The use of molecular techniques to evaluate the survival of a microorganism that was injected into an aquifer.

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Abstract:

An anonymous polymerase chain reaction (PCR) primer set and an internal probe were developed that are specific for *Pseudomonas* sp. strain B13, a 3-chlorobenzoate-metabolizing strain. Using this primer set and probe, we were able to detect *Pseudomonas* sp. strain B13 DNA sequences in DNA extracted from aquifer samples fourteen and a half months after *Pseudomonas* sp. strain B13 had been injected into a sand and gravel aquifer on Cape Cod. This primer-set and probe was also used to analyze isolates from 3-chlorobenzoate enrichments of the aquifer samples by Southern blot analysis. Hybridization of Southern blots with the *Pseudomonas* sp. strain B13-specific probe and a catabolic probe in conjunction with restriction fragment length polymorphism (RFLP) analysis of ribosome genes were used to determine that viable *Pseudomonas* sp. strain B13 persisted in this environment. We also isolated a new 3-chlorobenzoate-degrading strain from one of these enrichment cultures. The B13-specific probe does not hybridize to DNA from this isolate. The new strain could be an independent 3-chlorobenzoate degrader or the result of gene exchange between *Pseudomonas* sp. strain B13 and an indigenous bacterium. The later interpretation is supported by ~~This speculation is based on~~ an RFLP pattern of ribosome genes that differs from that of *Pseudomonas* sp. strain B13, identically sized restriction fragments hybridizing to the catabolic gene probe for the B13 chlorobenzoate pathway, and the absence of any enrichable 3-chlorobenzoate degrading strains in the aquifer prior to inoculation.

Introduction:

Much of the world's population relies on groundwater for domestic and industrial use. However, increasingly, groundwater has become contaminated by chemicals from various sources such as leaky underground gasoline storage tanks, landfill leachates, and industrial wastes. The use of microorganisms capable of degrading various toxic chemicals is a potential means for *in situ* remediation of these polluted sites (19, 37). One strategy for *in situ* biodegradation of pollutants is to stimulate the metabolism of the native microflora by the addition of nutrients or electron acceptors. Alternatively, if microorganisms capable

of degrading specific pollutants are not present or are ineffective, it would be necessary to attempt to inoculate the aquifer. This is more likely to be considered for remediation of oligotrophic aquifers where microbial biomass and hence diversity is low. In addition, microorganisms can be modified by natural gene transfer or through the use of molecular cloning techniques to improve their catabolic capabilities (for example see Rojo et al (28). One constraint on introducing foreign microorganisms into the environment is that it may be difficult to achieve long-term survival and establishment of a founding population (35).

Microcosms can be used as model ecosystems to make predictions about natural systems. However, there is little field data to confirm the validity of these predictions. To assess the survival of an introduced organism in a natural groundwater environment, *Pseudomonas* sp. strain B13, a naturally occurring 3-chlorobenzoate-degrading bacterium that has been used as a parent strain for genetically modified catabolic pathways (28), was injected at various depths into an unconfined sand and gravel aquifer on Cape Cod, MA. This aquifer contains a contaminant plume of municipal sewage, that has depleted oxygen within the aquifer. Because detection limits for plate count enumerations were undesirably high and because *Pseudomonas* sp. strain B13 might remain viable but unculturable after a prolonged period in an oligotrophic environment (6), we developed and evaluated nucleic acid-based methods for detecting and possibly enumerating *Pseudomonas* sp. strain B13 at low numbers in natural samples. Our approach was to extract total DNA from sediments and to use the polymerase chain reaction (PCR) for detection of *Pseudomonas* sp. strain B13-specific DNA sequences since the method has made it possible to detect small amounts of target sequences in a complex background (29). PCR amplification has been demonstrated to detect low levels of target DNA sequences in laboratory studies (3, 4, 34, 36), but reports of its successful use in soil and aquifer samples from the field are scarce.

In this report we describe the development of PCR primers and probes for an anonymous *Pseudomonas* sp. strain B13 gene fragment that serves as a *Pseudomonas* sp. strain B13-specific marker. We also describe the use the *Pseudomonas* sp. strain B13

anonymous primer and probe to detect *Pseudomonas* sp. strain B13 DNA in aquifer samples taken fourteen and a half months after *Pseudomonas* sp. strain B13 had been injected into an aquifer on Cape Cod. Selective enrichments, restriction fragment length polymorphisms (RFLPs) of ribosome genes, and a 3-chlorobenzoate catabolic probe (9) were used to confirm the presence of *Pseudomonas* sp. strain B13 in the aquifer. The *Pseudomonas* sp. strain B13-specific and catabolic probes and RFLP were also used to identify a new 3-chlorobenzoate-degrading strain in an enrichment culture. Limitations of the PCR technique for environmental monitoring were encountered and are discussed.

Materials and Methods:

Organisms and culture conditions. The strains and plasmids used in this study are listed in Table 1. *Pseudomonas* sp. strain B13 was maintained on M9 medium (22) supplemented with trace minerals and 5 mM 3-chlorobenzoate. *Escherichia coli* XL1-Blue was maintained on LB medium (22) with 12.5 μM tetracycline. All other strains were maintained on half strength tryptic soy broth medium (Difco Laboratories, Detroit, MI). Native aquifer microorganisms were isolated by plating on 1/10 strength Peptone-Tryptose-Yeast extract-Glucose (PTYG) medium. *E. coli* was incubated at 37°C; all other strains were cultured at 30°C

Field site and sampling. The study site was an unconfined sand and gravel aquifer on Cape Cod near Falmouth, MA. Regional ground water flow rates range between 0.4 and 0.6 m/da. The site contains a large plume of sewage-contaminated groundwater, which has affected dissolved organic carbon and oxygen concentrations within the aquifer (18). The microbial population distributions within the plume have been described (11, 12). The three depths at which *Pseudomonas* sp. strain B13 was injected had different geochemical characteristics and different degrees of contamination; level 1, in uncontaminated groundwater above the plume is aerobic and is low in nutrients, level 2, is within the plume but still contains measurable levels of oxygen (6-8 mg/L), and level 3, within the plume has no detectable oxygen (< 0.05 mg/L). Approximately 150 L of ground water containing

10^{10} culturable *Pseudomonas* sp. strain B13/L and 10^{11} DAPI (4',6-diamidino-2-phenylindole)-stained *Pseudomonas* sp. strain B13/L were injected at each of five well points at all three depths. Fourteen and a half months later, aquifer material was recovered by a split-spoon sampling device from the injection point and down gradient as described in Krumme et al. (16), as are further details concerning the field injection, well design and environmental characteristics of the different depths.

DNA isolation. Genomic DNA from pure cultures was prepared by standard techniques. In brief, bacteria grown to maximum densities were recovered from liquid cultures by centrifugation, resuspended in 25% w/v sucrose, and treated with 0.625 mg ml^{-1} lysozyme EC 3.2.1.17 (L 6876, Sigma) and 0.06 M EDTA at 37°C. After a 10 min incubation the suspension was diluted with water (7% sucrose final conc.) and SDS was added to a final concentration of 0.5%. The suspension was then incubated 30 min at 37°C with 0.13 mg ml^{-1} Ribonuclease A, EC 3.1.27.5 (Sigma) and an additional 30 min with 0.13 mg ml^{-1} predigested protease (*Streptomyces griseas*, Sigma). Following extraction with phenol and chloroform the DNA was recovered by precipitation with ethyl alcohol.

