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Phylogenetic Analyses of a New Group of Denitrifiers Capable of Anaerobic Growth on Toluene and Description of *Azoarcus tolulyticus* sp. nov.

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To understand the phylogeny and taxonomy of eight new toluene-degrading denitrifying isolates, we performed a 16S rRNA sequence analysis and a gas chromatographic analysis of their cellular fatty acids and examined some of their biochemical and physiological features. These isolates had 16S rRNA sequence signatures identical to those of members of the beta subclass of the *Proteobacteria*. The levels of similarity were as follows: 97.9 to 99.9% among the new isolates; 91.2 to 92.4% between the new isolates and *Azoarcus* sp. strain S5b2; 95.3 to 96.2% between the new isolates and *Azoarcus* sp. strain BH72; and 94.8 to 95.3% between the new isolates and *Azoarcus indigens* VB32^T (T = type strain). Phylogenetic trees constructed by using the distance matrix, maximum-parsimony, and maximum-likelihood methods showed that our eight denitrifying isolates form a phylogenetically coherent cluster which represents a sister lineage of the previously described *Azoarcus* species. Furthermore, the fatty acid profiles, the cell morphology, and several physiological and nutritional characteristics of the eight isolates and the previously described members of the genus *Azoarcus* were also similar. In contrast to the previously described members of the genus *Azoarcus*, the eight new isolates were capable of degrading toluene under denitrifying conditions. We concluded that these toluene-degrading denitrifiers are members of a new species of the novel nitrogen-fixing genus *Azoarcus*. We propose the name *Azoarcus tolulyticus* for these toluene-degrading denitrifying isolates and designate strain Tol-4 the type strain.

Benzene, toluene, ethylbenzene, and xylenes, compounds present in gasoline, are major environmental pollutants. Microbial decomposition of these compounds under aerobic conditions is well-known, but oxygen is often limited in zones of aquifers that are contaminated with benzene, toluene, ethylbenzene, and xylenes. Nitrate has been shown to stimulate the anaerobic degradation of some of these compounds in some soils and aquifers (11, 12, 14, 18), but little is known about the organisms responsible for this activity.

The following three toluene-degrading denitrifying strains have been described previously: *Pseudomonas* sp. strain K172 (22), *Pseudomonas* sp. strain T (6), and strain T1 (7). The phylogeny of these isolates has not been determined. Recently, we isolated eight new toluene-degrading denitrifiers from a variety of environments (3, 4, 9). The results of studies of partial 16S rRNA gene sequences (~280 bp) of these organisms and some of their physiological and morphological characteristics suggested that they are probably members of, or very closely related to, the novel nitrogen-fixing bacterial genus *Azoarcus* (4, 9). More importantly, these new isolates exhibit nitrogenase activity (4, 9), which is a key feature of the genus *Azoarcus* (19). However, while the previously described members of the genus *Azoarcus* were reported to be nondenitrifiers (19), the new isolates are able to grow on toluene under strictly anaerobic, denitrifying conditions. The previously described *Azoarcus* isolates were isolated principally from the rhizosphere of Kallar grass in tropical and subtropical regions (19), whereas the eight new isolates were isolated from a variety of nonrhizosphere habitats. In order to determine the phylogenetic position of these toluene-degrading denitrifiers, we sequenced almost the entire 16S rRNA gene of each isolate. In addition, we examined the cellular fatty acid compositions and some biochemical and physiological features of these organisms. Our results showed that our isolates are a closely related group that can be readily distinguished from the previously described *Azoarcus* strains. This led us to propose a new species in the genus *Azoarcus.*

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MATERIALS AND METHODS

Organisms and growth conditions. Eight unique, facultatively anaerobic toluene degraders were isolated under denitrifying conditions from a variety of environments (3, 4, 9). The geographic distribution and habitat characteristics of these isolates are listed in Table 1. These isolates were grown aerobically at 30°C on modified R2A (M-R2A) medium as described previously (9).

Genomic bacterial DNA isolation. Total DNA of each bacterial isolate was obtained by using the method of Zhou (25), with modifications. Cells from 15 ml of a late-exponential-phase culture were pelleted and suspended in 4 ml of extraction buffer containing 100 mM Tris-HCl (pH 8.0), 50 mM EDTA, 1.4 M NaCl, and 1% (wt/vol) CTAB (hexadecyltrimethylammonium bromide). Proteinase K was added to a final concentration of 50 μg/ml, and the sample was mixed vigorously; then 400 μl of 20% (wt/vol) sodium dodecyl sulfate was added, and the sample was incubated at 65°C for 2 h with gentle inversion every 15 to 20 min. The sample was purified once with 2 ml of phenol and 2 ml of chloroform-isoamyl alcohol (24:1) and once with 4 ml of chloroform and centrifuged at 12,000 × *g* for 10 min, and the DNA was precipitated from the aqueous phase with 0.6 volume of isopropanol. The precipitated DNA was spooled out, washed with cold 70% ethanol, dissolved in 500 μl of sterile distilled water which contained 10 μg of heat-treated RNase A, and incubated for 2 h at 37°C. The sample was then purified with an equal volume of chloroform. The DNA was precipitated from the aqueous phase with 0.1 volume of 3 M sodium acetate and 2 volumes of 95% ethanol, washed with 70% ethanol, dissolved in an appropriate volume of 1× TE, and stored at 4°C.

