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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*

HANS-JOACHIM ANDERS,[1] ANETTE KAETZKE,[1] PETER KÄMPFER,[2]† WOLFGANG LUDWIG,[3] AND GEORG FUCHS[1,4]*

Abteilung Angewandte Mikrobiologie und Mykologie, Universität Ulm, Ulm,[1] Fachgebiet Hygiene, Technische Universität Berlin, Berlin,[2] Lehrstuhl für Mikrobiologie, Technische Universität München, Munich,[3] and Lehrstuhl für Mikrobiologie, Institut für Biologie II, Universität Freiburg, Freiburg,[4] Germany

In the past workers have isolated several pseudomonad strains which have been used for studies of anaerobic aromatic metabolism. The best studied of these strains are strains KB 740^T (T = type strain) and K 172^T. The taxonomic positions of these two organisms were determined by classical methods, including experiments to determine substrate spectrum, quinone type, and total fatty acid composition. Our results clearly excluded these strains from the authentic genus *Pseudomonas*, which belongs to the gamma subclass of the *Proteobacteria*. Instead, the properties of these organisms indicated that they belong to the beta subclass of the *Proteobacteria*. The sequences of the 16S ribosomal DNA genes confirmed this conclusion and indicated that strain K 172^T represents a new species of the genus *Thauera*, *Thauera aromatica*, and that strain KB 740^T represents a new species of the genus *Azoarcus*, *Azoarcus evansii*.

The anaerobic metabolism of aromatic compounds is one of the major unsolved problems of bacterial metabolism. This process has been investigated mainly with pure cultures of known phototrophic bacteria (15) and with denitrifying bacteria (14) whose systematic position is uncertain. These organisms have often been referred to as pseudomonad strains which have some properties in common with members of the genus *Pseudomonas*; i.e., they have gram-negative, aerobic, rod-shaped cells with polar flagella. However, only the members of the *Pseudomonas fluorescens* branch, in the gamma subclass of the *Proteobacteria*, represent the authentic pseudomonads (46).

A number of different denitrifying bacteria have been isolated under denitrifying conditions by using aromatic substrates such as 2-aminobenzoate, phenol, and benzoate as sole carbon and energy sources (4, 43). A rapid genotyping method in which low-molecular-weight RNA profiles were used (17) indicated that these strains are not *Pseudomonas* strains belonging to rRNA group I (31) and that some of the strains might belong to the same genospecies. For example, strains KB 740^T (4) (T = type strain), KB 650 (4), and B4P (40) could form one genospecies, whereas strains K 172^T, S 100 (43), and S 2 (40) might form another, separate genospecies. This separation is also supported by physiological properties, such as utilization of certain aromatic substrates under denitrifying conditions. For example, the strains of the second genospecies (K 172^T, S 100, S 2) could use phenol (43) or toluene (1, 39) as sole organic substrates under denitrifying conditions, whereas the members of the first genospecies (KB 740^T, KB 650, B4P) could not.

* Corresponding author. Mailing address: Angewandte Mikrobiologie, Universität Ulm, D-89069 Ulm, Germany. Phone: 0731/5022710. Fax: 0731/5022719.

† Present address: Institut für Hygenie und Umweltmedizin, RWTH Aachen, Aachen, Germany.

In this study, a polyphasic strategy was used to classify strains KB 740^T and K 172^T, which are the most extensively studied members of these two groups. Phenotypic characteristics, such as fatty acid composition and quinone type, suggested that these strains belong to the beta subclass of the *Proteobacteria*. The exact phylogenetic positions of these organisms were further analyzed by determining 16S ribosomal DNA (rDNA) sequences.

MATERIALS AND METHODS

Materials. Chemicals and biochemicals were obtained from Merck (Darmstadt, Germany), Fluka (Neu-Ulm, Germany), Sigma (Heidelberg, Germany), Roth (Karlsruhe, Germany), Difco (Hamburg, Germany), Boehringer (Mannheim, Germany), United States Biochemicals (Braunschweig, Germany), and Pharmacia (Freiburg, Germany). Gases were obtained from Linde (Höllriegelskreuth, Germany). Pseudomonad strain KB 740^T (= DSM 6898^T) was a kind gift from Konstantin Braun (4), and pseudomonad strain K 172^T (= DSM 6984^T) was isolated in our laboratory (43). *Escherichia coli* JM 109 (= DSM 3423) was obtained from the Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany). Plasmid vector pBluescript SK+/−, which was obtained from Stratagene (Heidelberg, Germany), was used as a cloning vector.

