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Polymerase Chain Reaction-Restriction Fragment Length Polymorphism Analysis Shows Divergence among *mer* Determinants from Gram-Negative Soil Bacteria Indistinguishable by DNA-DNA Hybridization

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Mercury resistant (Hg^r) bacteria were isolated from four terrestrial sites: three containing high levels of mercury (sites T2, SE, and SO) and one uncontaminated site (SB). The frequencies of Hg^r bacteria in the total cultivable populations were 0.05% (SB), 0.69% (SO), 4.8% (SE), and 25% (T2). Between 35 and 100% of the isolates from the four sites contained DNA sequences homologous to a DNA probe from the mercury resistance (*mer*) operon of the Tn*501* Hg^r determinant. The *mer* sequences of 10 Tn*501*-homologous Hg^r determinants from each site were amplified by the polymerase chain reaction, with primers designed to consensus sequences of the *mer* determinants of Tn*501*, Tn*21*, and pMJ100, and were classified on the basis of the size of the amplified product and the restriction fragment length polymorphism pattern. Two main groups of amplification product were identified. The first, represented by the T2 and SB isolates and one SE isolate, gave an amplification product indistinguishable in size from that amplified from Tn*501* (~1,010 bp). The second group, represented by the SO isolates and the majority of the SE isolates, produced larger amplification products of 1,040 or 1,060 bp. Restriction fragment length polymorphism analysis revealed that each amplification product size group could be further subdivided into five subgroups.

Bacterial resistance to mercury compounds in soils and waters has been reported frequently (19, 22, 37). The molecular mechanisms of resistance to both inorganic mercury (Hg^{2+}) and to the more toxic organomercurials, e.g., phenyl mercuric acetate and methyl mercury, have been extensively studied (27). Several mercury resistance (*mer*) determinants have been sequenced, and of these most is known about the gram-negative, narrow-spectrum systems carried on the transposons Tn*501* and Tn*21* which confer resistance to Hg^{2+} ions but not to organomercurials. In these systems, Hg^{2+} ions are transported into the cell by the periplasmic and membrane-associated gene products of *merP* and *merT*, respectively. Once in the cell, mercuric reductase catalyzes an NADPH-dependent reduction of Hg^{2+} to the less toxic elemental mercury (Hg^0) which volatilizes from the cell. The structural genes and the *merR* gene are transcribed divergently, with the product of the *merR* gene both positively and negatively regulating *mer* gene transcription. Upon binding Hg^{2+}, MerR undergoes a conformational change allowing transcription of the *mer* operon by RNA polymerase. In the broad-spectrum *mer* determinant, pDU1358, resistance to organomercurials is encoded by organomercurial lyase (15), which cleaves the mercuric ion from the organo moiety, allowing subsequent reduction of Hg^{2+} to Hg^0 by mercuric reductase.

In many cases, mercury resistance (Hg^r) is associated with conjugative plasmids and/or transposons (19, 21, 34), which can facilitate the horizontal transfer and dissemination of *mer* genes through bacterial populations. These bacterial populations will be subject to selection if exposed to mercury compounds. The frequent presence of mercury in natural environments and the widespread occurrence of mercury resistance among bacteria from these environments provide an excellent opportunity to investigate the patterns of adaptation of microbial communities to environmental stress. To better understand this adaptive process, it would be valuable to know the extent of the genetic diversity of *mer* determinants within particular environments and additionally the degree of genetic diversity between environments.

Several different approaches have been used to investigate both the distribution and divergence of *mer* determinants in natural environments. Initially, Barkay et al. (1) used a 2.6-kb probe to detect Tn*21*-homologous determinants by DNA-DNA hybridization in natural isolates. This was followed by more-extensive hybridization studies using a series of *mer* probes against DNA isolated from bacteria both from culture collections (14) and from natural environments (37). Barkay et al. (2) also hybridized *mer* probes to DNA extracted directly from water to investigate the distribution of *mer* genes in the total biomass. More recently, oligonucleotide probes designed to consensus sequences of *mer* determinants were used to detect *mer* genes in bacteria isolated from the Rhine following mercury contamination of the river (26). Diversity of *mer* genes has also been demonstrated by restriction endonuclease and polypeptide analysis of *mer* determinants from conjugative plasmids (19, 20, 33) and by the immunological typing of mercuric reductases (5). It is likely that considerably more genetic diversity exists than has been detected by these methods. Furthermore, any analysis of bacterial population diversity must ultimately embrace the entire population and therefore be directed at the total bacterial DNA, rather than the DNA from cultivable bacteria. Increasing use is now being made of the polymerase chain reaction (PCR) in the study of gene divergence in bacteria (24, 29) and for detection of genes in natural populations (7). Together with the more refined

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levels of analysis provided by restriction fragment length polymorphisms (RFLP) or nucleotide sequence data, this technique should allow a much more comprehensive analysis of genetic diversity within bacterial populations.

