APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1985, p. 686–692
0099-2240/85/030686-07$02.00/0

Vol. 49, No. 3

Preparation of a DNA Gene Probe for Detection of Mercury Resistance Genes in Gram-Negative Bacterial Communities

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Received 6 August 1984/Accepted 12 December 1984

A DNA gene probe was prepared to study genetic change mechanisms responsible for adaptation to mercury in natural bacterial communities. The probe was constructed from a 2.6-kilobase *Nco*I-*Eco*RI DNA restriction fragment which spans the majority of the mercury resistance operon (*mer*) in the R-factor R100. The range of specificity of this gene probe was defined by hybridization to the DNA of a wide variety of mercury-resistant bacteria previously shown to possess the mercuric reductase enzyme. All of the tested gram-negative bacteria had DNA sequences homologous to the *mer* probe, whereas no such homologies were detected in DNA of the gram-positive strains. Thus, the *mer* probe can be utilized to study gene flow processes in gram-negative bacterial communities.

The effect of pollution on a given environment depends, to a great extent, on the response of the indigenous microbial community to the added pollutants. The determinant role played by microorganisms is a result of their large biomass, their participation in the cycling of essential elements, and their place at the lowest trophic level of food webs. Typically, microbial communities adapt to the presence of toxicants by the development of resistance and the ability to degrade or utilize these substances. As a result, new phenotypes are inherited in communities which develop under the influence of pollutants (13, 62).

At present, the molecular mechanisms which promote the development of new phenotypes in microbial communities are not fully understood. Davey and Reanney (12) have suggested that extrachromosomal elements play a critical role in genetic change mechanisms which allow adaptive evolution in bacteria. These mechanisms include spontaneous mutation, legitimate and illegitimate recombination, and DNA transfer between cells. Recombination and DNA transfer are often mediated by extrachromosomal elements in bacteria (12). The observations that genes advantageous in hostile environments are located on extrachromosomal elements (i.e., plasmids) further stress the participation of these elements in adaptive evolution. Such genes code for resistances to antibiotics, toxic metals and UV radiation (18), pathogenicity (53), and novel metabolic pathways for the utilization and degradation of recalcitrant hydrocarbons (12). In addition, the rate of point mutations and DNA rearrangments increases when organisms are subjected to environmental stress. The SOS system in *Escherichia coli* is an example of a mechanism which promotes this response. Thus, the induction of genetic change may be considered to be an adaptation mechanism in bacteria (30).

The classic example of plasmid-mediated genetic change is the coevolution of multiple drug resistance in bacteria in response to the therapeutic use of antibiotics (34). The molecular structure of multiple drug resistance plasmids suggested that replicons which previously existed in the cell had acquired new DNA sequences, each of which conferred a specific drug resistance (15). Such relationships between the presence of a stressor and the molecular structure of the bacterial genome have not been described in bacteria isolated from soils, waters, and sediments. However, the development of multiple drug and metal resistances in bacteria is enhanced in polluted environments (1, 46, 60). Further, plasmids are frequently isolated from these organisms, and some of these plasmids were shown to code for antibiotic and metal resistances (3, 31). DNA transfer between cells has been described previously to occur in waters (36), soils (22), and in a sewage treatment facility (2). All of these data strongly suggest that genetic change mechanisms play a role in the adaptive evolution of natural bacterial communities.

Available nucleic acid technology allows for the detection of specific DNA sequences in the genomes of a large number of bacteria (23). This approach can be utilized for the detection of genes coding for traits which enable bacterial survival in stressed environments. Following such genes in communities which develop under the influence of a specific stressor could aid our understanding of adaptive evolution in the natural environment. The development of a DNA gene probe for the detection of genes responsible for mercurial resistance in bacteria is described in this paper.

Mercury resistance is conferred by the inducible mercuric reductase enzyme which reduces the mercuric ion (Hg^{2+}) to the volatile elemental form (Hg^0) (57). This mechanism has been demonstrated in a diverse group of microorganisms, including enteric bacteria (49), *Staphylococcus aureus* (63), pseudomonads (8), *Bacillus* species (26, 60), *Thiobacillus ferrooxidans* (47), and *Mycobacterium scrofulaceum* (32). The genes coding for mercury resistance are organized in the *mer* operon, which includes at least four genes: *merR* (coding for the regulatory protein), *merT* (coding for membrane-bound mercury transport proteins), *merC* (coding for an additional positive regulatory function) (4), and *merA* (coding for the mercuric reductase enzyme) (18). The *mer* probe was constructed from this operon in the composite resistance plasmid R100 (NR1). This plasmid belongs to incompatibility group FII (IncFII), and it was originally isolated from *Shigella flexneri* (38). This operon has been extensively utilized in studies which have elucidated the genetics of mercury resistance (19, 37, 40), and it was therefore chosen for the preparation of the *mer* probe. Miki et al. (33) have shown that *mer* in plasmid R100 is located on

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TABLE 1. Bacterial strains and plasmids

Bacteria (plasmid)	Relevant phenotypic markers[a]	Reference
E. coli HMS174		
E. coli HMS174(pACNR25)	Tc^r Cm^s Hg^{ss}	This paper
E. coli KP245(pRR134)	Ap^r Hg^{ss}	33
E. coli HMS174(pACYC184)	Cm^r Tc^r	7
E. coli DU1040(R831)	Hg^r PMA^r	49
E. coli SK1592(pDB7)	Hg^r Tc^r	
E. coli UB281(pUB932)	Hg^r Tc^r	
E. coli CR34A(pRR12)	Cm^r Sm^- Sp^r Su^r Hg^r	35
E. coli W3110(R702)	Hg^r	49
B. cereus 5	Hg^r	26
B. cereus ATCC 14579		
T. ferrooxidans BA-4	Hg^r	47
T. ferrooxidans BA-4C	Hg^s	47
S. aureus RN453(pI258)	Hg^r Pc^r	41
S. aureus RN450	Hg^s	
M. scrofulaceum W262	Hg^r	32
M. scrofulaceum W262C	Hg^s	32

[a] r, Resistant; s, sensitive; ss, supersensitive; PMA, phenylmercuric acetate.

