APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1997, p. 4914–4919 Vol. 63, No. 12
0099-2240/97/$04.00+0

Analysis of *mer* Gene Subclasses within Bacterial Communities in Soils and Sediments Resolved by Fluorescent-PCR–Restriction Fragment Length Polymorphism Profiling

KENNETH D. BRUCE*

School of Biological Sciences, Donnan Labs, University of Liverpool, Liverpool, L69 7ZD, United Kingdom

Received 6 March 1997/Accepted 3 July 1997

Bacterial *mer* (mercury resistance) gene subclasses in mercury-polluted and pristine natural environments have been profiled by Fluorescent-PCR–restriction fragment length polymorphism (FluRFLP). For FluRFLP, PCR products were amplified from individual *mer* operons in mercury-resistant bacteria and from DNA isolated directly from bacteria in soil and sediment samples. The primers used to amplify DNA were designed from consensus sequences of the major subclasses of archetypal gram-negative *mer* operons within Tn*501*, Tn*21*, pDU1358, and pKLH2. Two independent PCRs were used to amplify two regions of different lengths (*merRTΔP* [ca. 1 kb] and *merR* [ca. 0.4 kb]) starting at the same position in *merR*. The oligonucleotide primer common to both reactions (FluRX) was labelled at the 5′ end with green (TET) fluorescent dye. Analysis of the *mer* sequences within databases indicated that the major subclasses could be differentiated on the basis of the length from FluRX to the first *Fok*I restriction endonuclease site. The amplified PCR products were digested with *Fok*I restriction endonuclease, with the restriction digest fragments resolved on an automated DNA sequencing machine which detected only those bands labelled with the fluorescent dye. For each of the individual *mer* operon sources examined, this single peak (in bases) position was observed in separate digests of either amplified region. These peak positions were as predicted on the basis of DNA sequence. *mer* PCR products amplified from DNA extracted directly from soil and sediment bacteria were studied in order to determine the profiles of the major *mer* subclasses present in each natural environment. In addition to peaks of the expected sizes, extra peaks were observed which were not predicted on the basis of DNA sequence. Those appearing in the restriction endonuclease digests of both study regions were presumed to be novel *mer* types. Genetic heterogeneity within and between mercury-polluted and pristine sites has been studied by this technique. Profiles generated were highly similar for samples taken within the same soil type. The profiles, however, changed markedly on crossing from one soil type to another, with gradients of the different groupings of *mer* genes identified.

Mercury resistance genes have provided a model system for a wide range of molecular ecological studies, such as the analysis of gene diversity and evolution within bacterial communities in natural environments (4, 19, 29). Bacterial *mer* (mercury resistance) operons have been identified across a wide range of bacterial genera and are distributed globally (27). The mechanism of bacterial resistance to mercurial compounds by reducing them to elemental mercury has been well characterized genetically (17). In the process of resistance, gene products from the *mer* operon transport into the cell and then reduce divalent mercury ions to the less toxic elemental mercury, which volatilizes from the cell (30).

mer genes show structural variation with a division made between narrow-spectrum and broad-spectrum types of *mer* operon, with the former conferring resistance to inorganic mercurial compounds alone and the latter effecting resistance to both inorganic and organomercurial compounds, due to the *merB* (organomercurial lyase) gene product (17). Variation also exists at the level of the DNA sequence. This variation can be used to examine the evolutionary relationships among different sequences. Previous work has indicated that the *mer* regions of gram-negative bacteria could be distinguished on the basis of PCR-restriction fragment length polymorphism (RFLP) into a number of different subclasses (25).

Despite their fundamental roles in ecosystem processes, work is still required to describe the distribution and diversity of the genetic component of bacterial communities in natural environments. Molecular biological techniques, as opposed to conventional microbiological approaches, are increasingly being used to study these bacterial communities to avoid the largely unrepresentative nature of bacterial cultivation (2). Most molecular studies have either used DNA probes to hybridize to nucleic acid within individual microbial cells (2) or analyzed nucleic acids extracted directly from noncultivated cells (9). For the latter, PCR is frequently used to amplify specific genes from mixed populations of target sequences.

The analysis of mixed-community PCR products has, however, proved time consuming and potentially biased due to the previous requirement for cloning prior to analysis. It has therefore been important to develop methods which resolve the diversity of the amplified products in a single electrophoretic profile. Of these methods, denaturing gradient gel electrophoresis (23, 24, 32) and those methods based on RFLP analysis (22) have been used successfully to study the diversity of 16S ribosomal DNA and other genes.

