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A Novel Means To Develop Strain-Specific DNA Probes for Detecting Bacteria in the Environment

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A simple means to develop strain-specific DNA probes for use in monitoring the movement and survival of bacteria in natural and laboratory ecosystems was developed. The method employed amplification of genomic DNA via repetitive sequence-based PCR (rep-PCR) using primers specific for repetitive extragenic palindromic (REP) elements, followed by cloning of the amplified fragments. The cloned fragments were screened to identify those which were strain specific, and these were used as probes for total genomic DNA isolated from microbial communities and subjected to rep-PCR. To evaluate the utility of the approach, we developed probes specific for *Burkholderia cepacia* G4 and used them to determine the persistence of the strain in aquifer sediment microcosms following bioaugmentation. Two of four probes tested were found to specifically hybridize to DNA fragments of the expected sizes in the rep-PCR fingerprint of *B. cepacia* G4 but not to 64 genetically distinct bacteria previously isolated from the aquifer. One of these probes, a 650-bp fragment, produced a hybridization signal when as few as 10 CFU of *B. cepacia* G4 were present in a mixture with 10^5 CFU nontarget strains, indicating that the sensitivity of these probes was comparable to those of other PCR-based detection methods. The probes were used to discriminate groundwater and microcosm samples that contained *B. cepacia* G4 from those which did not. False-positive results were obtained with a few samples, but these were readily identified by using hybridization to the second probe as a confirmation step. The general applicability of the method was demonstrated by constructing probes specific to three other environmental isolates.

Studies to understand the movement, survival, and growth of specific microbial populations in the environment require the use of methods to detect and estimate the relative abundance of specific strains when such strains are present in relatively low numbers in complex and diverse microbial communities such as those found in soils and aquifers. In recent years there have been numerous reports on the use of nucleic acid probes for detection of specific phylogenetic groups of microorganisms in situ (1, 6, 8, 12, 19, 23, 24, 31, 36, 40). While these probes enable detection of relatively broad groups of organisms, they are generally unable to distinguish between strains within a group. For certain applications, such as when one wants to monitor strains that possess specific characteristics that are uncommon or not universally shared by members of a broader group, greater selectivity or resolution is required. Similarly, probes specific for phylogenetic groups of organisms would be of limited utility in monitoring genetically engineered organisms in which the genotype of interest was unique to the strain. A second approach has been to clone random fragments of genomic DNA from the strain of interest and exhaustively test these to ensure that they are specificity for that strain (16, 35). Amplification of the target sequence from microbial communities can be achieved once the sequence has been determined and specific oligonucleotide primers can be developed. Although effective, this approach is quite laborious.

In this study, we sought to develop a generally useful procedure for producing strain-specific probes that was rapid and did not require the screening of large numbers of randomly selected clones. To provide sensitivity while maintaining simplicity, we sought to use PCR amplification of target sequences without imposing the need to determine the nucleic acid sequence of the probe to develop PCR primers. This aim was accomplished through the use of DNA fragments obtained by repetitive sequence-based (rep-PCR) (38) that were subsequently cloned and used as probes.

To evaluate this approach, strain-specific probes for *Burkholderia cepacia* G4 were developed and used to evaluate the effectiveness of bioaugmentation for removal of trichloroethylene (TCE) in an aquifer and in aquifer sediment microcosms.

MATERIALS AND METHODS

Construction of probes. Probes corresponding to *B. cepacia* G4 and three environmental bacterial isolates were constructed and tested as described below. All strains were grown on R2A agar (Difco, Detroit, Mich.) or broth at room temperature or 30°C and stored at −80°C in R2A broth supplemented with 15% glycerol.

rep-PCR was performed as described by de Bruijn (7). Primers specific for repetitive extragenic palindromic (REP) sequences were synthesized at the Macromolecular Structure Facility, Department of Biochemistry, Michigan State University. Templates for rep-PCRs consisted of 1 μl of liquid or frozen culture or a small amount of biomass from a colony grown on agar. It was not necessary to extract DNA prior to amplification by PCR since the cells were lysed under the amplification conditions used. The 25-μl PCR mix contained 50 pmol each of primers REPIR-I and REP2-1 (37), 1.25 mM each dATP, dCTP, dGTP, and dTTP (Pharmacia Biotech, Piscataway, N.J.), and 2 U of *Taq* DNA polymerase (Gibco-BRL, Gaithersburg, Md.) in Gitschier buffer (18) with 10% dimethyl sulfoxide. The template DNA was amplified by using the following temperature profile: 95°C for 6 min; followed by 35 cycles at 94°C for 1 min, 40°C for 1 min, and 65°C for 8 min; and then 1 cycle at 65°C for 16 min.

