APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1997, p. 2384–2390 Vol. 63, No. 6
0099-2240/97/$04.00+0

Sensitive Detection of a Novel Class of Toluene-Degrading Denitrifiers, *Azoarcus tolulyticus*, with Small-Subunit rRNA Primers and Probes

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Received 7 November 1996/Accepted 18 February 1997

***Azoarcus tolulyticus* is a new class of widely distributed, toluene-degrading denitrifiers of potential importance in remediating benzene, toluene, ethylbenzene, and xylene (BTEX)-contaminated environments. To detect these organisms in the environment, 16S rRNA gene-based phylogenetic probes were developed. Two sets of specific PCR amplification primers and two oligonucleotide hybridization probes were designed and tested against both closely and distantly related environmental isolates. All of these primers and probes were specific to the species *A. tolulyticus*. The sensitivity of the PCR amplification primer sets was evaluated with DNA isolated from *A. tolulyticus* Tol-4 pure culture and from sterile soils seeded with a known number of Tol-4 and *Escherichia coli* cells. These primer sets were able to detect 1 fg to 1 pg of template DNA from the pure culture and 1.11×10^2 to 1.1×10^8 Tol-4 cells per g of soil in the presence of 1.56×10^{10} *E. coli* cells. These two PCR amplification primers were also successfully tested at two field sites. The primers identified the *A. tolulyticus* strains among the toluene-degrading bacteria isolated from a low-O_2–high-NO_3^- aquifer at Moffett Field, Calif. Also, the presence of *A. tolulyticus* was detected in the groundwater samples from a BTEX-contaminated aquifer at an industrial site in Detroit, Mich., which showed anaerobic toluene degradation.**

Benzene, toluene, ethylbenzene, and xylenes (BTEX) are major contaminants in groundwater and can be troublesome to effectively remediate. In situ bioremediation remains potentially the most cost-effective cleanup technology for removing these compounds from contaminated sites. However, evaluating in situ bioremediation potential, especially under anaerobic conditions in the subsurface, is problematic (14). One approach for assessing in situ bioremediation is to monitor the microorganisms which are responsible for the degradation of the target compounds with molecular biological tools.

We recently isolated 15 organisms that degrade toluene under denitrifying conditions from a variety of habitats (3, 8). Phylogenetic analyses based on 16S rRNA gene sequences and certain physiological characteristics identified these organisms as a novel species, *Azoarcus tolulyticus* (20), of the genus of free-living nitrogen fixers, with strain Tol-4 designated as the type strain (3). These organisms were found in a variety of habitats, such as contaminated and noncontaminated soils, aquifers, and sediments, and appear to be widely distributed in nature (8), and thus, they may be potentially important to in situ bioremediation of BTEX-contaminated sites. To monitor these organisms in the environment and to evaluate in situ BTEX bioremediation potential, 16S rRNA gene-based phylogenetic probes specific to *A. tolulyticus* were developed and evaluated.

MATERIALS AND METHODS

Organisms and growth conditions. The bacterial strains used to evaluate the specificity of the PCR primers were primarily environmental isolates, capable of degrading toluene and other aromatic compounds, and phylogenetically related organisms (Table 1). Some toluene-degrading bacteria isolated from an aquifer amended with phenol and toluene at Moffett Field in California (7) were also used to test the designed primers. All of the isolates except *Azoarcus* sp. strains S5b2 and 6a3, *Azoarcus communis* SWub3^T, and *Azoarcus indigens* VB32^T were grown for genomic DNA isolation at 30°C on modified R2A medium as described previously (8). The genomic DNA from the other *Azoarcus* species was kindly provided by Barbara Reinhold-Hurek at the Max-Planck-Institut für Terrestrische Mikrobiologie, Marburg, Germany.

Anaerobic toluene degradation. The groundwater samples from four wells in BTEX-contaminated aquifers at an industrial site in Detroit, Mich., were provided by Susan Pfiffner of Oak Ridge National Laboratory. Bacterial samples from 250 ml of groundwater were collected by centrifugation. The supernatant was discarded, and the pellets were resuspended with 10 ml of basal salt medium (8). A suspension of 5 ml was used to inoculate a 50-ml serum bottle containing 25 ml of basal salt medium amended with 25 ppm of toluene and 5 mM KNO_3 in an anaerobic chamber. The samples were incubated at room temperature for 15 to 20 days. Aerobic toluene degradation enrichment was performed in a similar way except that all of the reagents were prepared aerobically. Toluene degradation was monitored by measuring the disappearance of toluene from the headspace as determined by using a gas chromatograph equipped with a flame ionization detector.

Genomic DNA isolation. Total DNA from pure bacterial cultures was isolated by a sodium dodecyl sulfate (SDS)-based method (20). The DNA concentration was measured spectrophotometrically and calibrated with known concentrations of lambda DNA (Pharmacia, Piscataway, N.J.).

Total DNA from the sterile soils seeded with *A. tolulyticus* Tol-4 and *Escherichia coli* DH5αF′ cells was isolated by a rapid purification method based on gel electrophoresis and a resin column (21). Tol-4 and *E. coli* cells in late exponential growth phase were seeded into sterile soil samples and enumerated by plate counting on modified R2A medium. One milliliter each of 10-fold serial dilutions of a Tol-4 cell suspension plus 0.5 ml of *E. coli* cells (1.56×10^{11} cells per ml) was mixed with 10 g of the sterile sandy soil from Wurtsmith Air Force Base, Wurtsmith, Mich., and incubated at room temperature for 1 h prior to DNA extraction. The sterile samples were produced by autoclaving the soil twice at 121°C for 1 h. The same DNA recovery method was used to extract DNA from the enrichment samples inoculated with the groundwater from the contaminated industrial site.