In this study we report the use of the PCR, coupled with RFLP analysis, to investigate the diversity of *mer* gene sequences in bacteria isolated from different terrestrial environments. The aim of this study was twofold: first, to define the extent of variation between *mer* sequences from cultivable bacteria, both within and between populations from different environments, and second, to validate the PCR-RFLP analysis by using cultivable organisms prior to a study of total biomass DNA and hence enable a comparison of the cultivable and total bacterial populations to be made.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* AB1157 (Sm^r) was the host to all plasmids used in this study. Plasmids used were: Col IBP9 (*cib cib^{imm}*) (9), R391 (Hg^r Km^r) (10), pSP200 (Hg^r Cm^r) (33), pACYC184::Tn*501* (Hg^r Cm^r) (6), pMJ100 (Hg^r Cm^r) (20), pHG106 (Hg^r [broad spectrum] Cm^r) (15), pMER419 (Hg^r) (19), and pMJ501 (Hg^r Cm^r) (20).

Soil descriptions. Soil samples were collected from four sites over the period from September 1991 to March 1992 and stored at 4°C prior to use. The samples were as follows: a soil sample (T2) from a disused copper mine in County Tipperary, Republic of Ireland, which when mined had high levels of associated mercury (43); and a sediment and soil sample (SE and SO, respectively) from Fiddlers Ferry, near Widnes on the banks of the River Mersey. The soil sample was taken 2 m inland from the sediment sample. The Mersey has high mercury levels in its sediment (8) because of industrial pollution, and Fiddlers Ferry is downstream of the estuaries' tidal limit. A soil sample (SB) from the banks of the River Etherow (a tributary of the Mersey) at Salter Brook Bridge in the Peak District National park was chosen as an example of a pristine unpolluted soil. Total mercury in the soil samples and the sediment was determined by neutron activation analysis (NAA) at the Centre for Analytical Research in the Environment, Imperial College, Ascot, United Kingdom.

Bacterial isolation and identification. Bacteria were isolated from sites within 3 days of sampling, by using a modified version of the method of Ramsay (36). Soil (10 g [wet weight]) was suspended in 90 ml of 50 mM Tris-HCl buffer (pH 7.5) by three 1-min cycles of homogenization in a blender, with 1 min of cooling on ice between each cycle. Serial dilutions of the homogenate in 25% (vol/vol) Ringer solution (2.25 g of NaCl, 0.15 g of KCl, 0.12 g of $CaCl_2$, and 0.05 g of $NaHCO_3$ per liter of sterile distilled water) were plated in duplicate on Luria agar (LA) and on LA supplemented with 50 μg of $HgCl_2$ ml^{-1} and incubated at room temperature. Bacteria were identified by Gram stain and by using API 20E and 20NE kits (Biomerieux).

Isolation of total genomic DNA. A bacterial cell pellet harvested by centrifugation of 0.5 ml of an overnight culture was resuspended in 100 μl of TE buffer (10 mM Tris-HCl–1 mM EDTA, pH 8.0) containing 5% (vol/vol) antifoam emulsion (Sigma) and 0.1 mg of xylene cyanol ml^{-1}. Cells were lysed by adding 20 μl of 1 M NaOH saturated with sodium dodecyl sulfate (SDS), with inversion for 1 min and vortexing for 1 min. DNA was visualized by electrophoresis of 20 μl of the lysate in a 0.7% agarose-TBE gel containing ethidium bromide (1 μg ml^{-1}) (42). Cell debris was extracted by the addition of a mixture of acid phenol (dissolved in distilled water), chloroform, and isoamyl alcohol (25:24:1) and centrifugation (17, 23). The upper aqueous phase was used directly in dot blot hybridization.

Preparation of *merRTPA* probe. A 2,217-bp *Hin*dIII-*Eco*RI fragment (nucleotide coordinates 136 to 2353) from Tn*501* containing intact *merT* and *merP* genes and flanking regions of *merR* and *merA* was gel purified from 0.7% low-melting-point agarose and labelled with [α-^{32}P]dCTP by random priming (11).