Growth of bacteria. Strain KB 740^T was cultivated anaerobically at 28°C with 5 mM benzoate and 20 mM nitrate as the sole sources of energy and cell carbon (4). Strain K 172^T was grown with 1 mM phenol and 4 mM nitrate as described elsewhere (43). Growth determination, cell harvesting, and storage at −70°C were performed as described previously (10). *E. coli* JM 109 cultures were grown in Luria-Bertani broth. *E. coli* JM 109 cells containing a plasmid were cultivated in Luria-Bertani broth supplemented with 100 μg of ampicillin per ml or on 1.5% agar plates supplemented with 100 μg of ampicillin per ml, 0.4 mM isopropyl-thio-β-D-galactoside, and 0.1 mM 5-bromo-4-chloro-3-indolyl-β-D-galactoside (2).

Phenotypic characterization. Morphological and biochemical characteristics and carbon substrate assimilation test results were determined as described elsewhere (4, 20, 43).

Chemotaxonomic characterization. Cellular fatty acids were extracted from cultures that had been cultivated aerobically at 25°C for 48 h in R2A medium. The fatty acids were methylated with methanolic HCl, and the fatty acid methyl esters obtained were analyzed by gas-liquid chromatography, using a Shimadzu model GC 9A chromatography apparatus equipped with a fused-silica capillary column (50 m by 0.32 μm), as described elsewhere (19). Ubiquinones were extracted and analyzed exactly as described by Collins (9).

Isolation of genomic DNA. A culture (1 liter) (A_{578}, 0.7) was harvested by

centrifugation at 10,000 × *g* and 4°C for 10 min. The cells were resuspended in 9.5 ml of 10 mM Tris-HCl (pH 8.0)–1 mM EDTA. The cells were lysed by adding 0.5 ml of 10% (wt/vol) sodium dodecyl sulfate and 50 μl of a proteinase K solution (20 mg/ml) and then incubating the preparation at 37°C for 1 h. After lysis, 1.8 ml of 5 M sodium chloride and 1.5 ml of 10% (wt/vol) hexadecyltrimethylammonium bromide in 0.7 M sodium chloride were added, and the crude extract was incubated at 65°C for 20 min. The hexadecyltrimethylammonium bromide-sodium chloride solution removed exopolysaccharides and proteins (2). The crude extract was purified with an equal volume of chloroform-isoamyl alcohol (24:1, vol/vol), and the nucleic acids were precipitated with 0.6 volume of ice-cold isopropyl alcohol. The pellet was washed with 70% ethanol, dried, and redissolved in 10 mM Tris-HCl (pH 8.0)–1 mM EDTA. This nucleic acid solution contained considerable amounts of RNA. The RNA was removed by treatment with an RNase A solution (20 ng/ml) for 1 h at 37°C. The DNA concentration was determined spectrophotometrically.

PCR amplification, cloning, and sequencing of the 16S rRNA genes. A 100-ng portion of bacterial DNA was used in each reaction mixture. Each reaction mixture (total volume, 100 μl) also contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM $MgCl_2$, 2.5 mM dATP, 2.5 mM dCTP, 2.5 mM dGTP, 2.5 mM dTTP, and amplification primers, each at a concentration of 0.3 μM. The reaction mixture was overlaid with 100 μl of mineral oil (25, 28, 36).

First, the chromosomal DNA was denatured at 94°C for 5 min. The samples were then cooled to 80°C, and 2.5 U of *Taq* DNA polymerase (Ampli Taq; Perkin-Elmer Cetus, Überlingen, Germany) was added. Each of the 30 amplification cycles included denaturation at 94°C for 1 min, primer annealing at temperatures between 48 and 64°C for 1.3 min, and extension at 72°C for 2 min; this was followed by a final extension step at 72°C for 10 min. The 16S rDNA gene was amplified between positions 28 and 1528 (*E. coli* numbering system [5]) with the following primer combination: forward primer 5′-ATA TGC GGC CGC AGA GTT TGA TYM GGC TCA G-3′ and reverse primer 5′-ATA TGC GGC CGC AGA AAG GAG GTG ATC C-3′ (Y = C or T; M = A or C). To purify the PCR products, the mineral oil was extracted with chloroform. The bacterial genomic DNA, excess primer, and nucleotides were separated from the aqueous phase with "Magic prep" spin columns (Promega/Serva, Heidelberg, Germany) according to the instructions of the distributor (26). The amplified and purified DNA fragments were cloned in the vector pBluescript (Stratagene). The resulting recombinant plasmids, K1 (strain K 172^T) and KB1 (strain KB 740^T), were transformed and amplified in *E. coli* JM 109. The amplified plasmid DNA was isolated with a Qiagen plasmid kit (Diagen, Hilden, Germany) according to the instructions of the distributor.