We employed a direct *in situ* lysis approach (23). Because the biomass in the aquifer sediments was low, ranging from 1.8×10^7 to 4×10^7 bacteria per cm^3 (12), in comparison with soils (ca 10^9 g^{-1}) we used 100 g of aquifer material for each extraction. To process a large number of samples efficiently, we concentrated the DNA by reverse dialysis in polyethylene glycol (PEG) as described previously (32). In brief, 100 g of aquifer material was mixed with ten ml of TE buffer (10 mM Tris, 1 mM EDTA), Sarkosyl detergent was added to a final concentration of 0.9%. The samples were incubated at 70°C for 1 h and were shaken by hand every 15 min. The samples were centrifuged 12,000 x *g* for 15 min and the supernatant collected. The samples were maintained at 4°C for the remaining procedures. The samples were then sequentially washed five times with 15 ml of TE buffer and the washes were pooled with the lysis solution. After clearing by centrifugation (12,000 X g for 15 min), the solution was transferred to 6,000-8,000-

molecular weight cutoff dialysis tubing (Spectra/por 132665; Spectrum Medical Industries, Los Angeles, CA) and buried in PEG (molecular weight 15,000-20,000) overnight (ca 14-16 h) at 4°C. DNA was purified from the concentrated samples (ca 2 ml reduced from 80 ml) by isopycnic CsCl centrifugation (208,000 X *g* for 20 h). Yields ranged from 1 to 10 µg per sample. Aquifer DNA for use as a background control was prepared by inoculating 100 ml of 1/10 strength PYTG with 10 g of sediment from the Cape Cod aquifer that had not been inoculated with *Pseudomonas* sp. strain B13. Following a two day incubation with shaking at 30°C, the sand was allowed to settle and the bacteria were harvested from 10 ml of supernatant by centrifugation for 10 min (12,000 X g). DNA was prepared from these enrichments as described above for pure cultures.

Construction of anonymous *Pseudomonas* sp. strain B13-specific PCR primer set. Bluescript KS- phagemid (Stratagene, Inc., La Jolla, CA) and *Pseudomonas* sp. strain B13 DNA were cleaved with restriction enzymes *Eco*RI, *Bam*HI, *Pst*I, or *Hin*dIII (New England Biolabs, Beverly, MA) following the manufacturer's directions. *Pseudomonas* sp. strain B13 genomic fragments were randomly ligated, using T4 DNA ligase (New England Biolabs, Beverly, MA), into Bluescript KS- vectors that had been cleaved with the same enzymes. Ligated DNA was used to transform *Escherichia coli* XL1-Blue (Stratagene, Inc., La Jolla, CA). Plasmid DNA from recombinants was isolated using standard methods (22). Plasmids were cleaved with the same enzymes that were used for their construction and analyzed by agarose gel electrophoresis. Recombinants with single inserts between 500 and 1000 base pairs were selected for further screening.

Recombinants containing inserts unique to *Pseudomonas* sp. strain B13 were selected by screening on colony blots. Approximately 1000 bacterial isolates from the Cape Cod aquifer as well as selected laboratory strains (Table I) were grown up on 1/10 strength PTYG agar plates, transferred to nitrocellulose filters, and lysed (22). *Pseudomonas* sp. strain B13 was used as a positive control and *E. coli* XL1-Blue as a negative control. *Pseudomonas* sp. strain B13 inserts from the recombinant phagemids

were labeled with α-[^{32}P] dCTP (3,000 Ci/mM; Dupont, NEN Research Products, Wilmington, DE) using a random hexamer priming kit from Boehringer Mannheim Biochemicals (Indianapolis, IN) and hybridized to the colony blots. Twelve different clones were used as probes for colony blots. Two inserts hybridized exclusively to *Pseudomonas* sp. strain B13 and were selected as potential *Pseudomonas* sp. strain B13 probes.

The two *Pseudomonas* sp. strain B13 clones, pGB13-1 and pGB13-6, were sequenced using the dideoxyribonucleotide chain termination method (30) using a Sequenase kit (U.S. Biochemical, Cleveland, OH). The sequences were used to design 20 base pair PCR primers corresponding to regions near the ends of each insert, such that the internal regions would be amplified. The oligonucleotide primers were synthesized by the Michigan State University Macromolecular Facility. Primer specificity for *Pseudomonas* sp. strain B13 DNA was further tested by amplifying *Pseudomonas* sp. strain B13 DNA or DNA isolated from the Cape Cod aquifer microoorganisms using the external primers. Finally, a set of internal primers was designed to amplify a smaller region interior to the first primer set of the cloned *Pseudomonas* sp. strain B13 DNA in pGB13-1. These primers were used to amplify a fragment from the cloned *Pseudomonas* sp. strain B13 DNA fragment (pGB13-1) to use as an internal probe to confirm the presence of amplified *Pseudomonas* sp. strain B13 DNA.

Polymerase chain reaction. Amplification was in a Thermal Cycler (Perkin-Elmer Cetus) using the following parameters: an initial 2 min. melting period at 94° C followed by 30 cycles of the following series: 2 min. 94°C, 15 s 60°C, 1 min. 72°C. The reaction conditions were 50 mM KCl, 10 mM Tris-Cl pH 8.3, 1.5 mM MgCl, 0.01% gelatin, 200 μM each nucleotide, 2 μM each primer, 1.25 U Amplitaq/50 μL reaction. Amplification controls were a set of universal 23S ribosomal primers S2301 (5' TTCTGCAGTACCGTGAGGGAAAGG 3') and S23R01 (5' GGCTGCTTCTAAGCCAAC 3') capable of amplifying 23S ribosome gene sequences

from eubacteria. The 23S primer set amplifies a 611 bp fragment in *E. coli*. The 23S primer sequences were provided by D. Dwyer (German Institute for Biotechnology, Braunschweig).

Enrichments. Aquifer samples were taken fourteen and a half months following *Pseudomonas* sp. strain B13 injection into a Cape Cod aquifer at 0.61 meter intervals below the surface, from 4.9-9.8 meters below the surface (mbs), at the injection site. Additional samples were taken at the same depths 0.91, 1.83, and 3.66 meters down gradient (relative to groundwater flow) from the injection site. Numbers of *Pseudomonas* sp. strain B13 in aquifer samples were estimated by the most probable number technique on 3-chlorobenzoate medium (1). For enrichment cultures, samples from each depth were mixed and 10 g was added to 90 ml of 3-chlorobenzoate medium in sterile 250 ml flasks. Each sample was done in triplicate. Enrichments were incubated with shaking at 25°C for 4 weeks. Samples were taken from the enrichments at one week intervals and tested for growth on 3-chlorobenzoate as the sole carbon source. Isolates growing on 3-chlorobenzoate were purified and their DNA isolated for comparison with *Pseudomonas* sp. strain B13 DNA.

Analysis of isolates obtained from 3-chlorobenzoate enrichment of aquifer sediments. DNA from aquifer isolates, *Pseudomonas* sp. strain B13, and control bacteria were cleaved with the restriction endonucleases, *Eco*RI, *Hind*III, or *Pst*I (New England Biolabs, Beverly, MA). Restriction fragments were separated by electrophoresis on 0.7 % agarose gels and immobilized on Zeta Probe nylon membranes (Biorad Inc, Richmond, CA) by alkaline blotting, following the manufacturers instructions. Blots were probed with ribosomal probes, pST32 comprising the 16S and 23S genes and intergenic sequences from *Salmonella typhimurium* (26) and pAR17 a universal 23S ribosome gene probe (27), a catabolic probe, *clc*D (9), or with the anonymous *Pseudomonas* sp. strain B13-specific probe. Probes were labeled with α-[^{32}P]dCTP (3,000 Ci/mM; Dupont, NEN Research Products, Wilmington, DE) either by nick translation or by the random priming

technique using a kit from Boerhinger Manheim Inc (Indianapolis, IN). The 16/23S ribosome probe, pST32, was labeled by nick translation. The 23S ribosome probe, pAR17, was prepared by first transferring the 23S fragment to pUC19. The fragment was then amplified by PCR using a set of M13 universal primers (M13F=5'CCCAGTCACGACGTTGTAAAACGAC3'; M13R=5'AGGAAACAGCTATGACCATGATTAC3'). The amplified fragment was gel purified and labeled by random priming. The *clc*D probe was made by cutting plasmid pDC100 with *Pvu*II and isolating the 1.1 bp fragment comprising the *clc*D gene from an agarose gel. The fragment was then labeled by random priming. Blots were hybridized at high stringency, 65°C in 6X SSC, 5X Denhardt's solution, 0.5% sodium dodecyl sulfate, 0.2 mg denatured salmon sperm DNA ml^{-1}, and 5% w/v dextran sulfate.

3-Chlorobenzoate metabolism. 3-Chlorobenzoate metabolism was determined by measuring the disappearance of 3-chlorobenzoate in the culture medium. Analysis was by high performance liquid chromatography using a C18 column (Supelco). The liquid phase was 33% acetonitrile, 67% H_2O, and 0.1% phosphoric acid with a flow rate of 1.5 ml/min. 3-Chlorobenzoate disappearance was measured using a *uv* detector set at 230 nm.