Dot blot hybridization. Extracts (20 μl) of total genomic DNA from each isolate were electrophoresed on an agarose-TBE gel. Control DNA from strains of *E. coli* AB1157 bearing the plasmids pACYC184::Tn*501*, pMER419, pMJ100, pHG106, and pMJ501 (positive controls) and R391, pSP200, and Col 1BP9 (negative controls) were similarly treated. By a comparison of band intensities against standards of a kilobase DNA ladder (GIBCO-BRL), equivalent amounts of DNA from each isolate were estimated with subsequent denaturation by the addition of 10 μl of 0.2 N NaOH. By using a dot blot manifold, DNA was transferred to a Genescreen hybridization transfer membrane (Dupont). Subsequent hybridization with the *mer* probe was performed as recommended by the Genescreen instruction manual, with duplicate 100-ml washes in (i) 0.3 M NaCl, 0.06 M Tris-HCl (pH 8.0), and 0.002 M EDTA (5 min); (ii) 0.3 M NaCl, 0.06 M Tris-HCl (pH 8.0), 0.002 M EDTA, and 1% SDS (30 min); (iii) 0.03 M NaCl, 0.006 M Tris-HCl (pH 8.0), and 0.0002 M EDTA (30 min), by using 65 instead of 60°C throughout the procedure, to increase stringency to detect homology greater than 70%. Membranes were exposed to Fuji RX film for 3 to 4 days at −70°C.

Oligonucleotide primers. Oligonucleotide primers R (5′-GGG AGA TCT AAA GCA CGC TAA GGC [GA]TA-3′) and P (5′-GGG GAA TTC TTG AC[TA] GTG ATC GGG CA-3′) were designed to regions conserved in the mercury resistance determinants: Tn*501* (28), Tn*21* (3), and pMJ100 (17). Primers amplify a region containing intact *merR* and *merT* genes and the 5′ end of *merP* (*merRTΔP*).

PCR amplification. Target DNA from cultivable organisms was prepared by the method of Gussow and Clackson (16), except that bacterial colonies resuspended in sterile distilled water were boiled for 10 instead of 5 min to ensure lysis. Tn*501* template DNA was prepared by using the alkaline lysis extraction procedure (4) on pACYC184::Tn*501* from *E. coli* AB1157. Amplification of *merRTΔP* regions was performed for 28 cycles (with a Genetic Research Instruments PTC-100 V2.0). Each cycle consisted of a denaturation step (1 min, 94°C), an annealing step (1 min, 62°C), and an extension step (2 min, 72°C). After the last cycle, extension was continued for a further 10 min to allow the reaction to go to completion. The PCR contained target DNA (50 μl of the bacterial lysate or 0.06 μg of Tn*501*), 30 pmol of each oligonucleotide primer, 50 μM each deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP), and 2.5 U of *Taq* DNA polymerase and 10 μl of the supplier's (Boehringer Mannheim) 10× *Taq* DNA polymerase buffer and was made up to a total volume of 100 μl with sterile distilled water and overlaid with 100 μl of mineral oil.

Hybridization and restriction analysis of the PCR products. PCR products (10 μl) were electrophoresed on a 0.7% agarose TBE gel containing ethidium bromide (1 μg ml^{-1}). Following Southern transfer, hybridization with the *mer* probe was carried out as described above. Membranes were then exposed to Fuji RX film for 1 to 4 h at −70°C. PCR product (15 μl) was digested with each of the following

TABLE 1. Distribution of Hg^r bacteria and Tn*501*-homologous *mer* sequences at the four research sites

Sample location	Total mercury[a] (ppm)	Total bacteria (CFU g^{-1})	Hg^r bacteria (CFU g^{-1})	% Hg^r bacteria	% Isolates hybridizing to *mer* probe[b]
Tipperary 2 (T2)	0.361 ± 0.03	2.4×10^7	6.0×10^6	25.0	37.5 (18/48)
Fiddlers Ferry sediment (SE)	0.161 ± 0.029	2.3×10^7	1.1×10^6	4.8	34.7 (17/49)
Fiddlers Ferry soil (SO)	0.441 ± 0.039	1.0×10^8	6.9×10^5	0.69	80 (40/50)
Salter Brook Bridge (SB)	<0.12	3.6×10^6	1.9×10^3	0.05	100 (50/50)

[a] The detection limit for total mercury was <0.12 ppm.
[b] Numbers in parentheses show the fraction of isolates bearing *mer* sequences homologous to Tn*501* over the total number tested.

restriction endonucleases: *Ava*I, *Bgl*I, *Bss*HII, and *Hin*dIII (GIBCO-BRL). Reaction conditions used were those recommended by the suppliers. Restriction products were electrophoresed in a 2% agarose-TBE gel containing ethidium bromide (1 μg ml^{-1}).