PCR amplification. Species-specific oligonucleotide primers were designed with the OLIGO program (16) based on the Tol-4 16S rRNA gene sequence (20) and other 16S rRNA gene sequences from the Ribosomal Database Project (RDP) (13). Primers A and B were designed to amplify an approximately 1.1-kb segment of 16S rRNA genes from *A. tolulyticus*, which corresponds to positions 205 to 22 and 1278 to 1263 in the *E. coli* 16S rRNA sequence (2). Primers C and D were designed to amplify a gene segment of about 600 bp from *A. tolulyticus*, which corresponds to positions 476 to 493 and 1136 to 1119 in the *E. coli* 16S rRNA sequence. Primer set C-D can also be coupled with primer set A-B for nested amplification. Oligonucleotides E and F (*E. coli* sequence positions 501 to 484 and 1129 to 1114, respectively) were designed as hybridization probes. All of

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TABLE 1. Bacterial strains used for specificity testing

Strain	Subgroup	BTEX degradation[a]	Accession no.[b]	No. of mismatches to primer/probe:					
				A	B	C	D	E	F
A. tolulyticus Tol-4[c]	Beta	T, E	L33694	0	0	0	0	0	0
A. tolulyticus Td-1	Beta	T, E	L33687	0	0	0	0	0	0
A. tolulyticus Td-2	Beta	T	L33691	0	0	0	0	0	0
A. tolulyticus Td-3	Beta	B, T	L33693	0	0	0	0	0	0
A. tolulyticus Td-15	Beta	T, E	L33688	0	0	0	0	0	0
A. tolulyticus Td-17	Beta	T, E	L33689	0	0	0	0	0	0
A. tolulyticus Td-19	Beta	T	L33690	0	0	0	0	0	0
A. tolulyticus Td-21	Beta	B, T, E	L33692	0	0	0	0	0	0
A. indigens VB32^T	Beta	ND	L15531	5	4	2	3	2	3
Azoarcus sp. strain S5b2	Beta	ND	L15532	7	4	3	4	4	3
Azoarcus sp. strain 6a3	Beta	ND							
A. communis SWub3^T	Beta	ND							
B. cepacia G4	Beta	B, T, E	L28675	8	5	4	5	4	4
B. cepacia ATCC 25416	Beta	ND	M22518	8	5	4	5	4	4
B. pickettii PK01	Beta	B, T, E	L37367	6	4	4	4	5	3
Alcaligenes eutrophus	Beta	ND	M32021	4	5	4	4	4	3
Pseudomonas stutzeri KC	Gamma	T	Unsubmitted	9	4	7	9	4	4
P. putida F1	Gamma	B, T, E	L37365	9	4	7	8	4	4
P. putida PaW1	Gamma	T, *m*, *p*	L28676	9	4	7	7	4	4
P. mendocina KR	Gamma	T, E	L37366	8	4	8	8	5	4
P. fluorescens RW 215[d]	Gamma	B, T, E, *p*	Unsubmitted	9	4	7	8	4	4
Acinetobacter sp. strain RW31[e]	Gamma	B, T, E	Unsubmitted	7	9	7	7	4	4

[a] Compounds degraded under aerobic conditions: B, benzene; E, ethylbenzene; T, toluene; X, xylene; *m*, *m*-xylene; *p*, *p*-xylene. ND, not determined.
[b] 16S rRNA gene sequence accession number in GenBank.
[c] All *A. tolulyticus* strains also degrade toluene under anaerobic denitrifying conditions.
[d] Strain RW 215 also degrades benzene, toluene, ethylbenzene, and *p*-xylene under microaerophilic conditions (24).
[e] Strain RW31 also degrades benzene, toluene, and ethylbenzene under microaerophilic conditions (24).

the oligonucleotides were synthesized at the Macromolecular Structure, Sequencing and Synthesis Facility at Michigan State University.

PCR amplification conditions with these two primer sets were first optimized for primer and $MgCl_2$ concentrations. All PCR amplifications were accomplished under the optimal conditions with "hot start" (4) in a 20-μl volume containing 1× *Taq* polymerase buffer (10× buffer is 100 mM Tris-Cl, 30 mM $MgCl_2$, and 500 mM KCl [pH 8.3]), 200 μM deoxynucleoside triphosphates, 14 pmol of each primer, 1 fg to 100 ng of template DNA, and 0.5 U of *Taq* polymerase (Boehringer Mannheim, Indianapolis, Ind.). All stocks for PCR amplification were made and procedures were performed with the precautions suggested by Kwok and Higuchi (10). The reaction conditions for all primer sets consisted of initial denaturation at 94°C for 2 min, 5 cycles of denaturation at 94°C for 30 s, primer annealing at 68°C (for primer set A-B) or 60°C (for primer set C-D) for 2 min, and extension at 72°C for 1 min, plus 30 additional cycles with annealing for 1 min instead of 2 min. In all cases, one additional cycle with a final 6 min of chain elongation at 72°C was carried out after the cycling was completed. Temperature was cycled in programmable temperature cyclers (GeneAmp PCR system 9600 [Perkin-Elmer Corp., Norwalk, Conn.]) (DNA Engine PT200 [MJ Research Inc., Watertown, Mass.]). The PCR products were stored at 4°C.

Primer set C-D was also coupled with primer set A-B for nested PCR amplification. The 16S gene fragments were first amplified from 10 ng of genomic DNA with primer set A-B. Then 1 μl of the PCR-amplified product was used as the template for the second amplification with primer set C-D.

Detection of amplified products. PCR-amplified DNAs were detected by gel electrophoresis and radiolabelled gene probes. Aliquots of amplified samples (10 μl) were separated by gel electrophoresis on 1.5% agarose gels, stained with ethidium bromide, visualized with a UV transilluminator, and photographed. Southern hybridization was carried out as described previously (23).

Oligonucleotide probe hybridization. Oligonucleotides were labelled at the 3′ end with [α-^{32}P]ATP (3,000 Ci/mmol, 10 μCi/μl; DuPont NEN, Wilmington, Del.) by using terminal transferase (Boehringer Mannheim). The reaction mixture (20 μl) contained 0.7 pmol of oligonucleotide, 2.0 μl of 10× reaction buffer, 1.5 μl of $CoCl_2$, 2.0 μl of terminal transferase, and 6.3 μl of [α-^{32}P]ATP. The mixture was incubated at 37°C for 15 min. After being labelled, the oligonucleotide probes were purified with Nensorb-20 columns (DuPont NEN) as specified by the manufacturer.