Results:

Construction of *Pseudomonas* sp. strain B13 PCR primers and probes. To construct a set of PCR primers and a probe specific for *Pseudomonas*. sp. strain B13, total *Pseudomonas* sp. strain B13 genomic DNA was cleaved with restriction endonucleases and ligated into a Bluescript KS vector. The sizes of the cloned fragments were determined by agarose gel electrophoresis. Recombinants with single inserts 0.5-1.5 kb in size were selected for further screening. To identify *Pseudomonas* sp. strain B13-specific DNA sequences, the cloned *Pseudomonas* sp. strain B13 sequences were hybridized to DNA from laboratory strains and Cape Cod aquifer isolates by colony blot hybridization. Two clones, pGB13-1 and pGB13-6, were identified that hybridized exclusively to *Pseudomonas* sp. strain B13 DNA and not to any of the aquifer isolates or laboratory

strains (data not shown). To further evaluate the suitability of each of these cloned fragments for sensitive detection of *Pseudomonas* sp. strain B13, the fragments were partially sequenced. The sequence data was used to design PCR primers for each end of the cloned fragments. To test the primers, PCR reactions were set up to attempt to amplify both *Pseudomonas* sp. strain B13 DNA and Cape Cod aquifer DNA. The reaction products were analyzed by agarose gel electrophoresis. Primers derived from pGB13-1 sequences amplified a 711 bp product from *Pseudomonas* sp. strain B13 DNA, but not from Cape Cod aquifer DNA. Primers derived from pGB13-6 sequences amplified products of similar size, approximately 240 bp, from both *Pseudomonas* sp. strain B13 DNA and from Cape Cod aquifer DNA. Because the *Pseudomonas* sp. strain B13 DNA fragment cloned in pGB13-1 appeared to represent a sequence that could distinguish *Pseudomonas* sp. strain B13 DNA from bulk aquifer DNA, the insert was completely sequenced and a second set of primers internal to the first pair were designed to amplify a 344 bp internal region to use as a DNA probe (Fig. 1). The DNA sequence and the predicted polypeptide sequences from translation all reading frames were used to screen the Genbank (version 71.0) and EMBL (version 30.0) data bases using the FASTA and TFASTA algorithms and the Wisconsin GCG Sequence Analysis (7). No similarities to sequences in the data bases were found.

The ability to amplify low amounts of *Pseudomonas* sp. strain B13 DNA in a background of non-target DNA was tested by diluting *Pseudomonas* sp. strain B13 DNA and adding it to a constant amount of DNA from enrichments of sediments from the aquifer on Cape Cod. The *Pseudomonas* sp. strain B13 target DNA sequence was amplified by PCR using the external primers, primer set 1 (Fig. 1). The PCR products were analyzed by gel electrophoresis, transferred to nylon membranes, and hybridized with the *Pseudomonas* sp. strain B13 internal probe, an internal sequence amplified from pGB13-1 using the internal primers, primer set 2 (Fig. 1). We were able to routinely detect 1 pg, $1x10^{-12}$g, of *Pseudomonas* sp. strain B13 DNA (Fig. 2). As positive controls for PCR

amplification, duplicate reactions were performed using 23S ribosome universal primers to amplify ribosome encoding DNA. In all reactions where DNA was present an amplification product of the correct size, approximately 600 bp, was observed. No amplification products were observed in controls without DNA (data not shown).

Next we tested the ability of the *Pseudomonas* sp. strain B13-specific primer set to detect *Pseudomonas* sp. strain B13 sequences from extracted aquifer sediments amended prior to extraction with *Pseudomonas* sp. strain B13 grown in liquid cultures. Aquifer sediments from all depths, that did not contain *Pseudomonas* sp. strain B13, were mixed together and divided into 100 g aliquots. Various amounts of *Pseudomonas* sp. strain B13 were added to the sediments. Sediments without added *Pseudomonas* sp. strain B13 were used as negative controls. Bacteria in the sediments were then lysed *in situ* and the DNA purified. Duplicate samples of DNA were amplified using either the *Pseudomonas* sp. strain B13 specific primer set 1 or the universal 23S ribosome primer set. The results of these experiments were inconsistent. In some experiments, although DNA was present, as determined by ethidium bromide staining of DNA electrophoretically separated on agarose gels, we were unable to amplify either *Pseudomonas* sp. strain B13 DNA with the *Pseudomonas* sp. strain B13 primers or total eubacterial DNA with the universal primers. When the DNA was successfully amplified, the detection sensitivity for *Pseudomonas* sp. strain B13 was seldom better than traditional methods such as selective plating, on average 10^3/g of aquifer material (data not shown).

The disparity in our ability to amplify DNA extracted from homogeneous aquifer samples using PCR, led us to investigate the possibility that there might be differences between aquifer samples taken at different times or at different depths. To test these possibilities, DNA was extracted from aquifer sediments that had been taken at 0.61 m intervals below the surface at three different times at the injection site. The DNA samples were amplified using the universal 23S ribosome primers. DNA extracted from all samples

hybridization (Fig. 3C). No hybridization was observed for the aquifer DNA control, although the DNA was amenable to amplification using the 23S primers (Fig. 3A).

Although bacteria from the aquifer samples capable of degrading 3-chlorobenzoate could not be enumerated by MPN, the ability to amplify *Pseudomonas* sp. strain B13-specific sequences from aquifer DNA and positive enrichment cultures suggested that *Pseudomonas* sp. strain B13 was still present in the aquifer. We investigated this possibility by analyzing 3-chlorobenzoate-degrading isolates from the enrichment cultures using other methods. Based on the successful characterization of environmental isolates from the subsurface using restriction fragment length polymorphism (RFLP) by Reeves et al. (26), we decided to try this technique with our enrichment isolates. We initially used pST32, comprised of the 16S and 23S ribosome genes and the intergenic region from *Salmonella typhimurium* , as a probe (26). For RFLP analysis, DNA from enrichment isolates was cleaved with restriction endonucleases, resolved on agarose gels, and transfered to nylon membranes. The Southern blots were hybridized to pST32 and complex band patterns were observed (data not shown). To try to achieve a simpler hybridization pattern for subsequent experiments, a smaller probe comprised of a portion of the 23S ribosome, pAR17, was used (27). The RFLP patterns for *Eco*RI digested DNA were different for each of the laboratory strains tested and none of them resembled *Pseudomonas* sp. strain B13 (Fig. 4, Panel A). The RFLP patterns of laboratory strains using *Pst*I digested DNA also differed from *Pseudomonas* sp. strain B13, however *P. mendocina* and *P. aeruginosa* had identical patterns (Panel C). When RFLP patterns from enrichment isolates were compared with *Pseudomonas* sp. strain B13 most of the isolates had hybridization patterns identical to *Pseudomonas* sp. strain B13 (Fig. 4). Notable exceptions were two isolates from a single enrichment culture from sediments 7.92-8.53 mbs (asterisks, Fig. 4). These isolates had 23S ribosome restriction patterns identical to each other, but different from *Pseudomonas* sp. strain B13 and the other laboratory strains tested.

from depth 3, 7.32-8.53 mbs, and below, 8.53-9.14 mbs, could be amplified by PCR (Table 2). DNA extracted from depth 1, 5.49-6.10 mbs, could not be amplified.

Evaluation of *Pseudomonas* sp. strain B13 survival in the aquifer on Cape Cod. Fourteen and a half months after *Pseudomonas* sp. strain B13 was introduced into the aquifer, samples were taken at 0.61 m intervals below the surface at the injection site starting at 4.88 mbs, as well as from the same depths 0.91, 1.83, and 3.66 meters down gradient from the injection site. The number of *Pseudomonas* sp. strain B13 remaining in the Cape Cod aquifer was evaluated by most probable number analysis on 3-chlorobenzoate medium. Positive results were obtained for only one sample. At depth 3, 7.92-8.53 mbs, at the injection site, MPN analysis indicated that there were approximately 33 bacteria g^{-1} capable of growing on 3-chlorobenzoate. However, 3-chlorobenzoate enrichment cultures showed growth in four samples 7.32-7.92, 7.92-8.53, 8.53-9.14, and 9.14-9.45 mbs at the injection site. In addition one of the samples, 9.14-9.75 mbs taken 0.91 m down gradient from the injection site had bacterial growth on 3-chlorobenzoate.

