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Isolation, Characterization, and Distribution of Denitrifying Toluene Degraders from a Variety of Habitats

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Enrichments capable of toluene degradation under O_2-free denitrifying conditions were established with diverse inocula including agricultural soils, compost, aquifer material, and contaminated soil samples from different geographic regions of the world. Successful enrichment was strongly dependent on the initial use of relatively low toluene concentrations, typically 5 ppm. From the enrichments showing positive activity for toluene degradation, 10 bacterial isolates were obtained. Fingerprints generated by PCR-amplified DNA, with repetitive extragenic palindromic sequence primers, showed that eight of these isolates were different. Under aerobic conditions, all eight isolates degraded toluene, five degraded ethylbenzene, three consumed benzene, and one degraded chlorobenzene. *meta*-Xylene was the only other substrate used anaerobically and was used by only one isolate. All isolates were motile gram-negative rods, produced N_2 from denitrification, and did not hydrolyze starch. All strains but one fixed nitrogen as judged by ethylene production from acetylene, but only four strains hybridized to the *nifHDK* genes. All strains appeared to have heme nitrite reductase since their DNA hybridized to the heme (*nirS*) but not to the Cu (*nirU*) genes. Five strains hybridized to a toluene *ortho*-hydroxylase catabolic probe, and two of those also hybridized to a toluene *meta*-hydroxylase probe. Partial sequences of the 16S rRNA genes of all isolates showed substantial similarity to 16S rRNA sequences of *Azoarcus* sp. Physiological, morphological, fatty acid, and 16S rRNA analyses indicated that these strains were closely related to each other and that they belong to the genus *Azoarcus*. The activity and isolation of at least one toluene-degrading denitrifier from the majority of the habitat types studied suggest that microbes with the capacity to grow anaerobically on toluene are common in nature.

The monoaromatic hydrocarbons known as BTEX (benzene, toluene, ethylbenzene, and xylenes) are one of the major problems in environmental pollution. Their presence in groundwater is a widespread problem because of the leakage of underground petroleum storage tanks and spills at petroleum production wells, refineries, pipelines, and distribution terminals. Many governments have established cleanup standards for these chemicals in groundwater because of their carcinogenic potential (4, 14).

Biodegradation of BTEX under aerobic conditions is well-known; oxygen is utilized for ring activation and cleavage and serves as the electron acceptor for the complete oxidation of this compounds (15). The availability of oxygen, due to its low solubility in water and its low rate of transport through saturated porous matrices such as soil and sediments, is usually the rate-limiting parameter for BTEX removal from contaminated sites. Therefore, BTEX biodegradation in the absence of oxygen would be a very beneficial remediation process. It has only been in recent years that anaerobic degradation of these compounds has been conclusively established. Of the BTEX class of compounds, toluene seems to be the most easily degraded under anaerobic conditions. The degradation of toluene under denitrifying (3, 8, 11, 12, 21, 25, 36), methanogenic (16, 40), sulfate-reducing (9, 32), and ferric iron-reducing (27, 28) conditions has been reported. For bioremediation, the most attractive electron acceptor is nitrate since it is water soluble, not costly, and not seriously toxic and does not react with other inorganic species present, such as ferric iron. However, very little is known about the organisms responsible and how widely they are distributed in nature.

We report here on a new group of bacteria that grow on toluene under denitrifying conditions and show that they appear to be widely distributed in nature.

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MATERIALS AND METHODS

Enrichments and isolations. Soils and sediments were collected independently from various locations and handled by procedures to prevent any cross-contamination. Samples (5 to 10 g) were incubated without shaking with 10 ml of basal salts (BS) medium (31) amended with 5 mM KNO_3 in sterile centrifuge tubes. After 3 days of incubation, the enrichments were centrifuged (1,300 × *g* for 10 min), the supernatant was removed, and fresh sterile medium was added to the samples. The samples were vortexed and reincubated. This protocol was repeated one more time to deplete easily oxidizable carbon from the samples that could potentially reduce the selection for anaerobic toluene degraders. After the third incubation period, the samples were centrifuged and the pellet was resuspended in fresh medium; the large soil particles were then allowed to settle. The tubes were transported to a Coy anaerobic chamber, and 5 ml of the supernatant was transferred with a sterile syringe to a serum bottle containing 45 ml of BS medium containing 5 mM KNO_3 and 5 ppm of toluene that had been prepared by strict anaerobic protocol. Toluene disappearance was evaluated by headspace analyses. Enrichments positive for toluene degradation were spiked again with toluene at a concentration of 5 ppm and then at 25 ppm. The bottles were inverted and incubated, and the production of bubbles on the surface of the Teflon-lined septa indicated denitrification activity. Once bubble production ceased, typi-

cally after 3 days at this last stage of enrichment, three serial transfers of a 10% inoculum were made per sample into fresh medium containing 25 ppm of toluene. Anaerobic manipulations throughout this work were done in the anaerobic chamber and the resultant headspace of the bottles, and agar plates was nominally 10% H_2-90% N_2. The incubation temperature throughout this work was 30°C.