Numerical analysis. Restriction endonucleases were chosen to yield either two or three DNA fragments upon digestion of the amplification product of Tn*501*. The small number of bands generated facilitated accurate size determination of the fragments and reduced the number of fragments smaller than 90 bp which were difficult to visualize. The sizes of fragments less than 90 bp were inferred by subtraction from the size of the initial PCR product and/or from Tn*501* sequence data. This assumption has since been supported by subsequent sequence analysis of *merR* genes which confirmed the position of *Hin*dIII sites in amplification products and hence the size of *Hin*dIII fragments. PCR products were compared on the basis of their RFLP profiles, with the resulting size and band patterns used to determine different classes. Similarity coefficients between classes were determined (see Table 2), and dendograms were constructed by using the following treeing algorithms in the PHYLIP suite of programs (12) on the Seqnet computer at Daresbury, United Kingdom: the Fitch and Margoliash method (13), the neighbor-joining method (38), the Kitch method (a modified Fitch and Margoliash method), and the neighbor-joining UPGMA option.

RESULTS

Distribution of cultivable Hg^r bacteria and *mer* sequences in soil. Total viable counts ranged from 1×10^8 CFU g^{-1} in SO to 3.6×10^6 CFU g^{-1} in SB. The frequencies of resistance to inorganic mercury (Hg^{2+}) varied from 25% in T2 to 0.05% in the pristine soil sample (SB) (Table 1). Total mercury levels for each soil sample are also shown in Table 1. Genomic DNA was isolated from about 50 cultivable Hg^r isolates from each soil sample. Dot blot hybridization of total DNA from these isolates to the *mer* probe showed a wide variation in the proportion of isolates from each soil sample whose *mer* sequences bore strong homology to Tn*501* (Table 1). The *merRTPA* probe hybridized to DNA from all five positive controls, whilst the probe failed to hybridize to DNA from any of the negative controls. A large difference was seen between the populations at the two distinct but adjacent Fiddlers Ferry sites (Table 1). In each of the Hg^r bacterial populations from polluted sites, there was a distinct subpopulation which possessed *mer* sequences which were homologous to the Tn*501 merRTPA* probe. All the Hg^r isolates from SB bore *mer* sequences which were homologous to the Tn*501 mer* probe.

Amplification of *mer* genes from cultivable Hg^r soil bacteria. From each of the four soil samples, 10 Hg^r isolates bearing Tn*501*-homologous *mer* sequences were chosen for further study. By using the primers R and P, PCR amplification of DNA was detected from all 40 isolates and from the positive control, Tn*501*, whereas no amplification was observed following PCR of sterile distilled water (Fig. 1). On the basis of PCR product size, two distinct subgroups were distinguished; amplification products from the SB and T2 isolates and from one SE isolate were indistinguishable in size from that amplified from Tn*501* (~1,010 bp), whilst amplified *merRTΔP* regions from the remaining SE and SO isolates were slightly larger (1,040 to 1,060 bp) (Fig. 1). PCR products from all 40 isolates and from Tn*501* hybridized to the 2,217-bp *mer* probe from Tn*501* (data not shown).

RFLP analysis of amplified *mer* sequences. Cleavage by the four restriction endonucleases, *Ava*I, *Bgl*I, *Bss*HII, and *Hin*dIII, revealed a variety of different DNA fragment patterns amongst amplified *mer* regions from different isolates (Fig. 2 and 3). Five isolates from SB yielded amplified *mer* regions which had restriction patterns identical to that of the amplified *mer* region from Tn*501* (Table 2, class A). The remaining isolates all possessed *mer* regions divergent from Tn*501* (Table 2). On the basis of PCR product and restriction fragment sizes, the 40 isolates were subdivided into 10 classes (A to J), and similarity coefficients were calculated