The presence of *Pseudomonas* sp. strain B13 in the aquifer samples was investigated by PCR amplification of DNA and Southern hybridization using the *Pseudomonas* sp. strain B13-specific primer sets. DNA was extracted from 100 g of each field sample and amplified using the 23S ribosome primers and the *Pseudomonas* sp. strain B13-specific primers. For each reaction, DNA from the equivalent of 10 g of sand was used. No amplification products were obtained using the 23S primer set from samples taken from 4.88-5.47, 5.47-6.10, 6.10-6.71, 8.53-9.14, or 9.14-9.75 mbs (Fig. 3A). Using the *Pseudomonas* sp. strain B13 specific primers amplification products were visible only for *Pseudomonas* sp. strain B13 DNA and the sample taken 7.92-8.53 mbs on ethidium bromide stained gels (Fig. 3B). The amplification products from *Pseudomonas* sp. strain B13 primers were transferred to nylon filters and hybridized to the internal probe. Hybridization to the internal probe was observed for amplification products from samples taken at 6.71-7.32, 7.32-7.92, 7.92-8.53 mbs on an autoradiogram of the Southern

To confirm that the isolates from the enrichment culture with the different RFLP profile were able to metabolize 3-chlorobenzoate, cultures were grown in M9 medium with 3-chlorobenzoate as the sole carbon source. Cultures of *Pseudomonas* sp. strain B13 were grown in parallel and the medium was sampled at 24 h intervals. The culture medium was analyzed by HPLC for the presence of 3-chlorobenzoate. The novel isolates completely metabolized the 3-chlorobenzoate in 72 h compared to 48 h for *Pseudomonas* sp. strain B13.

The RFLP data suggested that with the exception of the two novel isolates the 3-chlorobenzoate metabolizing bacteria obtained in enrichment cultures were *Pseudomonas* sp. strain B13. To further investigate this interpretation, we used two additional probes to characterize the enrichment isolates. First we used the *Pseudomonas* sp. strain B13-specific internal probe that we had developed for analyzing PCR amplification products. When the Southern blots of *Eco*RI digested DNA was probed with the *Pseudomonas* sp. strain B13-specific probe a 2.6 kb fragment was identified for *Pseudomonas* sp. strain B13 and for all of the enrichment isolates that had *Pseudomonas* sp. strain B13-specific RFLP patterns using the ribosome probe (Fig. 5). DNA from the other laboratory strains and the two novel isolates did not hybridize with the *Pseudomonas* sp. strain B13-specific probe. Southern blots of the *Pst*I digested DNA hybridized with the *Pseudomonas* sp. strain B13-specific probe revealed a major band of 0.9 kb and an array of larger bands for *Pseudomonas* sp. strain B13 and all of the enrichment isolates except the two novel isolates (data not shown). Because the two enrichment isolates with different RFLP patterns were able to metabolize 3-chlorobenzoate, we evaluated whether the catabolic genes were similar to those of *Pseudomonas* sp. strain B13. We used a probe for the dienlactone hydrolase gene, *clc*D, that has a predicted amino acid sequence identical to the purified enzyme from *Pseudomonas* sp. strain B13 (9). Hybridization of the *clc*D probe to *Pst*I digested DNA revealed a major band of approximately 1.5 kb, as well as several larger bands (Fig. 6). Bands hybridizing to the *clc*D probe were observed for *Pseudomonas* sp. strain B13 and

for all of the 3-chlorobenzoate metabolizing enrichment isolates including the two novel strains (Fig. 6).

Discussion:

To validate microcosm studies as predictive models for the survival of introduced microbes in the environment, a 3-chlorobenzoate degrading bacteria, *Pseudomonas* sp. strain B13, was injected into a sand and gravel aquifer on Cape Cod (16). Parallel studies employed aquifer microcosms to test the survival of *Pseudomonas* sp. strain B13, and its genetically modified derivatives over time (16, 17). The unconfined aquifer contains a plume of contaminated groundwater as a result of secondary sewage disposal. The contaminant plume influences microbial populations within the aquifer, bacteria are more abundant and a greater proportion are free-living close to the contaminant source, where quantities of dissolved organic carbon are higher, but populations decline and a greater proportion attach to surfaces with increasing distance from the contaminant source. Free-living bacterial numbers ranged from 4.50-0.07 x $10^9 L^{-1}$ (11). Approximately 50 volatile organic compounds have been detected in the plume, including halogenated compounds (2). However 3-chlorobenzoate is not a known contaminant in this aquifer. Using enrichment cultures, Krumme could not find any 3-chlorobenzoate degrading organisms in sediments from the study site prior to the introduction of *Pseudomonas* sp. strain B13 (15). Thus the fate of culturable *Pseudomonas* sp. strain B13 in both the aquifer and microcosms comprised of aquifer sediments could be evaluated by growth on 3-chlorobenzoate as a sole carbon source. The inoculated strain was detectable by culture methods after one month in the upper aerobic zone of the aquifer, and after 14 and a half months in the anaerobic zone. The microcosms predicted survival at all depths for up to 2.3 months (16).

Our goal in this study was to develop sensitive nucleic acid-based techniques that would allow us to evaluate the fate the non-native microorganism, *Pseudomonas* sp. strain B13, at low concentrations and in the event that it became unculturable. Initially we tried using oligonucleotide probes targeted to variable regions of the *Pseudomonas* sp. strain

no 3-chlorobenzoate-degrading microbes were detected in the the aquifer prior to this study, these data suggest that the 3-chlorobenzoate catabolic pathway genes may have been transferred to another microbe. Natural transformation of *P. stutzeri* by sand-adsorbed DNA has been reported (21). Low microbial populations and a lack of selective pressure in the Cape Cod aquifer, make it less likely that the transformation occurred in the aquifer. *Pseudomonas* sp. strain B13 isolates were also isolated from this enrichment (Fig. 4, B and D, Fig. 5). Therefore the most likely explanation for the appearance of the new 3-chlorobenzoate-degrading organism is that a transfer of the catabolic genes occurred during the 3-chlorobenzoate enrichment of aquifer sediments.by transformation of a native aquifer microorganism. However we cannot rule out the possibility that this is an indigenous 3-chlorobenzoate degrader that happens to carry a highly homologous *clc*D gene on the same restriction fragment.

When we analyzed DNA extracted directly from the aquifer samples by PCR amplification, we obtained *Pseudomonas* sp. strain B13-specific amplification products from the sample taken at 6.71-7.32 mbs (Fig. 3). However we were unable to enrich for any 3-chlorobenzoate degrading organisms from the same samples. Possible explanations for these observations are that either *Pseudomonas* sp. strain B13 was present in the sample but was not culturable or that *Pseudomonas* sp. strain B13 was no longer present but its DNA persisted in the environment and was available for amplification. We also found that *Pseudomonas* sp. strain B13 was present 0.91 m downgradient from the injection site at 9.14-9.75 mbs. This suggests that there was some transport of *Pseudomonas* sp. strain B13 down gradient from the injection point. Previous injection studies at this site demonstrated that microbes are transported from the site of injection in forced gradient tracer experiments (13).

Molecular techniques can be useful tools for studying microorganisms in the natural environment (25, 31). Previous reports of PCR amplification of DNA from sediments report the ability to detect 1-10 microorganisms using PCR (34, 36). The high sensitivity

demonstrated that additional purification of DNA isolated directly from some environmental samples by agarose gel electrophoresis could improve the ability to amplify it by PCR (20).

When we added *Pseudomonas* sp. strain B13 to aquifer sediments prior to extraction, we were not able to achieve the same sensitivity of detection as in our experiments using serially diluted DNA added to non-target aquifer DNA as background. We could routinely detect 10^{-12} g of *Pseudomonas* sp. strain B13 DNA spiked in aquifer DNA, or approximately 17 bacteria, but could only achieve detection of 10^3 *Pseudomonas* sp. strain B13 on average when the bacteria was added to aquifer material and subsequently extracted. This is most likely due to losses of DNA during the extraction procedure. Additional improvements in DNA extraction and recovery techniques are needed in order to achieve more efficient and reproducible recoveries of DNA from low-biomass environmental samples.