This study develops the use of RFLP-based analyses. Following database searches, it was found that one restriction endonuclease, *Fok*I, could be used to differentiate among the

* Phone: 0151 794 3621. Fax: 0151 794 3655. E-mail: kenbruce@liv.ac.uk.

major subclasses of the archetypal gram-negative *mer* operon (Tn*501* [8], Tn*21* [5], pKLH2 [20], and pDU1358 [15]) on the basis of the length from the start of *merR* (regulatory region) to the first *Fok*I restriction site. The requirement for detection of this band among the other products was solved by fluorescent labelling of the oligonucleotide at the common start position in *merR*. Following digestion with *Fok*I and separation by electrophoresis in an automated DNA sequencing machine, only those bands which are fluorescently labelled are detected. This fluorescence-based technology has been used for the analysis of eukaryotic genes, e.g., in microsatellite variation in populations (21), and recently for model systems of bacterial genes (31).

mer genes from the archetypal gram-negative bacterial system have been amplified from DNA extracted from bacterial communities at four sites: Fiddlers Ferry sediment, Fiddlers Ferry soil, Tipperary soil, and Salter Brook Bridge soil. Following cloning of individual *mer* PCR products, RFLP was used to discriminate among the different *mer* types and thus study the *mer* gene distribution at a single position at a given study site (10). In the present study restriction endonuclease digests of fluorescently labelled *mer* amplicons were used to study *mer* gene diversity in a range of soil and sediment samples. The objective of the study was to use the *mer* gene model system to study genetic diversity within and between sites in order to define the spatial profiles of *mer* genes in natural environments.

MATERIALS AND METHODS

Soil and sediment samples. Soil and sediment samples were taken at the industrially polluted Fiddler's Ferry site on the River Mersey. The total mean (± standard deviation) mercury levels at this site were estimated previously as 0.441 ± 0.039 μg/g for soil samples and 0.161 ± 0.029 μg/g for sediment samples (10). Samples were also taken at the pristine site of Salter Brook Bridge and at the Green site, an urban grassy parkland site in Liverpool.

DNA extraction and amplification. Total DNA was isolated as previously described (9) from soil and sediment bacteria but with the incorporation of a bead-beating step (B. Braun cell homogenizer) of 30 s to ensure cell lysis prior to the first sodium phosphate buffer extraction. DNA was also isolated (28) from bacteria containing representatives of each of the distinct *merR* sequences identified in an earlier study (26).

The oligonucleotide primer FluRX was synthesized, labelled with the green fluorescent label TET (4,7,2′,7′-tetrachloro-6-carboxyfluorescein), and purified (Oswel Laboratories, University of Southampton, Southampton, United Kingdom). To balance hydrophobicity, a tetraethylene glycol molecule was inserted between the fluorescence moiety and the oligonucleotide (7, 16). The primers PX and MARB were synthesized and purified by Applied Biosystems, Warrington, United Kingdom. Primer sequences were as follows: FluRX, 5′-ATA AAG CAC GCT AAG GC[G/A] TA; PX, 5′-TTC TTG AC[T/A] GTG ATC GGG CA; and MARB, 5′-GTC AA[C/T] GTG GAG AC[A/C/G] ATC CG.

For each PCR, 20 pmol of both oligonucleotide primers was added to 50 mmol of deoxynucleoside triphosphate, 1.25 U of *Taq* DNA polymerase (Gibco BRL), 5 μl of 10× PCR amplification buffer (PARR; Cambio), and sterile distilled water (sufficient to bring the reaction volume to 50 μl after the addition of template DNA). Each reaction mix, overlaid with mineral oil, was heated to 95°C for 4 min, during which time template DNA (ca. 0.05 μg) was added. Amplification conditions were 94°C (1 min), 55°C (1 min), and 72°C (4 min) for either 30 cycles for individual bacterial colonies or 35 cycles for soil or sediment DNA samples, followed by 72°C for 10 min. Thirty-five cycles were required to amplify *mer* genes from pristine environments (10). All reactions were carried out with a Perkin-Elmer 480 thermocycler.

Restriction endonuclease digestion. PCR products were digested with the restriction endonuclease *Fok*I (Boehringer Mannheim, Lewes, United Kingdom) for 7 h at 37°C with the reaction buffer supplied by the manufacturer. Prior to digestion, the enzyme in 1× reaction buffer was centrifuged at 13,000 rpm in a microcentrifuge for 5 min through a spin column (Microcon-30; Amicon).

Data collection and analysis. Digested amplification products were resolved on a 6% denaturing polyacrylamide gel (Sequagel; National Diagnostics) with a DNA sequencer (Applied Biosystems 373A) and Genescan-500 or -2500 TAMRA (*N*,*N*,*N*′,*N*′-tetramethyl-6-carboxyrhodamine; Applied Biosystems) internal markers within each lane. Electrophoresis was carried out with the voltage limited to 900 V for 5 h on plates with 12 cm from the well to the read position. The gel was analyzed by the local Southern method of size calling contained in the Genescan PCR analysis software (version 1.2.2-1). The positions and areas of individual peaks were calculated with Genotyper software (version 1.1) in relation to the TAMRA-labelled markers.

TABLE 1. Fragment sizes of the major *mer* subclasses identified

Subclass	*merRTΔP* RFLP class[a]	Fragment size (bases)
se3	B	157
pKLH2	C, D, F, G, I	171
Tn*501*	A, H	201
pDU1358		221
Tn*21*-Tn*501*	J	231
Tn*21*	E	333

[a] RFLP classes are designated as in reference 25.