A 1.0-μg sample of genomic DNA was heated at 95°C for 10 min, chilled on ice for 5 min, and immobilized on maximum-strength Nytran nylon membranes (pore size, 0.45 μm) (Schleicher & Schuell, Keene, N.H.) with a slot blotter under a slight vacuum. Each membrane was then soaked in 1.5 M NaOH–0.5 M NaCl for 5 min for complete denaturation and neutralized with 1.5 M NaCl–0.5 M Tris (pH 7.4). The DNA was fixed to the membranes with a UV Stratalinker and air dried at room temperature. The membranes were prewet in 6× SSPE buffer (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]) and prehybridized in a bag with 100 μl of prehybridization solution per cm^2 containing 6× SSPE, 10× modified Denhardt III (23), 1% SDS, 20 μg of poly(A) (Sigma Chemical Co., St. Louis, Mo.) per ml, and 50 μg of denatured herring sperm DNA per ml. The prehybridization was carried out in a water bath with shaking at 10 rpm at 42°C for 12 to 16 h. The prehybridization solution was removed, and the membrane was hybridized with 100 μl of hybridization solution per cm^2 containing 6× SSPE, 1% SDS, and one-fourth to one-sixth of the ^{32}P-labelled probe at an activity of 1×10^6 to 2×10^6 cpm per ml. The hybridization was carried out in a water bath at 42°C for 10 to 12 h. After hybridization, the membrane was washed three times with shaking at 10 rpm with 6× SSPE plus 1% SDS at room temperature for 5 to 10 min and once with 1× SSPE plus 1% SDS at the half-disassociation temperature (T_d) for each oligonucleotide for 2 min. Blots were exposed at −70°C for 1 to 2 days with an intensifying screen.

To achieve specific hybridization, the T_ds for the two oligonucleotide probes were determined experimentally with three replicates. After hybridization overnight, the membranes were washed with 1× SSPE plus 1% SDS at room temperature three times for 10 min each time. The portion of each membrane

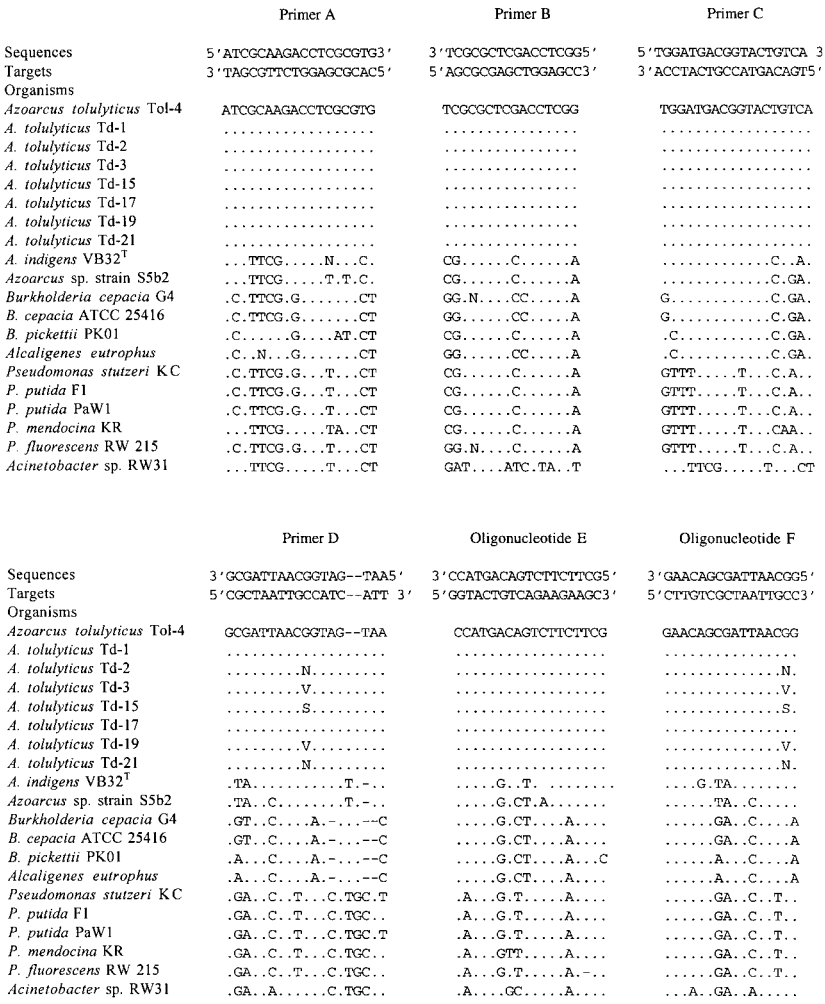

FIG. 1. 16S rRNA gene sequence alignments showing the target regions of the designed PCR amplification primers and oligonucleotide hybridization probes. A hyphen indicates a gap; a dot indicates the same nucleotide as that in the probe sequence.

containing a DNA sample was excised, placed in a 20-ml vial containing 10 ml of 1× SSPE plus 1% SDS, and incubated at a controlled temperature. The incubation temperature was increased in 2°C steps and ranged from 36 to 70°C. After the samples were incubated at the desired temperature for exactly 10 min, 0.5 ml of buffer was removed and placed in a 10-ml vial containing 5 ml of scintillation liquid and was kept for counting. Then, 0.5 ml of prewarmed fresh buffer was added to keep the volume constant. The process was repeated at every 2°C step until the incubation temperature reached 70°C. The radioactivity of the sample at each temperature was determined by liquid scintillation counting. The total cumulative counts were plotted against temperature, and the T_d was determined as the temperature corresponding to the release of half of the total cumulative counts.

rDNA sequencing and sequence analysis. The DNA sequences of the 16S rRNA genes (rDNA) were determined directly with the PCR-amplified DNA as the sequencing template. The amplified PCR products were purified by using the Wizard PCR Preps DNA purification system (Promega, Madison, Wis.) according to the manufacturer's instructions. DNA sequences were determined by automated fluorescent-*Taq* cycle sequencing with the ABI Catalyst 800 and ABI 373A sequencer (Applied Biosystems, Foster City, Calif.). Approximately 100 ng of the purified DNA was used for one automated fluorescent sequencing reaction. The sequencing primer (529R) for the 16S rRNA gene used in this study spanned *E. coli* 16S rRNA gene positions 529 to 515.