As an alternative method for assessing the survival of *Pseudomonas* sp. strain B13 in the Cape Cod aquifer, we examined isolates from 3-chlorobenzoate enrichments of aquifer sediments obtained a year and a half after *Pseudomonas* sp. strain B13 had been released into the environment by RFLP analysis. Analysis of RFLP patterns obtained using a universal ribosome probe, suggested that all but two 3-chlorobenzoate degrading isolates were *Pseudomonas* sp. strain B13. The two microbes with different RFLP patterns were obtained from the same enrichment culture suggesting that they were most likely clonal isolates. The *Pseudomonas* sp. strain B13-specific probe developed for PCR amplification studies hybridized to a unique EcoRI restriction fragment in *Pseudomonas* sp. strain B13 and in the enrichment isolates that had the same ribosomal RFLP patterns as *Pseudomonas* sp. strain B13. The *Pseudomonas* sp. strain B13-specific probe did not hybridize to the isolates with different RFLP patterns. However, a 3-chlorobenzoate catabolic probe hybridized to specific *Pst*I restriction fragments from all of the enrichment isolates. These data suggest that the two isolates with different ribosomal RFLP patterns are not *Pseudomonas* sp. strain B13, but have a very similar dienelactone hydrolase. Since

B13 23S ribosome gene. The oligonucleotides were tested for their ability to distinquish *Pseudomonas* sp, strain B13 sequences from PCR-amplified DNA from laboratory strains and from isolates from the aquifer on Cape Cod. Although we tested a wide variety of hybridization temperatures and conditions, the oligonucleotides were not specific enough to distinquish *Pseudomonas* sp. strain B13 DNA from the DNA isolated from aquifer microorganisms. The lack of specificity of the oligonucleotides for *Pseudomonas* sp. strain B13 was due, at least in part, to constraints on comparing Pseudomonas sp. strain B13 23S ribosome sequences with other species due to a limited data base. Because we felt that it was unlikely that we could design an oligonucleotide sequence specific enough to distinguish a single strain of *Pseudomonas*, even from the larger data base available for 16S ribosome sequences, we sought an alternative probe for identifying *Pseudomonas* sp. strain B13 DNA in the aquifer material.

We used a fragment of DNA of unknown function that appeared to be unique to *Pseudomonas* sp. strain B13 DNA to develop a set of PCR primers that could be used to specifically amplify *Pseudomonas* sp. strain B13 DNA from a background of non-target DNA. A second set of primers internal to the first set was used to generate a probe that increased the sensitivity for detecting *Pseudomonas* sp. strain B13 sequences (Fig. 1 and 3). Although the primer and probe sets worked well using DNA from pure cultures and from enrichment cultures, we encountered difficulties amplifying DNA extracted directly from the aquifer sediments even though ethidium bromide stained gels indicated that DNA was present in all samples. The ability to amplify directly extracted aquifer DNA was correlated to the depth at which the sediment sample was taken (Table 2). The reason for this inhibition of PCR amplification is not clear. One possibility could be interference caused by binding of organic matter, silts, or mineral ions to the DNA (24), since there are differences in chemical and physical characteristics at different depths. For example, phosphate levels are quite low at level one but increase with depth (33). A recent report

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for detecting target genes from sediment samples in these reports were for sediments amended with the target organism and used multiple copy genes as targets. In contrast, in this study, we examined samples in which the target microbes were present for over a year, more closely reflecting the conditions in which these detection methods would be used in the field. PCR amplification techniques have great promise as detection tools and for obtaining a greater understanding of microbes in the natural environment. However, before PCR techniques can be used routinely to examine soils and sediments there is a need to develop less costly and more efficient techniques for isolating DNA from these types of environmental samples. In addition, the inability to quantify organisms detected by PCR amplification is a limitation for ecological studies.

We were unable to track *Pseudomonas* sp. strain B13 routinely in the aquifer using PCR amplification of DNA extracted from sediment samples. However, by employing a combination of additional techniques, we were able to determine the fate of *Pseudomonas* sp. strain B13 in the aquifer on Cape Cod. By combining PCR with RFLP analysis and Southern hybridization analysis using gene probes, we were able to determine that *Pseudomonas* sp. strain B13 was still present in the aquifer at the deeper depths and still viable fourteen and a half months after it had been injected.

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Figure Legends:

Figure 1. The sequence of the cloned DNA fragment from *Pseudomonas sp* strain B13 used to generate the *Pseudomonas* sp. strain B13-specific PCR primer sets. The sequences of the external primers, primer set 1, and the internal primers, primer set 2, are shown above the complete sequence. The primer binding-sites are indicated by underlining on the complete sequence. The sizes of the amplified external fragment and the amplified internal probe fragment are shown diagrammatically at the bottom of the figure.

Figure 2. Southern hybridization of *Pseudomonas* sp. strain B13 DNA amplified by PCR from background DNA using *Pseudomonas* sp. strain B13 external primers, resolved by agarose gel electrophoresis, and hybridized with the 344 bp *Pseudomonas* sp. strain B13 internal probe (Fig. 1). Serially diluted *Pseudomonas* sp. strain B13 DNA 10 ng to 1 fg, as indicated at the tops of the first five lanes, against a background of 500 ng of Cape Cod aquifer DNA, was amplified with primer set 1 (Fig. 1). Controls shown in the next five lanes comprise PCR amplifications of Cape Cod aquifer DNA without *Pseudomonas*

sp. strain B13 DNA amendment, reaction mix with no added DNA, and *Pseudomonas* sp. strain B13 DNA alone. The lane adjacent to *Pseudomonas* sp. strain B13 DNA was left blank.

Figure 3. PCR Amplified aquifer DNA extracted from the injection site fourteen and a half months after the introduction of *Pseudomonas* sp. strain B13. Panel A. Ethidium bromide stained gel of PCR amplification products from DNA extracted from different depths in the aquifer (mbs=meters below surface), using 23S universal primers. Pure *Pseudomonas* sp. strain B13 DNA and Cape Cod aquifer DNA without *Pseudomonas* sp. strain B13 addition are used as controls. The arrow indicates the 611 bp PCR-product. Panel B. PCR amplification products using the same DNA and the *Pseudomonas* sp. strain B13 external primers, primer set 1 (Fig. 1). The arrow indicates the 711 bp PCR-product. Panel C. Southern blot of the gel shown in panel B hybridized with the 344 bp Pseudomonas sp. strain B13 internal probe (Fig. 1). The arrow indicates the 711 bp PCR-amplification product.

Figure 4. RFLP analysis of *Pseudomonas* sp. strain B13 DNA, DNA from selected laboratory strains, and isolates from 3-chlorobenzoate enrichments of Cape Cod aquifer sediments. Panels A and B. Southern blots of *Eco*RI digested DNA from *Pseudomonas* sp. strain B13, laboratory strains, 3-chlorobenzoate-enrichment isolates, and total aquifer DNA hybridized to a universal 23S ribosome probe (27). Panels C and D. The same DNA samples shown in panels A and B digested with *Pst*I and hybridized with the 23S ribosomal probe. dg=isolate from the down-gradient enrichment.

Figure 5. Southern hybridization analysis of 3-chlorobenzoate-enrichment isolates using the *Pseudomonas* sp. strain B13 anonymous probe. The blots of *Eco*RI-digested DNA (Fig. 4 A and B) were stripped and hybridized with the 344 bp *Pseudomonas* sp. strain

B13 internal probe (Fig. 1). The arrow indicates a 2.6 kb fragment specific for *Pseudomonas* sp. strain B13.