*Eco*RI restriction fragments H and I. Fragment H contains the junction (which consists of insertion sequence IS*1*b) between the r-determinant and the resistance transfer factor in R100 (59). The current model of the *mer* operon (18) implies that *mer* sequences start immediately to the right of IS*1*b, and the minimal size of the operon is estimated to be 3 kilobases (kb) (19). The *Eco*RI restriction site that separates fragments H and I is located within *merA*, leaving 2.7 kb of the operon which carries *merR* and *merA* on *Eco*RI fragment H (19). The *mer* probe described here encompasses the majority of these 2.7 kb.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids and their relevant phenotypic markers are listed in Table 1. *Bacillus cereus* 5 (26) was received from K. Izaki. *T. ferrooxidans* BA-4 and BA-4C (47) were sent by G. Olson. *E. coli* KP245(pRR134) (33) and CR34A(pRR12) (35) were supplied by R. Rownd and B. Petterson. *E. coli* strains harboring the following plasmids were provided by A. Summers: R831, R702 (49), pDB7, and pUB932. *S. aureus* RN450 and RN453(pI258) (41) were from I. Edelman and R. Novick, and *M. scrofulaceum* W262 and W262C (32) were from S. Meissner and J. Falkinham. Cultures were routinely maintained throughout this study on media amended with the drugs which selected for the plasmids.

Media and growth conditions. L broth and L agar (6) were routinely employed for the cultivation of *E. coli*, *Bacillus* species, and *S. aureus* strains. *E. coli* and *S. aureus* were grown at 37°C, and *Bacillus* cultures were grown at 30°C. *T. ferrooxidans* strains were grown chemolithotrophically in 9K medium as described by Silverman and Lundgren (52). *M. scrofulaceum* strains were cultivated as previously described (32).

Plasmid DNA preparation. Large-scale plasmid DNA preparations were obtained by the method described by Clewell and Helinski (9). *E. coli* KP245(pRR134) was grown in the presence of 50 μg of ampicillin (Sigma Chemical Co., St. Louis, Mo.) per ml, and *E. coli* HMS174(pACNR25) was grown in a medium amended with 12.5 μg of tetracycline (Sigma) per ml. Plasmid amplification in preparations of KP245(pRR134) and HMS174(pACNR25) was achieved by the addition of 100 μg of chloramphenicol (Sigma) per ml and 300 μg of spectinomycin (The Upjohn Co., Kalamazoo, Mich.) per ml, respectively, to cultures with optical densities of 0.85 at 600 nm. The cultures were then incubated overnight at 37°C before harvesting.

Preparation and isolation of DNA restriction fragments. DNA was digested with the appropriate restriction enzyme as recommended by the manufacturer. About 100 μg of the digested DNA was loaded into a 1% agarose gel (Bethesda Research Laboratories Inc., Gaithersburg, Md.) which contained 0.05 μg of ethidium bromide per ml and electrophoresed in TBE buffer (89 mM Tris base, 89 mM boric acid, 2.5 mM EDTA [pH 8.2]) at 40 mA overnight at room temperature. DNA fragments were electroeluted and recovered from the gel by either one of two methods. The gel slice containing the desired DNA band was placed in dialysis tubing, and the DNA was electrophoresed (70 mA for ca. 40 min) into 0.5× TBE buffer until it completely migrated out of the gel (as observed under UV light). The DNA was then purified and concentrated by using an Elutip column (Schleicher & Schuell Inc., Keene, N.H.) as recommended by the manufacturer. Alternatively, DNA bands were electroeluted into DE81 filter paper (Whatman Ltd.) and recovered as described by Fouts et al. (20).

Restriction map. *Eco*RI restriction fragment H was obtained, as described above, from digests of pRR134, and its restriction map was constructed by using the following enzymes: *Pst*I, *Hin*dIII, *Nco*I, *Ava*II, *Bst*EII, *Pvu*II, and *Hin*cII (Fig. 1). The following enzymes did not cut fragment H: *Bal*I, *Hpa*I, *Sal*I, *Sst*I, *Xho*I, *Bgl*II, *Sma*I, *Bcl*II, *Ava*I, and *Tag*I. Restriction enzymes were purchased from Bethesda Research Laboratories; New England BioLabs Inc., Beverly, Mass.; Boehringer Mannheim Biochemicals, Indi-