The mixture of rep-PCR products was ligated into the pCRII vector (Invitrogen, Carlsbad, Calif.) and then transformed into *Escherichia coli* One Shot competent cells, using a TA cloning transformation kit (Invitrogen) as recommended by the manufacturer. Transformants containing recombinant plasmids were selected on LB agar containing 50 μg of kanamycin per ml and 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml (30). Plasmids were extracted by alkaline lysis and digested with *Eco*RI to excise cloned fragments, whose sizes were measured by electrophoresis in 0.8% agarose stained with ethidium bromide (30). The DNA fragments were gel purified by

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using 1% SeaPlaque (FMC BioProducts, Rockland, Maine) low-melting-temperature agarose, extracted with a Geneclean II kit (Bio 101, Inc., La Jolla, Calif.), and labeled with alkali-labile digoxigenin (DIG)-dUTP, using a DIG DNA labeling and detection kit (Boehringer Mannheim Corp., Indianapolis, Ind.). Probes were stored at −20°C.

Sample preparation and analysis. Probes from each strain were screened against Southern blots of rep-PCR-amplified fragments from axenic bacterial cultures and also environmental samples of microbial communities. The pure cultures used were 64 bacterial strains previously isolated from a TCE-contaminated aquifer (11) at Moffett Field (Mountain View, Calif.). Colony blots of these isolates were prepared as described by Sambrook et al. (30). rep-PCR fragments of the pure cultures were obtained in a manner similar to that described above. However, some strains were resistant to lysis and did not yield amplification products when cultures were used as templates. In those cases, total genomic DNA was extracted (3), and approximately 50 ng was added to each rep-PCR mix. In addition, genomic DNA was extracted from a mixture containing 10 ml each of 60 different Moffett Field isolates grown individually in R2A broth. This complex mixture of community DNA was subjected to rep-PCR and used for preliminary screening of probes. Environmental samples consisted of effluents from aquifer column microcosms (25), some of which had been amended with G4, and also groundwater samples taken from the Moffett Field aquifer prior to injection of G4. One microliter of liquid from each sample was added directly to a rep-PCR mixture for amplification of the bacterial community DNA.

After PCR, 12 μl of each reaction mixture was electrophoresed in 1.5% agarose with 0.5× Tris-acetate-EDTA buffer (30). The DNA was transferred to a Hybond-N nylon membrane (Amersham, Arlington Heights, Ill.) by capillary blotting (33) and cross-linked to the membrane with a UV Stratalinker 1800 (Stratagene, La Jolla, Calif.). Prehybridization and hybridization solutions were as recommended in the Genius system user's guide for membrane hybridization (Boehringer Mannheim) and contained 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (3), 0.1% *N*-lauroylsarcosine, 0.02% sodium dodecyl sulfate, 5% blocking reagent, and 50% formamide. Filters were prehybridized at least 2 h and hybridized overnight in a solution containing heat-denatured, DIG-labeled probe. Prehybridization and hybridization incubation temperatures were increased from the recommended 42°C to 62°C to achieve high-stringency conditions (90 to 100% homology). Filters were washed twice in 2× SSC and then developed for chemiluminescence detection with Lumigen PPD according to the manufacturer's instructions. Filters were exposed to Kodak X-Omat film for up to 3 h during maximum luminescence for autoradiographic detection of signals.

Membranes that were hybridized sequentially to more than one probe were stripped by being washed briefly in water, washed twice for 10 min each time at 37°C in 0.4 N NaOH–0.1% SDS, and then rinsed thoroughly in 2× SSC as recommended by the manufacturer. These conditions effectively removed alkali-labile DIG-labeled probes.

Detection sensitivity. To determine sensitivity of detection of strain G4 by using rep-PCR amplification followed by hybridization analysis, strain G4 was grown overnight in R2A broth at 30°C and 225 rpm. A 10-fold dilution series was prepared with R2A broth containing 15% glycerol. From each dilution, 1 μl was added to a rep-PCR mixture, and 100 μl was plated on R2A agar. Dilutions were stored at −80°C. Plates were incubated at room temperature until colonies had developed. rep-PCR products were electrophoresed, blotted, and hybridized to fragment G4A, one of the probes developed as described above. CFU per microliter corresponding to each rep-PCR was calculated from the plate counts.