RESULTS AND DISCUSSION

Rationale. Two *mer* gene PCR products were amplified in separate PCRs from each source of template DNA. These products were of different length, yet they started from the same position in each *mer* operon. The rationale behind the use of two products was that digestion of either would produce the same fragment (i.e., peak position) when amplified from a single source of *mer*. This allows discrimination between informative (*mer*-specific) fragments and spurious (non-*mer*-specific) PCR products, as fragments generated from spurious products would be seen in only one digestion profile. Such spurious PCR products were generated with *mer* primers from soil and sediment community DNA in an earlier study (10) and were found here for both FluRX-PX and FluRX-MARB reactions. The ability to discriminate between *mer*-specific and spurious PCR products was important, as a failure to do so would lead to the misinterpretation of *mer* gene diversity.

In addition to discriminating between *mer*-specific and spurious *mer* sequences, this approach can also be used to identify particular *mer* subclasses based on fragment sizes relative to the sizes predicted from DNA sequence information. *Fok*I was found to be the only commercially available restriction endonuclease which would discriminate each of the distinct archetypal gram-negative subclasses of *mer*, providing Tn*501*-, Tn*21*-, pKLH2-, and pDU1358-like classes and additional size classes related to both se3 (26) and Tn*501*-Tn*21*. These are shown in Table 1.

Technical approach. The technical approach chosen here was related to that used to analyze microsatellite variation within eukaryotic populations (21). Results generated by fluorescent-PCR–RFLP (FluRFLP) are shown in Fig. 1a and b. FluRFLP products are shown in green (TET dye) (Fig. 1a and b) coelectrophoresed with red (TAMRA) fluorescent internal size markers. The even-numbered lanes are loaded and electrophoresed for five min prior to the loading of the odd-numbered lanes. The characteristic alternating pattern formed by this procedure (Fig. 1a) is used to detect any leakage of samples into adjacent lanes. Following electrophoresis, the PCR digests in each lane are analyzed independently by using the calibration provided by the internal size markers. The gel image is converted to a series of peaks (Fig. 1b). From these calibrations, the size in bases of each green peak is then calculated with the Genotyper software.

Preliminary studies showed that band profiles were profoundly distorted. This problem was overcome by the use of spin columns, which diluted or removed a compound—possibly glycerol—in the restriction endonuclease solution while retaining the activity of the restriction endonuclease.