PCR. The oligonucleotides used for amplifying 16S rRNA genes were the primer set consisting of fD1 and rP1 as described by Weisburg et al. (23), with modifications of the linker sequences, which contain restriction sites for *Cla*I and *Sal*I or *Bam*HI and *Spe*I. The 5′ and 3′ primers were 5′-CCATCGATGTCGACAGAGTTTGATCCTGGCTCAG-3′ and 5′-GACTAGTGGATCCACGGTTACCTTGTTACGACTT-3′, respectively. The oligonucleotides were synthesized at the Macromolecular Structure, Sequencing and Synthesis Facility at Michigan State University.

All stock solutions used for PCR amplification were prepared and all procedures were performed with the precautions suggested by Kwok and Higuchi (15).

TABLE 1. Sources of the strains used in this study

Strain	Habitat from which the strain was isolated	Geographic origin
Tol-4^T	Petroleum-contaminated freshwater aquifer sediment, 24 to 25 m deep	Northern Michigan
Td-1	Petroleum-contaminated soil	Western Washington
Td-2	Petroleum-contaminated soil	Western Washington
Td-3	Petroleum-contaminated marine sediments	Huntington Beach, Calif.
Td-15	Home compost pile, primarily lawn clippings	Lansing, Michigan
Td-17	Soil from industrial area	Jabaquara, Sao Paulo State, Brazil
Td-19	Soil from a landfill	Cubatao, Sao Paulo State, Brazil
Td-21	Noncontaminated organic (muck) soil	Clinton County, Michigan

Approximately 200 to 300 ng of genomic DNA was added to a standard 100-μl PCR mixture containing 1× *Taq* polymerase buffer (10 mM Tris-Cl, 1.5 mM $MgCl_2$, 50 mM KCl [pH 8.0]), 2.5 U of *Taq* polymerase (Promega, Madison, Wis.), 200 μmol of each deoxynucleoside triphosphate, and 100 pmol of each primer. The reaction conditions were as follows: denaturation at 92°C for 2 min, 30 cycles consisting of 94°C for 15 s, 55°C for 30 s, and 72°C for 2 min, and one additional 6-min cycle for chain elongation. The samples were stored at 4°C. Temperature cycling was performed with a programmable temperature cycler (GeneAmp PCR System 9600; Perkin Elmer Corp., Norwalk, Conn.).

Cloning. Amplified PCR products were precipitated with 0.1 volume of 3 M sodium acetate and 2 volumes of 95% ethanol, washed with 70% ethanol, and dissolved in an appropriate volume of 1× TE. Approximately 1 μg of the amplified PCR products was digested with 20 U of *Bam*HI for 24 h, precipitated with 95% ethanol, and digested with 20 U of *Cla*I for 24 h. The doubly digested DNA was then purified by gel electrophoresis on a 1% low-temperature agarose gel. The gel band corresponding to a 16S rRNA gene fragment was excised, and the DNA was recovered with GELase (Epicentre Technologies, Madison, Wis.) by using the "fast protocol" described in the manufacturer's instructions.

PCR products were cloned by using standard methods (1). Briefly, the purified *Bam*HI-*Cla*I-digested DNA was ligated to pBluescript SK+ (Stratagene, La Jolla, Calif.) by using T4 ligase (Boehringer Mannheim, Indianapolis, Ind.) with a ratio of plasmid vector to inserts of 1:10. The recombinant DNA was transformed into *Escherichia coli* DHα5F′ competent cells by electroporation by using *E. coli* Pulser (Bio-Rad Laboratories, Hercules, Calif.). The positive clones were screened by blue-white selection and were confirmed by the fragment size of *Bam*HI-*Cla*I-digested plasmid DNA.

Determination of nucleotide sequences. The template DNA was isolated from bacteria grown on Terrific broth (which contained [per liter] 12 g of Bacto Tryptone [Difco Laboratories, Detroit, Mich.], 24 g of Bacto Yeast extract [Difco], and 5 g of glycerol, as well as 17 mM KH_2PO_4 and 72 mM K_2HPO_4) by using a Wizard Minipreps DNA purification system (Promega) according to the manufacturer's instructions. DNA sequences were determined by automated fluorescent *Taq* cycle sequencing by using ABI Catalyst 800 and a model 373A sequencer (Applied Biosystems, Foster City, Calif.). The forward sequencing primers used in this study spanned the following *E. coli* 16S rRNA gene positions: 342 to 357, 519 to 533, 787 to 802, and 1099 to 1114. The reverse primers spanned positions 357 to 342, 529 to 515, 802 to 787, and 1115 to 1100. In addition, M13 forward and reverse primers were used to determine the sequences of both ends of the cloned 16S rRNA gene.