The recombinant plasmids were sequenced by the dideoxy chain termination method (37), and the purified PCR products were sequenced directly (8). We used universal or reverse primers that bound to vector sequences or synthetic oligonucleotides complementary to specific regions of the 16S rDNA gene. Sequencing was carried out by using the instructions of the manufacturer and a Sequenase 2.0 sequencing kit (United States Biochemicals). The termination reaction mixtures were separated on a 6% polyacrylamide gel with 8 M urea by using 89 mM Tris-borate–89 mM boric acid–2 mM EDTA (24). After drying, the gels were analyzed by autoradiography overnight at room temperature with Kodak X-Omat film (Sigma, Deisenhofen, Germany).

Analysis of data. The new 16S rDNA sequences were added to an alignment of about 1,800 homologous primary structures from bacteria (21, 29) by using the alignment tool of the ARB program package (42). Phylogenetic analyses were performed by using distance matrix maximum-parsimony and maximum-likelihood methods and the corresponding tools of the ARB (42) and PHYLIP (12) program packages, as well as the fastDNAml program (21). The compositions of the data sets varied with respect to the reference sequences as well as the alignment positions included. Variability at individual positions was determined by using the tools in the ARB package; the resulting data were used as criteria to remove or include variable positions. Data sets comprising all of the available, almost complete 16S rRNA sequences of members of the beta subclass of the *Proteobacteria* and selected representatives of other major bacterial lines of descent were analyzed by using distance matrix and maximum-parsimony methods. Subsets that included the corresponding sequences of the closest relatives of strains KB 740^T and K 172^T, as well as selected representatives of the major phylogenetic groups of the beta subclass of the *Proteobacteria*, were used for maximum-likelihood analyses.

Nucleotide sequence accession numbers. The rDNA sequences of strains K 172^T and KB 740^T have been deposited in the EMBL database under accession numbers X77118 and X77679, respectively. "*Azoarcus denitrificans*" sequences have been deposited by Zhou et al. in the EMBL data library. The accession numbers of these sequences and other sequences used to determine levels of 16S rRNA similarity are as follows: *Azoarcus evansii*, X77679; "*A. denitrificans*" Td-1, L33687; "*A. denitrificans*" Td-2, L33691; "*A. denitrificans*" Td-3, L33693; "*A. denitrificans*" Tol-4, L33694; "*A. denitrificans*" Td-15, L33688; "*A. denitrificans*" Td-17, L33689; "*A. denitrificans*" Td-19, L33690; "*A. denitrificans*" Td-21, L33692; "*Azoarcus*" sp. strain BH72, L15530; *Azoarcus indigens*, L15531; *Thauera aromatica*, X77118; *Thauera selenatis*, X68491; *Zoogloea ramigera* ATCC 19544^T, X74913; *Rhodocyclus purpureus*, M34132; *Rhodocyclus tenuis*, D16208; strain ToN1, X83534; strain EbN1, X83531; strain PbN1, X83532; and strain mXyN1, X83533.