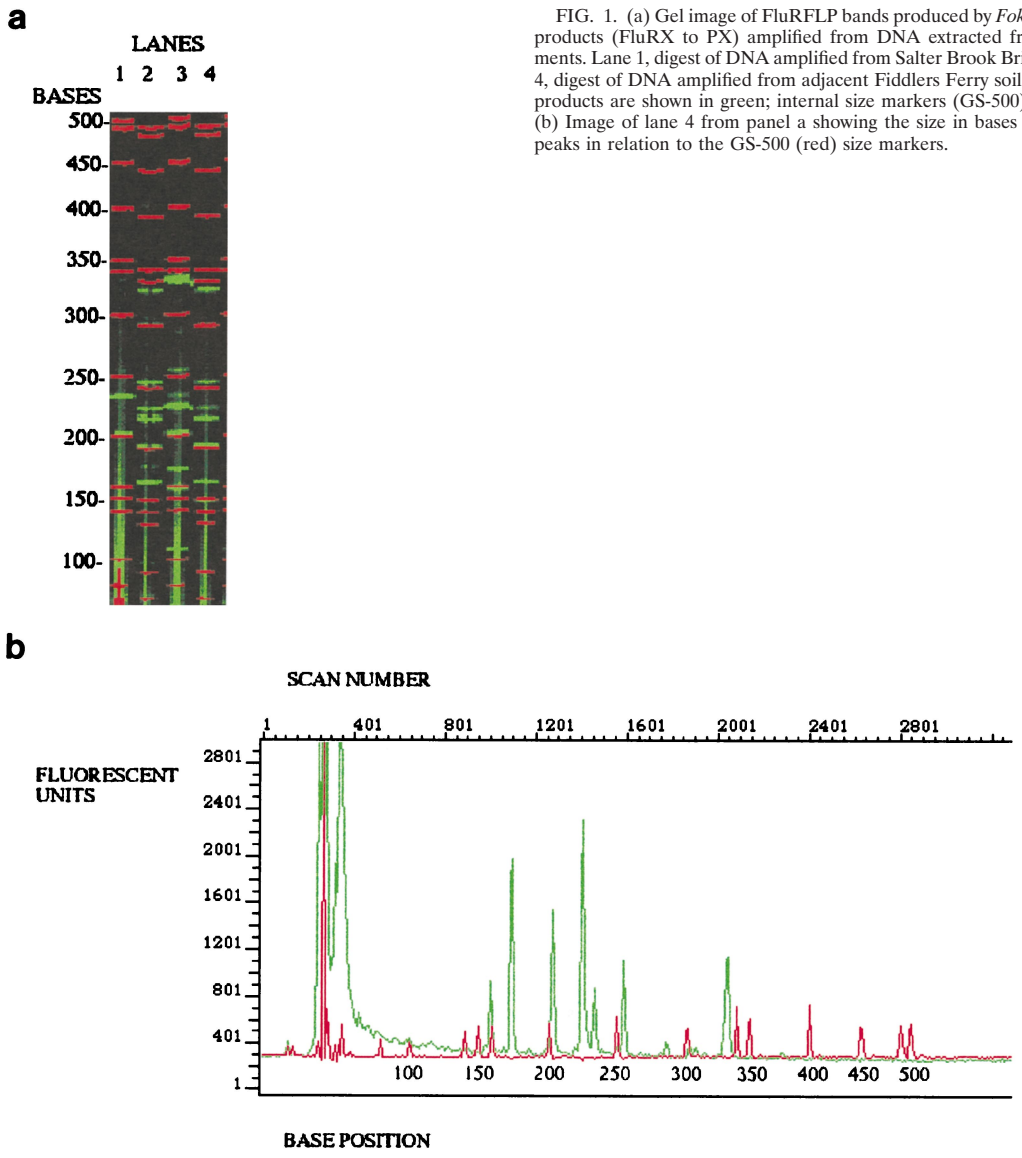

FIG. 1. (a) Gel image of FluRFLP bands produced by *Fok*I digestion of PCR products (FluRX to PX) amplified from DNA extracted from soils and sediments. Lane 1, digest of DNA amplified from Salter Brook Bridge; lanes 2, 3, and 4, digest of DNA amplified from adjacent Fiddlers Ferry soil samples. *mer* PCR products are shown in green; internal size markers (GS-500) are shown in red. (b) Image of lane 4 from panel a showing the size in bases of the *mer* (green) peaks in relation to the GS-500 (red) size markers.

FluRFLP of individual *mer* sequences. FluRFLP was used to study amplification products generated from the distinct sequence types of *mer* genes present in individual cultures of mercury-resistant bacteria. Osborn et al. (26) demonstrated that 9 of the 10 RFLP classes identified in an earlier study could be placed in one of the four major subclasses of archetypal gram-negative *mer* operons on the basis of their *merR* DNA sequences.

In this study, two regions (*merR* and *merRTΔP*) were amplified in separate PCRs from bacteria in each of the 10 distinct RFLP classes by using primer FluRX in combination with either PX or MARB. No significant bias was detected for either PX or MARB primer in terms of the amount of PCR product visualized in agarose gels containing ethidium bromide (data not shown). Following digestion by *Fok*I of either FluRX-PX or FluRX-MARB PCR products, the positions of the fluorescent fragments generated were noted (Fig. 2 and Table 1). Each distinct RFLP class of a known *mer* sequence gave the same fragment size by FluRFLP as was predicted on the basis of DNA sequence analysis, varying at most by 4 bases. This variation was most likely due to differences in size calling between electrophoretic runs.

*Fok*I was the only restriction endonuclease identified, following extensive analysis with the MAPSORT program (12), which would distinguish the major subclasses of *mer* in this region of the operon. Other criteria which were important in the selection of the restriction endonuclease were that the predicted fragment sizes had to differ by >10 bases and that the major subclasses had to be distinguishable in a region which could be sized by the automated DNA sequencer software. As FluRFLP analyses are performed automatically, this makes the generated data less subjective than that from many other methods.

FluRFLP on *mer* amplified from community DNA. The majority of the fragment sizes identified in Table 1 were present in every DNA sample extracted from bacterial communities in soil and sediment. However, distinct profiles were obtained for each environmental sample type studied (Fiddlers Ferry soil, Fiddlers Ferry sediment, and Green and Salter Brook Bridge soils). The FluRX primer was used alone in PCR amplifica-