Computer analysis. The sequences were assembled by using the gel-assembling programs in the Genetics Computer Group software package (5) and were aligned with the sequences in the Ribosomal Database Project database (16) on the basis of both primary- and secondary-structure information. The resulting alignment was edited for appropriate analyses by using the SUBALIGN and GDE programs from the Ribosomal Database Project.

The evolutionary distances between strains were estimated by the method of Jukes and Cantor (13). The 16S rRNA gene sequences were compared by using the following three methods: unweighted pair group method of analysis (UPGMA), maximum parsimony, and maximum likelihood using the programs in PHYLIP 3.5 (8). For the UPGMA and maximum-parsimony methods we used a bootstrap procedure with 500 and 100 replicates. The SEQBOOT program was used to obtain confidence levels for the UPGMA and maximum parsimony analyses. For the maximum-likelihood analysis, the transition/transversion ratio used was 2.0, and the jumble option was used.

Gas chromatography of cellular fatty acids. Bacterial isolates were precultured on M-R2A medium and streaked onto plates containing 0.3% (wt/vol) tryptic soy broth supplemented with 1.5% (wt/vol) Bacto Agar (Difco). The plates were incubated at 30°C for 72 to 96 h. The cells used for gas chromatographic analysis were collected from at least three plates by scraping them with a sterile loop. Saponification, methylation, and extraction of fatty acids were performed by using a previously described procedure (20). Numerical comparisons of the fatty acid profiles were carried out by using the program for principal-component analysis in SYSTAT 5.0 (SYSTAT, Inc., Evanston, Ill.).

Physiological tests. Inocula for all of the physiological tests were obtained from cells grown aerobically on M-R2A medium at 30°C for 24 to 48 h. Gram staining and tests for catalase and cytochrome *c* oxidase activities and starch hydrolysis were carried out by using standard methods (21) with known strains as positive and negative controls. Other biochemical tests were performed by using a GN MicroPlate (BIOLOG, Hayward, Calif.) and an API NFT kit (bioMerieux, Montalieu Vercieu, France) according to the manufacturers' instructions. The preparations were incubated at 37°C, and results were recorded at 24 and 120 h and at various times between 24 and 120 h.

Additional tests for growth on substrates were performed by using aerobic BS medium (9) adjusted to pH 7 and incubated at 30°C. The inoculum for each test was prepared from cells grown on M-R2A broth, harvested by centrifugation, washed twice with sterile saline, and resuspended in sterile BS medium before they were inoculated into BS medium amended with the test substrate. *Azoarcus indigens* VB32^T (T = type strain) and *Azoarcus* sp. strain S5b2 were also included in the tests for glucose, maltose, and mannose utilization. Growth was measured by determining the increase in optical density at 600 nm, and data were recorded after 1 week of incubation. Strains were also tested for growth in M-R2A broth adjusted to pH 3, 4, 5, 6, 7, 8, and 9 and in M-R2A broth containing 0, 0.5, 1, 2, 5, and 10% NaCl. The temperature range for growth was determined by culturing organisms in standard M-R2A broth (pH 7) at 4, 15, 25, 30, 37, and 45°C. Strains were also tested for growth on nutrient agar, tryptic soy agar (TSA), and semisolid, nitrogen-free, malate-containing medium (19). The growth of each strain on complex media was compared with the growth of the organism on standard M-R2A medium. All tests were done in duplicate.

Nucleotide sequence accession numbers. The GenBank nucleotide sequence accession numbers for our isolates are as follows: *Azoarcus tolulyticus* Tol-4^T, L33694; *A. tolulyticus* Td-1, L33687; *A. tolulyticus* Td-2, L33691; *A. tolulyticus* Td-3, L33693; *A. tolulyticus* Td-15, L33688; *A. tolulyticus* Td-17, L33689; *A. tolulyticus* Td-19, L33690; and *A. tolulyticus* Td-21, L33692.

RESULTS AND DISCUSSION

Sequence data. Using automated fluorescence sequencing, we determined almost the entire 16S rRNA gene sequences (1,457 or 1,458 bp) for the eight new isolates; these sequences corresponded to positions 28 to 1491 on the *E. coli* 16S rRNA gene. Although the isolates were obtained from a variety of environments (Table 1), their 16S rRNA genes showed that they were very closely related, with levels of similarity of 97.9 to 99.9% (Table 2). Three of these isolates, Tol-4^T, Td-1, and Td-2, had sequences that were extremely similar (levels of similarity, 99.7 to 99.9%) but not identical (Table 2). This finding was supported by the results of repetitive extragenic palindromic PCR analyses, which showed that the eight isolates had distinct repetitive extragenic palindromic PCR patterns (9). The G+C contents of the 16S rRNA genes of these isolates ranged from 56.1 to 56.6 mol%.

Phylogenetic analyses. An analysis of the helices between positions 180 and 220 of the *E. coli* 16S rRNA gene sequence (24) indicated that the new isolates belong to the beta or gamma subdivision (data not shown). The signature sequences of these isolates matched the signature sequences of members of the beta subdivision of the *Proteobacteria* (24) (Table 3).