These enriched samples were serially diluted and plated on two different solid media; (i) BS medium plus 5 mM NO_3^- plus toluene vapors and (ii) modified R2A (M-R2A), based on the original composition provided by Difco (Detroit, Mich.). M-R2A had the following salt mixture composition per liter: KH_2PO_4, 0.25 g; K_2HPO_4, 0.4 g; KNO_3, 0.505 g; $CaCl_2 \cdot 2H_2O$, 0.015 g; $MgCl_2 \cdot 6H_2O$, 0.02 g; $FeSO_4 \cdot 7H_2O$, 0.007 g; Na_2SO_4, 0.005 g; NH_4Cl, 0.8 g; $MnCl_2 \cdot 4H_2O$, 5 mg; H_3BO_3, 0.5 mg; $ZnCl_2$, 0.5 mg; $CoCl_2 \cdot 6H_2O$, 0.5 mg; $NiSO_4 \cdot 6H_2O$, 0.5 mg; $CuCl_2 \cdot 2H_2O$, 0.3 mg; and $NaMoO_2 \cdot 2H_2O$, 0.01 mg. For solid medium, 15 g of Bacto agar (Difco) was added. The pH was adjusted to 7.0 before autoclaving. The carbon sources and their concentrations, per liter, were yeast extract, 0.5 g; peptone, 0.5 g; Casamino Acids, 0.5 g; dextrose, 0.5 g; soluble starch, 0.5 g; and sodium pyruvate, 0.5 g. Glass petri dishes were used for toluene vapor-based growth, and toluene was added to a small vial inside an incubation jar. The amount of toluene added to provide a final concentration of 25 ppm was calculated in the basis of the total volume of agar in the incubation jar. After 1 week of incubation, sufficient toluene to provide 25 ppm was again added. The jar used in the toluene vapor experiments was sealed with a Teflon-lined aluminum sheet (Cole-Palmer, Chicago, Ill.) to prevent toluene absorption by the rubber sealer of the jar. The plates were incubated under anaerobic and aerobic conditions.

For purification of isolates, single colonies from different plates and different dilutions were selected and purified at least three times on M-R2A before further evaluation. Confirmation of toluene degradation under denitrifying conditions was done by transferring a heavy inoculum of each isolate to sterile 20-ml vials, recapping the vials with foam plugs, and transporting the vials to an anaerobic chamber for headspace gas exchange (at least 6 h), after which 10 ml of anaerobic BS medium–NO_3^-–25 ppm of toluene was added. The vials were sealed with sterile butyl rubber Teflon-lined septa and incubated for at least 2 weeks before toluene disappearance was evaluated. As a control, medium without nitrate containing cells and toluene was incubated at the same time as the medium with nitrate and was used as a reference to determine nitrate-dependent toluene removal.

Characterization of isolates. Cell size and shape were observed by phase-contrast microscopy for cells grown anaerobically on toluene in liquid medium and for cells grown on M-R2A agar. The latter were resuspended in water, and 5 μl was added to a microscopic slide containing 5 μl of molten 0.4% agarose solution, mixed gently, and covered with a coverslip. The medium for the nitrogen fixation assay consisted of BS free of nitrogen sources supplemented with filter-sterilized solutions of (final concentration per liter) biotin (0.1 mg), $Na_2MoO_4 \cdot 2H_2O$ (0.002 g), malic acid (5 g), and KOH (4.5 g), adjusted to pH 7. Agarose (0.07%) was added to provide for establishment of microaerobic conditions (7). Inocula were grown on liquid M-R2A, and cells washed in saline solution were inoculated into 20-ml serum vials containing 5 ml of medium. The vials were incubated for 48 h, and nitrogenase activity was evaluated by the acetylene reduction assay (1) after 6 and 24 h of incubation with acetylene. *Azospirillum brasiliense* sp7 was used as a positive control.

Denitrification was evaluated by observing gas bubble formation in inverted tubes and by measuring the presence of N_2O in the vial headspace by gas chromatography (GC). The denitrification medium consisted of the salt mixture and the following carbon sources (final concentration per liter): Proteose peptone (0.5 g), yeast extract (0.5 g), Casamino Acids (0.5 g), potassium acetate (1 g), and sodium succinate (1 g). Argon was the headspace for the samples analyzed by GC.

Starch hydrolysis was performed by the standard method (37) on M-R2A plates supplemented with 0.2% starch.

The isolates analyzed for cellular fatty acids were precultured on M-R2A and streaked onto plates containing 0.3% (wt/vol) tryptic soy broth solidified with 15 g of tryptic soy agar (TSA) per liter (24). At least three plates of each isolate were incubated for 72 to 96 h in order to obtain enough biomass for the analysis. Cells were harvested from the plates by scraping with a sterile loop. Saponification, methylation, and extraction were performed by the procedure described previously (35). Cluster analysis was carried out by using an in-house cluster program and the MIDI software.

Detectable plasmids were screened for in isolates cultured anaerobically on toluene-BS-NO_3^- medium. Cells were lysed, and plasmids were screened as described by Kado and Liu (23).

The anaerobic growth rate of the isolates in BS (containing one-fifth of the original EDTA concentration)–5 mM NO_3^-–50 ppm of toluene was determined by measuring the optical density at 600 nm. The inoculum was grown under the same conditions. Growth in TSA was evaluated every 12 h, and the colony size was compared with that of M-R2A-grown cells.

To test for the isolates' capabilities to degrade benzene; ethylbenzene; *o*-, *m*-, and *p*-xylenes; and chlorobenzene, inocula were grown on BS-NO_3^--toluene medium under both denitrifying and aerobic conditions. Inocula (0.5 ml) were transferred to 20-ml of sterile auto sampler vials. For aerobic growth, 4.5 ml of BS–NO_3^-–25 ppm of the aromatic substrate was added to the vials, and the vials were sealed with Teflon-lined stoppers. For anaerobic growth, the vials were prepared similarly except that the media with the aromatic substrates were added in the anaerobic chamber. Positive degradation activity was defined as at least 80% loss of substrate in the headspace as measured by GC analysis, compared with that for noninoculated controls.