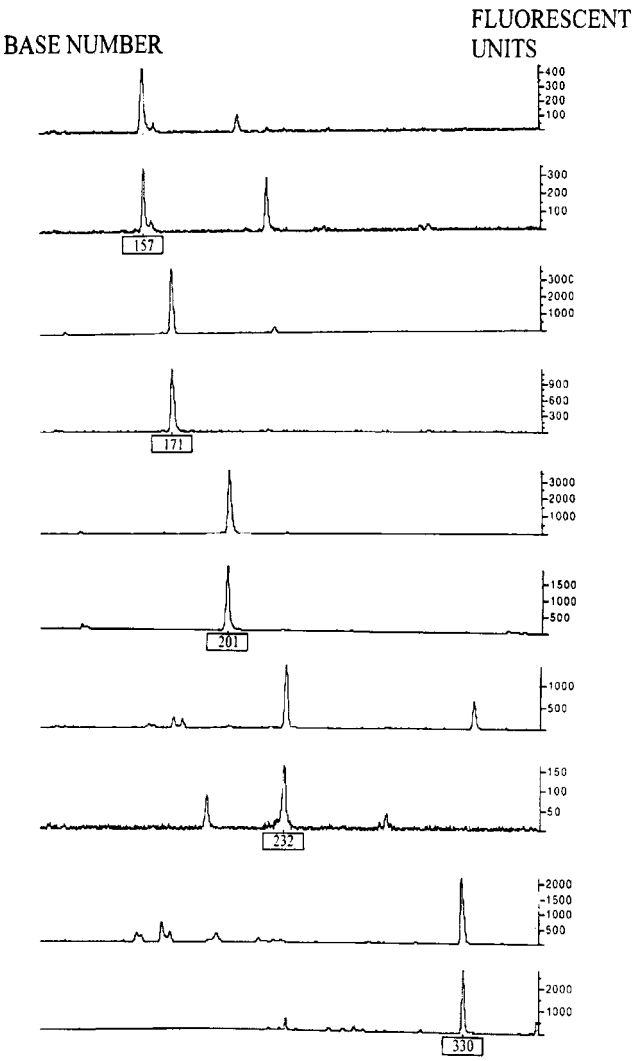

FIG. 2. Processed image of FluRFLP fragment sizes produced by *Fok*I digestion of PCR products amplified from bacteria containing distinct *mer* FluRFLP subclasses. Track 1, pDU1358 (FluRX and MARB primers); track 2, pDU1358 (FluRX and PX); track 3, pKLH2 (FluRX and MARB); track 4, pKLH2 (FluRX and PX); track 5, Tn*501* (FluRX and MARB); track 6, Tn*501* (FluRX and PX); track 7, Tn*21*-Tn*501* (FluRX and MARB); track 8, Tn*21*-Tn*501* (FluRX and PX); track 9, Tn*21* (FluRX and MARB); and track 10, Tn*21* (FluRX and PX).

tions with DNA isolated from soil samples as a target. No products were detected with this single FluRX primer in these control PCR amplifications. Therefore, the profiles resulted from the PCR amplifications with both primers in a given reaction.

Each DNA sample extracted from natural environments was analyzed at least three times. Furthermore, as a test of the variation in the process, the *mer* gene subclass FluRFLP profiles obtained following extraction of DNA from three 1-g replicate samples of soil taken only centimeters apart at the Fiddlers Ferry site (Fig. 1a, lanes 2, 3, and 4) were observed; little variation was seen.

Other fragments were observed which are not shown in Table 1. Of these, some (e.g., 252 and 285) were found in digestion analysis of both of the amplified *mer* regions (*merRTΔP* and *merR*). Given that they were present in both digests, one likely explanation is that they represent novel *mer* sequences present in the natural environment which have not yet been described. Such novel classes of *mer* have been identified previously (10, 25). Subsequent studies will examine mercury-resistant bacteria cultured from these natural environments to first isolate the subclasses and then determine the DNA sequences, from which their evolutionary relationship to the other major *mer* subclasses will be elucidated. These data will be incorporated in an iterative manner for the next phases of these studies. It is also possible, however, that these fragments have been generated from *mer* gene homologs in environmental bacteria which may or may not be resistant to mercury. The possibility of the novel fragments having resulted from incomplete digestion of PCR products was also considered. On analysis with MAPSORT, a variety of potential partial restriction products were predicted for five of the six recognized classes, including sizes of 227, 281, and 329 bases for Tn*501*; 285 bases for Tn*501*-Tn*21*; 217 and 301 bases for pKLH2; 302 and 350 bases for pDU1358; and 222, 252, 306, and 360 bases for se3. There are several facts which indicate that partial digestion of PCR products caused little significant alteration in the profiles generated: no evidence was found for these fragments in the work with pure bacterial cultures, the same profiles were reproducibly generated over a number of experiments, and many of the predicted bands do not appear in the profiles.

The relationship between the profiles generated by *Fok*I for either the *merRTΔP* or the *merR* region, with FluRX-PX or FluRX-MARB, respectively, was examined with respect to the areas of the peaks produced for the same soil or sediment DNA sample. As Suzuki and Giovannoni have shown, care must be taken when making inferences about the starting concentrations of genes based on the final ratio of PCR products (31). This was found to be true to an extent here. While generally similar profiles from the digestion of the *merRTΔP* or the *merR* regions were produced for the samples tested, a fragment size of 157 bases (se3-like) was either underrepresented following amplification with the MARB primer or overrepresented by the use of the PX primer in mixed-community DNA. However, despite the complex interactions of primers and template during amplification, it can be said that if the final ratios of fragments produced by two independent DNA samples originally amplified with the same set of primers are similar, then the communities from which the samples were taken were also similar.

Spatial heterogeneity of *mer* genes. The present study focused on the analysis of the soil and sediment DNA samples with the primers FluRX and PX. The final concentration of PCR products obtained for the series shown in Fig. 3 prior to digestion differed by less than a factor of 2. An example of the FluRFLP profiles obtained following the digestion of *merRTΔP* PCR products amplified from DNA directly extracted from Fiddlers Ferry soil samples is shown in Fig. 1. FluRFLP analyses of *mer* genes amplified from the sediment, the intermediate boundary between sediment and soil (referred to as the interface sample), and the soil are shown in Fig. 3. It is important to note that these profiles should be compared as relative as opposed to absolute numbers of fluorescent units between the tracks. From these profiles, profound differences in *mer* gene profiles were identified over as little as 1 m in the transition from sediment to soil at the Fiddlers Ferry site (Fig. 3). The extreme changes in the profiles confirmed the difference between the *mer* gene populations present in Fiddlers Ferry sediment and soil found in an earlier study (10).

