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Taxonomic characterization of denitrifying bacteria that degrade aromatic compounds and description of *Azoarcus toluvorans* sp. nov. and *Azoarcus toluclasticus* sp. nov.

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A taxonomic characterization of twenty-one strains capable of degrading aromatic compounds under denitrifying conditions, isolated from ten different geographical locations, was performed on the basis of general morphological and physiological characteristics, cellular fatty acids, DNA base composition, small ribosomal (16S) subunit DNA sequences, whole-cell protein patterns and genomic DNA fragmentation analysis, in addition to DNA similarity estimations using hybridization methods. The collection of strains was subdivided into a number of different groups. A first group, consisting of four strains, could be assigned to the previously described species *Azoarcus tolulyticus*. A second group (five strains) had DNA which reannealed highly to that of strains of the first group, and it is considered to represent a genomovar of *A. tolulyticus*. The third and fourth groups, composed of a total of five strains, represent a new species of *Azoarcus*, *Azoarcus toluclasticus* (group 3) and a genomovar of this species (group 4), respectively. Finally, the fifth group, with two strains, corresponds to another new species of the genus *Azoarcus*, *Azoarcus toluvorans*. In addition to these five groups, the collection includes five individual strains perhaps representing as many different new species. The above classification is partially consistent with the results of approaches other than DNA–DNA hybridization (electrophoretic patterns of whole-cell proteins and of the fragments obtained after digestion of total DNA with infrequently cutting restriction enzymes). On the other hand, no correlation of these groupings was found in terms of the cellular fatty acid composition. It is also unfortunate that no simple sets of easily determinable phenotypic properties could be defined as being characteristic of each of the groups.

Keywords: *Azoarcus toluvorans* sp. nov., *Azoarcus toluclasticus* sp. nov., aromatic degradation, taxonomy

INTRODUCTION

In recent years many bacterial strains capable of growing at the expense of aromatic compounds under denitrifying conditions have been isolated from a variety of environments and subjected to studies on the metabolic pathways operating in the degradation of substrates (Altenschmidt & Fuchs, 1991; Biegert & Fuchs, 1996; Chee-Sanford *et al.*, 1996; Frazer *et al.*, 1995; Gorny *et al.*, 1992; Heider & Fuchs, 1997; Migaud *et al.*, 1996). A number of these isolates have been assigned to the genera *Azoarcus* and *Thauera* (Anders *et al.*, 1995; Fries *et al.*, 1994; Rabus & Widdel, 1996; Rhee *et al.*, 1997; Seyfried *et al.*, 1994; Springer *et al.*, 1998; Zhou *et al.*, 1995). Interestingly, although these two genera represent a major group of organisms of this type, the identification of the first *Azoarcus* species, *Azoarcus indigens*, was based on the capacity for dinitrogen fixation that it had as a member of the rhizosphere microflora of Kallar grass, which grows in arid regions of Pakistan (Reinhold-Hurek *et al.*, 1993). A second species, *Azoarcus communis*, was

identified for strains that shared the property of nitrogen fixation, but differed in morphological and physiological characteristics from the type species (Reinhold-Hurek *et al.*, 1993). Additional strains with the basic properties of the genus *Azoarcus* and characterized by genomic fingerprinting criteria, were added to the genus (Hurek *et al.*, 1997).

The property of denitrification was negative in the type species, *A. indigens*, but it was present in the strains later assigned to the same genus, which suggested a change in the generic definition (Anders *et al.*, 1995). A denitrifying strain KB 740^T was assigned to the genus under the new name *Azoarcus evansii*. Aside from being able to fix dinitrogen, this strain was able to degrade 2-aminobenzoate, 2-fluorobenzoate and *p*-cresol under denitrifying conditions (Anders *et al.*, 1995). Eight strains degrading toluene under denitrifying conditions were assigned to the new species *Azoarcus tolulyticus* (Zhou *et al.*, 1995). They are also able to grow in a medium free of combined nitrogen. Recently, a new species able to degrade resorcinol and grow under obligate denitrifying conditions has been described under the name *Azoarcus anaerobius* (Springer *et al.*, 1998). Additional *Azoarcus* strains capable of degrading aromatic compounds or cyclic compounds under denitrifying conditions have been isolated, but have not been assigned to any of the known species (Harder, 1997; Rabus & Widdel, 1996; Rhee *et al.*, 1997; van Schie & Young, 1998).

Strains degrading toluene under conditions of denitrification were assigned to the species *Azoarcus tolulyticus* on the basis of their 16S rRNA gene sequences and their fatty acid composition (Zhou *et al.*, 1995). A large number of isolates obtained from an aquifer at Moffett Field, California, USA, were added to the collection on the basis of fatty acid profiles characteristic of *Azoarcus* (Fries *et al.*, 1997a). Their properties included the degradation of toluene and phenol in combination with the capacity of co-metabolizing trichloroethylene. Lately, new strains isolated from aquifer sediments in Northern Michigan were reported as toluene degraders under denitrifying conditions. Their taxonomic position was not determined (Chee-Sanford *et al.*, 1996).

In this paper, we report the results of polyphasic study of strains previously assigned to the species *Azoarcus tolulyticus*, together with recently isolated strains which are able to degrade toluene and phenol. The collection was found to include three species and, in addition, five strains not closely related to one another or to the other members of the collection, and which may perhaps be members of as many additional species.