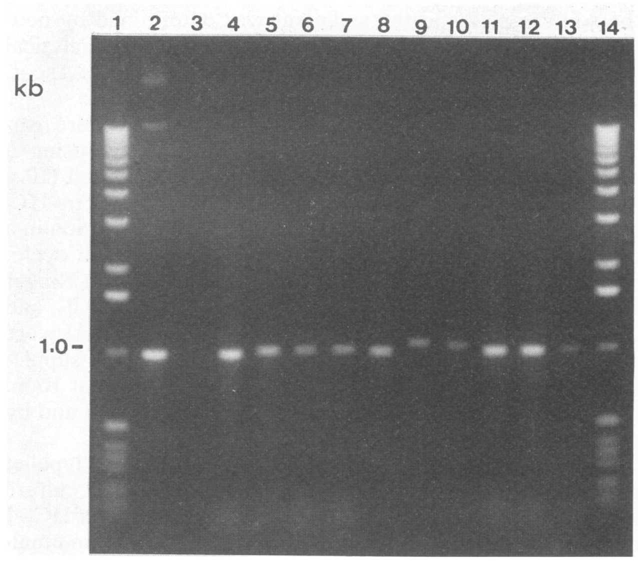

FIG. 1. Agarose gel electrophoresis of the PCR products amplified from Hg^r cultivable isolates with the primers R and P. Lanes: 1 and 14, kilobase ladder; 2, 0.06 μg of pACYC184::Tn*501* DNA after PCR; 3, sterile distilled water after PCR; 4 to 13, crude extracts of DNA after PCR of SB29, SE35, SE6, SE12, SE20, SE31, SO1, SB3, T2:7, and T2:38 (classes A to J), respectively.

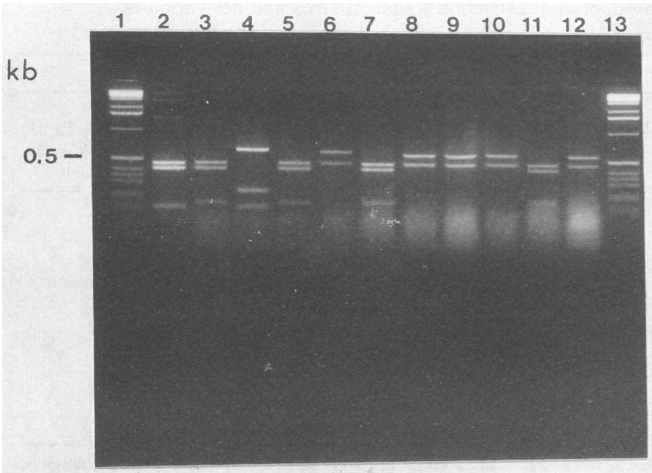

FIG. 2. Agarose gel electrophoresis of *Bss*HII RFLPs of PCR products amplified from Hg^r cultivable isolates with the primers R and P. Lanes: 1 and 13, kilobase ladder; 2, 0.06 μg of pACYC184::Tn*501* after PCR; 3 to 12, crude extracts of DNA after PCR of SE isolates 18, 20, 23, 31, and 35 and SO isolates 7, 8, 9, 12, and 13, respectively.