Earlier studies using a cloning approach showed Tn*501*-like *mer* genes to predominate in Fiddlers Ferry soil, whereas in

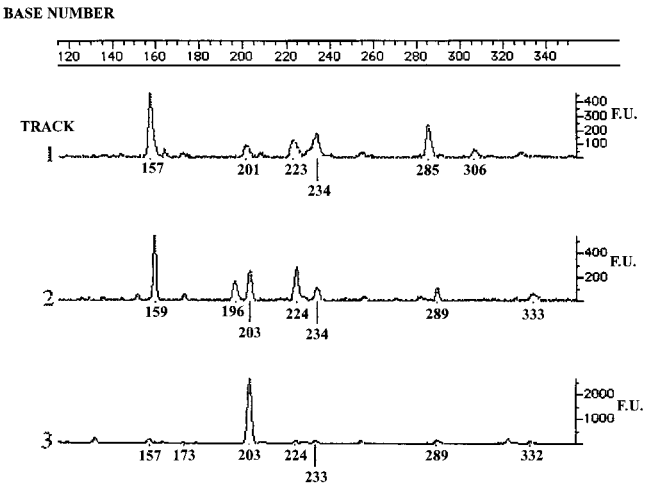

FIG. 3. Processed image of FluRFLP fragments produced by *Fok*I digestion of PCR products (FluRX to PX) amplified from DNA extracted from Fiddlers Ferry (see text) sediment and soil, with the *mer* fragments shown in bases. Track 1, digest of DNA amplified from sediment; track 2, digest of DNA amplified from the sediment-soil interface taken 0.5 m away; track 3, digest of DNA amplified from soil taken 1 m away. F.U., fluorescent units.

Fiddlers Ferry sediment, there was a predomination of pDU1358-like sequences (10). The profiles in Fig. 3 correlated well with these previously published cloning studies in that a fragment size of 157 bases (se3-like) was dominant in the sediment and a fragment size of 201 bases (Tn*501*-like) dominated in the soil sample. This provides little evidence of a cloning bias for these sequences.

In addition to these "between soil type" comparisons, "within soil type" samples were also studied. Table 2 shows a numerical representation of the relative areas of the *merRTΔP* region FluRFLP-generated fragments for samples collected 50 cm and 20 m from the initial sampling point. Other intermediate sample points (at 10 cm and 6 m; data not shown) produced profiles similar to those for samples from 0 cm and 50 cm. Similarity in *mer* subclass profiles was therefore seen in DNA samples collected over many meters in Fiddlers Ferry soil. The *mer* gene subclass profiles taken at the Green study site were distinct from those taken at the Fiddlers Ferry site (data not shown). Although distinct from the Fiddlers Ferry soil, samples from the Green study site showed a high degree of similarity to each other.

Spatial structures are being increasingly studied in microbial ecology. The spatial scales studied range widely, from the in situ detection of members of the *Proteobacteria* in activated sludge (1) through BIOLOG-based studies of spatial gradients within soil and estuarine sites (14) to the geographical differentiation of strains of the rice blight pathogen in different agroecosystems along a 310-km transect (3). Given the frequent location of *mer* genes on mobile genetic elements, such as transposons and/or plasmids, it is potentially surprising to find similar *mer* populations over 20 m within the same soil horizon while identifying profound vertical differences over as little as 50 cm when crossing between different soil or sediment horizons. The reasons for the spatial conservation or variation over these distances may be due to the physiochemistry of the natural environment imposing similar or different selection pressures and so maintaining a given *mer* population. For example, sediment sites are frequently associated with higher levels of methylmercury (13). Although a link is not conclusive, it should be noted that certain members of the se3-like subclass carry a recognition sequence for organomercurial compounds. This link between gene type and environment is under investigation. Of the differences in physiochemical environment which may exist, the variations in the levels of the particular chemical species of mercury which are present in environments (6) may be the most significant in shaping the population structure of the *mer* genes present. The potential association between environment and bacterial genetic structure has been strengthened by the finding of Croston et al. that the spatial and temporal distribution of methylmercury in sediment is influenced by the nature of the indigenous microbes (11).

TABLE 2. *merRTΔP* FluRFLP profiles of fragments[a]

Distance (m)	Relative area					
	se3	pKLH2	Tn*501*	pDU1358	Tn*21*-Tn*501*	Tn*21*
0	1	3.6	17.9	25.0	10.0	23.4
0.5	1	2.1	15.0	26.9	10.4	23.1
20	8.3	9.0	4.9	1	5.8	51.4

[a] Displayed as relative areas of the subclasses for soil samples taken at intervals 50 cm and 20 m from the initial sampling point at Fiddlers Ferry.