Molecular methods. Repetitive extragenic palindromic (REP)-PCR patterns were obtained from cells with Rep-1 and Rep-2 primers by PCR (5). Amplification was performed with a model 9600 Perkin-Elmer Cetus thermocycler. Products (10 μl) were separated by electrophoresis in 1.5% agarose gels and stained with ethidium bromide. Amplification was primarily done by using individual colonies grown on M-R2A, but for isolates with poor or no amplification, DNA extracted from cells was used as the template for PCR amplification. For isolates with very similar patterns, the same stock of primers and reagents were used in the analysis.

The gene probes used are described in Table 1. *Escherichia coli* cultures carrying the plasmids with probes were grown for plasmid amplification in the presence of the appropriate antibiotic. Plasmids were extracted by a standard protocol (29). The probes were isolated as restriction fragments from their respective vectors in 1% low-melting-point agarose, purified with the Gene Clean kit (Bio 101, Inc., La Jolla, Calif.), and labelled with [α-^{32}P]dCTP (3,000 Ci/mM; Dupont, NEN Research Products, Wilmington, Del.) by using a random hexamer priming kit from Boehringer Mannheim Biochemicals. Labelled probes were separated from unincorporated nucleotides prior to use with a spun column (29). The probes were used at approximately 10^6 cpm/ml of hybridization fluid.

Genomic DNA was obtained by standard methods (10) from

TABLE 1. List of DNA probes used in this study

Organism (probe source)	Gene encoded and probe size	Plasmid	Laboratory source and reference[a]
Pseudomonas putida PaW1	Methyl monooxygenase (hydroxylase and NADH-ferredoxin reductase), 2.35 kb (*Sal*I-*Hin*dIII)	pG5H2836	S. Harayama (18)
Pseudomonas putida F1	Toluene dioxygenase (large and small subunits of oxygenase, ferrodoxin, and part of reductase), 3.5 kb (*Eco*RI-*Bgl*II)	pDTG601	D. Gibson (44)
Pseudomonas mendocina KR	Toluene *para*-hydroxylase (monooxygenase and ferredoxin), 3.6 kb (*Eco*RI-*Eco*RI)	pMY421	M. DeFlaun (42)
Pseudomonas pickettii PKO1	Toluene *meta*-hydroxylase (α subunit of monooxygenase), 0.68 kb (*Apa*I-*Ava*I)	pAB14ΔAva I	R. Olsen (2, 30)
Pseudomonas sp. strain JS-150	Toluene *ortho*-hydroxylase, 2.2 kb (*Eco*RV–*Hin*dIII)	pRO20116	R. Olsen (22)[b]
Pseudomonas stutzeri JM300	Heme containing nitrite reductase, 0.7 kb(*Dde*I-*Dde*I)	pBsGTh 2.4	J. Tiedje (38)
Pseudomonas sp. strain 179	Copper containing nitrite reductase, 1.9 kb (*Eco*RI-*Bam*HI)	pRTc1.9	J. Tiedje (41)
Pseudomonas stutzeri ZoBell	Nitrous oxide reductase, 1.2 kb (*Pst*I-*Pst*I)		W. Zumft (39)
Micrococcus luteus	23S rRNA, 0.47 kb (*Eco*RI-*Hin*dIII)	pAR17	K. Schleifer (33)
Rhizobium sp.	Nitrogenase (*nifHDK* genes), 3.6 kb (*Bgl*II-*Xho*I)	pRS2	F. de Bruijn (10)

[a] Laboratories from which the clones were obtained from.
[b] This strain was provided by J. C. Spain (17). The probe has strong hybridization to *P. cepacia* G-4 (22) (Fig. 3C) and likely reflects G-4-like sequences as well.

pure cultures of isolates and selected strains grown on M-R2A broth under aerobic conditions. Restriction endonuclease digestion of DNA was performed according to the manufacturer's specifications. Digested DNA was size fractioned by electrophoresis in 0.7% agarose gels and transferred to nitrocellulose (polyester-supported BAS 68380; Schleicher & Schuell, Keene, N.H.) as previously described (29) with 20× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4 [pH 7.04], and 1 mM EDTA). The DNA on the filters was cross-linked by UV light (Stratagene, La Jolla, Calif.). The solutions used for DNA hybridization analysis have been described elsewhere (19). The membranes were prehybridized for at least 24 h in heat-sealed bags containing 100 μl of prehybridization fluid per cm^2 of filter. Prehybridization fluid contained 5× Denhardt solution, 5× SSPE, 50% formamide, and 200 μg of sonicated and denatured salmon sperm DNA per ml. The hybridization solution was the same as that for prehybridization but included 10% (wt/vol) dextran sulfate and was added at 50 μl/cm^2 of filter. The membranes were incubated for at least 24 h. Two hybridization temperatures, 30°C (low stringency) and 42°C (high stringency), were used. After hybridization, the filters were washed once for 15 min with agitation at 30°C with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS). For low-stringency hybridization, a second wash for 30 min with 0.5× SSC–0.1% SDS was performed. For high-stringency hybridization, a second wash for 15 min with 0.5× SSC–0.1% SDS followed by a third wash with 0.1× SSC–0.1% SDS was performed. This was followed by a final wash at 55°C for 30 min with the last solution. After the washes, hybridization signals were visualized by using the Betascope radioactive blot analyzer (Betagen Corp., Waltham, Mass.) or by autoradiography with X-Omat AR film (Kodak, Rochester, N.Y.) exposed at −70°C with a Quanta III (Sigma, St. Louis, Mo.) intensifying screen. Exposure times were 1 to 3 days, depending on the intensity of the radioactive signal. For reuse of the same blot for another probe, the blots were stripped by washing for 10 min in boiling water–0.1% SDS at least two times, depending on the signal left on the blot after evaluation with the Betascope. A final wash with 2× SSPE–0.1% SDS for 10 min completed the stripping protocol.