To further study the phylogenetic relationships of our isolates, their 16S rRNA gene sequences were compared with those of members of the beta subclass of the *Proteobacteria* obtained from the Ribosomal Database Project. Our isolates exhibited substantial levels of similarity to members of the novel nitrogen-fixing bacterial genus *Azoarcus* (12). The levels of similarity between our isolates and *Azoarcus* sp. strain S5b2

TABLE 2. Pairwise levels of sequence identity and evolutionary distances between the toluene-denitrifying isolates and members of the beta subclass of the *Proteobacteria*, as well as *E. coli* as an outgroup

Strain or species	% Sequence identity or evolutionary distance (10^2)[a]														
	A. tolulyticus Tol-4^T	*A. tolulyticus* Td-1	*A. tolulyticus* Td-2	*A. tolulyticus* Td-3	*A. tolulyticus* Tol-15	*A. tolulyticus* Td-17	*A. tolulyticus* Td-19	*A. tolulyticus* Td-21	*A. indigens* VB32^T	*Azoarcus* sp. strain BH72	*Azoarcus* sp. strain S5b2	*R. purpureus*	*N. europaea*	*Alcaligenes eutrophus*	*E. coli*
A. tolulyticus Tol-4^T		99.9	99.7	98.3	98.8	98.4	98.1	98.2	95.3	96.1	92.4	89.6	88.3	89.6	82.1
A. tolulyticus Td-1	0.1		99.7	98.3	98.8	98.4	98.1	98.2	95.3	96.2	92.4	89.6	88.3	89.6	82.1
A. tolulyticus Td-2	0.4	0.4		98.3	98.6	98.2	97.9	98.1	95.1	95.6	92.1	89.6	88.2	89.4	81.9
A. tolulyticus Td-3	1.7	1.7	1.7		98.9	98.5	99.0	98.8	95.1	95.8	91.5	89.7	88.6	89.0	81.7
A. tolulyticus Td-15	1.3	1.3	1.5	1.0		99.4	98.9	99.0	95.1	95.8	92.0	89.5	88.4	89.4	81.9
A. tolulyticus Td-17	1.6	1.6	1.8	1.5	0.6		98.4	99.0	94.9	95.7	91.8	89.4	88.2	89.5	81.7
A. tolulyticus Td-19	2.0	2.0	2.1	1.0	1.1	1.6		98.8	94.9	95.3	91.2	89.3	88.2	88.8	81.4
A. tolulyticus Td-21	1.9	1.9	2.0	1.1	1.0	1.0	1.3		94.8	95.3	91.4	89.4	88.1	89.0	81.3
A. indigens VB32^T	4.6	4.6	4.8	4.8	4.9	5.0	5.1	5.2		97.5	93.5	90.9	88.3	89.7	83.0
Azoarcus sp. strain BH72	3.7	3.7	4.0	4.1	4.1	4.3	4.7	4.7	2.6		93.4	90.9	88.9	90.2	82.2
Azoarcus sp. strain S5b2	7.9	7.9	8.2	8.9	8.3	8.5	9.3	9.0	6.8	7.0		92.0	88.5	90.0	82.7
R. purpureus	10.3	10.3	10.4	10.1	10.5	10.5	10.7	10.6	9.6	9.8	8.3		88.2	89.4	81.3
N. europaea	12.4	12.4	12.6	12.0	12.4	12.6	12.6	12.8	12.8	12.2	12.5	13.0		88.7	80.5
Alcaligenes eutrophus	10.5	10.5	10.8	11.1	10.7	10.6	11.5	11.2	10.6	10.2	10.2	11.0	11.8		81.2
E. coli	20.4	20.4	20.7	20.9	20.7	20.9	21.4	21.5	19.3	20.6	20.0	21.2	22.4	21.4	

[a] The values on the upper right are levels of sequence identity, and the values on the lower left are evolutionary distances.

were 91.2 to 92.4%, the levels of similarity between our isolates and *Azoarcus* sp. strain BH72 were 95.3 to 96.2%, and the levels of similarity between our isolates and *A. indigens* VB32^T were 94.8 to 95.3% (Table 2). More distantly related were the photosynthetic bacterium *Rhodocyclus purpureus*, the ammonia-oxidizing bacterium *Nitrosomonas europaea*, and *Alcaligenes eutrophus* (Table 2). The nearest known phylogenetic relative other than a member of the genus *Azoarcus* appeared to be the newly described selenate-respiring species *Thauera selenatis* (17), whose 16S rRNA sequence was 93% similar to the 16S rRNA sequences of our strains. While *T. selenatis* does denitrify, it does not fix nitrogen.

In 100% of the UPGMA analyses, all eight toluene-degrading denitrifying isolates appeared in one cluster, which formed a sister lineage of the previously described *Azoarcus* species. Tol-4^T, Td-1, and Td-2 formed a cluster that was separate from the other five isolates. The branches leading to Td-3, Td-15, Td-17, Td-19, and Td-21 had lower confidence levels, ranging from 56 to 82% (data not shown).

Our parsimony analysis yielded 12 equally most-parsimonious trees that differed only in the relative positions of Td-3, Td-15, Td-17, Td-19, and Td-21 and in the placement of *N. europaea* and *Alcaligenes eutrophus*. We also analyzed the data by using 100 replicates. The tree obtained (Fig. 1) provided confidence intervals for the nodes. The results shown in Fig. 1 are consistent with those based on our previous partial sequence analyses (4, 9).