RESULTS AND DISCUSSION

Well-studied pseudomonad strains K 172^T and KB 740^T were preliminarily placed in the genus *Pseudomonas* on the basis of some common physiological and morphological properties. These organisms were gram negative and oxidase positive and were not able to ferment sugars. A large variety of organic acids and aromatic compounds and some amino acids could be oxidized both aerobically and with nitrate as an electron acceptor. Under aerobic conditions strain K 172^T used benzoate, 2-aminobenzoate, 3-hydroxybenzoate, 4-hydroxybenzoate, 4-hydroxyphenylacetate, phenylacetate, gentisate, phenylalanine (slow growth), butyrate, isobutyrate, propionate, isovalerate, adipate, glutarate, acetate, glycerophosphate, glycolate, D-lactate, L-lactate, DL-lactate, L-malate, oxaloacetate, L-asparagine, L-glutamate, L-isoleucine, L-leucine, L-serine, and β-alanine as sole carbon sources, but did not use 4-aminobenzoate, vanillate, fructose, 2-oxoglutarate, D-tartrate, *m*-tartrate, L-tartrate, L-aspartate, L-lysine, L-ornithine, L-phenylalanine, L-proline, or L-valine; under anaerobic conditions this strain used benzoate, toluene, phenol, phenylacetate, phenylglyoxylate, 2-hydroxybenzoate, 3-hydroxybenzoate, 4-hydroxybenzoate, protocatechuate, 2-aminobenzoate (slow growth), 4-hydroxyphenylacetate, phenylalanine (poor or uncertain growth), *p*-cresol, benzaldehyde, benzyl alcohol, indole, indolylacetate, glutarate, succinate, fumarate, L-malate, acetate, and ethanol but did not use *o*-cresol, *m*-cresol, 2-fluorobenzoate, *o*-phthalate, adipate, pimelate, cyclohexanecarboxylate, D-glucose, D-fructose, D-maltose, D-ribose, or D-lactose (1, 43). Under aerobic conditions strain KB 740^T used benzoate, 2-aminobenzoate, 4-aminobenzoate, 3-hydroxybenzoate, 4-hydroxyphenylacetate, phenylacetate, vanillate, gentisate, phenylalanine, fructose, butyrate, isobutyrate, propionate, isovalerate, glutarate, acetate, glycolate, D-lactate, L-lactate, DL-lactate, L-malate, oxaloacetate, 2-oxoglutarate, D-tartrate, *m*-tartrate, L-tartrate, L-asparagine, L-aspartate, L-glutamate, L-isoleucine, L-leucine, L-lysine, L-ornithine, L-phenylalanine, L-serine, L-proline, L-valine, and β-alanine as sole carbon sources but did not use 4-hydroxybenzoate, adipate, or glycerophosphate; under anaerobic conditions this strain used benzoate, phenylacetate, phenylglyoxylate, 3-hydroxybenzoate, 4-hydroxybenzoate, 2-aminobenzoate, 4-hydroxyphenylacetate, phenylalanine, *p*-cresol, 2-fluorobenzoate, benzaldehyde, benzyl alcohol, indolylacetate, *o*-phthalate, adipate, pimelate, glutarate (slow growth), cyclohexanecarboxylate, succinate, fumarate, L-malate, acetate, acetone, D-glucose (slow growth), D-fructose, and D-maltose but did not use toluene, phenol, 2-hydroxybenzoate, protocatechuate, *o*-cresol, *m*-cresol, indole, ethanol, D-ribose, or D-lactose (4, 27, 38). Growth with some of these compounds was very slow. A comparison of the reaction profile with previously published data did not allow unambiguous identification, although most of the other properties (see below) indicated that these strains were more closely related to the genera *Acidovorax*, *Comamonas*, *Variovorax*, and *Hydrogenophaga*, than to the genus *Pseudomonas*. These genera have been recognized as members of a new bacterial family, the *Comamonadaceae*, in rRNA superfamily III (in the beta subclass of the *Proteobacteria*); members of this family reportedly are closely related genotypically and rather diverse phenotypically (44). Accumulation of poly-β-hydroxybutyrate (e.g., by strain K 172^T) is also a characteristic that distinguishes the β-subgroup species from authentic *Pseudomonas* species, which do not accumulate this compound.

Fatty acid patterns and quinone type. The fatty acid profiles of strains K 172^T and KB 740^T were very similar (Table 1). In addition to the two main fatty acids, *cis*-9-hexadecenoate (16:1)

TABLE 1. Characteristics of pseudomonad strains K 172^T and KB 740^T, *T. selenatis*, *A. indigens*, *Z. ramigera* ATCC 19544^T, and *P. aeruginosa* ATCC 10145