The 16S rRNA gene was amplified from genomic DNA by PCR and cloned into a plasmid vector as described previously (43). The 16S rRNA gene was sequenced from both directions with an automated fluorescence sequencer (model 373A; Applied Biosystems, Foster City, Calif.) with the forward and reverse primers, which span *E. coli* 16S rRNA gene positions 785 to 805 and 1115 to 1100, respectively. The DNA sequences were compared with those in the Ribosomal Database Project (26) and analyzed by using the programs in the Genetics Computer Group software package (6) and in PHYLIP phylogeny inference package (13).

Analytical methods. Toluene; ethylbenzene; *o*-, *m*-, and *p*-xylenes; benzene; and chlorobenzene concentrations were measured with a GC equipped with an flame ionization detector, a DB-624 capillary column (J&W Scientific, Folson, Calif.), and a headspace sampler. The vials and bottles were equilibrated at 30°C, the column was equilibrated at 90°C, and the injector and detector were equilibrated at 200°C. He was the carrier gas. Acetylene and ethylene were assayed by a GC/-flame ionization detection by using a DB-23 megabore column (J&W Scientific) at 75°C, an injector at 160°C, and a detector at 320°C with N_2 as the gas carrier. NO_3^- and NO_2^- concentrations in culture supernatant were determined by high-performance liquid chromatography (HPLC) analysis using a Partisil 10 SAX column (Whatman, Clifton, N.J.), UV detection at 210 nm, and 50 mM phosphate (pH 3.0) as the eluant. N_2O was measured by GC-electron capture detection with a Poropak Q column at 55°C, 300°C detector temperature, and 95% argon–5% methane as the carrier gas.

Nucleotide sequence accession numbers. The partial 16S rRNA sequences of all new isolates have been placed in Genbank under accession no. L 33687 to L33694.

RESULTS

Enrichments. Successful enrichments for denitrifying toluene degraders were obtained from about half of the samples from both chemically contaminated and noncontaminated sites (Table 2). Enrichments positive for toluene degradation were obtained from widely separated and dissimilar habitats. Ten isolates that were confirmed as pure cultures and were able to grow on toluene in the presence of nitrate and the complete absence of oxygen were obtained (Table 2). The isolates also came from a wide range of environments. Additional clones that appeared to have toluene-degrading denitrifying activity were obtained, but they were not studied further because it was too difficult to confirm purity, growth was too variable, or they appeared to be identical or closely related to isolates already in pure culture by REP-PCR analysis.

TABLE 2. Source of inoculum, number of enrichments with positive activity for toluene degradation under denitrifying conditions, and number of isolates obtained with this activity from each sample

Source of inoculum and description of sample	Enrichments (no. positive/ total no. tested)	Isolates obtained[a] (strain no.)
Noncontaminated		
Cameroon, rainforest soil	1/2	IU
Michigan, muck soil	2/2	2 (Td-20, Td-21)
Michigan, compost pile	1/1	1 (Td-15)
Michigan, agricultural soil	1/2	1 (Td-16)
Hawaii, Big Island forest soil	2/3	INA
Hawaii, Kauai sugar cane soil	0/1	
Siberia, Kolyma Valley permafrost soil	0/2	
Total	7/13	4
Contaminated with organic pollutants		
Sao Paulo, Brazil; industrial waste	2/3	2 (Td-17, Td-19)
Rio Grande do Sul, Brazil; industrial sludge	2/3	INA
Ontario, Canada; pulp mill lagoon sediment	1/4	IU
Bear Lake, Michigan; aquifer, sand, petroleum (24 to 26-m deep)	1/1	1 (GR-3)
Wexford, Michigan; aquifer, sand, petroleum	0/1	
Huntington Beach, California; marine, petroleum	1/1	1 (Td-3)
Washington State	2/2	2 (Td-1, Td-2)
Six undescribed chemically contaminated soils	1/6	IU
Total	10/21	6

[a] IU, isolation unsuccessful; INA, isolation not attempted.

Successful enrichment and isolation were strongly dependent on the use of relatively low toluene concentrations, typically 5 ppm. This strategy was used because our early isolation and enrichment attempts were unsuccessful after extensive effort with 250 ppm and because we had noted a considerable increase in the most probable number estimate of aerobic toluene degraders when the toluene concentration was decreased from 250 to 50 ppm and again when reduced to 5 ppm. Thus, we reasoned that toluene toxicity could also be an important factor for successful enrichment and isolation of anaerobic toluene degraders. Once isolated, the cultures were routinely cultured in 25 ppm of toluene and, once growing, could be fed 50 ppm to obtain higher cell yields.

All isolates were obtained from plates of the M-R2A medium incubated aerobically. Some isolates were obtained from the anaerobic toluene vapors plus nitrate or M-R2A plus nitrate medium, but by REP-PCR they were identical to the ones isolated from the same source on the aerobic medium. In all cases, the denitrifying toluene degraders were pinpoint colonies. Larger colonies were also picked, but these isolates were either denitrifiers or toluene degraders. Some of them were capable of both functions but did not carry out both under the same conditions. In some cases, it was very difficult to separate contaminating cells from the denitrifying toluene degrader. Purity was based primarily on uniform and repeated colony morphology after at least 2 weeks of incubation.