The maximum-likelihood analysis yielded a topology (Fig. 2) that differed from the topology of the parsimony tree only in the position of *N. europaea* relative to *Alcaligenes eutrophus* and in the position of *Azoarcus* sp. strain BH72 relative to *A. indigens* VB32^T. The maximum-likelihood dendrogram was

TABLE 3. Results of a signature sequence analysis performed with the eight new toluene-degrading denitrifying isolates and other taxa[a]

Position[b]	Signature base[c]			
	Consensus	*A. tolulyticus*	Beta *Proteobacteria*	Other *Proteobacteria*
50	A	U	U	A
108	G/C	A	A	G/C
640	A	U	U/G	A
690	G	A	A	G
722	G	A	A	G
812	G	C	C	G
871	U	G	G	U
929	G	A	A	G
947	G	U	U	G
976	G	A	A/g	G
1234	C	A	A	C

[a] Signature sequences were obtained from reference 24.
[b] Standard *E. coli* 16S rRNA numbering.
[c] A capital letter indicates a major base which accounts for >90% of assayable cases. A lowercase letter indicates a minor base which is found in <15% of the assayable cases or in only one sequence in the group (24).

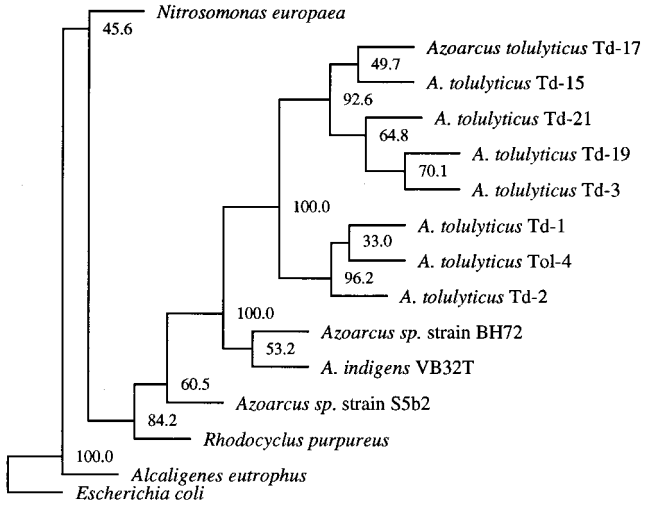

FIG. 1. Bootstrap parsimony tree obtained by using DNAPAR and SEQBOOT with different *A. tolulyticus* strains and related species. The percentages indicate the bootstrap confidence estimates on the branches obtained with 100 replicates.

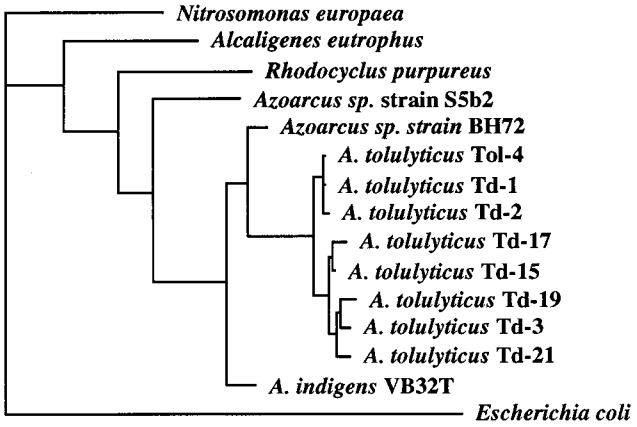

FIG. 2. Maximum-likelihood topology for different *A. tolulyticus* strains and related bacteria as determined by the jumble option based on 10 orderings of the data (log likelihood, −5971). The relative distances between nodes and tips and between internodes are shown. All branch lengths are significant at the $P = 0.01$ level.

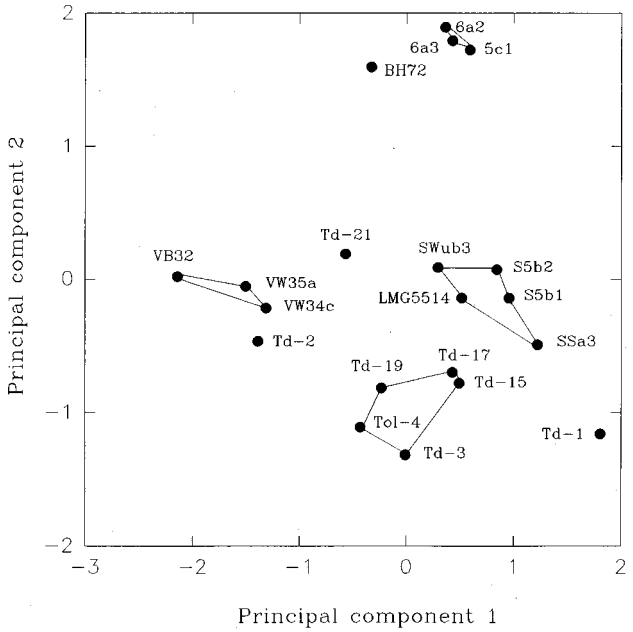

FIG. 3. Two-dimensional plot obtained from a principal-component analysis of the cellular fatty acid profiles of the eight toluene-degrading denitrifiers, as well as 12 other *Azoarcus* strains. The previously described strains of the genus *Azoarcus* formed three clusters, as recognized by Reinhold-Hurek et al. (19). Most of the new isolates appeared to belong to a new cluster.