Figure 6. Southern hybridization analysis of 3-chlorobenzoate-enrichment isolates using a catabolic gene probe clcD, the dienelactone hydrolase gene (9). The blot of the *Pst*I digested DNA (Fig. 4B) stripped and hybridized with the clcD probe. The arrow indicates a 1.5 kb fragment found in all 3-chlorobenzoate-degrading isolates. The non-*Pseudomonas* sp. strain B13 isolates are indicated by *.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference[a]
Strains		
Pseudomonas sp. strain B13	3Cba$^+$	(8)
P aeruginosa PAO1	3Cba$^-$	(14)
P. mendocina KR	3Cba$^-$ TOL$^+$	R. Olsen(UMI)
Pseudomonas sp. B8	3Cba$^-$rif^r	Lloyd Elliot (WSU) (10)
P. cepacia PCO 1224	3Cba$^-$ nal^r	R. Olsen(UMI)
Streptomyces coelicolor	3Cba$^-$	W. Champness(MSU)
Agrobacterium rhizogenes	3Cba$^-$	M. Thomashow(MSU)
Escherichia coli K12 Strain XL1-Blue	3Cba$^-$ recA$^-$	Stratagene (5)
Plasmids		
pDC100	3Cba$^+$ Ap^r p_{tac} lacI^q	(9)
pAR17	23S ribosome universal probe	(27)
pST32	16S-23S ribosome probe	(26)
pKS$^-$	Ap^r	Stratagene
pGB13-1	3Cba$^-$ Ap^r B13 specific probe	This work
pGB13-6	3Cba$^-$ Ap^r B13 specific probe	This work

[a]UMI, University of Michigan; WSU, Washington State University; MSU, Michigan State University

Primer Set 1 Forward 5'CCGCCTCGTCTCGCTGCTAG3'

Revers 5'TGTCCTGCCCAGGCTCGAAG3'

Primer Set 2 Forward 5'CTGGGAAACGCGAACCGACC3'

Reverse 5'CGGCAGTCTGCTGGTGCCAC3'

```
  1  CGAGGCCGCC TCGTCTCGCT GCTAGAGAAC ATCAGCCGCC AAACACCGCG
                Primer 1F
 51  GTGTGGGCGC TTTATCGCAG CAGCGCATCT GTCGCCGAAG TTCGCCAATT

101  GGTGGACTTC CTGAAGGATG GCCTGCAACG CATTCATCCA GGAACGGCTT

151  GAGTCCAACG CCGCTAGTGG CTCCGGGCCG AATCGTCGGC TGCCAGCATG

201  CCGACCGTGG CGATGCAGGA AGTCATGGAT ATGCGCTGGG AAACGCGAAC
                                                Primer 2F
251  CGACCCAGGC GAAGAGGCCG TACTTGAGCA GCTTGCCGAC GAAGACCACC

301  AGTAGCGCGC CTGGATAGTC CAGCGGGGCG TTGGCCAGCA CCACCAACGC

351  AGGCGTCTGC GGTACGGCGA GATGGATACG AGGAAGAGTG CCCAGCAAGT

401  GGTAGCTGTG GGTCCAGTCC ATCACCCGCT GCCAGGTCGG GTCCTGCAAC

451  ATCTGCGGGA AGTGAGCGTA GAGGCTGGCC CAGCCCATGT GGTGGGCAAC

501  GATCATCAGC AACAGCGCCC CCAGACTGCT TCCCAGCACA TGCGATGCCC

551  AGCCAACGCC GTGGCACCAG CAGACTGCCG GCACCACCAC GGAGGTGATG
                       Primer 2R
601  GGGAGATGGC GAACAGGGTG CCGACGAGGC CAGCCCCAAG CACAGCCAGG

651  GCAACTTCCA GCCGTCTCCG GCCCAGCGCA ACATTCGTGC AGAACGCCTT

701  CGAGCCTGGG CAGGACATCG CGAGGCGCTT CATGTCAGCC TCAGCGCCTG
        Primer 1R
751  CTGAAAGTTT GCTGCAAGCG
```

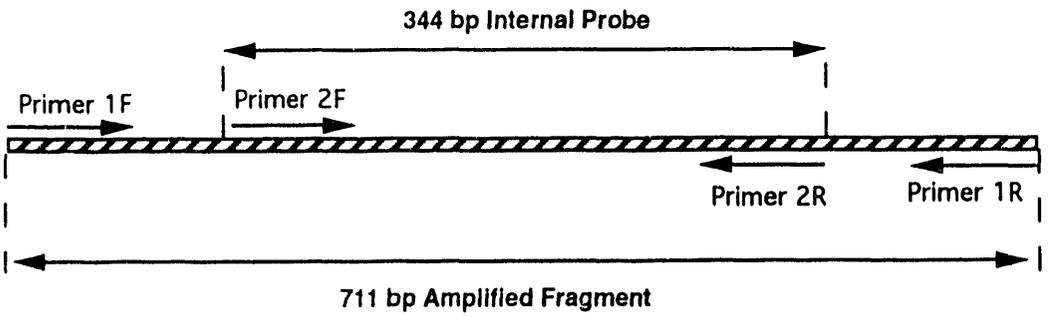


Fig. 1. The use of molecular techniques to evaluate the survival of a microorganism that was injected into an aquifer. Suzanne M. Thiem, Richard L. Smith, and James M. Tiedje

TABLE 2. The ability to amplify DNA extracted from different aquifer samples by PCR using a universal 23S ribosome primer set

		Sampling Time		
Depth (mbs)[a]	conditions[b]	1	2	3
(1)				
5.49-6.10	aerobic	-	-	-
(2)				
6.10-6.71	low O_2	-	-	-
6.71-7.32		-	+	+
(3)				
7.32-7.92	anaerobic	+	+	+
7.92-8.53		+	+	+
8.53-9.14	anaerobic	nd[b]	+	+

[a]mbs=meters below surface

[b]from Krumme (17)

[c]nd =not determined

Fig. 2. The use of molecular techniques to evaluate the survival of a microorganism that was injected into an aquifer. Suzanne M. Thiem, Richard L. Smith, and James M. Tiedje

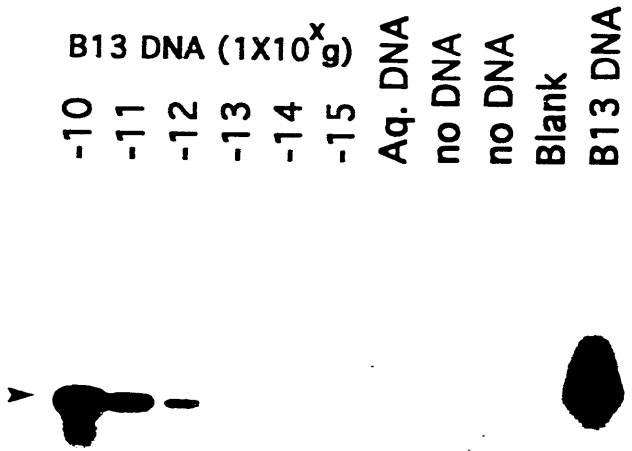


Fig. 3. The use of molecular techniques to evaluate the survival of a microorganism that was injected into an aquifer. Suzanne M. Thiem, Richard L. Smith, and James M. Tiedje