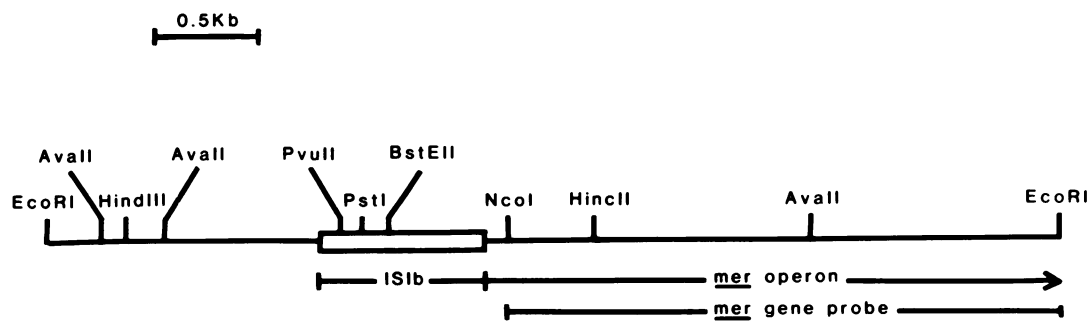

FIG. 1. Endonuclease restriction map of *Eco*RI fragment H from plasmid R100. The arrow at the right-hand side of the line describing the *mer* operon indicates the continuation of the operon beyond the RI site.

anapolis, Ind.; and Codon, San Francisco, Calif. Digests (0.15 μg of DNA digested with 5 U of each enzyme) were carried out by using the low-, medium-, and high-salt solutions according to the directions of Maniatis et al. (28). Digested DNA samples were electrophoresed in 1 or 1.5% agarose gels (depending on the expected size of the fragments) in TBE at 70 mA for 2 to 4 h. Restriction fragment sizes were measured from photographs of gels. *Hin*dIII digests of bacteriophage lambda DNA were employed as size markers.

Cloning of a 2.6-kb *Nco*I-*Eco*RI fragment. The 2.6-kb *Nco*I-*Eco*RI fragment, which spans the right-hand side of fragment H (Fig. 1), was cloned into the *Eco*RI site of the vector pACYC184(7). Synthetic *Eco*RI linkers (600 pmol) (Bethesda Research Laboratories) were ligated by T4 DNA ligase to 1 μg of the blunt-ended 2.6-kb fragment. Blunt ending was performed in nick translation buffer (28) supplemented with 2 mM deoxynucleoside 5'-triphosphate and 50 μg of bovine serum albumin per ml by the Klenow fragment of DNA polymerase I (Bethesda Research Laboratories) at 22°C for 25 min. All preparations of the vector (1 μg) and the DNA fragment (ca. 0.4 μg) and their ligation were carried out by using common cloning procedures (28).

Transformation and selection of clones containing recombinant plasmids. Competent *E. coli* HMS174 cells were prepared from a 100-ml culture (optical density at 600 nm of 0.6) by the method of Dagert and Ehrlich (11). Competent cells (100 μl) were mixed with the ligated DNA, incubated on ice for 20 min, and then transferred to a 37°C water bath for 10 additional min. Two milliliters of L broth was added, and the suspensions were further incubated at the same temperature for 2 h with shaking. Samples (25 μl) were plated on L agar containing 12.5 μg of tetracycline per ml. Colonies grown after 24 h of incubation at 37°C were transferred to a medium containing chloramphenicol (10 μg/ml). Sixteen Cm^s Tc^r clones were selected and tested for the presence of recombinant plasmids. Plasmid DNA was isolated from 1 ml of overnight cultures by the method of Holmes and Quigley (25). DNA preparations were digested with *Eco*RI and electrophoresed as described above. *Eco*RI digests of 2 of the 16 tested clones were apparently identical and contained two fragments of ca. 2.6 and 4.3 kb. Digestion of the DNA of these two clones with *Hin*cII yielded three fragments of ca. 1.1, 2.5, and 3.0 kb. These values corresponded to the number and size of *Hin*cII fragments expected for a recombinant plasmid composed of pACYC184 and the 2.6-kb *Nco*I-*Eco*RI fragment (7; Fig. 1). The recombinant plasmid was designated pACNR25 (Table 1).

DNA-DNA hybridization. The 2.6-kb fragment (obtained by digesting pACNR25 with *Eco*RI as described above) was labeled in vitro with 5'-[α-^{32}P]dCTP with a nick translation kit (Bethesda Research Laboratories) as recommended by the manufacturer. The DNA of the tested strains was denatured and fixed to nitrocellulose paper (BA85, 0.45 μm) (Schleicher & Schuell) by several methods. The colony hybridization procedure (23) was employed with all *E. coli* derivatives (Table 1). Crude DNA extracts were prepared from those strains which could not be analyzed by colony hybridization. Crude extracts of *S. aureus* strains were prepared from 30-ml cultures grown overnight as described by Forbes and Schaberg (17). *T. ferrooxidans* strains were grown to cell densities of 3.0×10^7 cells per ml. Cells were directly counted with an Improved Hausser-Neubauer counter (C. A. Hausser & Son, Philadelphia, Pa.). Strain BA-4 was grown in the presence of 5 μM Hg. Cultures (500 ml) were harvested as previously described (52), and their crude extracts were prepared by the method of Martin et al. (29). Crude extracts of *B. cereus* 5 and ATCC 14579 were obtained as described by Silberstein and Cohen (51), except that lysis was achieved with 1% sodium dodecyl sulfate without the addition of sodium perchlorate. Crude extracts of *M. scrofulaceum* W262 and W262C were prepared from cultures with cell density corresponding to an optical density at 550 nm of 0.5 by the method of Crawford and Bates (10). Crude extracts were sheared by five passages through a 23-gauge syringe needle to allow pipetting. One microliter of NaOH (1 N) was added to 10 μl of these solutions, which were then placed in a boiling water bath for 5 min. Solutions were brought to a final concentration of 0.2 M Tris (pH 7.5), and the cooled samples were spotted on nitrocellulose paper which was previously treated with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Prehybridization (2 h at 68°C) and hybridization (40 h at 68°C) were carried out as described by Grunstein and Hogness (23). Filters were washed and prepared for autoradiography as described by Maniatis et al. (28). Autoradiography was carried out at −70°C with XRP-5 X-ray film (Eastman Kodak Co., Rochester, N.Y.).