also very similar to the UPGMA dendrogram. Use of the jumble option yielded several different solutions with similar likelihood scores. These solutions always supported the following conclusions: (i) the eight isolates formed a phylogenetically coherent unit that was a sister lineage of *Azoarcus* species, which was also a sister lineage of *R. purpureus*; and (ii) the new isolates were more closely related to *Azoarcus* sp. strain BH72 and *A. indigens* than to *Azoarcus* sp. strain S5b2.

The results of all three methods of analysis suggested that the eight toluene-degrading denitrifying isolates which we studied were (i) phylogenetically very closely related to each other and (ii) members of the genus *Azoarcus.*

Cellular fatty acid methyl ester patterns. The major fatty acids of all of the new isolates were 16:0, *cis*-7 16:1, 3-OH 10:0, 12:0, and 18:1 fatty acids (Table 4), but we observed a number of variations in the minor fatty acids present in the isolates. For example, Td-15 lacked 15:0, cyclo 17:0, and 18:0 fatty acids, and Td-17 did not contain 10:0, 15:0, and 18:0 fatty acids. Td-2, Td-3, and Td-21 contained small amounts of 19:0, 3-OH 17:0, and 19:1 fatty acids, respectively. The absence or presence of small amounts of some fatty acids could be regarded as characteristics of different isolates. None of the fatty acid patterns of our isolates matched the fatty acid patterns in the MIDI database. There were no fatty acid profiles of *Azoarcus* sp. strains in the MIDI database.

The fatty acid profiles of the eight isolates which we studied were very similar to those of previously described members of the genus *Azoarcus* (19). 16:0 and *cis*-7 16:1 fatty acids were predominant in both the previously described and new isolates. In contrast to the previously described members of the genus *Azoarcus*, however, all eight new isolates contained a considerable amount of 12:0 fatty acid but lacked 3-OH 8:0 fatty acid. A numerical comparison of the profiles of the 16:0, *cis*-7 16:1, and 18:1 fatty acids of the eight new isolates and the previously described members of the genus *Azoarcus* revealed four clusters (Fig. 3). While the previously described members of the genus *Azoarcus* were in three clusters (19), five of our eight isolates (all of the isolates except Td-1, Td-2, and Td-21) ap-

TABLE 4. Cellular fatty acid compositions of the eight toluene-degrading denitrifying isolates

Strain	% of total fatty acids												
	10:0[a]	3-OH 10:0	12:0	14:0	15:0	*cis*-7 16:1	16:0	cyclo 17:0	18:1[b]	18:0	19:0	3-OH 17:0	19:1
A. tolulyticus Tol-4^T	0.53	7.74	9.49	1.54	0.49	44.67	30.95	1.21	2.63	0.50	ND[c]	ND	ND
A. tolulyticus Td-1	0.42	1.72	6.75	1.30	0.73	61.89	21.54	2.21	3.45	ND	ND	ND	ND
A. tolulyticus Td-2	0.64	5.52	12.8	0.90	0.61	44.01	23.41	4.11	7.02	ND	1.00	ND	ND
A. tolulyticus Td-3	0.56	4.91	12.8	2.13	0.51	50.29	25.11	0.80	2.22	ND	ND	0.80	ND
A. tolulyticus Td-15	0.47	6.24	7.28	0.95	ND	49.69	30.62	ND	4.42	ND	ND	ND	ND
A. tolulyticus Td-17	ND	4.91	4.89	1.55	ND	44.7	38.61	1.45	3.90	ND	ND	ND	ND
A. tolulyticus Td-19	0.38	5.93	7.93	1.23	0.86	42.79	35.95	0.90	3.60	0.44	ND	ND	ND
A. tolulyticus Td-21	0.36	4.02	4.34	1.18	0.87	42.33	33.59	1.19	9.31	0.28	ND	ND	0.45

[a] Number of carbon atoms:number of unsaturated carbon bonds. *cis* refers to the configuration of the double bond, and the position is indicated relative to the aliphatic ends of the molecule; cyclo and OH indicate cyclopropane and hydroxyl substitutions, respectively.
[b] Mixture of 7c/9ω/12ω, 9c/12ω/7c, and 12ω/9ω/7c.
[c] ND, not detected.