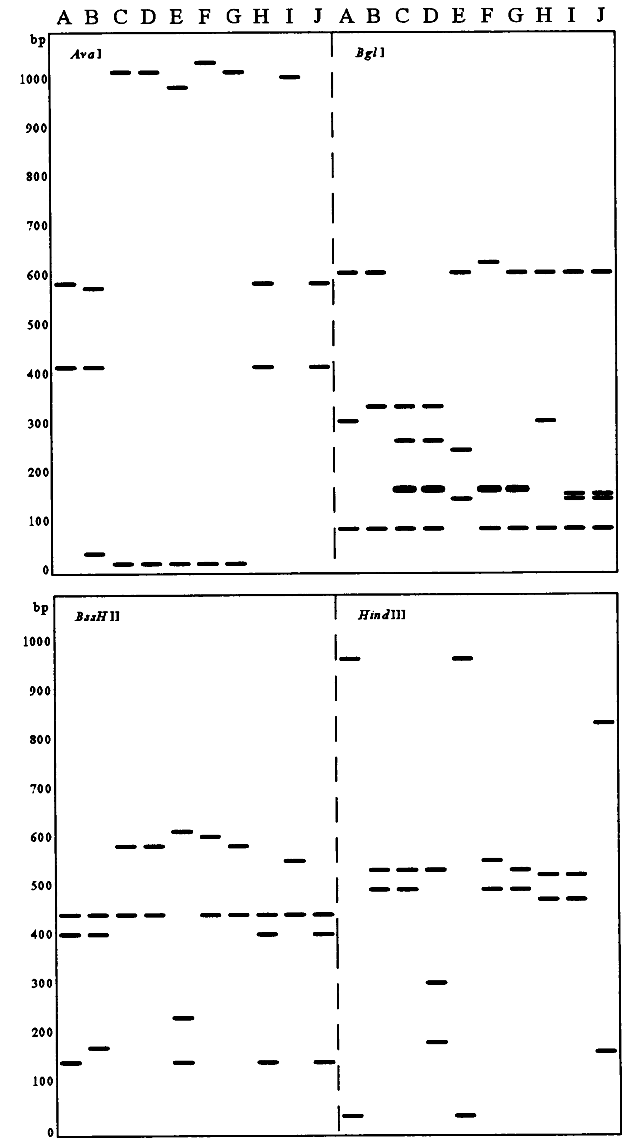

FIG. 3. Schematic representation of restriction patterns of amplified *mer* regions from cultivable Hg^r bacteria. A to J represent classes based upon PCR product and restriction fragment size (Table 2). NB class A includes Tn*501*. For the *Bgl*I digests, bands of double thickness indicate doublets.

(Fig. 3 and Table 2). Treeing algorithms derived from these values consistently grouped *mer* sequences from these isolates into two distinct subsets. This division is seen clearly in the neighbor-joining UPGMA dendogram (Fig. 4).

API identification of Hg^r isolates. The 40 environmental isolates were identified to at least genus level by Gram staining and subsequent biochemical tests (API 20E and 20NE kits). Seven genera were identified, and 30 isolates were identified to species level (Table 3). Species diversity was greatest amongst the T2 and the SE isolates (five and four different species, respectively). SO and SB bacterial populations showed little diversity amongst the isolates studied. Of the 10 isolates from SO, 9 were *Enterobacter cloacae* and composed the class G isolates. Class C also consisted of bacteria from one species only; however, classes B, H, I, and J exhibited species diversity (Tables 2 and 3).

DISCUSSION

The proportion of cultivable Hg^r bacteria in each soil sample was determined. Values ranged from 0.05 to 25% and were within the range of Hg^r frequencies (0.007 to 49%) cited by Rochelle et al. (37). Much of this variation may be due to differences in mercury levels amongst the soil samples studied and the bacterial populations therein. However, a direct comparison of these values with those from previous studies is not possible, as variation may be due to differences between the metal-binding properties of the media used (35) and to the different $HgCl_2$ concentrations used in the media. As expected, frequencies of Hg^r isolates were lowest in the population at SB, chosen originally as a control site believed to have low levels of mercury. Hg^r isolate levels in the other soil samples, which are known to be contaminated with mercury, were higher, although the levels observed in the SO population were lower than expected in view of the higher mercury soil concentration. Previous studies have shown a marked correlation between the number of Hg^r bacteria and the environmental mercury content (22, 41). The low numbers of resistant isolates amongst the SO population may be a result of levels of available mercury being low in this soil compared with the total mercury content, thereby reducing the selection pressure. Alternatively, the use of direct selection with high levels of $HgCl_2$, without prior induction, may have inhibited the growth of some Hg^r bacteria (34).

DNA hybridization to the Tn*501* *mer* probe showed that all of the SB Hg^r isolates and a large majority of the SO Hg^r isolates carried *mer* genes homologous to the archetypal Tn*501* and Tn*21* resistance determinants. This lack of variation amongst SB isolates directly contradicts the results of Olson et al. (30), who suggest *mer* gene variation is greater in soils with lower total mercury levels. This lack of observed diversity may be due in part to the limited species diversity observed in both soils amongst the Hg^r cultivable isolates

TABLE 2. Classification of *mer* determinants based on PCR product and restriction fragment sizes and derivation of similarity coefficients[a]