Sequences were assembled with assembling programs in the Genetics Computer Group software package (5) and preliminarily analyzed by searching the current databases (GenBank release 91.0 and EMBL release 44.0) with the program FASTA. Sequences were then aligned manually to the 16S rDNA sequences of the species, which showed high similarity scores in the outputs of FASTA, in the previously aligned 16S rDNA sequence database (13) with the GDE multiple-sequence editor program from the RDP. Initial phylogenetic screening was constructed with the neighbor-joining DNA distance program in the PHYLIP package (6) based on all 16S rDNA sequences of the bacterial groups, such as the beta and gamma subdivisions of the class *Proteobacteria*. Appropriate subsets of 16S rDNA sequences were selected and subjected to final phylogenetic analysis through the maximum-likelihood method with the program fastDNAml in the RDP.

RESULTS

Selection of species-specific primers and oligonucleotides. 16S rRNA gene sequences from *A. tolulyticus* were compared with those from reference strains, most of them belonging to the beta subclass of the *Proteobacteria*. We selected primers from the regions which appeared to be most suitable with respect to sequence conservation within the *A. tolulyticus* species versus divergence from the other *Azoarcus* species and other members of the beta subclass of the *Proteobacteria*. For PCR amplification primers, mismatches near the 3′ ends of the primers were designed to be minimal for the *A. tolulyticus* strains and maximal for the reference strains, especially for the other *Azoarcus* species, whereas for oligonucleotide hybridization probes, mismatches within the central regions of the probes were designed to be maximal for the reference strains (Fig. 1). There were two to nine mismatches between all the

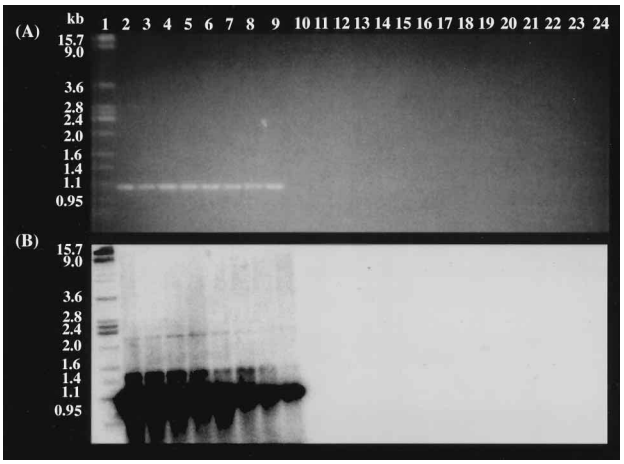

FIG. 2. Specific detection of *A. tolulyticus* by PCR with primer set A-B on an agarose gel (A) and by Southern hybridization (B). Lanes: 1, lambda molecular size marker; 2 to 9, *A. tolulyticus* Tol-4, Td-1, Td-2, Td-3, Td-15, Td-17, Td-19, and Td-21, respectively; 10, *A. indigens* VB32^T; 11, *Azoarcus* sp. strain S5b2; 12, *Azoarcus* sp. strain 6a3; 13, *A. communis* SWub3^T; 14, *B. cepacia* G4; 15, *B. cepacia* ATCC 25416; 16, *Burkholderia pickettii* PK01; 17, *Alcaligenes eutrophus*; 18, *Pseudomonas stutzeri* KC; 19, *P. putida* F1; 20, *P. putida* PaW1; 21, *P. mendocina* KR; 22, *P. fluorescens* RW215; 23, *Acinetobacter* sp. strain RW31; 24, negative control (no DNA).

probe sequences and the reference sequences (Table 1). When the PCR primers and the oligonucleotide probes were run against the GenBank and EMBL databases with the program FASTA in the Genetics Computer Group package and against the RDP Small-Subunit database by using the RDP CHECK _PROBE service (11), no exact matching complements were found, except for the strain KB740, which had perfect matches with all of the PCR amplification primers and oligonucleotide hybridization probes.

Species-specific amplification of 16S rDNA segments. The specificity of the species-targeted primer sets (A-B and C-D) was tested with eight *A. tolulyticus* strains and with other members of the beta and gamma subgroups of the *Proteobacteria* listed in Table 1. At annealing temperatures of 68°C (for primer set A-B) and 60°C (for primer set C-D), an amplification product of the appropriate size was obtained for all strains of *A. tolulyticus* on agarose gels. In contrast, no amplification signal was observed for any of the reference strains or for the negative control of no DNA (Fig. 2A and 3A).

To confirm the specificity of these primers by PCR amplification, the same gels were blotted and hybridized with a 1.1-kb 16S rRNA gene fragment from *A. tolulyticus* Tol-4. A strong positive signal was detected with a 2-h exposure for all *A. tolulyticus* strains, but no signal was detected for the negative control or any of the other reference strains (Fig. 2B and 3B). However, a faint band was observed with a 20-h exposure for *Azoarcus* sp. strain 6a3, *Burkholderia cepacia* ATCC 25416, *Pseudomonas stutzeri* KC, *Pseudomonas putida* F1 and PaW1, and *Pseudomonas fluorescens* RW215 with primer set A-B and for *P. putida* F1, *Pseudomonas mendocina* KR, and *P. fluorescens* RW215 with primer set C-D (data not shown), indicating that poor amplification occurred for these strains.