TABLE 5. Characteristics of the eight toluene-degrading denitrifying isolates

Test	Strain							
	Tol-4^T	Td-1	Td-2	Td-3	Td-15	Td-17	Td-19	Td-21
Gram stain	−[a]	−	−	−	−	−	−	−
Catalase	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+
Nitrogen fixation	+	+	+	+	+	+	+	+
Denitrification[b]	+	+	+	+	+	+	+	+
Growth on nutrient agar	+	+	+	+	+	+	+	+
Growth on TSA	+w	+w	+w	+w	+w	+w	+w	+w
Growth on SM[c]	+w	+w	+w	+w	+w	+w	+w	+w
Growth at pH 5[d]	−	−	−	−	−	−	−	+
Growth in the presence of 2% NaCl[d]	−	+	+	+	+	+	+	+
Growth at 15°C[d]	+	+	+	+	+	+	+	+w
Growth at 45°C[d]	−	+	+	+	+	+	−	−
Substrate utilization tests[e]								
Glucose	+	+	+	+	+w	+w	+	+
Maltose	+	+	+	+	+	+w	+	+w
Mannose	−	−	−	−	−	−	−	−
Malate	+	+	+	+	+	−	+	−
Ethanol	+	+	+w	+	+	+	+	+w
Acetate	+	+	+	+	+	−	+	−
Succinate	+	+	+	+	+	+w	+	−
Lactate	+	+	+	+w	+	+	+	−
Pyruvate	+w	+w	−	−	+w	−	+	−
Benzoate	+	+	+	+	+	+	+	+w
API NFT tests[f]								
NO_2^-	−	−	+v	−	−	−	−	−
N_2	+v	+	+	+	−	+	+	+
Glucose	+	+v	−[g]	+v	+	−[h]	+	−
Maltose	+	+	+	+	+	−	+	−[i]
Adipate	+	+	+	−	−	−	−	−[i]
Malate	+	+	+	+	+	+	+	+
Phenylacetate	−	−	−	−	−	+	−	−

[a] −, negative; +, positive; +w, weak growth; +v, variable (one of the duplicate tests was positive).
[b] Denitrification was determined by examining gas production by standard methods (4, 9).
[c] SM, semisolid, nitrogen-free, malate-containing medium.
[d] Strains were tested in M-R2A broth adjusted to the appropriate pH or NaCl concentration. Standard M-R2A medium was used to test for growth at different temperatures. All strains grew at pH 6 to 9, in the presence of 0, 0.5, and 1% NaCl, and at 25, 30, and 37°C. None of the strains grew at pH 3 or 4, in the presence of 5 or 10% NaCl, or at 4°C.
[e] Aerobic growth on different substrates in BS medium after 1 week of incubation at 30°C. Glucose, maltose, mannose, malate, and benzoate were tested at a concentration of 1 mM; ethanol, succinate, and lactate were tested at a concentration of 3 mM; and acetate and pyruvate were tested at a concentration of 5 mM.
[f] Data were recorded after 48 h of incubation. Duplicate tests were performed. NO_2^- and N_2 were the products tested for in the nitrate reduction assay. None of the isolates grew on L-arabinose, D-mannose, D-mannitol, *N*-acetyl-D-glucosamine, D-gluconate, caprate, or citrate after 48 h of incubation. Td-21 was positive for utilization of mannitol and gluconate after 120 h of incubation. No strain fermented glucose. No tryptophanase, arginine dihydrolase, urease, esculin hydrolysis, gelatinase, and β-galactosidase activities were observed after incubation for 48 h. Strains Tol-4^T, Td-1, and Td-19 were positive for esculin hydrolysis after incubation for 72 h. Strains Td-2, Td-3, Td-17, and Td-21 were positive for esculin hydrolysis after incubation for 120 h. Strains Td-17 and Td-21 were positive for gelatinase activity after incubation for 72 and 96 h, respectively.
[g] Positive after incubation for 72 h.
[h] Positive after incubation for 96 h.
[i] Positive after incubation for 120 h.

peared to form a new cluster that was separated from the previously described members of the genus *Azoarcus.*

Physiological and biochemical analysis. All of the new isolates are gram negative, cytochrome *c* oxidase positive, and catalase positive (Table 5). We previously showed that all of these isolates are capable of fixing nitrogen by growing on N_2 (9). Most of the isolates exhibited esculin hydrolysis activity after 48 h (Table 5). No β-galactosidase, tryptophanase, urease, and arginine dihydrolase activities were observed. All of the isolates except Td-15 produced N_2 gas in the NO_3^- reduction assay when the reagents supplied by an API NFT kit were used (Table 5). Td-15, as well as the other new strains, did produce N_2 gas from nitrate when a more specific and sensitive assay was used to test for denitrification (4, 9). The difference in N_2 production obtained in the two tests for denitrification with Td-15 was probably due to differences in the compositions of the test media.

All of the isolates grew well on M-R2A medium and nutrient agar and poorly on TSA and semisolid, nitrogen-free, malate-containing medium compared with growth on M-R2A medium (Table 5). All of the strains grew at pH 6 to 9, in the presence of 0, 0.5, and 1% NaCl, and at 25, 30, and 37°C. Some of the strains grew under conditions outside the ranges described above, but none grew at a pH of 4 or less or in the presence of an NaCl concentration of 5% or more. Although the results obtained for growth on glucose and maltose differed depending on the substrate assay used (Table 5), all or most of the isolates grew on glucose, maltose, malate, ethanol, acetate, succinate, and benzoate. The API NFT data obtained after the recommended 48 h of incubation are reported below, but pos-

itive results for growth were obtained with several substrates after longer incubation periods. No starch hydrolysis or glucose fermentation was detected. The substrate oxidation profiles obtained by using BIOLOG GN MicroPlates were negative for each isolate after 24 h of incubation, and only after 120 h did some of the isolates show a positive or weak response to some carbon substrates (data not shown).