Class	*s*									
	A Tn*501*, SB2, SB4, SB8, SB22, SB29	B SE3, SE9, SE18, SE23, SE35, SO12	C SE6, SE11	D SE12	E SE20	F SE31	G SO1, SO2, SO3, SO5, SO6, SO7, SO8, SO9, SO13	H SB3, SB5, SB12, SB13, SB24, T2:13	I T2:7, T2:17	J T2:12, T2:19, T2:23, T2:37, T2:38, T2:41, T2:46
A		43.5	17.4	16.7	45.5	18.2	27.3	81.8	38.1	69.6
B			50.0	40.0	8.7	26.1	52.2	43.5	27.3	41.7
C				88.0	8.7	52.2	87.0	17.4	18.2	16.7
D					8.3	41.7	75.0	16.7	17.4	16.0
E						9.1	18.2	27.3	28.6	34.8
F							54.5	18.2	19.0	17.4
G								27.3	28.6	26.1
H									57.1	69.6
I										54.5
J										

[a] Similarity coefficients (s) were calculated by using the formula $s = 100\ (n_{xy}/n_x + n_y)$, where n_{xy} is the number of bands shared between a pair of isolates (x and y), and n_x and n_y are the number of bands of strains x and y, respectively.

(Table 3). A minority of Hg^r isolates from SE and T2 bore *mer* sequences that were homologous to Tn*501*. The majority of isolates from these locations may employ a resistance mechanism other than volatilization, such as methylation, or their *mer* determinants may be divergent from archetypal sequences. Given the proximity of the two samples and the possibility of bacterial movement due to tidal activity, it is of particular note that the two Fiddlers populations (SE and SO) should vary so much with respect to frequencies of Hg^r isolates and *mer* gene diversity and shows that soils and their microbial populations can differ markedly over very small distances. Hybridization to a single DNA probe gives only a very simple picture of genetic diversity within bacterial populations. To overcome this limitation, Olson et al. (31) and Rochelle et al. (37) used a series of probes to both gram-negative and gram-positive *mer* determinants to give a more complete picture of *mer* gene diversity in natural populations. At the same time, they stressed the importance of using other approaches, in addition to phenotypic expression and probe analysis, to study bacterial diversity and in particular *mer* gene variation.

Sequence data from both gram-negative and gram-positive *mer* determinants enables preliminary evolutionary relationships to be drawn, especially amongst the gram-negative *mer*

FIG. 4. Dendogram (neighbor-joining UPGMA) of genotypic relationships between *mer* determinants from cultivable Hg^r isolates derived by PCR-RFLP analysis. Divergence between classes of *mer* determinants is expressed as percent similarity. For composition of classes, see Table 2.

TABLE 3. Identification of Hg^r bacterial isolates from Tipperary, Fiddlers Ferry, and Salter Brook Bridge

Isolate	API identification	Level of certainty for identification[a]
T2:7	*Acinetobacter calcoaceticus*	Good
T2:12	*Aeromonas hydrophila*	Very good
T2:13	*Aeromonas hydrophila*	Very good
T2:17	*Agrobacterium radiobacter*	Very good
T2:19	*Aeromonas hydrophila*	Good
T2:23	*Aeromonas hydrophila*	Excellent
T2:37	*Aeromonas salmonicida*	Acceptable
T2:38	*Enterobacter aerogenes*	Very good
T2:41	*Aeromonas hydrophila*	Very good
T2:46	*Aeromonas hydrophila*	Excellent
SE3	*Pseudomonas testosteroni*	Acceptable
SE6	*Acinetobacter calcoaceticus*	Good
SE9	*Alcaligenes faecalis*	Good
SE11	*Acinetobacter calcoaceticus*	Good
SE12	*Acinetobacter calcoaceticus*	Good
SE18	*Pseudomonas testosteroni*	Good
SE20	*Alcaligenes faecalis*	Good
SE23	*Pseudomonas testosteroni*	Good
SE31	*Klebsiella oxytoca*	Very good
SE35	*Alcaligenes faecalis*	Good
SO1	*Enterobacter cloacae*	Excellent
SO2	*Enterobacter cloacae*	Excellent
SO3	*Enterobacter cloacae*	Good
SO5	*Enterobacter cloacae*	Excellent
SO6	*Enterobacter cloacae*	Excellent
SO7	*Enterobacter cloacae*	Excellent
SO8	*Enterobacter cloacae*	Excellent
SO9	*Enterobacter cloacae*	Excellent
SO12	*Pseudomonas* sp.	Good
SO13	*Enterobacter cloacae*	Good
SB2	*Pseudomonas* sp.	Good
SB3	*Pseudomonas* sp.	Good
SB4	*Pseudomonas fluorescens*	Good
SB5	*Pseudomonas* sp.	Good
SB8	*Pseudomonas* sp.	Good
SB12	*Pseudomonas* sp.	Good
SB13	*Pseudomonas* sp.	Good
SB22	*Pseudomonas* sp.	Good
SB24	*Pseudomonas* sp.	Good
SB29	*Pseudomonas* sp.	Good