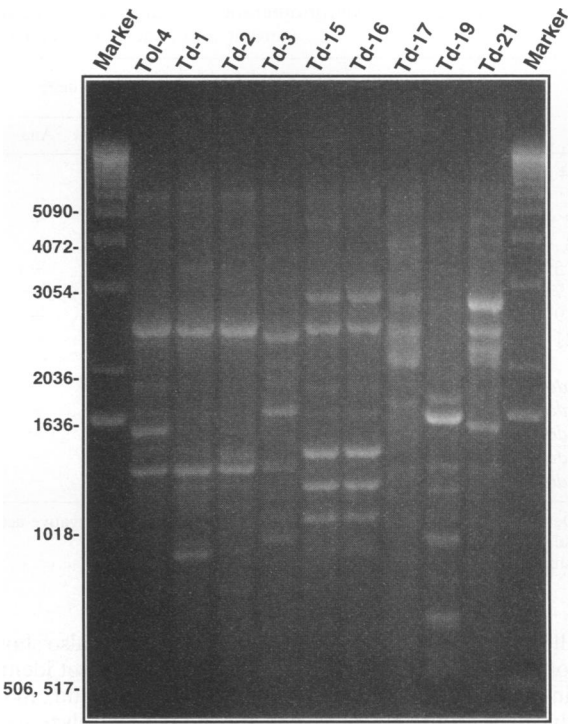

FIG. 1. REP-PCR fingerprint patterns of toluene-degrader denitrifier isolates generated by using chromosomal DNA. Lanes 1 and 11 show size markers, with the base pairs indicated on the left.

Consistent REP-PCR patterns and consistent cell morphology were used as confirmatory methods for purity.

We also carried out parallel enrichments for denitrifying benzene degraders, using the same enrichment conditions and environmental samples as those used for the toluene enrichments. No activity could be confirmed by measuring benzene removal from any of the enrichments.

We used REP-PCR to screen for sufficiently different strains for further study. There were eight distinct profiles from the 10 confirmed denitrifying toluene degraders (Fig. 1). One of the isolates, GR-3, had a REP-PCR pattern identical to that of our previous isolate, Tol-4 (3), and was not studied further. One set of isolates with identical patterns came from the same site (Michigan muck soil) and may be siblings; therefore, only isolate Td-21 was studied further. The other set of isolates with identical patterns came from Michigan agricultural soil and a compost pile; only the compost isolate, Td-15, was studied further since it came from a very different environment.

Characteristics. The ability of the seven new isolates to degrade related aromatic compounds under aerobic or anaerobic conditions is very limited, except that all isolates can also degrade toluene aerobically (Table 3). Several of the cultures were initially negative for aerobic toluene use; but after repeated experiments and optimizing conditions, all were shown to be capable of aerobic toluene consumption, albeit some showed weak ability. Five strains could use the alkylated analog, ethylbenzene, aerobically, but none could use it anaerobically. Three isolates used benzene, and one used chlorobenzene aerobically. *meta*-Xylene was the only substrate used anaerobically other than toluene, and it was used by only one isolate. The anaerobic pathway seems very specific for toluene, and the aerobic substrate range is much more limited than it is for the well-studied aerobic toluene degraders (Table 3).

TABLE 3. Removal of different substrates by toluene-degrading isolates and well-known aerobic strains in BS medium under aerobic and anaerobic (denitrifying) conditions after 2 weeks of incubation[a]

Isolate	Benzene		Toluene		Ethyl benzene		*o*-Xylene		*m*-Xylene		*p*-Xylene		Chlorobenzene	
	Ae[b]	Ana[c]	Ae	Ana	Ae	Ana	Ae	Ana	Ae	Ana	Ae	Ana	Ae	Ana
Tol-4	−	−	+	+	+	−	−	−	−	−	−	−	−	−
Td-1	−	−	+	+	+	−	−	−	−	−	−	−	−	−
Td-2	−	−	±	+	−	−	−	−	−	−	−	−	−	−
Td-3	±	−	±	+	−	−	−	−	−	−	−	−	−	−
Td-15	−	−	+	+	+	−	−	−	−	+	−	−	−	−
Td-17	+	−	+	+	+	−	−	−	−	−	−	−	−	−
Td-19	−	−	±	+	−	−	−	−	−	−	−	−	+	−
Td-21	+	−	+	+	+	−	−	−	−	−	−	−	−	−
Pseudomonas cepacia G4	+	−	+	−	+	−	+	−	−	−	+	−	+	−
Pseudomonas mendocina KR	+	−	+	−	+	−	+	−	+	−	+	−	+	−
Pseudomonas pickettii PKO1	+	−	+	−	+	−	+	−	+	−	+	−	+	−
Pseudomonas putida F1	+	−	+	−	+	−	+	−	+	−	+	−	−	−
Pseudomonas putida PaW1	+	−	+	−	+	−	+	−	−	−	+	−	−	−

[a] Denotes more than 80% removal from headspace vial; −, negative activity; ±, activity often delayed. All substrates at 25 ppm concentration.
[b] Ae, aerobic conditions.
[c] Ana, anaerobic (denitrifying) conditions.

All seven isolates had similar major features but also showed minor differences which confirmed that they were not identical strains (Table 4). All were gram-negative rods and, motile, produced N_2 from denitrification, and did not hydrolyze starch. They did not grow well aerobically on complex media such as TSA. Growth could begin to be seen on 1/10 strength TSA as very sparse tiny colonies only after 48 h of incubation. M-R2A is the best medium that we have found for growth on plates. Colonies of 1 to 4 mm in diameter can be obtained after 36 to 72 h of incubation. N_2 fixation was shown by subsurface pellicle formation in semisolid medium free of combined nitrogen, by ethylene production from acetylene (all but isolate Td-15), and by DNA from four of seven strains hybridized to the *nifHDK* genes (Table 4).

The major fatty acids for all strains studied and their concentration ranges were cis:9 16:0 (42.3 to 61.9%), 16:0 (21.5 to 38.6%), 12:0 (4.4 to 12.9%), 3-OH-10:0 (1.7 to 7.7%), 14:0 (0.9 to 1.55%), cyclo 17:0 (0 to 4.11%), and 18:0 (less than 1%). Species identification based on total fatty acids and cluster analysis was not possible since the analysis gave a similarity index of less than 0.4 by using the MIDI database.