More recent samples (Table 2), however, have shown a change in the profiles present in Fiddlers Ferry soil. These samples were taken as close as possible to the original locations. The reason for the change in profiles may lie in the recent renovation and redevelopment around the previously disused Fiddlers Ferry Inn site.

Conclusion. FluRFLP differs from other RFLP methods for studying mixed-community PCR products (e.g., Martinez-Murcia et al. [22]) in that a single fluorescent fragment forms the sole focus of the analysis in contrast to the pattern of multiple fragments in a conventional RFLP analysis. Alone, these fluorescent fragments are not very informative. This approach works due to the coupling of the knowledge of the different *mer* DNA sequences and a restriction endonuclease which is specific for the analysis of each subclass or major type of DNA sequence under investigation; this information had been developed previously for the *mer* systems under investigation. Similar studies may be performed with other sequence types, e.g., transposon sequences (18).

By using PCR to study the gene composition of environmental samples, a range of biases can be introduced, such as reannealing biases of primers to a range of different template structures and biases due to the effect of differential levels of degeneracy and GC content between primers. This system will be extended in subsequent studies to determine the effects of these factors on the overall assessment of subclass distribution in environments by the study of complex models comprising multiple target sequences. The ultimate aim of this research is the quantification of the abundance of each subclass in a range of natural environments.

The sensitivity of the DNA sequencing machine and software facilitates high resolution of digested DNA. Since these machines are present in many laboratories, variations on this technique are possible, e.g., by using other primer labels, such as ^{32}P. In this sense, the technique is truly generic, with many applications in microbial ecology. Studies are under way to determine the sensitivity of the system in comparison to other profiling methods, such as denaturing gradient gel electrophoresis. However, even from the present study alone, FluRFLP appears to have detected previously uncharacterized *mer* sequences.

The iterative process described above, coupled to quantifi-

cation, will extend these studies, which have suggested differential spatial dimensions to the distribution of a specific bacterial gene in soil and sediment environments. As such, the resolving power of FluRFLP has been demonstrated; this resolving power will be particularly valuable to determine the effect of environmental change on microbial communities.

ACKNOWLEDGMENTS

I acknowledge Donald Ritchie and Peter Strike, for the initial formulation of the ideas of this work and for subsequent discussion, and the assistance of Mark Hughes.

K.D.B. is supported by a NERC Postdoctoral Fellowship (GT5/94/TLS), and this work was partly funded by NERC grant GR3/9081. The work benefited from the use of the SEQNET facility at Daresbury, United Kingdom.