Sensitivity tests with pure cultures. To determine the lower limit of detection of *A. tolulyticus* DNA, serial 10-fold dilutions of genomic DNA from *A. tolulyticus* Tol-4 were analyzed by PCR with the two primer sets. On agarose gels, the detection levels were 100 fg for primer set A-B (Table 2 and Fig. 4A) and 1 ng for primer set C-D (Table 2 and data not shown). The sensitivity of detection was increased 100-fold by Southern hybridization to as low as 1 fg of DNA for primer set A-B

TABLE 2. Detection limit of template DNA for different primer sets

Primer set(s)	Detection limit for template DNA from:			
	Pure culture		Seeded soils[a]	
	Agarose gel	Southern blot	Cells/ PCR	Cells/g of sandy soil
A-B	100 fg	1 fg	0.9	1.11×10^2
C-D	1 ng	1pg	9×10^5	1.11×10^8
A-B + C-D	1 ag	1ag	ND	ND

[a] ND, not determined.

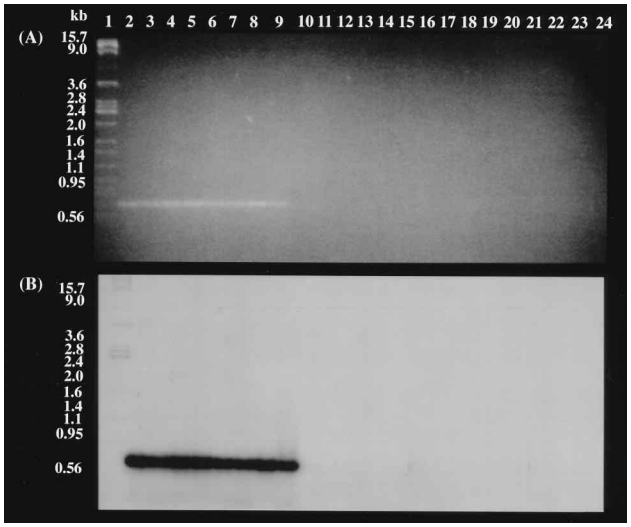

FIG. 3. Specific detection of *A. tolulyticus* by PCR with primer set C-D on an agarose gel (A) and by Southern hybridization (B). The lanes are as in Fig. 2.

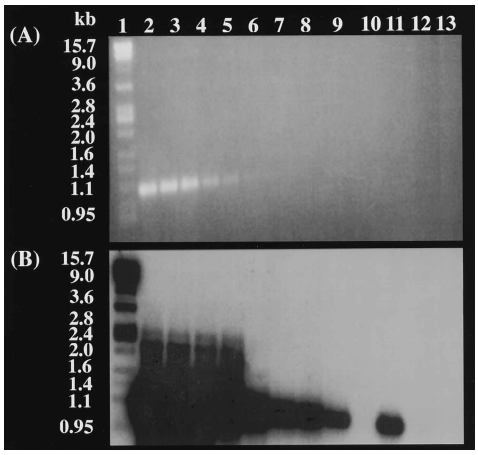

FIG. 4. Sensitivity of detection by PCR with primer set A-B on template DNA isolated from an *A. tolulyticus* Tol-4 pure culture on an agarose gel (A) and by Southern hybridization (B). Lanes: 1, lambda molecular size marker; 2, 10 ng; 3, 1 ng; 4, 100 pg; 5, 10 pg; 6, 1 pg; 7, 100 fg; 8, 10 fg; 9, 1 fg; 10, 100 ag; 11, 10 ag; 12, 1 ag; 13, negative control (no DNA).

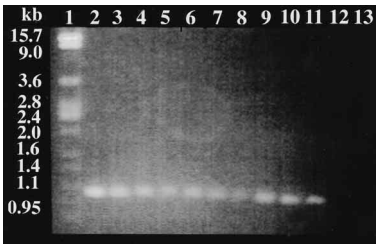

FIG. 5. Sensitivity of detection by PCR with primer set A-B on template DNA isolated from sterile soil seeded with *A. tolulyticus* Tol-4 and *E. coli* on an agarose gel. Lanes: 1, lambda molecular size marker; 2, 1.11×10^{11} cells per g of soil (9×10^8 cells); 3, 1.11×10^{10} cells per g of soil (9×10^7 cells); 4, 1.11×10^9 cells per g of soil (9×10^6 cells); 5, 1.11×10^8 cells per g of soil (9×10^5 cells); 6, 1.11×10^7 cells per g of soil (9×10^4 cells); 7, 1.11×10^6 cells per g of soil (9×10^3 cells); 8, 1.11×10^5 cells per g of soil (9×10^2 cells); 9, 1.11×10^4 cells per g of soil (9×10^1 cells); 10, 1.11×10^3 cells per g of soil (9 cells); 11, 1.11×10^2 cells per g of soil (0.9 cells); 12, 10 ng of pure *E. coli* DNA; 13, negative control (no DNA). Lanes 2 to 11 were also seeded with 1.56×10^{10} *E. coli* cells per g of soil (1.25×10^8 cells).

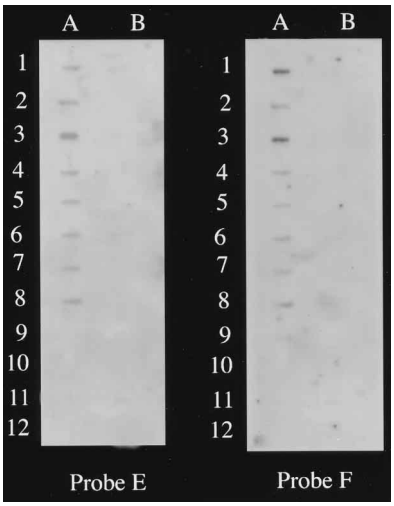

FIG. 6. Specificity of oligonucleotide hybridization probes E and F. Lanes A: rows 1 to 8, *A. tolulyticus* Tol-4, Td-1, Td-2, Td-3, Td-15, Td-17, Td-19, and Td-21, respectively; row 9, *A. indigens* VB32^T; row 10, *Azoarcus* sp. strain S5b2; row 11, *Azoarcus* sp. strain 6a3; row 12, *A. communis* SWub3^T. Lanes B: row 1, *B. cepacia* G4; row 2, *B. cepacia* ATCC 25416; row 3, *B. pickettii* PK01; row 4, *A. eutrophus*; row 5, *P. stutzeri* KC; row 6, *P. putida* F1; row 7, *P. putida* PaW1; row 8, *P. mendocina* KR; row 9, *P. fluorescens* RW215; row 10, *Acinetobacter* sp. strain RW31; row 11, *Rhodococcus* sp. strain Ea 39; row 12, *Rhodococcus erythropolis*.