Characteristic	Strain K 172Ta	Strain KB 740^{Tb}	*T. selenatis*[c]	*A. indigens*[d]	*Z. ramigera* ATCC 19544Te	*P. aeruginosa* ATCC 10145^{Tf}
Cell morphology	Coccoid rods	Rods	Rods	Rods	Rods	Rods
Cell size (μm)	1.0-2.5 × 0.5-1.5	1.5-3.0 × 0.6-0.8	0.56 × 1.4	0.5-0.7 × 2.0-4.0	1.0-1.3 × 2.1-3.6	1.5-4.0 × 0.5-1.0
Motility	(+)	+	+	+	+	+
Flagella	ND[g]	Single, subpolar	Single, polar	Single, polar	Single, polar	Single, polar
Catalase reaction	+	+	+	+	(+) (weak)	+
Oxidase reaction	+	+	+	+	+	+
Denitrification	+ (mainly N_2O)	+ (mainly N_2O)	+ (to N_2O)	–	+ (to N_2)	+ (to N_2 or N_2O)
Selenate respiration	–	–	+	ND	ND	ND
Oxidative metabolism	+	+	+	+	+	+
G+C content (mol%)	67 ± 1	67.5 ± 1	66	66.6	65.3	67
Optimum pH for growth	7.0-7.4	7.8	8.0 with nitrate, 7.0 with selenate	7.0-7.5	7.0-7.5	7.2-7.4
Optimum temp for growth (°C)	28	28-37	25-30	40	28-37	37
Growth factors required	–	–	+	+	–	ND
Quinone(s)	ND	Ubiquinone 8	Ubiquinone 8	ND	Ubiquinone 8, rhodoquinone 8	Ubiquinone 9
Fatty acids in lipids[h]						
3-Hydroxydecanoate (3-OH 10:0)	4.9	5.2	ND	5	89[i]	2.6
Dodecanoate (12:0)	8.5	8.6	ND	6.5	6[j]	3.4
cis-9-Hexadecenoate (16:1)	53.4	54.9	ND	33.5	68[j]	6
Hexadecanoate (16:0)	25.9	24.9	ND	34.6	15[j]	28
cis-9,11-Octadecenoate (18:1)	3.7	4.5	ND	8.3	6[j]	35.6
Hydrolysis of:						
Gelatin	ND	ND	–	–	+	+
Starch	ND	ND	–	–	–	–
Urea	ND	+ (growth)	ND	–	+	ND
Poly-β-hydroxy-butyrate	+	ND	+	ND	+	–

[a] Some data were obtained from reference 43.
[b] Some data were obtained from reference 4.
[c] Data from reference 23.
[d] Data from reference 34.
[e] Data from references 16, 18, 31, and 35.
[f] Data from references 3, 22, and 30.
[g] ND, not determined.
[h] Unless indicated otherwise, values are percentages of the total fatty acids.
[i] Percentage of 3-OH fatty acids.
[j] Percentage of nonpolar fatty acids.

and hexadecanoate (16:0), small amounts of 3-hydroxydecanoate (3-OH 10:0) were also found. These fatty acid profiles were similar to the profiles described for other members of the beta subclass of the *Proteobacteria* (45) and were different from the profiles described for members of the authentic genus *Pseudomonas* (41). Notably, the lack of hydroxylated fatty acids other than 3-OH 10:0 is a strong argument against assignment to the genus *Pseudomonas* whose members contain more than one hydroxylated fatty acid. The fatty acid profiles are consistent with placement of the strains in the acidovorans rRNA cluster sensu De Ley (11) but not in the authentic genus *Pseudomonas.*

Also, the quinone type found, ubiquinone 8, is consistent with this conclusion. Ubiquinone 8 is the major quinone found in members of the beta subclass, whereas ubiquinone 9 is the characteristic quinone found in the authentic pseudomonads (6, 7). A summary of these properties and other features of the strains which we studied and related members of the beta subclass of the *Proteobacteria*, is shown in Table 1.

Phylogeny and taxonomic position. The rDNA sequences of strains K 172^T and KB 740^T were determined and compared with about 1,800 homologous sequences from other bacteria. This comparison showed that both organisms are members of the beta subclass of the *Proteobacteria* and that neither is related to the authentic pseudomonads, which belong to the gamma subclass. Among the beta subclass of the *Proteobacteria*, strains K 172^T and KB 740^T cluster with a major phylogenetic group that includes representatives of the genera *Azoarcus*, *Rhodocyclus*, *Thauera*, and *Zoogloea*. The closest relative of strain K 172^T is *T. selenatis* (23), whereas strain KB 740^T clusters with toluene-degrading "*A. denitrificans*" strains (13); the overall levels of sequence similarity are 97.7 and 98.5 to 99.4%, respectively (Table 2). Two other denitrifying toluene-degrading strains, mXyN1 and ToN1 (32), cluster with K 172^T and KB 740^T, respectively. The levels of sequence similarity with two members of the gamma subclass, *Pseudomonas aeruginosa* and *Chromatium vinosum*, are approximately 81% for both strains.