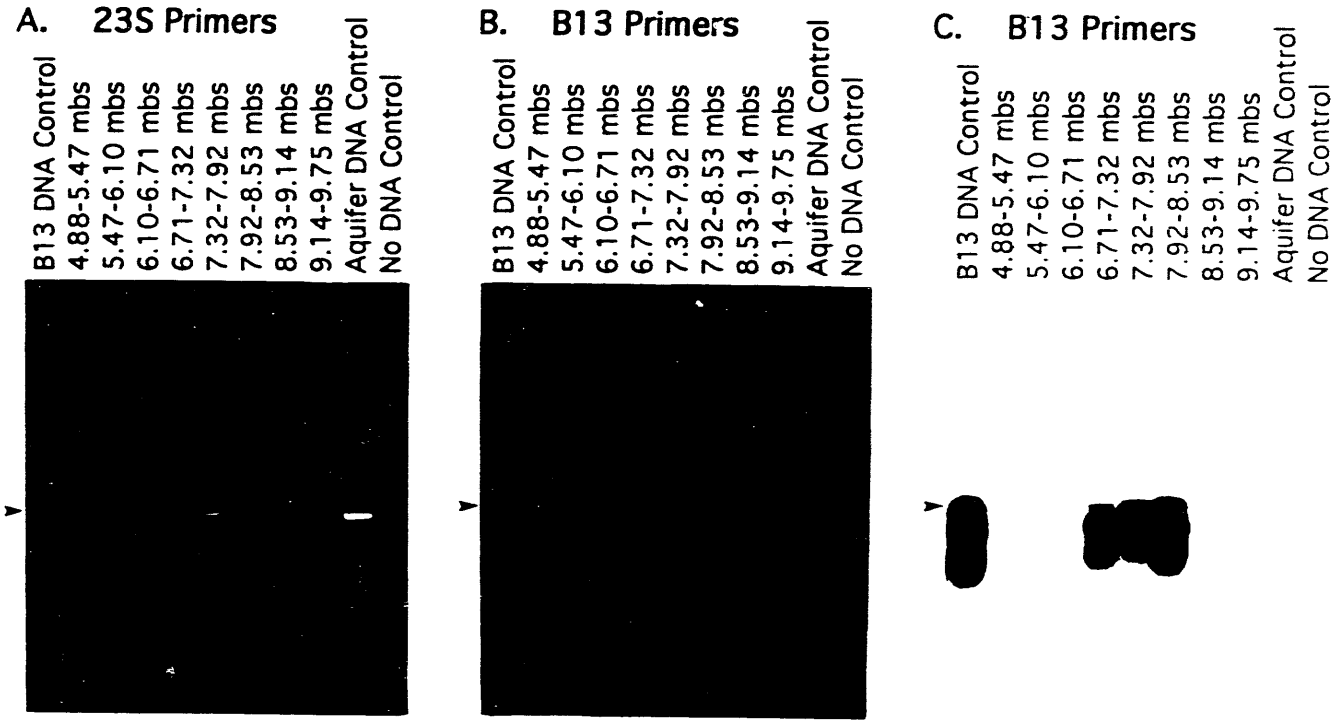


Fig. 4. The use of molecular techniques to evaluate the survival of a microorganism that was injected into an aquifer. Suzanne M. Thiem, Richard L. Smith, and James M. Tiedje

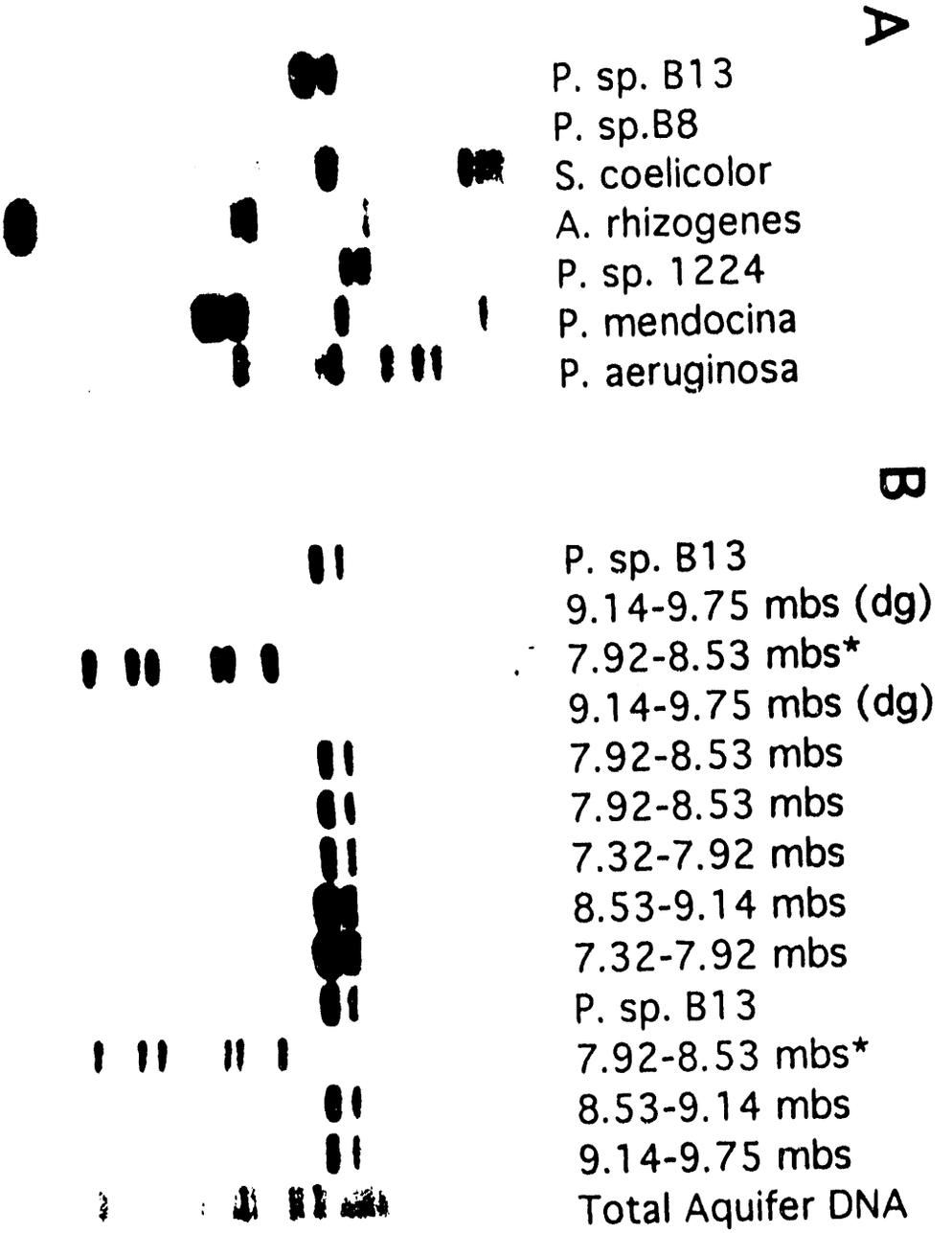


Fig. 4. The use of molecular techniques to evaluate the survival of a microorganism that was injected into an aquifer. Suzanne M. Thiem, Richard L. Smith, and James M. Tiedje