Stringency control. The stringency of DNA hybrid formation was controlled by carrying out hybridizations at different formamide concentrations. Crude extracts of *E. coli* HMS174 and HMS174(pACNR25) were prepared as described by Clewell and Helinski (9). DNA was spotted on nitrocellulose filter paper as described above. Filters were prehybridized for 3.5 h at 37°C with shaking in a solution containing 5× SSC, 10× Denhardt (1× Denhardt is 0.2 g of Ficoll, 0.2 g of polyvinylpyrrolidone, 0.2 g of bovine serum albumin in 1 liter of 3× SSC), 0.1% sodium dodecyl sulfate, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.0), and 100 μg of calf thymus DNA. Hybridization was carried out for 40 h under the same conditions, except that the hybridization solutions included formamide (MCB Reagents, Cincinnati, Ohio) at final concentrations of 0, 10, 20, 30, 40, and 50%. After hybridization, the filters were washed four times at 52°C with 2× SSC–0.1% sodium dodecyl sulfate solution, once with 0.1× SSC–0.1% sodium dodecyl sulfate, and the last time with 0.1× SSC. Autoradiography was performed as described above.

RESULTS

Construction of the *mer* probe. A restriction enzyme map of fragment H was constructed (Fig. 1) to ensure that the *mer* probe did not contain any additional DNA sequences. This step was essential to ensure the specificity of the probe (see below). Fragment H was obtained from the recombinant plasmid pRR134 (33). IS1b was positioned by Ohtsubo and Ohtsubo (43) between 1.4 and 2.1 kb to the right of the *Eco*RI restriction site; this site creates the left border of fragment H (Fig. 1). These authors have also determined the *Pst*I and *Hin*dIII restriction sites which lie in this region (43). Digestions with these enzymes were, therefore, performed to place IS*1*b on fragment H. This position was further confirmed by digests with *Bst*EII and *Pvu*II, each of which has a single restriction site in fragment H (Fig. 1) as well as in IS*1*b (43). Placing IS*1*b between 1.4 and 2.1 kb to the right of the left end of fragment H leaves the expected 2.7-kb *mer* sequences which span the right part of fragment H (Fig. 1). Of the 17 restriction enzymes which were employed in this study, only 3 were found to cut in the *mer* region of fragment H. Single and double digests located an *Ava*II site, an *Hin*cII site, and an *Nco*I site 1.08, 2.2, and 2.6 kb, respectively, to

the left of the right border of fragment H (Fig. 1). The restriction site closest to the right end of IS1b was that of *Nco*I, which divides fragment H into two fragments of 2.6 and 2.2 kb. The 2.6-kb fragment was the largest fragment to contain *mer* sequences exclusively, and it was chosen to be used as the *mer* probe.

Range of specificity of the *mer* gene probe. The specificity of the *mer* gene probe to bacterial DNA sequences which code for mercury resistance was determined by DNA-DNA hybridization between radioactively labeled probe sequences and DNA of bacteria previously shown to possess the mercuric reductase enzyme. The colony hybridization procedure was employed for those strains which could be grown as colonies on nitrocellulose filter paper and lysed by the common procedure described by Grunstein and Hogness (23). Crude cell extracts of strains which did not form colonies (i.e., *T. ferrooxidans*) or did not lyse on the filter paper (*S. aureus* and *B. cereus* strains) were prepared and spotted on the filter paper as described above. The stringency of hybridization was determined by carrying out hybridization of strains HMS174 (negative control) and HMS174(pACNR25) (positive control) in the presence of increased formamide concentrations. Hybridization to pACNR25 occurred under all tested conditions. The negative control hybridized with the *mer* probe in the presence of 30 to 50% formamide. Hybridization in high-salt solution at 68°C (see above) reproducibly differentiated between the positive (clear strong signals) and the negative (no signal) controls, and this procedure was therefore routinely employed throughout this study. Hybridization was carried out in the presence of 25 μg of cold pACYC184 DNA per ml because preliminary experiments indicated that the probe preparation contained contaminating vector DNA. This contamination resulted in hybridization to sequences other than *mer*. Hybridization was observed between the *mer* probe and DNA obtained from the following *E. coli* strains: W3110(R702), SK1592(pDB7), UB281(pUB932), CR34A(pRR12), DU1040(R831), and HMS174(pACNR25) and from *T. ferrooxidans* BA-4 and BA-4C (Fig. 2). DNA of *S. aureus* RN450 and RN453, *B. cereus* 5 and ATCC 14579, and *M. scrofulaceum* W262 and W262C did not hybridize with the *mer* gene probe.