The phylogenetic tree in Fig. 1 shows the positions of denitrifying strains KB 740^T and K 172^T in the beta subclass of the *Proteobacteria*. The positions of close relatives of these organisms and selected representatives of the major phylogenetic groups of the beta subclass are also shown. The tree is based on the results of maximum-likelihood analyses. To avoid treeing artifacts in the lower branches, data for highly variable positions were successively removed from the data set used to perform various analyses with the sequences. Such artifacts may result from ambiguous alignment of variable regions or from false identities as a consequence of multiple base changes during the course of evolution. The tree was further evaluated by performing distance matrix and maximum-parsimony anal-

TABLE 2. Overall levels of 16S rDNA sequence similarity for *A. evansii*, *T. aromatica*, and related reference organisms belonging to the beta subclass of the *Proteobacteria*[a]

	Ae	A19	A3	A21	A17	A15	ToN1	A1	A4	A2	EbN1	PbN1	AB	Ai	Ta	mXyN1	Ts	Zr	Rp
A19	99.0																		
A3	99.2	99.0																	
A21	99.4	98.8	98.8																
A17	99.2	98.3	98.4	99.1															
A15	99.4	98.9	99.0	99.0	99.2														
ToN1	99.8	99.0	99.2	99.2	99.0	99.2													
A1	98.8	98.1	98.2	98.3	98.4	98.6	98.8												
A4	98.8	97.9	98.2	98.3	98.4	98.6	98.8	99.9											
A2	98.5	97.8	98.2	98.2	98.2	98.4	98.6	99.6	99.6										
EbN1	95.6	95.7	96.2	96.1	96.5	96.4	96.4	97.2	97.2	97.0									
PbN1	96.8	96.1	95.6	96.3	96.5	96.5	96.7	97.2	97.2	97.0	98.3								
AB	95.3	95.3	95.9	95.5	95.9	95.9	95.9	96.4	96.4	96.2	95.6	95.5							
Ai	95.5	94.9	95.3	95.0	95.1	95.1	95.5	95.5	95.5	95.3	95.5	95.3	97.4						
Ta	94.3	93.2	93.7	93.9	94.2	93.8	94.2	93.9	93.9	93.6	92.6	93.6	94.3	94.5					
mXyN1	94.6	93.6	94.1	94.2	94.4	94.1	94.5	94.2	94.2	93.9	94.6	93.9	94.8	94.8	99.6				
Ts	93.8	93.1	93.7	93.4	93.5	93.2	93.8	93.6	93.5	93.3	93.5	93.2	94.0	94.2	97.7	98.0			
Zr	93.0	92.1	92.4	92.6	93.1	92.7	93.1	92.7	92.7	92.5	91.4	92.6	93.1	92.4	92.7	92.8	91.7		
Rp	90.9	90.4	90.8	90.1	90.7	90.5	91.0	90.8	90.9	90.6	90.6	91.4	91.3	91.6	91.0	91.3	91.8	91.5	
Rt	90.7	90.3	90.7	90.5	90.7	90.5	90.8	90.9	91.1	90.7	90.3	90.6	91.2	90.7	90.4	90.6	90.8	91.3	96.4

[a] The complete sequences (length, 1495 bp) between the PCR primers were determined; these sequences corresponded to positions 28 to 1528 in the *E. coli* system (5). Abbreviations: Ae, *A. evansi*; A1, "*A. denitrificans*" Td-1; A2, "*A. denitrificans*" Td-2; A3, "*A. denitrificans*" Td-3; A4, "*A. denitrificans*" Tol-4; A15, "*A. denitrificans*" Td-15; A17, "*A. denitrificans*" Td-17; A19, "*A. denitrificans*" Td-19; A21, "*A. denitrificans*" Td-21; AB, "*Azoarcus*" sp. strain BH72; Ai, *A. indigens*; Ta, *T. aromatica*; Ts, *T. selenatis*; Zr, *Z. ramigera* ATCC 19544^T; Rp, *R. purpureus*; Rt, *R. tenuis*.

yses of comprehensive data sets that differed with respect to the inclusion of variable positions and outgroup reference organisms.

In Fig. 1, multifurcations indicate branches for which a relative order could not be determined unambiguously or was not supported by the results obtained when different treeing methods were used. The affiliations of the strains which we studied and their closest relatives are shown in Fig. 2. Because we wanted to resolve close relationships, all sequence positions were included for construction of this tree. The highly variable positions provided valuable phylogenetic information useful for the elucidation of close relationships. As described above, artifacts are unlikely to occur when very similar sequences are compared. In the case of the "*A. denitrificans*" strains (13) and the two other toluene-degrading strains (ToN1 and mXyN1) (32) the relationships are too close (Table 2) for all of these organisms to be properly resolved by comparative 16S rDNA analysis. To indicate the depth of the group, only strains Td-1 and strain KB 740^T were included.