To determine sensitivity of detection of G4 against a background of nontarget organisms, the following mixture of nontarget strains was prepared. Undiluted effluents (100 μl) from aquifer column microcosms which had not been amended with G4 were plated on R2A agar and incubated at room temperature. The resulting lawn of growth was harvested in 2 ml of R2A broth containing 15% glycerol. This thick suspension was immediately diluted and plated to determine CFU per milliliter by standard plate counts. The remaining suspension was stored at −80°C. A dilution series of the nontarget suspension was mixed with a constant amount of G4 approaching the detection limit for the latter as determined above. These mixtures were then subjected to rep-PCR and hybridization to probe G4A.

RESULTS

Identification of strain-specific probes. Fragments of genomic DNA from *B. cepacia* G4 that served as candidates for strain-specific probes were obtained by rep-PCR amplification of genomic DNA and ligation of the products into pCRII. The ligation mixture was used to transform *E. coli*, and 17 clones that remained white or light blue on X-Gal were obtained. Eight of these were found to harbor inserts of four different sizes that were comparable to the rep-PCR fragments of strain G4 (Fig. 1). Each recombinant plasmid contained a single rep-PCR amplification product. There are two fragments in

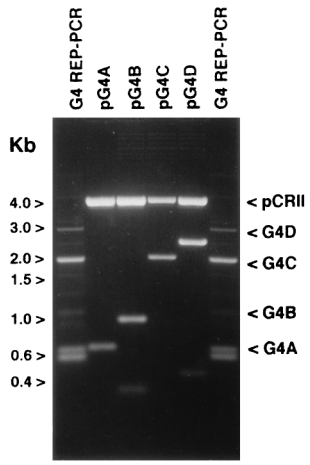

FIG. 1. Ethidium bromide-stained agarose gel of the *B. cepacia* G4 rep-PCR amplification product mixture and the four purified G4 rep-PCR amplification products used as probes. Products cloned into the pCRII vector were excised by digestion with *Eco*RI. Recombinant plasmids are identified above each lane, and probe designations are shown on the right. Arrows indicate G4 rep-PCR products which hybridize to the corresponding probe.

the lanes designated G4B and G4D because these inserts contain *Eco*RI cleavage sites and *Eco*RI was the enzyme used to excise the insert from the pCRII vector. Southern hybridization analyses (not shown) showed that fragment G4A corresponds to the major 650-bp rep-PCR product, whereas the fragment designated G4B corresponds to the faint 1-kb rep-PCR band. Similarly, fragment G4C hybridized to the 1.9-kb band, and fragment G4D hybridized to the faint band just below 3 kb. These four fragments were tested to determine if they could serve as strain-specific probes.

Selectivity of probes. Hybridization to nontarget strains by the four probe fragments was examined in assays using 64 bacterial strains that had previously been isolated from a TCE-contaminated aquifer (11) and three other strains of *B. cepacia*, including RASC, DBO1, and ATCC 17762. When the strains were subjected to rep-PCR prior to hybridization, the probes were found to be specific to *B. cepacia* G4 amplification products, and strong hybridization signals were consistently obtained (Fig. 2). Identical results were obtained for the other 44 aquifer isolates and for the other *B. cepacia* strains. Hybridization of the 650-bp probe to multiple bands appears to be an artifact resulting from excess PCR template, since dilution of the template resulted in hybridization to a single band (Fig. 3). Of the four probes tested, fragments G4A and G4C produced the most intense hybridization signals, presumably because they correspond to the most highly amplified rep-PCR products. It was postulated that G4A would be a more specific probe since it is smaller than G4C and less likely to have DNA sequence that would cross-react with nontarget strains. Fragments G4B and G4D were less useful as probes because of weak hybridization signal obtained and large size, respectively. Thus, G4A was used as the primary probe in subsequent analyses, and G4C was used to confirm the results obtained with G4A.