[a] Reliability of identification classifications are as follows: excellent, 99.9%; very good, 99.0%; good, 90.0; and acceptable, 80.0.

determinants, of which most is known. On the basis of *merR* sequence data, a whole series of related determinants can be grouped together (39). The sequences of the determinants Tn*501* (6, 28), Tn*21* (3), pDU1358 (15), pKLH2 (25), and pMJ100 (17), which is also borne on a transposon (18), share homologies over the *merR* gene of between 85 and 94% at the protein level. Given the DNA sequence homologies, all these sequences would be expected to hybridize to the Tn*501* probe at the stringencies used in this study, To investigate divergence within this subset of gram-negative *mer* determinants, oligonucleotide primers were designed to consensus regions in the *merR* and *merP* genes of Tn*501*, Tn*21*, and pMJ100. The 1-kb region flanked by these primers provided a substrate for rapid restriction analysis to permit the evolutionary relationships amongst a large number of *mer* determinants to be estimated. By using these primers, *mer* sequences were routinely amplified from the majority of isolates tested, all of which had previously hybridized to the Tn*501 mer* probe. Amplification products from all 40 isolates studied were confirmed as being *mer* sequences by hybridization.

RFLP analysis of the PCR products has revealed diversity amongst isolates from all four populations, although isolates from SB only differed from each other upon *Hin*dIII digestion, whilst five of these isolates were indistinguishable from Tn*501* (Table 2, class A; Fig. 3 and 4). The SB isolates were all of the genus *Pseudomonas* (Table 3), and although this genus is particularly diverse, it is perhaps of note that Tn*501* was originally isolated from a strain of *Pseudomonas aeruginosa* (40). The greatest variation was observed between the isolates from SE, with 10 isolates divided across five different classes and amplification products of three different sizes (Table 2; Fig. 1 and 3). The SO isolates exhibited the least variation, with 9 of the 10 isolates sharing an amplified product with the same restriction pattern. These nine isolates were all presumptively identified as *E. cloacae* (Table 3). However, in SE and T2, single bacterial species bear divergent *mer* determinants, whilst common *mer* determinants are found in more than one species (Tables 2 and 3), suggesting that *mer* determinant sequence variation is not species specific.

The PCR-RFLP analysis also showed that there were two distinct subgroups of gram-negative *mer* determinant between these populations (Fig. 4), one comprising the isolates from T2 and SB and one SE isolate (classes A, E, H, I, and J), and the other subgroup comprising the 19 remaining Fiddlers Ferry isolates (classes B, C, D, F, and G). This observation was immediately obvious from the difference in PCR product size (Fig. 1) and was confirmed upon subsequent RFLP analysis. Initial sequence analysis of these isolates (32) suggests that the two subgroups may be divided into Tn*501*-Tn*21*-type sequences and pKLH2-pDU1358-pMJ100-type sequences. This division is consistent with a dendogram based on MerR protein homologies (39). Thus, it would seem that this division has a geographical basis, with cultivable Mersey isolates bearing pKLH2-pDU1358-pMJ100-type *mer* determinants, whilst Tn*501*-Tn*21*-type determinants are predominant amongst T2 and SB cultivable isolates. It is of note that some of the *mer* determinants isolated from the river Mersey by Jobling et al. (19, 20), e.g., pMJ100 and pMJ501, bear considerable homology to the transposon Tn*5053* and to pKLH2, respectively (18). The latter two determinants were isolated from a Russian antimony and mercury mine.

This study has revealed wide-ranging genetic diversity among the *mer* genes from bacteria isolated from a wide range of mercury-contaminated and uncontaminated sites. Diversity has been observed in both the length and sequence of the *mer* genes and is completely masked when these sequences are screened by DNA-DNA hybridization with a Tn*501* probe. Moreover, it is clear that the level and type of sequence diversity are population specific and that considerable variation exists between populations. We conclude that PCR-RFLP analysis is a very sensitive method for detecting divergence. Coupled with the extraction of native DNA directly from bacterial soil populations (7), this method is being used to define and analyze the divergence and distribution of *mer* genes in the total bacterial populations of terrestrial ecosystems.

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