(Table 2 and Fig. 4B). With nested amplification, the sensitivity of detection for primer set A-B on agarose gels was 1 ag of DNA, equivalent to that by Southern hybridization (Table 2).

Sensitivity tests for Tol-4 added to soils. To determine the sensitivity of different primer sets for detecting *A. tolulyticus* in a heterogeneous DNA background, a known number of Tol-4 cells, ranging from 0 to 5.55×10^{11}, and *E. coli* cells (7.8×10^{10}) were inoculated into 5 g of sterile sandy soil and DNA was then extracted from the soil. Following direct DNA extraction and purification from each seeded soil, a 1-μl aliquot from 500 μl of purified DNA was used as the template for hot start. PCR amplification was observed with 1.11×10^2 Tol-4 cells per g of soil for primer set A-B (Fig. 5) and 1.11×10^6 Tol-4 cells per g of soil for primer set C-D (data not shown). Assuming a DNA recovery efficiency from soils of 80% (24), the actual detection limit for PCR amplification on agarose gels was 0.9 cells for primer set A-B and 9×10^5 cells for primer set C-D (Table 2).

Species-specific detection of *A. tolulyticus* by oligonucleotide hybridization. For 16S rRNA-targeted oligonucleotide hybridization, we used oligonucleotides E and F, which target similar regions of the 16S rRNA gene as do primers C and D. The experimentally determined T_d was 46°C for oligonucleotide probe E and 45°C for oligonucleotide probe F. The empirically determined T_d was used as the final wash temperature in the subsequent hybridization studies.

The specificity of these oligonucleotide probes was determined from slot blots with both closely related and distantly related bacterial strains. At the wash temperatures of 46°C (for probe E) and 45°C (for probe F), strong hybridization signals were obtained with both of the oligonucleotide probes for the different *A. tolulyticus* strains, but no signals were obtained for the other *Azoarcus* species or the other reference strains (Fig. 6), indicating that these two oligonucleotide probes are specific to *A. tolulyticus* under such hybridization conditions.

Identification of toluene-degrading isolates from phenol-injected aquifers. Eleven toluene-degrading strains isolated from aquifers (3, 7) were used to test these specific primers. Some of the strains were closely related to *A. tolulyticus* as indicated by fatty acid methyl ester (FAME) analysis (7). While no amplification signal was observed with the two primer sets for some of these 11 isolates, amplification products of the appropriate size were obtained for the isolates MF-23, MF-107, MF-92, MF-H-4-6, and BL-11 (Table 3), suggesting that these isolates are members of *A. tolulyticus*, and the others are not. This was confirmed by 16S rDNA sequence analysis. Phylogenetic analysis based on partial 16S rDNA sequences (~350 bp) revealed that isolates MF-23, MF-107, MF-92, MF-H-4-6, and BL-11 are closely related to *A. tolulyticus*, with similarities of 99.2 to 99.8% (Table 3), but the other isolates are only distantly related to *A. tolulyticus*. These results indicate that the designed primers are specific to *A. tolulyticus* and can be useful tools for isolate identification.

Detection of *A. tolulyticus* in BTEX-contaminated aquifers. The applicabilities of these primer sets were also tested with environmental samples from a BTEX-contaminated aquifer. PCR amplifications were achieved with the two primer sets for the genomic DNA sample from well I-2, whereas no amplification was observed for the genomic DNA samples from the other three wells (Table 4). These results suggested that *A. tolulyticus* and related strains were present in well I-2 but not in the other wells. Well I-2 was used as an injection well for a nitrate addition experiment, and thus, bacteria near this well would have been exposed to high nitrate concentrations. The presence of toluene-degrading, denitrifying bacteria in well I-2 was supported by the enrichment experiments. Although aerobic toluene degradation was observed for all four samples, anaerobic toluene degradation under denitrifying conditions was detected only for the sample from well I-2 (Table 4). Furthermore, PCR products were also observed with the two primer sets for the anaerobic enrichment samples from well I-2, but no amplification was achieved for the anaerobic enrichment samples from the other three wells or for any of the aerobic enrichment samples. These results indicate that *A. tolulyticus* and related strains existed in well I-2.

DISCUSSION

Specificity and sensitivity are the two most important criteria for any assay to become a useful and reliable tool for identification, detection, and tracking of organisms in environments. In the present study, two sets of PCR primers and two oligonucleotide hybridization probes specific to the species *A. tolu-*

TABLE 3. Identification of toluene-degrading strains isolated from aquifers based on FAME analysis, phylogenetic probes for *A. tolulyticus*, and 16S rDNA sequence analysis

Isolate	Close relative based on FAME analysis	Amplification by primer sets A-B and C-D	Close relative based on 16S rDNA sequence (% similarity)
MF-23	*A. tolulyticus* Td-1	+	*A. tolulyticus* Td-1 (99.3)
MF-107	*Hydrogenophaga pseudoflava*	+	*A. tolulyticus* Tol-4 (99.2)
MF-92	*A. tolulyticus* Td-21	+	*A. tolulyticus* Tol-4 (99.7)
MF-H-4-6	*A. tolulyticus* Td-1	+	*A. tolulyticus* Td-1 (99.3)
BL-11	ND[a]	+	*A. tolulyticus* Tol-4 (99.8)
MF-11	No match	−	*Variovorax paradoxus* (89.3)
MF-80	*Pseudomonas syringae*	−	*P. putida* (98.1)
MF-168	No match	−	*P. putida* (98.0)
MF-175r	*B. pickettii*	−	*Burkholderia solanacearum* (95.3)
MF-182	*P. putida*	−	*P. putida* (98.7)
MF-415	*A. tolulyticus* Td-21	−	*V. paradoxus* (94.6)

[a] ND, not determined.

lyticus were designed and evaluated. Specific amplification of DNA from *A. tolulyticus* was observed on agarose gels with primer set A-B at the annealing temperature of 68°C and with primer set C-D at the annealing temperature of 60°C. These results suggest that these PCR primers are specific to *A. tolulyticus*.