When the probes were screened against colony blots (i.e., total genomic DNA) of the 64 aquifer isolates, hybridization to nontarget strains occurred (data not shown). When used in this way, the hybridization signal was weak and sometimes absent from positive controls, suggesting that detection of the unamplified target was poor. Amplification of the target by rep-PCR overcame both of these limitations and was thus considered a

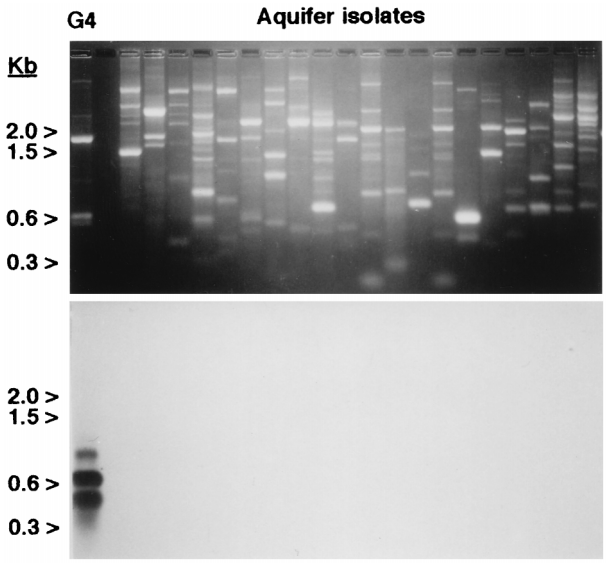

FIG. 2. Ethidium bromide-stained agarose gel of rep-PCR amplified DNA from *B. cepacia* strain G4 and 20 aquifer isolates. Each lane contains amplification products from a single isolate. The corresponding Southern blot was hybridized to probe G4A.

critical step in the processing of samples to be hybridized to rep-PCR-derived probes.

Sensitivity of strain-specific probes. To determine the sensitivity of probe G4A, known amounts of *B. cepacia* G4 were mixed in different ratios with nontarget organisms. The nontarget organisms were from samples of aquifer microcosm effluents and were used without prior enrichment or isolation of individual species. The numbers of *B. cepacia* G4 and nontarget organisms were determined by viable cell counts using R2A medium. Probe G4A consistently detected 10 CFU of G4 alone and against a background of 10^5 CFU nontarget microorganisms contained in 1 μl of PCR template (Fig. 3). Interestingly, the ratio of target to nontarget organisms was critical, but the absolute number of target cells present in the sample was not. Thus, G4 must represent 1/10,000, or 0.01%, of the PCR template DNA in order to be detected. The data show that the target sequence is detectable with probe G4A even at concentrations too low to be visible on an ethidium bromide-stained agarose gel. In some experiments, 1 CFU per μl of template occasionally produced a detectable hybridization signal (data not shown), suggesting that the detection limit was less than 10 CFU.

Microcosm samples. The ability of the probes to detect *B. cepacia* G4 in microbial communities to which it had been added was evaluated by using samples of effluent from microcosms containing aquifer material. These microcosms had been maintained with an influent feed of groundwater to which TCE and either phenol or lactate had been added (25). Selected microcosms had been amended with either *B. cepacia* G4 or *B. cepacia* $PR1_{301}$; the latter is a constitutive mutant derived from *B. cepacia* G4 (26). *B. cepacia* G4 and $PR1_{301}$ have the same rep-PCR pattern and hybridize identically to the probes derived from strain G4 (data not shown). The patterns of DNA fragments amplified from the microcosm effluent samples were complex relative to that of *B. cepacia* G4 alone, indicating the presence of multiple strains in the microbial communities. When leachate was plated on R2A agar, we could distinguish at least eight different colony morphologies that were present in numbers ranging from 10^4 to 10^6 CFU per ml. Hybridization to probes G4A and G4C was observed only in samples from microcosms that had been seeded with either *B. cepacia* G4 or $PR1_{301}$ (Fig. 4, lanes 3, 5, 6, 7, 9, and 10). The intensity of hybridization observed was consistent with the observed rate of TCE degradation (25); samples with low rates of TCE degradation hybridized weakly to the probe, whereas strong hybridization signals were observed with samples from microcosms in which relatively high rates of TCE degradation occurred.