R702 is an IncP plasmid originally isolated in New Jersey from *Proteus mirabilis* (49), and its mercury resistance system was studied by Summers and Kight-Olliff (56). The mercury resistance transposon 501 (Tn*501*), which was originally found in *Pseudomonas aeruginosa* PAT (55), was included in this study in the recombinant plasmid pUB932. R831 is an IncM broad-spectrum mercury resistance plasmid, conferring resistance to inorganic mercury salts and organomercurial compounds. It was initially isolated in Boston from *Serratia marcescens* (49), and its mercuric reductase and organomercurial hydrolase enzymes were investigated by Schottel (50). Two of the tested strains carried the mercury resistance system of plasmid R100. pRR12 is a copy number mutant of R100 (35) carrying the complete r-determinant region of this plasmid, and pDB7 is a recombinant plasmid in which the R100 *mer* operon has been cloned into the *Pst*I site of pBR322. The observed signals varied in their intensities, suggesting that *E. coli* W3110(R702) had more copies of the *mer* genes than the other strains (Fig. 2). However, any quantitation of gene copy number is questionable due to the crudeness of the colony hybridization method which permits variability as a result of colony size and age.

T. ferrooxidans BA-4 is a mercury-resistant strain which

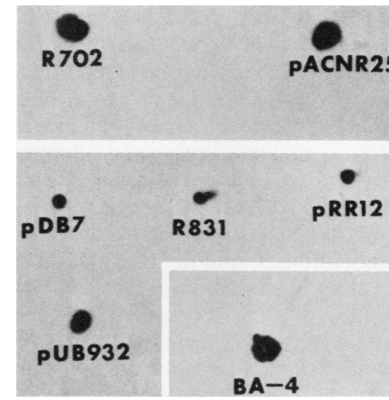

FIG. 2. Specificity range of the *mer* gene probe. Colonies of bacterial strains containing the indicated plasmids and crude extract of *T. ferrooxidans* BA-4 were prepared for hybridization as described in the text. DNA of *E. coli* strains was hybridized overnight in 5 ml of hybridization buffer containing 25 μg of cold pACYC184 DNA per ml. Radioactively labeled probe was then added to ca. 8.0×10^6 cpm (specific activity, 18 Ci/μmol). Autoradiograms of R702 and pACNR25 were obtained after an overnight exposure and of R831, pDB7, pRR12, and pUB932 after 3 days. Dotted DNA of *T. ferrooxidans* strains was hybridized with ca. 7.0×10^6 cpm (specific activity, 147.5 Ci/μmol). The autoradiogram was obtained after 3 days of exposure at −70°C.

was isolated from a coal strip mine in Wyoming and whose mercuric reductase enzyme has been described (47). The *mer* gene in this organism has not been shown yet to be on a plasmid. A hybridization signal (Fig. 2) indicated the existence of sequences homologous to the R100 *mer* operon in *T. ferrooxidans* BA-4. A much weaker signal was observed with DNA of the mercury-sensitive cured derivative strain BA-4C (data not shown). The crude extracts of these strains were obtained from cultures with similar cell masses. It is therefore concluded that this difference in hybridization signal intensities indicates a higher occurrence of the *mer* gene in strain BA-4. Thus, the *mer* probe hybridized with the DNA of all of the gram-negative mercury-resistant organisms which were included in this study. These strains were isolated from a variety of geographical regions and represented a wide range of bacterial genera, and the *mer* genes were carried on plasmids of different incompatibility groups.

The mercury resistance systems of three gram-positive bacteria have been investigated to date. *mer* in *S. aureus* is carried on penicillinase plasmids (41). Izaki (26) has isolated and characterized the mercuric reductase enzyme of *B. cereus* 5, and Meissner and Falkinham (32) have studied this system in *M. scrofulaceum*. Crude extracts of *S. aureus* RN450 and RN453 (same as RN450, except harboring the penicillinase plasmid pI258), *B. cereus* 5 and ATCC 14579, and *M. scrofulaceum* W262 and its cured derivative strain W262C did not hybridize with the *mer* probe under any of the described hybridization conditions. Hybridization in the presence of gradually increasing concentrations of formamide, utilized to control the stringency required for hybrid formation, yielded negative results (data not shown). Thus, if any homology exists between the *mer* probe which originated in a gram-negative organism and the DNA of the tested mercury-resistant gram-positive bacteria, it is below the level detected by conventional hybridization procedures.

DISCUSSION

The utilization of DNA-DNA hybridization for the detection of specific DNA sequences in bacteria isolated from

natural samples has been previously described. However, these studies were mostly concerned with the detection of pathogenic microorganisms in foods (16, 24) and waters (14). The preparation of the *mer* gene probe described in this paper was aimed at the development of a means for the study of genetic change mechanisms in the adaptation of natural bacterial communities to a stressor. Several reasons suggest that bacterial mercury resistance is an appropriate model system for this type of study. (i) Mercury is a deleterious environmental pollutant, and the role played by microorganisms in its cycling in the environment has been extensively investigated (27, 39, 45, 54). (ii) The genes coding for mercury resistance (i.e., the *mer* operon) have been shown to be carried on plasmids (58) and transposons (55). (iii) The biological evolution of elemental mercury (supposedly by the mercuric reductase enzyme) from water (39) and soils (48) has been demonstrated in situ, and microorganisms isolated from these environments reduced mercurials to the elemental form (44, 60, 61).

The successful development of a gene probe required that any nonspecific DNA sequences be separated from *mer* sequences to prevent background hybridization. In addition, the range of specificity of the probe was determined to define what component of the bacterial community can be studied with this probe.