The morphology of all strains grown anaerobically on toluene was small rods, typically 1.4 to 2.1 μm in length (Fig. 2A). When grown on M-R2A agar, however, all formed longer cells, 2.1 to 2.8 μm (Fig. 2B), and some isolates (i.e., Td-3, Td-15, Td-17, and Td-19) had a tendency to form chains (Fig. 2C).

DNA isolated from the seven new isolates and five well-studied aerobic toluene degraders was digested and hybridized on Southern blots to various probes to determine which strains carried similar sequences. Hybridization with a universal 23S rRNA probe confirmed that sufficient DNA was in all lanes and that all strains were different (Fig. 3A). The probe for the denitrifying Cu-nitrite reductase gene (*nirU*) showed no hybridization (data not shown), but the heme-nitrite reductase probe (*nirS*) hybridized to all strains, suggesting that they are all denitrifiers with the heme-type enzyme (Fig. 3B). The nitrous oxide reductase probe (*nosZ*) hybridized to all strains and in different positions (data not shown). Probes for the first steps in all five aerobic toluene-degrading pathways were used, but only the probe for the *ortho*-hydroxylase (*Pseudomonas* sp. strain JS150) (Fig. 3C) and the *meta*-hydroxylase (*P. pickettii* PKO1) (Fig. 3D) genes showed hybridization. Five strains (all

TABLE 4. Summary of characteristics of the different denitrifying toluene degraders[a]

Isolate	Growth on M-R2A for 48 h	Growth on 1/10 TSA		Growth (h) rate[b]	Denitrification			Nitrogen fixation			Starch hydrolysis	Detectable plasmids
		48 h	96 h		N_2[c]	N_2O	NO_2^-	Pellicle[d]	Acetylene[e]	Probe[f]		
Tol-4	+	±	+	8–13	+	+	+	+	+	+	−	−
Td-1	+	±	+	6–7	+	+	±	+	+	+	−	−
Td-2	+	±	+	5–7	+	+	+	+	+	+	−	−
Td-3	+	−	+	7–8	+	+	+	+	+[g]	−	−	+
Td-15	+	−	+	6–7	+	+	+	+	−	−	−	−
Td-17	+	−	+	5–7	+	+	+	+	+	+	−	−
Td-19	+	±	+	5–7	+	+	+	+	+[g]	−	−	+
Td-21	+	−	+	5–7	+	+	+	+	+	+	−	−

[a] +, positive activity; −, negative activity; ±, variable activity.
[b] Cells grown on BS–50 ppm of toluene under denitrifying conditions. Doubling time is expressed in hours.
[c] Observation of bubbles on the surface of the Teflon-lined septum.
[d] Formation of growth pellicle on nitrogen-free medium.
[e] Detection of ethylene from acetylene after at least 24 h of incubation.
[f] Presence of distinct band on Southern blot hybridized with *nifHDK* probe.
[g] Ethylene production detectable only after 24 h of incubation with acetylene.

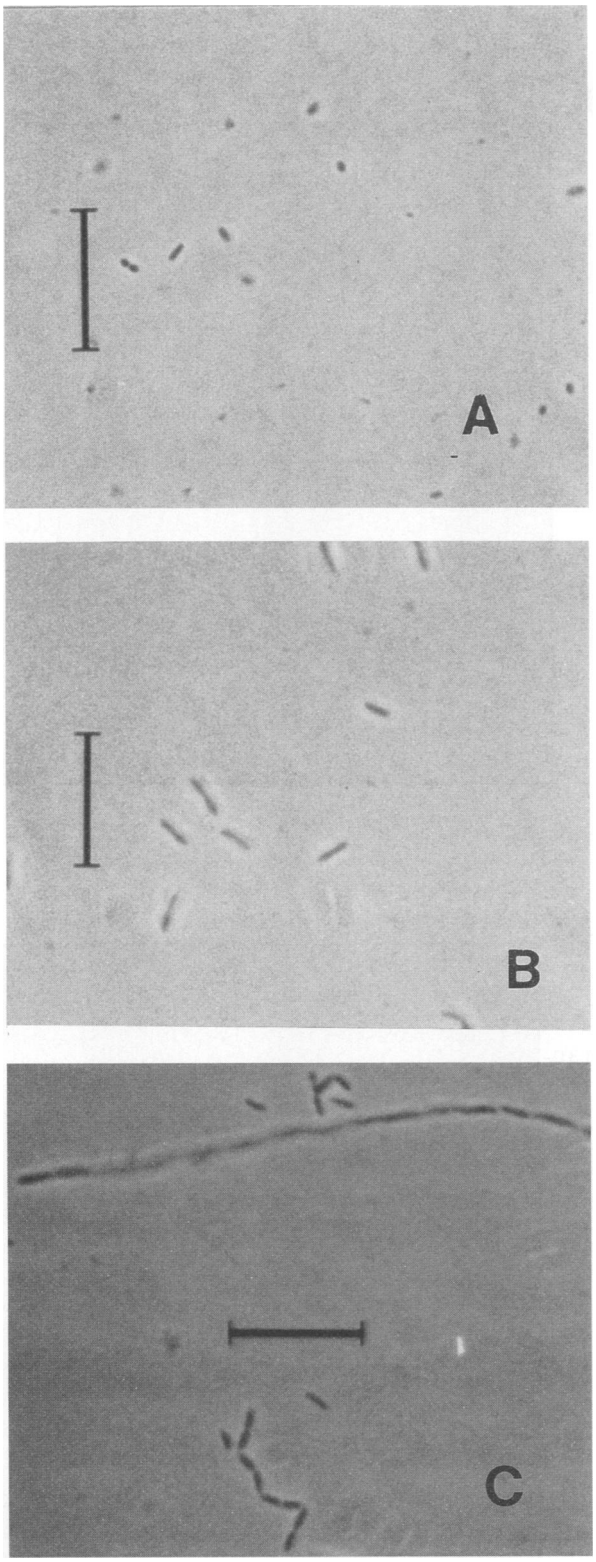

FIG. 2. Phase-contrast photomicrographs of isolates Td-2 (A), Td-3 (B), and Td-17 (C). Isolate Td-2 was grown on BS-toluene liquid medium to late exponential phase. Isolates Td-3 and Td-17 were grown on M-R2A solid medium for 48 h. Bars, 14 μm.