The substrate use patterns, particularly the substrate use patterns for organic acids, obtained with our isolates were similar to the patterns obtained with the previously described members of the genus *Azoarcus* (19). The new isolates, however, grew on glucose and maltose, while the previously described members of the genus *Azoarcus* reportedly do not grow on carbohydrates (19). Indeed, when *A. indigens* VB32^T and *Azoarcus* sp. strain S5b2 were assayed under our conditions, they did not grow on glucose, maltose, or mannose, while our strains grew on glucose and maltose. On the basis of the results of API NFT tests, the substrate range for the new isolates is limited, although this limitation may be due to the conditions under which they were tested. Also, in contrast to the previously described *Azoarcus* strains, the eight new isolates grew poorly on TSA. M-R2A medium and nutrient agar are solid media that support good growth of the new *Azoarcus* isolates. In addition, two of the previously described isolates (*A. indigens* VB32^T and *Azoarcus* sp. strain S5b2) did not degrade toluene under denitrifying conditions (unpublished data).

The morphology of isolates Td-1, Td-2, Td-3, Td-15, Td-17, Td-19, and Td-21 was described by Fries et al. (9), and the morphology of strain Tol-4^T was similar (4). The cells are short, motile rods (length, 1.4 to 2.1 μm) when the organisms are grown anaerobically on toluene; the cells are longer motile rods (length, 2.1 to 2.8 μm) or occur in long chains when the organisms are grown on M-R2A agar. Growth on M-R2A medium resulted in uniform, translucent yellowish colonies that were approximately 2 to 3 mm in diameter and had dark centers. The cellular morphology of the new isolates is similar to the cellular morphology of the previously described *Azoarcus* species (19). Additional details concerning characterization of type strain Tol-4 and its anaerobic metabolism of toluene have been determined by Chee-Sanford et al. (4).

The ability to fix nitrogen, the fatty acid methyl ester profiles, and the physiological, biochemical, and morphological features strongly support the conclusion based on the results of 16S rRNA sequence analyses that the new isolates are members of the nitrogen-fixing bacterial genus *Azoarcus*. However, the toluene-degrading ability of these organisms under denitrifying conditions, their nonrhizosphere niche, several physiological, biochemical, and nutritional differences, and differences of 3 to 8% between their 16S rRNA sequences and the 16S rRNA sequences of other *Azoarcus* species suggest that the new isolates are members of a new species in the genus *Azoarcus*. Hence, we formally propose that these toluene-degrading denitrifiers should be placed in a new species of the genus *Azoarcus*. Since toluene-degrading ability under denitrifying conditions is the key feature that differentiates these isolates from the previously described members of the genus *Azoarcus*, we propose the name *Azoarcus tolulyticus* for them.

Description of *Azoarcus tolulyticus* sp. nov. *Azoarcus tolulyticus* (to.lu.ly′ti.cus. N. L. n. Fr. Sp. *tolu*, balsam from Santiago de Tolu, toluene; Gr. adj. *lyticus*, dissolving; N. L. masc. adj. *tolulyticus*, toluene dissolving). Cells are gram-negative, short, motile rods (length, 1.4 to 2.1 μm) when the organisms are grown anaerobically on toluene; cells are longer motile rods (length, 2.1 to 2.8 μm) or occur in long chains when the organisms are grown on M-R2A agar. Growth on M-R2A medium results in uniform translucent yellowish colonies that are approximately 2 to 3 mm in diameter and have dark centers. Oxidase and catalase positive. Cells lack tryptophanase, β-galactosidase, urease, and arginine dihydrolase activities. Nitrate and oxygen can serve as terminal electron acceptors. Capable of growth on nitrogen-free media under a nitrogen (N_2) atmosphere. Grows well on M-R2A medium, nutrient agar, a few aromatic substrates, including toluene, and organic acids such as malate. Grows poorly on TSA. Limited substrate range. All strains grow at pH 6 to 9, in the presence of up to 1% NaCl, and at 25 to 37°C. All strains have *cis*-7 16:1 and 16:0 fatty acids as their predominant cellular fatty acids. This organism belongs to the beta subclass of the *Proteobacteria* as determined by 16S rRNA gene sequence analyses. In contrast to the previously described species of the genus *Azoarcus*, the strains are able to degrade toluene under denitrifying conditions, grow on glucose and maltose, contain a considerable amount of 12:0 fatty acid, and lack 3-OH 8:0 fatty acid.

Strain Tol-4, the type strain, has been deposited in The American Type Culture Collection as strain ATCC 51758. This strain was isolated from a petroleum-contaminated core taken from the Bear Lake aquifer in Northern Michigan.

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