Aquifer samples. Groundwater samples from the Moffett Field aquifer were analyzed to determine whether G4 could be detected in the aquifer prior to injection of the organism. Ten replicate samples of groundwater collected from the Moffett Field aquifer were subjected to rep-PCR amplification and then hybridized sequentially with probes G4A and G4C (Fig. 5). Positive controls consisted of either 10 CFU of *B. cepacia* G4 per μl of R2A broth or 10 CFU of *B. cepacia* G4 added per μl of groundwater prior to PCR amplification. The pattern of DNA fragments in each lane is barely visible on the ethidium bromide-stained gel due to the small amount of bacterial DNA template present in the groundwater used for amplification and the resulting low yield of PCR-amplified DNA (Fig. 5). The only samples that hybridized to both probes were the two positive controls. Probe G4A hybridized to a 650-bp fragment in one of the groundwater samples, but there was no hybridization with probe G4C. Thus, the hybridization observed with probe G4A constitutes a false positive, but the absence of *B. cepacia* G4 was evident by the lack of hybridization to probe G4C. These data indicate that *B. cepacia* G4 was not present in the aquifer water samples. This finding was consistent with thorough bacterial community analyses which did not detect G4 in the aquifer prior to injection (11). The data also indicate that in nature, we will occasionally encounter rep-PCR products from nontarget strains that hybridize to a probe, but misleading false-positive results are easily resolved by use of a second probe to confirm the data.

Strain specificity of rep-PCR fragments. To test the general applicability of the use of rep-PCR for rapid development of strain-specific probes, three rep-PCR fragments were cloned and purified from each of three randomly selected bacterial strains designated 1, 2, and 3. The fragments cloned ranged in size from 450 bp to 3 kb. The three cloned DNA fragments

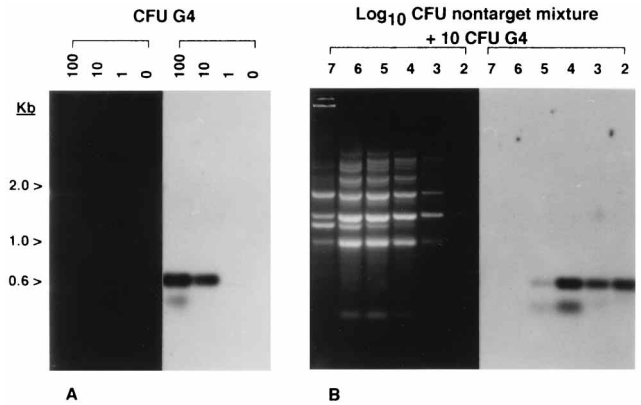

FIG. 3. Ethidium bromide-stained agarose gels of rep-PCR reactions of *B. cepacia* G4 alone (A) and in a mixture of nontarget organisms (B). The corresponding Southern blots were hybridized to probe G4A from strain G4. (A) Numbers above lanes refer to number of CFU of strain G4 added as the template before rep-PCR. (B) Numbers above lanes refer to the $\log_{10}$ CFU of nontarget organisms mixed with 10 CFU of strain G4 prior to rep-PCR.