The *mer* operon in R100 is adjacent to IS*1*b (33), which is widely distributed among some gram-negative bacteria (42). Therefore, the preparation of a probe required the careful elimination of this element. Utilizing the *Nco*I restriction site, which is located about 100 base pairs from the right end of IS*1*b, as the left border of the gene probe (Fig. 1) clearly secured a complete separation between *mer* and any additional nonspecific DNA sequences. Although the gene probe consists exclusively of *mer* DNA, hybridization with other sequences in the bacterial genome cannot be ruled out. This point is especially important in light of recent reports indicating that the mercuric reductase polypeptide shares an extensive amino acid homology with other enzymes, such as glutathione reductase (5) and lipoamide dehydrogenase (21). Obviously, if these homologies were also present in the DNA sequences of the genes coding for these enzymes, they could result in hybridization with the *mer* probe. Indeed, the observed hybridization of the negative control strain (HMS174) under some stringencies may be explained by these homologies. However, it seems that at the stringency conditions which were routinely used in this study, these homologies did not interfere with the specificity of the probe.

The specificity range of the *mer* probe was determined by hybridization with DNA isolated from resistant bacteria which had been previously shown to possess the reductase activity. The results strongly suggested (Fig. 2) a relatedness among the DNA sequences which code for mercury resistance in the tested gram-negative bacteria. The observation that DNA of gram-positive resistant bacteria did not hybridize with the probe DNA indicated little (if any) sequence homology between the two mercury resistance systems. Although the number of tested strains was small, these results suggested that bacterial mercury resistance systems evolved after the divergence of gram-negative from gram-positive bacteria. However, nucleic acid or amino acid sequence analyses are needed for the determination of evolutionary pathways of enzyme systems.

The distinction between the mercury resistance systems of gram-negative and gram-positive organisms which was revealed by DNA-DNA hybridization in this study is in full agreement with previously published reports (18, 40, 47). These investigators based their conclusions on the physiological properties and serological interactions of the reductases (47) and, more recently, on DNA sequence analysis (5, 40). For example, mercuric reductases originating in gram-negative organisms are heat stable (or even heat stimulated), whereas those produced by gram-positive organisms are inactivated at 60°C (47). Antisera prepared against reductases of gram-negative organisms reacted, at least partially, with the enzymes of other gram-negative organisms but not with those of gram-positive organisms (47). However, the reductases of gram-negative bacteria are divided based upon their serological behavior to type I and type II, which are prototyped by the reductases of Tn*501* and R831, respectively. Despite these differences, the reductases of gram-negative bacteria are closely related, as is indicated by serological cross-reactivity (47), complementation experiments (56), and DNA sequence homologies. The DNA sequences of the Tn*501* reductase and that of R100s share about 60% homology (A. O. Summers, personal communication), and the results presented in Fig. 2 suggest that homology extends to other gram-negative reductases.

Olson et al. (47) reported that although the mercuric reductase from *T. ferrooxidans* BA-4 had all the properties of the gram-negative enzymes, it was not inactivated by antisera prepared against the reductases coded by Tn*501* or R831. The hybridization observed between the *mer* probe and the DNA of this strain (Fig. 2) indicates that sequence homology between these two mercury resistance systems occurs. These results could be explained if regions determining antigenic specificity were non-homologous in the *merA* genes of *T. ferrooxidans* and Tn*501* and R831. Alternatively, the *mer* probe consists of additional sequences to *merA* (Fig. 1) which could bear base homology with the mercury resistance gene(s) of *T. ferrooxidans* BA-4.

The demonstrated specificity range of the *mer* gene probe suggests a model system for the study of the role of genetic change in adaptation to mercury among gram-negative natural bacterial communities. The investigation of this process in gram-positive bacteria and other groups of microorganisms would require the development of other probes specific for the mercury resistance genes of these organisms.

ACKNOWLEDGMENTS

We thank Jerry Manning and the staff of his laboratory from Molecular Biology and Biochemistry, School of Biological Sciences, University of California, Irvine, for the hospitality, expertise, and support which made this work possible. Gratitude is extended to the numerous individuals who provided bacterial strains and advice regarding their cultivation. The help of A. O. Summers is especially appreciated. In addition, the authors acknowledge A. O. Summers and J. Watterson for critical review of this report.

LITERATURE CITED

1. **Allen, D. A., B. Austin, and R. R. Colwell.** 1977. Antibiotic resistance patterns of metal-tolerant bacteria isolated from an estuary. Antimicrob. Agents Chemother. **12**:545–547.
2. **Altherr, M. R., and K. L. Kasweck.** 1982. In situ studies with membrane diffusion chambers of antibiotic resistance transfer in *Escherichia coli*. Appl. Environ. Microbiol. **44**:838–843.
3. **Barkay, T., and R. R. Colwell.** 1983. Altered mercury transport in a mutant of *Pseudomonas fluorescens* B69. J. Gen. Microbiol. **129**:2945–2950.
4. **Barrineau, P., and A. O. Summers.** 1983. A second positive regulatory function in the *mer* (mercury resistance) operon. Gene **25**:209–221.