All of the PCR amplification primers and oligonucleotide probes match the sequence of strain KB 740, a benzoate-degrading denitrifier. Phylogenetic and physiological analyses have shown that KB 740, described as *Azoarcus evansii* (1), is closely related to *A. tolulyticus* (1, 20), but it does not degrade toluene anaerobically (1). Hence, these primers and oligonucleotides could also detect *A. evansii.*

Specific detection of *A. tolulyticus* was also obtained with oligonucleotide hybridization probes E and F. These probes could be coupled with the specific PCR amplification primer set A-B for identifying and detecting *A. tolulyticus* in the environment. Such a combination will improve the specificity and sensitivity of 16S rRNA gene-based detection methods. Based on our experiences, specific detection of 16S rRNA genes with oligonucleotide hybridization is more easily achieved than with PCR amplification, but PCR amplification is much more sensitive than oligonucleotide hybridization.

With these PCR primers, 10 fg to 1 pg of template DNA from a pure culture can be reliably detected, which is equivalent to less than 2 to 200 cells, assuming 5 fg of DNA per cell. The sensitivity was further increased with nested amplification. The sensitivity of PCR amplification with these primers was higher than or similar to those achieved in other studies based on 16S rRNA genes (12, 18, 19, 22). For primer set A-B, the sensitivity of detection with the heterogeneous template DNA (from Tol-4 and *E. coli*) isolated from a soil matrix was comparable to that with the template DNA from the pure culture (Table 2), while for primer set C-D the sensitivity was significantly lower. The lower sensitivity could result from insufficient purity of the template DNA or the presence of a large amount of heterogeneous *E. coli* DNA.

TABLE 4. Detection of *A. tolulyticus* in a BTEX-contaminated aquifer at an industrial site

Well no.	Anaerobic toluene degradation	Aerobic toluene degradation	PCR amplification by primer set A-B or C-D for template DNA isolated from community:		
			Prior to enrichment	After anaerobic enrichment	After aerobic enrichment
I-2	+	+	+	+	−
KV-11B	−	+	−	−	−
KV-13	−	+	−	−	−
R-2	−	+	−	−	−

The sensitivity of PCR amplification was primer dependent, and the basis for this is not understood (9, 22). Also, we found that fresh reagents were very important to achieving highly sensitive amplification. In this probe study, we generally used freshly made deoxynucleoside triphosphates, but in no case were they more than 10 days old. Furthermore, primer concentration is also important for high-sensitivity PCR amplification. Because primer dimers may occupy the active sites of *Taq* polymerase (15), the primer concentration must be optimized and should be kept as low as possible.

In the first cycles of PCR amplification with genomic DNA as the template, primers must perform a genomic screening (15) until they find the complementary annealing sites. The probability of successful primer annealing in the very first cycles is mainly determined by the target copy number and whether there is enough genomic screening time to find a target (15). Rolfs et al. (15) showed that longer annealing times in the first cycles were advantageous to the genome screening process. In this study, we found that the use of a longer annealing time (2 min) in the first five cycles did improve the sensitivity of the amplification with primer set A-B.

Although these PCR amplification primers have been tested successfully with field samples, problems with specificity could still occur. Because the database from which these primers were designed is limited in sequences of these groups, it may not be representative of 16S rRNA sequences of environmental strains. Hence, the specificity of these and often other phylogenetic probes for environmental isolates cannot be fully evaluated. Thus, when using phylogenetic probes to study environmental samples, it should be kept in mind that potential cross-amplification or hybridization may occur. Furthermore, other environmental strains may have identical 16S rRNA sequences but differ in toluene-degrading ability or other ecological traits.

With certain precautions, these primer sets and oligonucleotide hybridization probes can be used to detect *A. tolulyticus* and related strains in environmental samples. A diagnostic test based on the combination of the multiple PCR amplification primer sets and oligonucleotide hybridization probes makes false-positive conclusions less likely, but the possibility of their occurrence is not completely excluded because different strains can have identical 16S rRNA gene sequences. False-negative

results may occur if the number of *A. tolulyticus* cells is below the detection limit or if the template DNA from environmental samples is not pure enough. To avoid drawing false-positive or false-negative conclusions, it is advisable to couple these PCR-based phylogenetic probes with some other tools for detection, such as functional gene probes and randomly cloned specific DNA fragments (17). These probes can also be used in the preliminary identification of new isolates, which then can be confirmed by partial sequencing of the hypervariable regions of the 16S rRNA gene, such as the region containing primer D, and by other group-specific tests. To achieve high specificity and sensitivity, we optimized the conditions for these primer sets on particular thermocyclers (GeneAmp PCR system 9600 and DNA Engine PT200). The optimal conditions for the primer sets could be slightly different for other thermocyclers.

ACKNOWLEDGMENTS

We are grateful to Marcos Fries for the toluene-degrading strains from Moffett Field; Barbara Reinhold-Hurek for strains S5b2, 6a3, VB32^T, and SWub3^T; Susan Pfiffner for the Detroit aquifer samples; and Emily Alexander for technical assistance.