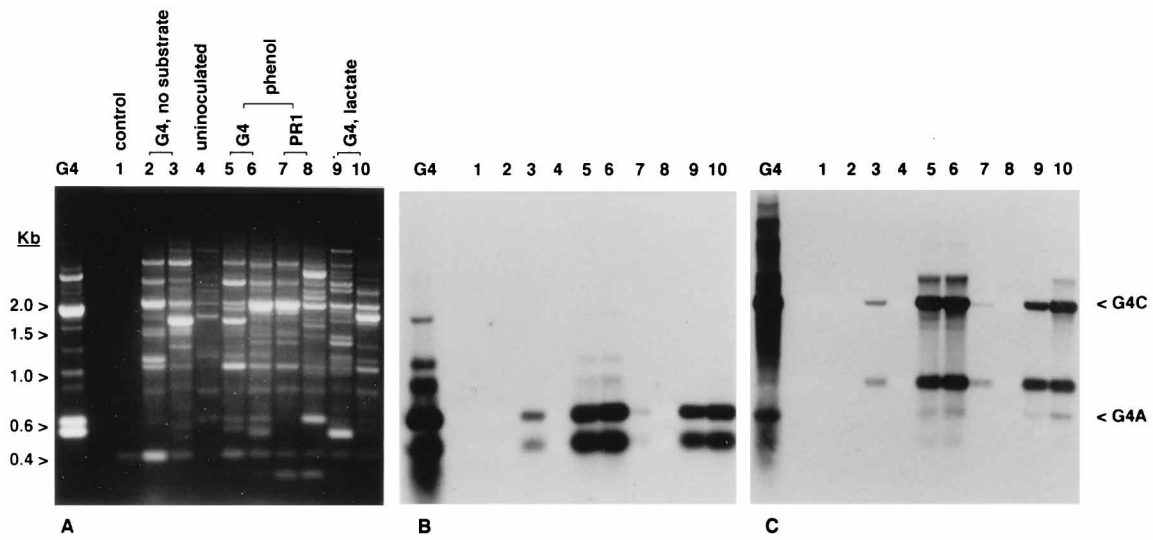

FIG. 4. (A) Ethidium bromide-stained agarose gel of rep-PCR amplification products of microbial communities present in effluents from aquifer column microcosms. Column treatments (organism and substrate amendments) are indicated above each lane. (B) Detection of *B. cepacia* G4 and its derivative $PR1_{301}$ on the corresponding Southern blot, using the 650-bp probe G4A. (C) The same Southern blot hybridized to the 1.9-kb probe G4C after removal of probe G4A. Positions of hybridization signals arising from probes G4A and G4C are indicated on the right.

from each strain that were evaluated further were designated A, B, and C in order of increasing size and preceded by the strain number. The selectivity of the probes was initially assessed by hybridization to DNA amplified by rep-PCR from the parental strains and a mixture of genomic DNAs extracted from 60 of the aquifer isolates (not shown). Only one probe, 3C, hybridized to DNA amplified from the nontarget mixture. This probe is 3 kb and since it is large may contain nonspecific DNA sequence. One probe from each isolate was further tested by hybridization against rep-PCR products from the 64 individual Moffett Field aquifer isolates. Hybridizations of probes 1B (900 bp), 2B (600 bp), and 3A (900 bp) to 20 of these isolates are shown in Fig. 6. All three probes hybridized specifically to DNA amplification products from their corresponding parent strains. The only exception was cross-hybridization of probe 3A to a rep-PCR product from one of the 64 nontarget aquifer isolates (not shown).

DISCUSSION

Here we report the development of a rapid and sensitive method to obtain strain-specific nucleic acid probes that have the sensitivity and specificity required for the detection of specific organisms in complex microbial communities. The method developed has several advantages over previously described approaches for obtaining strain-specific molecular probes and PCR primers.

First, these probes can be developed and used without any prior knowledge of the host strain or the probe sequence. The PCR primers used here are based on the 38-bp REP element that has been shown to be widely distributed among phylogenetically diverse eubacterial species (for a review, see reference 22). Dimri et al. (9) showed that *E. coli* K-12 has more than 500 copies of the element distributed throughout the genome. Although the number of REP elements in the ge-

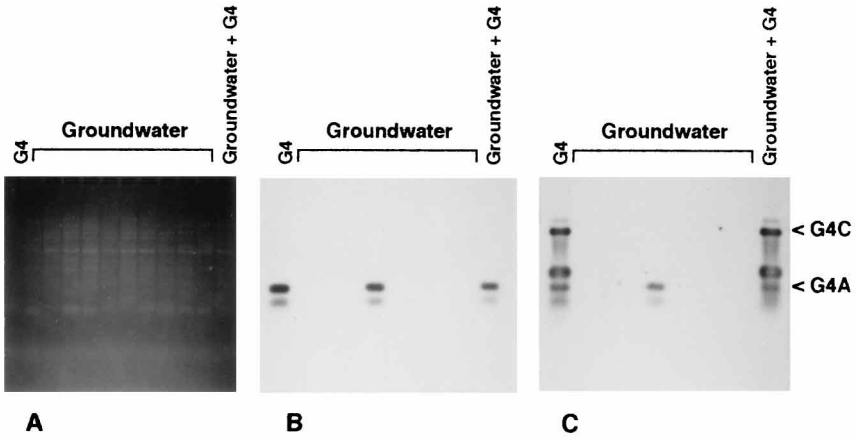

FIG. 5. (A) Ethidium bromide-stained agarose gel of rep-PCR amplification products from ten groundwater samples (one per lane) taken from the Moffett Field aquifer prior to injection of *B. cepacia* G4. Positive controls include rep-PCR amplification products from 100 CFU of strain G4 alone and 100 CFU of G4 mixed with groundwater. The Southern blot was hybridized sequentially to G4A (B) and to G4C (C), so that signals corresponding to both probes appear in panel C. Positions of hybridization signals arising from probes G4A and G4C are indicated on the right.