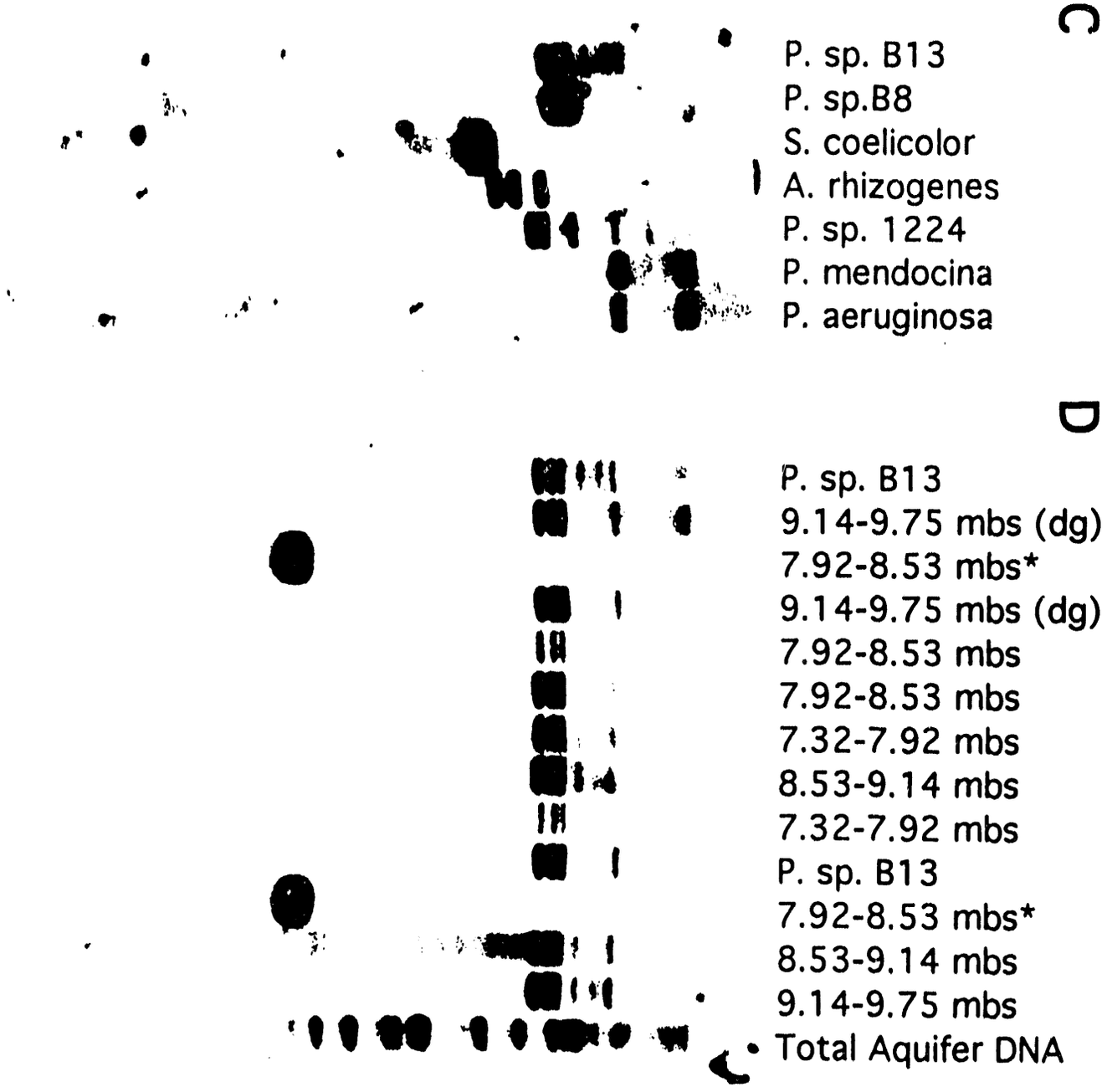


Fig. 5. The use of molecular techniques to evaluate the survival of a microorganism that was injected into an aquifer. Suzanne M. Thiem, Richard L. Smith, and James M. Tiedje

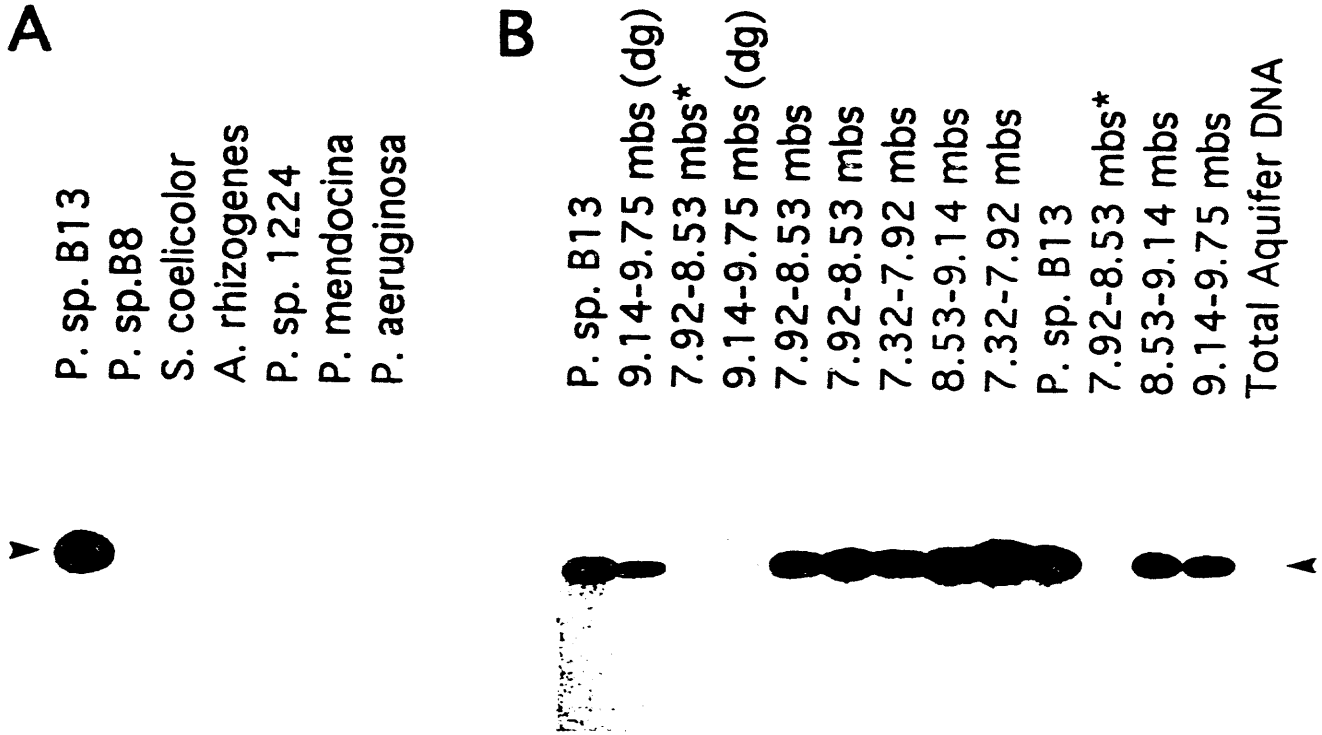
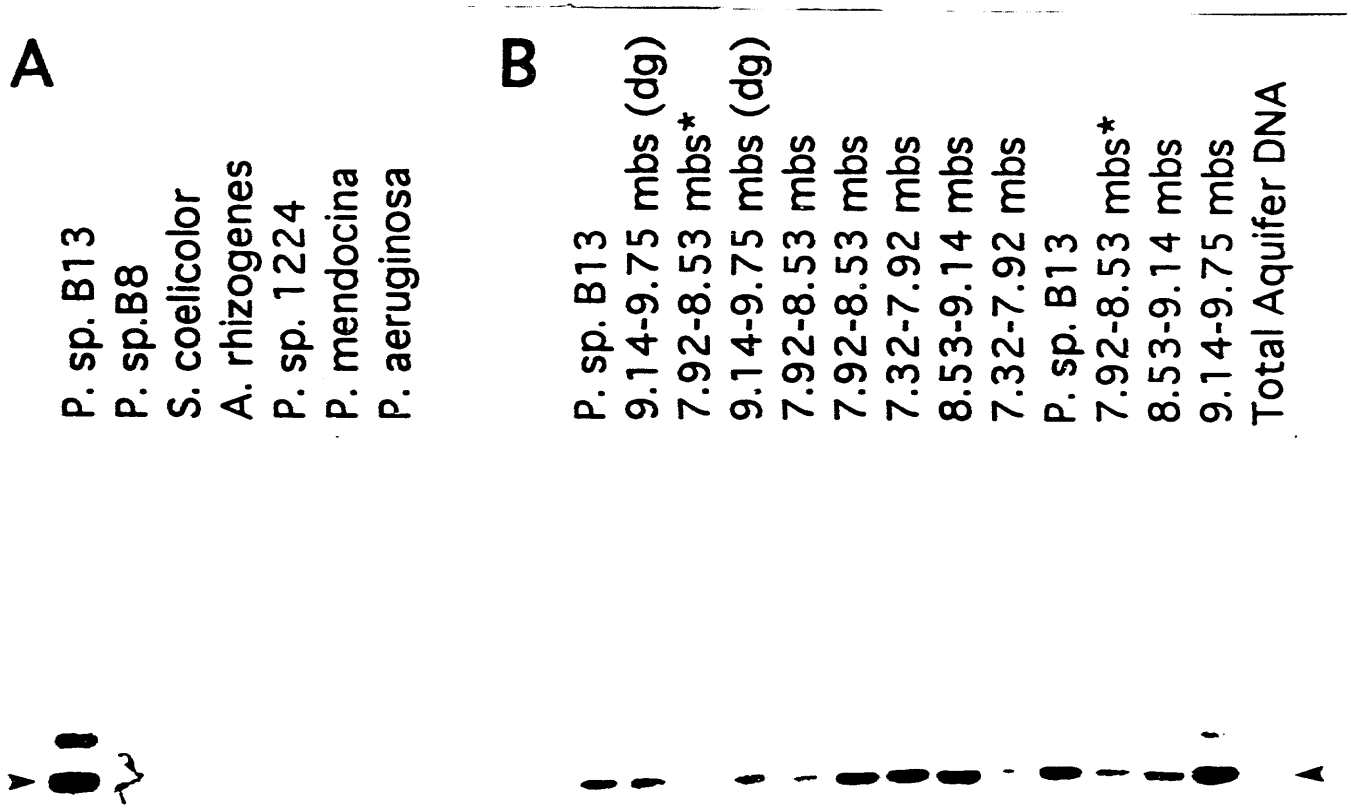


Fig. 6. The use of molecular techniques to evaluate the survival of a microorganism that was injected into an aquifer. Suzanne M. Thiem, Richard L. Smith, and James M. Tiedje



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