REFERENCES

1. **Amann, R., J. Snaidr, M. Wagner, W. Ludwig, and K.-H. Schleifer.** 1996. In situ visualization of high genetic diversity in a natural microbial community. J. Bacteriol. **178:**3496–3500.
2. **Amann, R. I., W. Ludwig, and K.-H. Schleifer.** 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. **59:**143–169.
3. **Ardales, E. Y., H. Leung, C. M. V. Cruz, T. W. Mew, J. E. Leach, and R. J. Nelson.** 1996. Hierarchical analysis of spatial variation of the rice bacterial-blight pathogen across diverse agroecosystems in the Philippines. Phytopathology **86:**241–252.
4. **Barkay, T., C. Liebert, and M. Gillman.** 1989. Hybridization of DNA probes with whole-community genome for detection of genes that encode microbial responses to pollutants: *mer* genes and Hg^{2+} resistance. Appl. Environ. Microbiol. **55:**1574–1577.
5. **Barrineau, P., P. Gilbert, W. J. Jackson, C. S. Jones, A. O. Summers, and S. Wisdom.** 1984. The DNA sequence of the mercury resistance operon of the Inc FII plasmid NR1. J. Mol. Appl. Genet. **2:**601–619.
6. **Bozongo, J. C. J., K. J. Heim, Y. A. Chen, W. B. Lyons, J. J. Warwick, G. C. Miller, and P. J. Lechler.** 1996. Mercury pathways in the Carson River-Lahontan Reservoir system, Nevada, USA. Environ. Toxicol. Chem. **5:**677–683.
7. **Brown, D. J. S., and T. Brown.** 1995. The synthesis of chemically labelled PCR primers, p. 57–70. *In* C. R. Newton (ed.), PCR: essential data. Wiley Interscience, New York, N.Y.
8. **Brown, N. L., S. J. Ford, R. D. Pridmore, and D. C. Fritzinger.** 1983. Nucleotide sequence of a gene from the *Pseudomonas* transposon Tn*501* encoding mercury resistance. Biochemistry **22:**4089–4095.
9. **Bruce, K. D., W. D. Hiorns, J. L. Hobman, A. M. Osborn, P. Strike, and D. A. Ritchie.** 1992. Amplification of DNA from native populations of soil bacteria by using the polymerase chain reaction. Appl. Environ. Microbiol. **58:**3413–3416.
10. **Bruce, K. D., A. M. Osborn, A. J. Pearson, P. Strike, and D. A. Ritchie.** 1995. Genetic diversity within *mer* genes directly amplified from communities of non-cultivated soil and sediment bacteria. Mol. Ecol. **4:**605–612.
11. **Croston, N. J., J. M. Bubb, and J. N. Lester.** 1996. Spatial-distribution and seasonal-changes in methylmercury concentrations in shallow lakes. Hydrobiologia **321:**35–45.
12. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence-analysis programs for the Vax. Nucleic Acids Res. **12:**387–395.
13. **Devereux, R., M. R. Winfrey, J. Winfrey, and D. A. Stahl.** 1996. Depth profile of sulfate reducing bacteria ribosomal RNA and mercury methylation in an estuarine sediment. FEMS Microbiol. Ecol. **20:**23–31.
14. **Garland, J. L., and A. L. Mills.** 1991. Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. Appl. Environ. Microbiol. **57:**2351–2359.
15. **Griffin, H. G., T. J. Foster, S. Silver, and T. K. Misra.** 1987. Cloning and DNA sequence of the mercuric- and organomercurial-resistance determinants of plasmid pDU1358. Proc. Natl. Acad. Sci. USA **84:**3112–3116.
16. **Grzybowski, J., F. McPhilips, and T. Brown.** 1995. The use of 5′ chemically labelled primers in PCR, p. 93–98. *In* C. R. Newton (ed.), PCR: essential data. Wiley Interscience, New York, N.Y.
17. **Hobman, J. L., and N. L. Brown.** 1996. Bacterial mercury resistance genes, p. 527–568. *In* H. Sigel and A. Sigel (ed.), Metal ions in biological systems. Marcel Dekker, Inc., New York, N.Y.
18. **Holt, R. J., P. Strike, and K. D. Bruce.** 1996. Phylogenetic analysis of *tnpR* genes in mercury resistant soil bacteria. FEMS Microbiol. Lett. **144:**95–102.
19. **Jobling, M. G., S. E. Peters, and D. A. Ritchie.** 1988. Restriction pattern and polypeptide homology amongst plasmid-borne mercury resistance determinants. Plasmid **20:**106–112.
20. **Kholodii, G. Y., Z. M. Gorlenko, O. L. Lomovskaya, S. Z. Mindlin, O. V. Yurieva, and V. G. Nikiforov.** 1993. Molecular characterisation of an aberrant mercury resistance transposable element from an environmental *Acinetobacter* strain. Plasmid **30:**303–308.
21. **Levitt, R. C., M. B. Kiser, C. Dragwa, A. E. Jeducka, J. Xu, D. A. Meyers, and J. R. Hudson.** 1994. Fluorescence-based resource for semiautomated genomic analyses using microsatellite markers. Genomics **24:**361–365.
22. **Martinez-Murcia, A. J., S. G. Acinas, and F. Rodriguez-Valera.** 1995. Evaluation of prokaryotic diversity by restriction digestion of 16S rDNA directly amplified from hypersaline environments. FEMS Microbiol. Ecol. **17:**247–256.
23. **Muyzer, G., E. C. De Waal, and A. G. Uitterlinden.** 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. **59:**695–700.
24. **Muyzer, G., A. Teske, C. O. Wirsen, and H. W. Jannasch.** 1995. Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. Arch. Microbiol. **164:**165–172.
25. **Osborn, A. M., K. D. Bruce, P. Strike, and D. A. Ritchie.** 1993. Polymerase chain reaction-restriction fragment length polymorphism analysis shows divergence among *mer* determinants from gram-negative soil bacteria indistinguishable by DNA-DNA hybridization. Appl. Environ. Microbiol. **59:**4024–4030.
26. **Osborn, A. M., K. D. Bruce, P. Strike, and D. A. Ritchie.** 1995. Sequence conservation between regulatory mercury resistance genes in bacteria from mercury polluted and pristine environments. Syst. Appl. Microbiol. **18:**1–6.
27. **Osborn, A. M., K. D. Bruce, P. Strike, and D. A. Ritchie.** 1997. Distribution, diversity and evolution of the bacterial mercury resistance operon. FEMS Microbiol. Rev. **19:**239–262.
28. **Pitcher, D. G., N. A. Saunders, and R. J. Owen.** 1989. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett. Appl. Microbiol. **8:**151–156.
29. **Rochelle, P. A., M. K. Wetherbee, and B. H. Olson.** 1991. Distribution of DNA sequences encoding narrow- and broad-spectrum mercury resistance. Appl. Environ. Microbiol. **57:**1581–1589.
30. **Silver, S., and M. Walderhaug.** 1992. Gene regulation of plasmid-determined and chromosome-determined inorganic ion transport in bacteria. Microbiol. Rev. **56:**195–228.
31. **Suzuki, M. T., and S. J. Giovannoni.** 1996. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl. Environ. Microbiol. **62:**625–630.
32. **Wawer, C., and G. Muyzer.** 1995. Genetic diversity of *Desulfovibrio* spp. in environmental samples analyzed by denaturing gradient gel electrophoresis of [NiFe] hydrogenase gene fragments. Appl. Environ. Microbiol. **61:**2203–2210.