5. **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.
6. **Carlton, B. C., and B. J. Brown.** 1981. Gene mutation, p. 222–242. *In* P. Gerhardt (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
7. **Chang, A. C. Y., and S. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the p15A cryptic miniplasmid. J. Bacteriol. **134**: 1141–1156.
8. **Clark, D. L., A. A. Weiss, and S. Silver.** 1977. Mercury and organomercurial resistances determined by plasmids in *Pseudomonas*. J. Bacteriol. **132**:186–196.
9. **Clewell, D. B., and D. R. Helinski.** 1969. Supercoiled circular DNA-protein complex in *Escherichia coli*: purification and induced conversion to an open circular DNA form. Proc. Natl. Acad. Sci. U.S.A. **62**:1159–1166.
10. **Crawford, J. T., and J. H. Bates.** 1979. Isolation of plasmids from mycobacteria. Infect. Immun. **24**:979–981.
11. **Dagert, M., and S. D. Ehrlich.** 1979. Prolonged incubation in calcium chloride improves the competence of *Escherichia coli* cells. Gene **6**:23–28.
12. **Davey, R. B., and D. C. Reanney.** 1981. Extrachromosomal genetic elements and the adaptive evolution of bacteria, p. 113–147. *In* M. K. Heacht, W. C. Steere, and B. Wallace (ed.), Evolution biology, vol. 13. Plenum Publishing Corp., New York.
13. **Doelman, P., and L. Haanstra.** 1979. Effects of lead on the soil bacterial flora. Soil Biol. Biochem. **11**:4487–4491.
14. **Echeverria, P., J. Seriwatana, O. Chityothin, W. Chaicumpa, and C. Tirapat.** 1982. Detection of enterotoxigenic *Escherichia coli* in water by filter hybridization with three enterotoxin gene probes. J. Clin. Microbiol. **16**:1086–1090.
15. **Farrar, W. E., Jr.** 1981. Evolution among antibiotic resistance plasmids in the hospital environment, p. 1–10. *In* S. B. Levy, R. C. Clowes, and E. L. Koenig (ed.), Molecular biology, pathogenicity and ecology of bacterial plasmids. Plenum Publishing Corp., New York.
16. **Fitts, R., M. Diamond, C. Hamilton, and M. Neri.** 1983. DNA-DNA hybridization assay for detection of *Salmonella* spp. in foods. Appl. Environ. Microbiol. **46**:1146–1151.
17. **Forbes, B. A., and D. R. Schaberg.** 1983. Transfer of resistance plasmids from *Staphylococcus epidermidis* to *Staphylococcus aureus*: evidence for conjugative exchange of resistance. J. Bacteriol. **153**:627–634.
18. **Foster, T. J.** 1983. Plasmid-determined resistance to antimicrobial drugs and toxic metal ions in bacteria. Microbiol. Rev. **47**:361–409.
19. **Foster, T. J., H. Nakahara, A. A. Weiss, and S. Silver.** 1979. Transposon A-generated mutations in the mercuric resistance genes of plasmid R100-1. J. Bacteriol. **140**:167–181.
20. **Fouts, D. L., J. E. Manning, G. M. Fox, and C. W. Schmid.** 1981. A complex repeated DNA sequence within the Drosophila transposable element copia. Nucleic Acids Res. **9**:7053–7064.
21. **Fox, B. S., and C. T. Walsh.** 1983. Mercuric reductase: homology to glutathione reductase and lipoamide dehydrogenase. Iodoacetamide alkylation and sequence of the active site peptide. Biochemistry **22**:4082–4088.
22. **Graham, J. B., and C. A. Istock.** 1979. Gene exchange and natural selection cause *Bacillus subtilis* to evolve in soil culture. Science **240**:637–639.
23. **Grunstein, M., and D. S. Hogness.** 1975. Colony hybridization: a method for the isolation of cloned DNAs that contain a specific gene. Proc. Natl. Acad. Sci. U.S.A. **72**:3961–3965.
24. **Hill, W. E., W. L. Payne, and C. C. G. Aulisio.** 1983. Detection and enumeration of virulent *Yersinia enterocolitica* in food by DNA colony hybridization. Appl. Environ. Microbiol. **46**: 636–641.
25. **Holmes, D. S., and M. Quigley.** 1981. A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. **114**: 193–197.
26. **Izaki, K.** 1981. Enzymatic reduction of mercurous and mercuric ions in *Bacillus cereus*. Can. J. Microbiol. **27**:192–197.
27. **Jensen, S., and A. Jernelov.** 1969. Biological methylation of mercury in aquatic organisms. Nature (London) **223**:753–754.
28. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. **Martin, P. A. W., P. R. Dugan, and O. H. Tuovinen.** 1981. Plasmid DNA in acidophilic, chemolithotrophic thiobacilli. Can. J. Microbiol. **27**:850–853.
30. **McDonald, J. F.** 1983. The molecular basis of adaptation: a critical review of relevant ideas and observations. Annu. Rev. Ecol. Syst. **14**:77–102.
31. **McNicol, L. A., T. Barkay, M. J. Voll, and R. R. Colwell.** 1982. Plasmid carriage in *Vibrionaceae* and other bacteria isolated from the aquatic environment. J. Wash. Acad. Sci. **72**:60–66.
32. **Meissner, P. S., and J. O. Falkinham III.** 1984. Plasmid-encoded mercuric reductase in *Mycobacterium scrofulaceum*. J. Bacteriol. **157**:669–672.
33. **Miki, T., A. M. Easton, and R. H. Rownd.** 1978. Mapping of the resistance genes of the R plasmid NR1. Mol. Gen. Genet. **158**:217–224.
34. **Mitsuhashi, S.** 1971. Epidemiology of bacterial drug resistance, p. 1–23. *In* R. S. Mitsuhashi (ed.), Transferable drug resistance factor. University Park Press, Baltimore.
35. **Morris, C. F., H. Hashimoto, S. Mickel, and R. Rownd.** 1974. Round of replication mutant of a drug resistance factor. J. Bacteriol. **118**:855–866.
36. **Morrison, W. D., R. V. Miller, and G. S. Sayler.** 1978. Frequency of F116-mediated transduction of *Pseudomonas aeruginosa* in a freshwater environment. Appl. Environ. Microbiol. **36**:724–730.
37. **Nakahara, H., T. G. Kinscherf, S. Silver, T. Miki, A. M. Easton, and R. H. Rownd.** 1979. Gene copy number effects in the *mer* operon of plasmid NR1. J. Bacteriol. **138**:284–287.
38. **Nakaya, R., A. Nakamura, and Y. Murata.** 1960. Resistance transfer agents in *Shigella*. Biochem. Biophys. Res. Commun. **3**:654–659.
39. **Nelson, J. D., and R. R. Colwell.** 1975. The ecology of mercury-resistant bacteria in the Chesapeake Bay. Microb. Ecol. **1**:191–218.
40. **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.
41. **Novick, R. P., and C. Roth.** 1968. Plasmid-linked resistance to inorganic salts in *Staphylococcus aureus*. J. Bacteriol. **95**:1335–1342.
42. **Nyman, K., K. Nakamura, H. Ohtsubo, and E. Ohtsubo.** 1981. Distribution of the insertion sequence IS1 in gram-negative bacteria. Nature (London) **289**:609–612.
43. **Ohtsubo, H., and E. Ohtsubo.** 1978. Nucleotide sequence of an insertion element, IS1. Proc. Natl. Acad. Sci. U.S.A. **75**: 615–619.
44. **Olson, B. H., T. Barkay, and R. R. Colwell.** 1979. Role of plasmids in mercury transformation by bacteria isolated from the aquatic environment. Appl. Environ. Microbiol. **38**:478–485.
45. **Olson, B. H., and R. C. Cooper.** 1974. *In situ* methylation of mercury in estuarine sediments. Nature (London) **252**:682–683.
46. **Olson, B. H., and I. Thornton.** 1982. The resistance patterns to metals of bacterial populations in contaminated land. J. Soil Sci. **33**:271–277.
47. **Olson, G. J., F. D. Porter, J. Rubinstein, and S. Silver.** 1982. Mercuric reductase enzyme from a mercury-volatilizing strain of *Thiobacillus ferrooxidans*. J. Bacteriol. **151**:1230–1236.
48. **Rogers, R. D., and J. C. McFarlane.** 1979. Factors influencing the volatilization of mercury from soil. J. Environ. Qual. **8**:255–260.
49. **Schottel, J., A. Mandal, D. Clark, S. Silver, and R. W. Hedges.** 1974. Volatilisation of mercury and organomercurials determined by inducible R-factor systems in enteric bacteria. Science **251**:335–337.
50. **Schottel, J. J.** 1978. The mercuric and organomercurial detoxifying enzymes from a plasmid-bearing strain of *Escherichia coli*.