but isolates Td-3 and Td-15) showed strong signals to the *ortho*-hydroxylase probe. The *meta*-hydroxylase probe hybridized to DNA from two strains that also showed hybridization to the *ortho*-hydroxylase probes, but the hybridizing bands were in different positions for the two probes.

The sequence of approximately 280 nucleotide bases, corresponding to the *E. coli* 16S rRNA gene sequence from nucleotide 810 to 1090, was obtained for the seven isolates. The percent similarities among these isolates ranged from 97.8 to 100%. The partial sequences of these isolates showed strong similarity to 16S rRNA gene sequences of the nitrogen-fixing genus *Azoarcus* (20). The similarities are *Azoarcus* sp. strain Sb52 (90.7 to 91.5%), Azoarcus sp. strain BH72 (92.6 to 94.1%), and *Azoarcus indigens* (93.4 to 94.9%). The phylogenetic tree constructed by the maximum parsimony method showed that all the toluene-denitrifying isolates form a phylogenetically coherent unit clustered with *A. indigens* and *Azoarcus* sp. strain BH72 (Fig. 4). Very similar tree topologies were also obtained by distance matrix and maximum likehood methods (data not shown).

DISCUSSION

Pure cultures of anaerobic toluene degraders have not been easy to isolate. After a decade of effort by many capable laboratories, seven isolates that use nitrate as an electron acceptor (3, 8, 12, 36), one that uses Fe(III) (28), and one that uses sulfate have been reported (32). This study yielded 10 new isolates and additional active enrichments. We believe that the most important reason for the improved success rate of enrichment and isolation was the strategy of avoiding toxicity by never exposing the culture to more than 5 ppm of toluene during the initial enrichment process. Also important were exhausting the residual available carbon before adding toluene, isolating cells by aerobic growth on M-R2A, and being sure to select the pinpoint colonies. This procedure, however, may have selected for only a certain group of denitrifying toluene degraders and may explain why we were unsuccessful in obtaining isolates from some of the active enrichments.

The seven new isolates appear to be closely related to each other and to be members of the genus *Azoarcus*. The identification is based on the fact that these isolates share the following key features with the described *Azoarcus* strains (20, 34): all 16S rRNA sequences fall within the cluster for this genus, they fix nitrogen, they have the same type and proportion of cellular fatty acids as do the described strains, and they have similar morphology when grown on complex medium. They do have some phenotypic and ecologic differences from the described *Azoarcus* strains. The new strains all denitrify and grow poorly on TSA, which are not characteristics of the previously described strains (34). Also, 11 of 12 previously described strains were isolated from the roots of tropical grasses and are considered to be rhizosphere-associative nitrogen fixers. None of our isolates came from plant rhizospheres. Three came from environments in which plants had recently grown (Michigan muck and agricultural soil), but four came from soils contaminated with chemical wastes in industrial areas, one from an aquifer 24 to 26 m underground and one from a compost pile. Thus, the ecological niche of the new isolates may be very different from that described for the previous isolates.

All our isolates are able to use toluene aerobically as well as anaerobically, which would not be expected if these are independent traits. Perhaps the pathways share some common steps, reflect a common phylogenetic heritage, or were a result of concurrent selection resulting from continued use of the