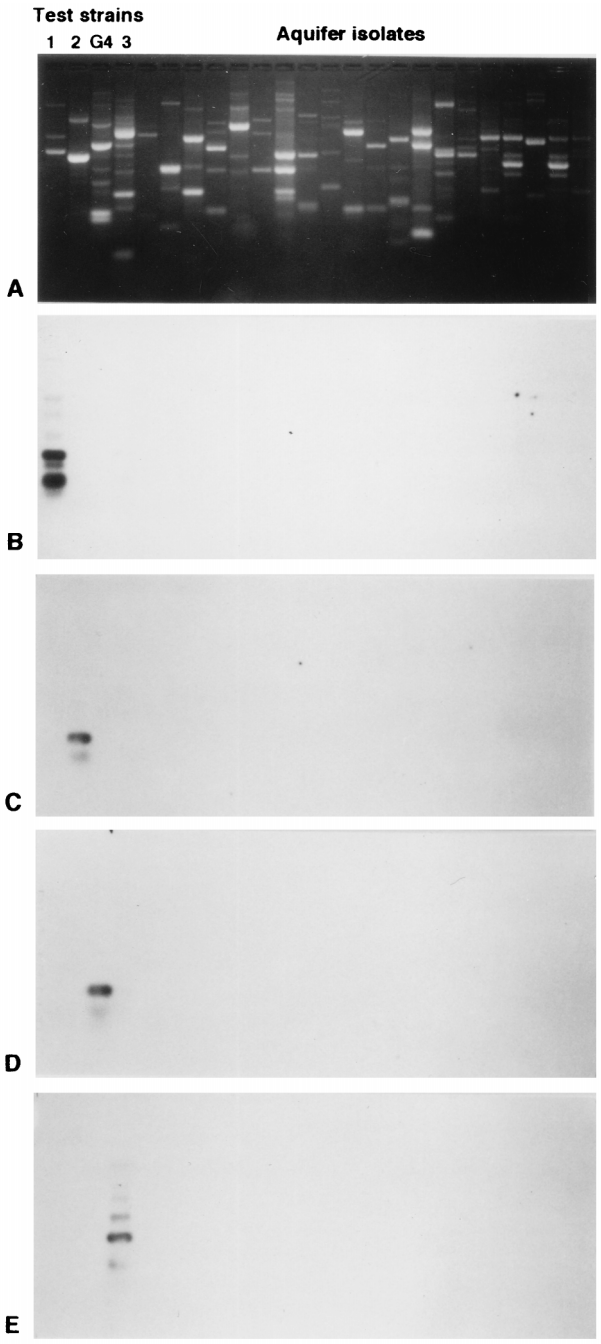

FIG. 6. (A) Ethidium bromide-stained agarose gel of rep-PCR amplification products from 20 of the 64 aquifer isolates. Corresponding Southern blots were hybridized to probes 1B (B), 2B (C), G4A (D), and 3A (E).

nomes of other bacterial species is unknown, empirical data obtained from rep-PCR fingerprinting of diverse bacterial species (7, 10, 15, 21, 39, 41) suggest a similar number and distribution of the REP element. Thus, it is likely that rep-PCR amplification can be used to amplify random fragments of DNA from most, if not all, eubacterial species. Alternatively, there are other repeated sequences that are conserved among eubacteria such as the enterobacterial repetitive intergeneric consensus (7, 13, 15, 22, 32) or BOX elements (17, 21, 38). Consensus PCR primers have been developed for these elements as well and would be expected to yield amplification products that could serve as strain-specific probes. Our data suggest that probes that are small (<900 bp) might offer greater selectivity than larger ones and that major products from the rep-PCR amplification of the target strain DNA might offer greater sensitivity.

Second, the physical arrangements of REP elements differ among the genomes of closely related strains. Thus, when the outwardly directed REP primers are used to amplify regions of DNA flanked by REP elements, a unique collection of products that differ in size is obtained for each strain. Indeed, previous ecological, agricultural, and epidemiological studies utilizing rep-PCR fingerprinting have shown that it is possible to distinguish subspecies of organisms (7, 10, 15, 21, 39, 41). Hence, the DNA fragments obtained by rep-PCR are likely to have a size as well as sequence that is unique for a particular strain. Thus, while it is conceivable that a given DNA sequence might be conserved among closely related strains, the additional requirement that the probe hybridize to DNA of a specific size increases the selectivity of the method.