J. Biol. Chem. **253**:4341–4349.
51. **Silberstein, Z., and A. Cohen.** 1978. Hybridization analysis of restriction endonuclease DNA fragments of *Bacillus cereus* transcribed during spore outgrowth. J. Bacteriol. **134**:1081–1088.
52. **Silverman, M. P., and D. G. Lundgren.** 1959. Studies on the chemoautotrophic iron bacterium *Ferrobacillus ferrooxidans*. I. An improved medium and a harvesting procedure for securing high cell yields. J. Bacteriol. **77**:642–647.
53. **So, M., F. Heffron, and B. J. McCarthy.** 1979. The *E. coli* gene encoding heat stable toxin is a bacterial transposon flanked by inverted repeats of IS1. Nature (London) **277**:453–456.
54. **Spangler, W. J., J. L. Spigarelli, J. M. Rose, and H. M. Miller.** 1973. Methylmercury: bacterial degradation in lake sediments. Science **180**:192–193.
55. **Stanisich, V. A., P. M. Bennett, and M. H. Richmond.** 1977. Characterization of a translocation unit encoding resistance to mercuric ions that occurs on a nonconjugative plasmid in *Pseudomonas aeruginosa*. J. Bacteriol. **129**:1227–1233.
56. **Summers, A. O., and L. Kight-Olliff.** 1980. Tn1 generated mutants in the mercuric ion reductase of the *Inc*P plasmid, R702. Mol. Gen. Genet. **180**:91–97.
57. **Summers, A. O., and E. Lewis.** 1973. Volatilization of mercuric chloride by mercury-resistant plasmid-bearing strains of *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. J. Bacteriol. **113**:1070–1072.
58. **Summers, A. O., and S. Silver.** 1978. Microbial transformations of metals. Annu. Rev. Microbiol. **32**:637–672.
59. **Tanaka, N., J. H. Cramer, and R. H. Rownd.** 1976. *Eco*RI restriction endonuclease map of the composite R plasmid NR1. J. Bacteriol. **127**:619–636.
60. **Timoney, J. F., J. Port, J. Giles, and J. Spanier.** 1978. Heavy-metal and antibiotic resistance in the bacterial flora of sediments of New York Bight. Appl. Environ. Microbiol. **36**:465–472.
61. **Tonomura, K., and F. Kanazaki.** 1969. The reductive decomposition of organic mercurials by cell-free extract of a mercury-resistant pseudomonad. Biochim. Biophys. Acta **184**:227–229.
62. **Wassel, R. A., and A. L. Mills.** 1983. Changes in water and sediment bacterial community structure in a lake receiving acid mine drainage. Microb. Ecol. **9**:155–169.
63. **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.