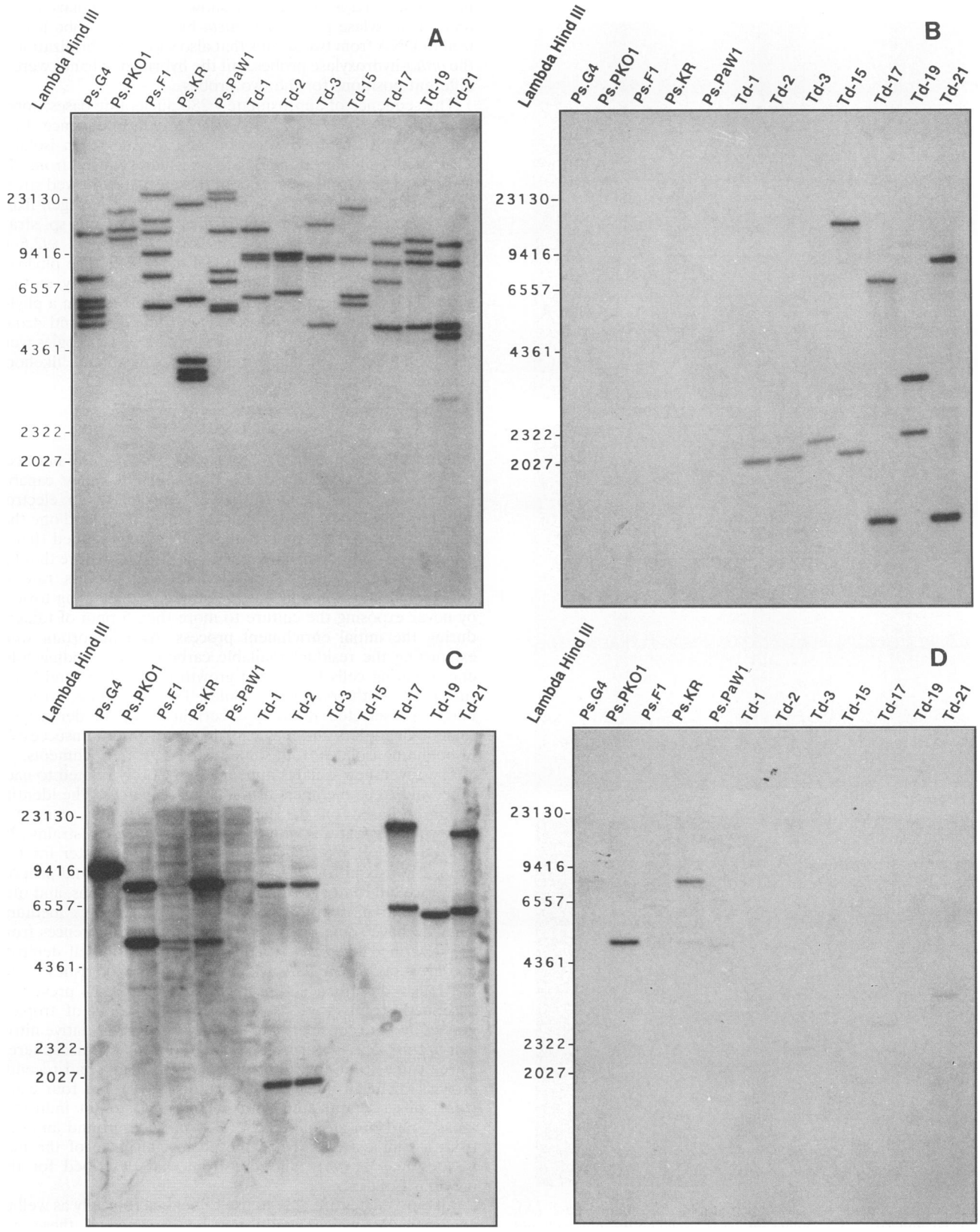

FIG. 3. Southern hybridization of genomic DNA from pure cultures of toluene degraders digested with *Eco*RI and hybridized with the following gene probe: universal 23S rRNA (A), heme nitrite reductase (B), toluene *ortho*-hydroxylase (C), and toluene *meta*-hydroxylase (D). Hybridizations were done under high (A and B) and low (C and D) stringency conditions. Size markers (in base pairs) are indicated on the left.

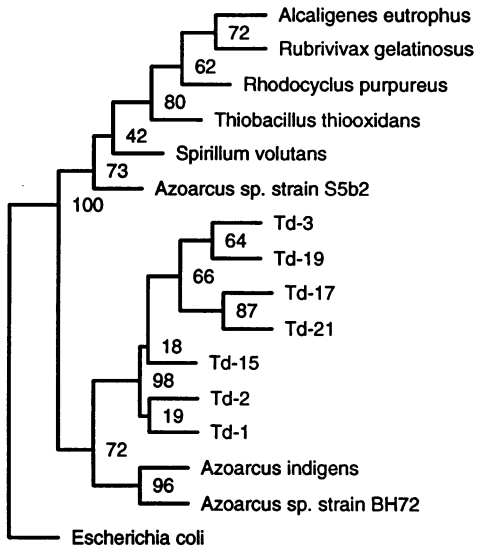

FIG. 4. Phylogenetic position of the toluene-degrading denitrifier (Td) isolates. This tree was constructed by using the programs SEQBOOT, DNAPARS, and CONSENSE in PHYLIP 3.5 and rooted by reference to *E. coli*. The numbers under the nodes are the bootstrap confidence estimates on the branches in 100 replicates. All other 16S rRNA gene sequences were obtained from the Ribosomal Database Project (26).

same substrate under both aerobic and anaerobic conditions. The phylogenetic heritage of the aerobic pathway at least is not necessarily expected because the aerobic toluene pathway has often been found on transmissible plasmids. The aerobic substrate range, however, is far more limited than that found for the well-studied aerobes, suggesting that the *Azoarcus* aerobic pathway(s) has unique features.

Hybridization at high stringency of the subunit probe for the toluene *ortho*-hydroxylase pathway to DNA from five of the *Azoarcus* isolates suggests the presence of this gene in these strains and hence that the *ortho*-hydroxylase pathway may be responsible for aerobic toluene metabolism. Two of the strains may also have the *meta*-hydroxylase pathway since they also hybridized to this probe at different positions on the Southern blot. Having three different toluene pathways, two aerobic and at least one anaerobic, in one strain is perhaps unexpected. Two of the strains had detectable plasmids, but the aerobic toluene pathway probes did not hybridize to the plasmids.

This study indicates that denitrifying toluene degraders are widely distributed in nature; and thus if nitrate were present or added, toluene should be removed. This conclusion is based on finding anaerobic toluene removal in such diverse and widely distributed environments as a pristine rain forest in Cameroon, industrial sites in two states of Brazil, a forest preserve in the young geographically isolated island of Hawaii, a marine beach in California, a deep sandy aquifer in Michigan, a wood pulp treatment lagoon in Ontario, and a compost pile in Michigan. The only different environment that did not yield an enrichment was permafrost soils collected from a region adjacent to the Arctic Ocean in eastern Siberia. Many samples did not yield enrichments, however. This may be due to the difficulty of successfully enriching these organisms or to the fact that such organisms may not be present in every gram of nature. The poor enrichment record for the undescribed contaminated samples is likely due to general toxicity from the chemical contaminants since chemical odors were apparent in these samples.

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