Third, the levels of DNA probe sensitivity and selectivity achieved in this study compare favorably to those reported for other PCR-based methods (8, 19, 24, 27, 31, 35, 36), and the use of rep-PCR offered the advantage of increasing the amount of target DNA present without requiring prior knowledge of the target sequence. Moreover, the analysis was simplified because it was not necessary to extract DNA prior to rep-PCR amplification. This overcomes the problems associated with extraction of DNA from some environmental samples which may include low yield and selective recovery (14) in addition to being time-consuming and laborious. Sensitivity could potentially be further increased by concentrating bacteria from water samples onto a filter prior to PCR (2) or by preincubation of the samples in enrichment media to increase cell numbers. However, results from the latter may be confounded if over time the strains become viable but nonculturable (1, 20). The data indicate that most probes derived from rep-PCR amplification are specific to their parent strains and do not cross-hybridize with rep-PCR products amplified from nontarget organisms. As a result, only a few fragments must be tested to obtain an adequate probe. This may result from the fact that REP elements are found in extragenic regions of the chromosome, and therefore there may be a greater probability of amplifying DNA that lies outside structural or regulatory genes. One might expect that such regions are more divergent among bacterial species than either structural or regulatory genes and therefore are more likely to be unique to a given bacterial strain.

Finally, the method provides a semiquantitative estimate of the number of target organisms present in a sample. Estimates of relative abundance can be made by comparison of the signal to those obtained when increasing and known amounts of the target organism are used to seed a control sample. A more quantitative measure could be taken by combining PCR with a standard most-probable-number (MPN) procedure (29). This would simply involve scoring MPN tubes for presence of the target strain using a rep-PCR reaction followed by hybridization to the probe. Since sample preparation and analysis are simple, a large number of samples could be handled efficiently.

Several recent studies describe the development and use of bacterial strain-specific probes (4, 5, 16, 20, 34, 35). The two challenges faced in the development of such methodologies are to obtain nucleic acid sequences of suitable specificity to distinguish between strains of a single species and to achieve adequate detection sensitivity by specific amplification of the target DNA sequence from mixed microbial communities. Two

basic approaches have been used. One approach is to use variable regions of conserved and universally distributed genes, such as those encoding rRNA (43) or DNA gyrase (42), to design strain-specific probes as well as PCR primers for amplification of the target sequence from microbial communities. Similarly, oligonucleotide probes directly targeted to rRNA of actively growing cells have been used to detect specific strains by in situ hybridization to whole cells (5, 20). These approaches require isolation and sequence analysis of the target DNA segment from the strain of interest, followed by comparison to other sequences available in databases. There are at least two serious limitations to this approach. First, the rRNA genes are so highly conserved that identification of sequences which differentiate closely related strains may be difficult or impossible (35, 42). Indeed, the variable region is sometimes identical between different species and even genera (12), increasing the likelihood of obtaining false positives upon amplification from complex mixtures of DNA. Second, the rRNA sequence databases, although relatively extensive, currently represent only about 25% of described species (1) and a much smaller fraction of those found in nature. This is a potentially serious limitation to comparative sequence analysis since the vast majority of organisms in the environment have not been characterized (1). This increases the probability that a false-positive signal could by chance be obtained with what appeared to be a unique probe based on comparison with available rRNA sequence information. The base substitution frequency is much higher for *gyrB*, so that greater differences exist between strains (42). However, fewer sequences are available for comparison, and so more empirical screening is necessary.

A second approach previously used to obtain strain-specific fragments of DNA is trial and error. Specifically, random DNA fragments isolated by cloning restricted genomic DNA from the strain of interest are screened exhaustively against nontarget strains until a unique fragment is identified (16, 35). Subtractive hybridization has been used to simplify the screening procedure by enriching for unique sequences (4, 6, 34). However, the additional procedures required seem to negate any benefits derived from decreasing the number of candidate fragments to be screened. Once the strain-specific fragment is obtained, sequence analysis is necessary for the purpose of designing PCR primers (27, 34, 35). Primers must then be synthesized and tested for specificity and reliable PCR amplification.

Our approach is also based on cloning of random fragments, but the number of candidate fragments is greatly reduced by using the subset amplified in the rep-PCR reaction. Most probes derived from rep-PCR fragments are specific to their parent strains when hybridized against rep-PCR products from nontarget organisms. These probes can be developed and used without any prior knowledge of the host strain or the probe sequence. And, the target sequence can be amplified from a mixed community without the need for strain-specific PCR primers. Thus, it appears that the method reported here offers significant advantages over those previously described.

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