Comparison with species belonging to the genera *Thauera* and *Azoarcus*. The high level of sequence similarity between strain K 172^T and the type strain of *T. selenatis* suggests that strain K 172^T is a *Thauera* strain. *T. selenatis* is characterized by a unique property—it reduces selenium oxyanions under anaerobic conditions (33). Strain K 172^T was tested for selenate reduction by using benzoate or acetate as an electron donor, but no selenate reduction was detected under these conditions. In addition, the rDNA gene sequences exhibited a difference of 2.3% (Table 2). Therefore, we suggest that strain K 172^T is a new species of this genus, for which we propose the name *Thauera aromatica*. Recently, Rabus and Widdel (32) described a closely related toluene-degrading denitrifier, strain mXyN1, which was not able to use phenol.

The 16S rDNA sequence data revealed that strain KB 740^T exhibited high levels of similarity (98.5 to 99.4%) (Table 2) with homologous sequences from "*A. denitrificans*" strains (13) deposited by Zhou et al. in the EMBL data library and with the homologous sequence of isolate ToN1 (99.8%) (Table 2) described by Rabus and Widdel (32), strains which use toluene as a substrate under denitrifying conditions. This suggests that these strains are members of a common genus and that they belong to the same species. However, this group is only moderately closely related to the validly described type species *A. indigens* (34), as shown in Fig. 2 and indicated by mean overall levels of sequence similarity of 94.9 to 95.5%. There are also some major phenotypic differences. For instance, it has been

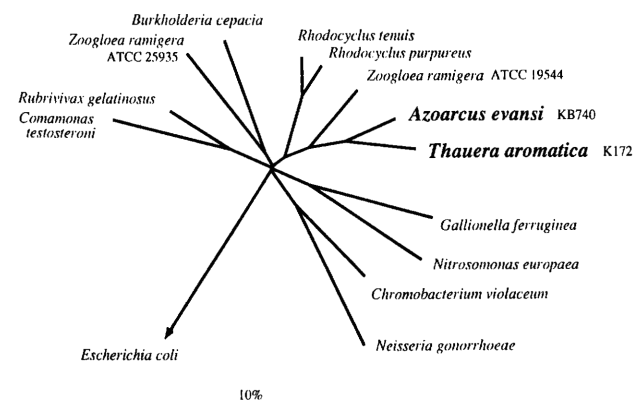

FIG. 1. Phylogenetic tree showing the phylogenetic positions of the denitrifying organisms *A. evansii* KB 740^T and *T. aromatica* K 172^T, as well as selected relatives in the beta subclass of the *Proteobacteria*, as determined from 16S rRNA sequences. The topology was evaluated by performing maximum-parsimony and distance matrix analyses of comprehensive data sets. Bar = 10% estimated sequence divergence.

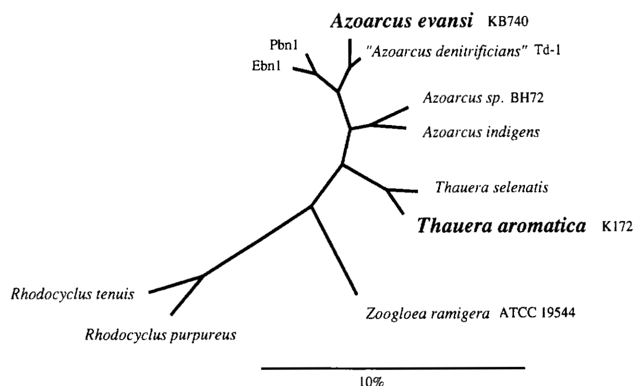

FIG. 2. Phylogenetic tree showing the relationships of *A. evansii*, *T. aromatica*, and related strains. All sequence positions were included for the calculations. Strains Pbn1 and Ebn1 are denitrifiers that grow anaerobically with ethylbenzene (32). Bar = 10% estimated sequence divergence.

reported that members of the genus *Azoarcus* do not denitrify, are not able to use benzoate aerobically, and grow with monosaccharides aerobically. However, recent investigations have shown that *Azoarcus* strains in fact do denitrify, yet none of the strains was able to grow with benzoate under denitrifying conditions (33a). Strain KB 740^T clearly denitrifies and grows aerobically and under denitrifying conditions with benzoate and fructose, and preliminary tests have indicated that it fixes molecular nitrogen, in contrast to strain K 172^T (unpublished data). We suggest that strain KB 740^T, the related "*A. denitrificans*" strains, and strain ToN1, which have not been validly described yet, should be grouped together in one species. As long as information concerning differentiating characteristics is limited, the new species may be placed in the genus *Azoarcus*. Since strain KB 740^T and the other strains mentioned above differ in many properties from members of the genus *Thauera* and from members of the validly described *Azoarcus* species, we suggest that strain KB 740^T belongs to a new species of the genus *Azoarcus*, for which we propose the name *Azoarcus evansii*.

