic acids extracted from environmental samples. We have used a similar approach to relate the abundance of functional bacterial genes (i.e., those that encode Hg^{2+} resistance and volatilization) to the ecological process (i.e., Hg^{2+} resistance) that is mediated by bacteria carrying these genes. Identification of functional genes and their relatedness to ecological processes could be used to predict the fate and, indirectly, the effects of pollutants in the environment.

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