This project was supported by the Center for Microbial Ecology, the National Science Foundation (grant BIR9120006), the National Institute of Environmental Health Sciences Superfund Research and Education Grant ES-04911, and the U.S. Department of Energy under funding to Oak Ridge National Laboratory, which is managed by Lockheed Martin Energy Research Corp. under contract no. DE-AC05-96OR22464. J.Z. acknowledges support of the Alexander Hollaender Distinguished Postdoctoral Fellowship Program, sponsored by the U.S. Department of Energy, Office of Health and Environmental Research, and administered by the Oak Ridge Institute for Science and Education.

REFERENCES

1. **Anders, H.-J., A. Kaetzke, P. Kämpfer, W. Ludwig, and G. Fuchs.** 1995. Taxonomic position of aromatic-degrading denitrifying pseudomonad strains K 172 and KB 740 and their description as new members of the genera *Thauera*, as *Thauera aromatica* sp. nov., and *Azoarcus*, as *Azoarcus evansii* sp. nov., respectively, members of the beta subclass of the *Proteobacteria*. Int. J. Syst. Bacteriol. **45:**327–333.
2. **Brosius, J., T. J. Dull, D. D. Sleeter, and H. F. Noller.** 1981. Gene organization and primary structure of a ribosomal RNA operon from Escherichia coli. J. Mol. Biol. **148:**107–127.
3. **Chee-Sanford, J. C., J. W. Frost, M. R. Fries, J. Zhou, and J. M. Tiedje.** 1996. Evidence for acetyl coenzyme A and cinnamoyl coenzyme A in the anaerobic toluene mineralization pathway in *Azoarcus tolulyticus* Tol-4. Appl. Environ. Microbiol. **62:**964–973.
4. **D'Aquila, R. T., L. J. Bechtel, J. A. Videler, J. J. Eron, P. Gorczca, and J. C. Kaplan.** 1991. Maximizing sensitivity and specificity of PCR by preamplification heating. Nucleic Acids Res. **19:**3749.
5. **Devereaux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
6. **Felsenstein, J.** 1989. PHYLIP—Phylogeny inference package (version 3.2). Cladistics **5:**164–166.
7. **Fries, M. R., G. D. Hopkins, P. L. McCarty, L. J. Forney, and J. M. Tiedje.** 1997. Microbial succession during a field evaluation of phenol and toluene as the primary substrates for trichloroethene cometabolism. Appl. Environ. Microbiol. **63:**1515–1522.
8. **Fries, M. R., J. Zhou, J. Chee-Sanford, and J. M. Tiedje.** 1994. Isolation, characterization, and distribution of denitrifying toluene degraders from a variety of habitats. Appl. Environ. Microbiol. **60:**2802–2810.
9. **He, Q., M. Marjamaki, H. Soini, J. Mertsola, and M. K. Viljanen.** 1994. Primers are decisive for sensitivity of PCR. BioTechniques **17:**82–87.
10. **Kwok, S., and R. Higuchi.** 1989. Avoiding positives with PCR. Nature **339:** 237–238.
11. **Larsen, N., G. J. Olsen, B. L. Maidak, M. J. McCaughey, R. Overbeek, T. J. Macke, T. L. Marsh, and C. R. Woese.** 1993. The ribosomal database project. Nucleic Acids Res. **21**(Suppl.)**:**3021–3023.
12. **Mahony, J. B., K. E. Luinstra, J. W. Sellors, and M. A. Chernesky.** 1993. Comparison of plasmid- and chromosome-based polymerase chain reaction assays for detecting *Chlamydia trachomatis* nucleic acids. J. Clin. Microbiol. **31:**1753–1758.
13. **Maidak, B. L., N. Larsen, M. J. McCaughey, R. Overbeek, G. J. Olsen, K. Fogel, J. Blandy, and C. R. Woese.** 1994. The ribosomal database project. Nucleic Acids Res. **22:**3485–3487.
14. **National Research Council.** 1993. In situ bioremediation, when does it work? National Academy Press, Washington, D.C.
15. **Rolfs, A., I. Schuller, U. Finckh, and I. Weber-Rolfs.** 1992. PCR: clinical diagnostics and research. Springer-Verlag, New York, N.Y.
16. **Rychlick, W., and R. Rhoads.** 1989. A computer program for choosing optimal oligonucleotides for filter hybridization, sequencing and in vitro amplification of DNA. Nucleic Acids Res. **17:**8543–8551.
17. **Thiem, S. M., M. L. Krumme, R. L. Smith, and J. M. Tiedje.** 1994. Use of molecular techniques to evaluate the survival of a microorganism injected into an aquifer. Appl. Environ. Microbiol. **60:**1059–1067.
18. **Tsai, Y.-L., and B. H. Olson.** 1992. Detection of low numbers of bacterial cells in soils and sediments by polymerase chain reaction. Appl. Environ. Microbiol. **58:**754–757.
19. **Tsai, Y.-L., and B. H. Olson.** 1992. Rapid method for separation of bacterial DNA from humic substances in sediments for polymerase chain reaction. Appl. Environ. Microbiol. **58:**2292–2295.
20. **Zhou, J., M. R. Fries, J. C. Chee-Sanford, and J. M. Tiedje.** 1995. Phylogenetic analyses of a new group of denitrifiers capable of anaerobic growth on toluene and description of *Azoarcus tolulyticus* sp. nov. Int. J. Syst. Bacteriol. **45:**500–506.
21. **Zhou, J., M. A. Bruns, and J. M. Tiedje.** 1996. DNA recovery from soils of diverse composition. Appl. Environ. Microbiol. **62:**316–322.
22. **Zhou, J.-Z., and J. M. Tiedje.** 1995. Development of phylogenetic probes specific to *Burkholderia cepacia* G4, p. 65–75. *In* R. E. Hinchee, G. S. Douglas, and S. K. Ong (ed.), Monitoring and verification of bioremediation. Battelle Press, Columbus, Ohio.
23. **Zhou, J.-Z., and J. M. Tiedje.** 1995. Gene transfer from a bacterium injected into an aquifer to an indigenous bacterium. Mol. Ecol. **4:**613–618.
24. **Zhou, J.-Z., J. Chee-Sanford, R. Weller, and J. M. Tiedje.** Unpublished data.