Unfortunately, none of the previously described *Azoarcus* strains was available for comparison; these organisms have not been deposited in culture collections even though they are members of a new genus. The only species of the genus *Thauera* that has been described previously, *T. selenatis*, has been deposited in the American Type Culture Collection; however, the type strain has been patented and therefore was not available for comparison. Other denitrifying strains, such as strains SP, S 100, U 120, S 2, B4P, which were isolated in our laboratory, have somewhat different morphological properties, differ in their substrate spectra, or have been enriched with media containing aromatic substrates different from those used for strain KB 740^T (anthranilate) and strain K 172^T (phenol). These organisms will be investigated in a future comprehensive study which includes other newly isolated denitrifying, aromatic-degrading strains and, we hope, the previously described *Azoarcus* and *Thauera* strains.

Amendment of the description of the genus *Thauera*. Selenate is used as the electron acceptor by some strains. Vitamins are required for growth by only some strains. The substrates used for growth given in the original genus description are the substrates used by *T. selenatis*. Since strain K 172^T cannot use all of the substrates used by the type strain, the list of substrates should be redefined when more representatives of the genus *Thauera* are known.

Description of *Thauera aromatica* sp. nov. *Thauera aromatica* (ar.o.ma′ ti.ca. M. L. adj. *aromatica*, aromatic, because the organism was isolated on media containing aromatic compounds as sole carbon sources). Gram-negative, rod-shaped cells are 1.0 to 2.5 μm long and 0.5 to 1.5 μm wide, have rounded ends, and accumulate poly-β-hydroxybutyrate. Cells are slightly motile. Colonies on defined medium containing benzoate are 1 mm in diameter and white after 4 to 5 days of aerobic growth at 28°C. No growth or poor growth occurs at 37°C and on complex media. Grows aerobically or anaerobically with nitrate on minimal media. Cells do not reduce selenate or ferment sugars. Many aromatic compounds are used as sole carbon sources under denitrifying conditions. The aromatic substrates are metabolized to CO_2. During growth with aromatic compounds nitrite is an intermediate and is reduced mainly to N_2O. No vitamins are required for growth. The optimum growth temperature is around 28°C. The optimum pH for growth is between 7 and 7.4. Cells are catalase and oxidase positive. Phylogenetically, *T. aromatica* is a member of the beta subclass of the *Proteobacteria*. This organism was isolated from anaerobic sludge by using media containing phenol and nitrate. The type strain is K 172 (= DSM 6984). The DNA base composition is 67 mol% G+C. For additional characteristics of the type strain see above and Table 1.

Description of *Azoarcus evansii* sp. nov. *Azoarcus evansii* (e′van.si.i. L. gen. n. *evansii*, of Evans, in honor of the late W. C. Evans, a pioneer in studies of anaerobic aromatic metabolism). Cells are rods with rounded ends and are 1.5 to 3 μm long and 0.6 to 0.8 μm wide. Each cell is motile by means of one subpolarly inserted flagellum. Gram negative and KOH positive. Oxidase and catalase positive. Colonies on minimal media containing benzoate are 1 to 2 mm in diameter and white or beige after 3 to 4 days of incubation at 28°C. Yeast extract (0.1%, wt/vol) inhibits growth. The optimum growth temperature is around 37°C, and the optimum pH is around 7.8. No growth occurs at 41°C. Strictly oxidative metabolism occurs with oxygen or nitrate as the terminal electron acceptor. During growth with aromatic compounds nitrite is an intermediate and is reduced mainly to N_2O. Positive in the oxidative oxidation-fermentation test with different sugars. No fermentation occurs. In the presence of nitrate a variety of aromatic substrates are used anaerobically as sole carbon sources as are some sugars, dicarboxylic acids, and cyclohexanecarboxylate.

Several L-amino acids are growth substrates for aerobic growth. The major fatty acids are *cis*-9 16:1, 16:0, 12:0, 3-OH 10:0, and *cis*-9,11 18:1. The major quinone is ubiquinone 8. *A. evansi* is a member of the beta subclass of the *Proteobacteria*. The type strain is KB 740 (= DSM 6898). Isolated by K. Braun and D. T. Gibson from creek sediment in the United States with media containing 2-aminobenzoate and nitrate (4). The base composition of the DNA is 67.5 mol% G+C. Additional morphological, biochemical, and nutritional features are summarized above and in Table 1.

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