METHODS

Organisms and growth conditions. A total of 21 denitrifying strains capable of degrading aromatic compounds was used for this study (Table 1). The strains were isolated using BS medium plus 5 mM NO_3^- plus toluene vapours or phenol and modified R2A (M-R2A) medium from various environments including agricultural soils, composts, aquifers and contaminated soils from different geographic regions (Chee-Sanford *et al.*, 1996; Fries *et al.*, 1994, 1997b). For the purpose of culturing these strains, M-R2A medium was used as described previously (Fries *et al.*, 1994).

Table 1. Geographical origins of strains and DNA G+C content

Strain	Geographical origin	G+C (mol%)
2a1	Aquifer sediment, Wexford, Northern Michigan, USA	68·1
3a1	Aquifer sediment, Kalkaska, Northern Michigan, USA	67·9
7a1	Aquifer sediment, Kalkaska, Northern Michigan, USA	68·6
BL2	Aquifer sediment, Bear Lake, Northern Michigan, USA	68·2
BL11	Aquifer sediment, Bear Lake, Northern Michigan, USA	67·4
MF7	Shallow aquifer sediment, Moffett Field, California, USA	67·7
MF23	Shallow aquifer sediment, Moffett Field, California, USA	67·8
MF58	Shallow aquifer sediment, Moffett Field, California, USA	66·1
MF62	Shallow aquifer sediment, Moffett Field, California, USA	61·8
MF63^T	Shallow aquifer sediment, Moffett Field, California, USA	66·8
MF66	Shallow aquifer sediment, Moffett Field, California, USA	67·6
MF107	Shallow aquifer sediment, Moffett Field, California, USA	67·4
MF118	Shallow aquifer sediment, Moffett Field, California, USA	67·8
MF441	Shallow aquifer sediment, Moffett Field, California, USA	68·0
Tol-4^T	Aquifer sediment, Bear Lake, Northern Michigan, USA	66·9
Td1	Soil from petroleum-contaminated site, Washington, USA	69·3
Td2	Soil from petroleum-contaminated site, Washington, USA	67·8
Td3	Marine sediment, Hunting Beach, California, USA	68·5
Td17	Soil from industrial area, Jabaquara, Sao Paulo State, Brazil	66·9
Td19	Soil from landfill, Cubatao, Sao Paulo State, Brazil	67·3
Td21^T	Muck soil, Clinton County, Michigan, USA	68·6

Morphology and physiological tests. Cell dimensions and morphology were determined by phase-contrast microscopy. Flagellation was determined using Leifson's method (Leifson, 1951). The presence of catalase and oxidase was tested as described by Smibert & Kreig (1994). Growth on

nutrient, brain-heart infusion and trypticase soy media was tested at 30 °C under aerobic conditions. Growth on toluene was tested in the presence of nitrate and an anaerobic atmosphere with toluene as described previously (Evans *et al.*, 1991). Growth on various substrates was determined at 30 °C under aerobic and denitrifying conditions, using minimal salts medium plates (Tschech & Fuchs, 1987) containing 1 mM final concentration of acetate, benzoate, butyrate, caproate, fructose, fumarate, glucose, 2-hydroxybenzoate, 3-hydroxybenzoate, 4-hydroxybenzoate, 4-hydroxyphenylacetate, phenol, phenylacetate, phthalate, propionate, pyruvate, succinate, valerate, D-xylose, L-arabinose, D-ribose, D-galactose, sucrose, lactose, maltose, starch, adipate, tartrate, lactate, mannitol, aspartate, proline, phenylalanine, arginine, acetamide or histamine.

Determination of cellular fatty acids. All of the strains, including strains KB 740^T, T1 and K 172^T, were precultured under denitrifying conditions in minimal salts medium with 10 mM succinate and 30 mM nitrate (Tschech & Fuchs, 1987). After the OD_{620} of the cultures reached 0·4, the cells were used as an inoculum (10%) for 50 ml fresh liquid medium containing 10 mM succinate and 30 mM nitrate in anaerobic serum bottles and cultivated for 24 h at 30 °C under an argon atmosphere. The cells were collected by centrifugation at room temperature. The fatty acids were saponified, methylated, extracted and analysed by GC using the SHERLOCK Microbial Identification System (MIDI). Identification was confirmed by GC-MS using a HP 5890 series II GC (Hewlett Packard) equipped with an HP 5971 mass selective detector and an HP5MS column (30 m × 0·25 mm, 0·25 μm film thickness; Hewlett Packard). Helium was used as the carrier gas. The injector was heated to 250 °C with an initial column temperature of 60 °C for 1 min, increasing at the rate of 20 °C min^{-1} to 300 °C.

Genomic bacterial DNA isolation. Chromosomal DNA of each strain was isolated as previously described (Song *et al.*, 1998). The extracted DNA was sheared by two passages through a French press at 20000 p.s.i. (138 MPa) and diluted to 0·4 mg DNA ml^{-1}. The purity of the DNA was determined by measuring absorbance at 230, 260 and 280 nm. The A_{260}/A_{280} and A_{260}/A_{230} ratios were in all cases close to 2.

Measurement of G+C content. The G+C content of DNA was measured by HPLC following the method of Mesbah *et al.* (1989) with salmon sperm DNA as a standard. The nucleotides were analysed by HPLC (Beckman Instruments) with a reversed phase C18 column (250 × 4·6 mm; Beckman Instruments) and a solvent of 12% HPLC-grade methanol and 20 mM triethylamine at a flow rate of 1·5 ml min^{-1}. Absorbance was monitored at a wavelength of 254 nm.

Determination and analysis of 16S rRNA gene sequence. The 16S rRNA gene was PCR amplified with the universal eubacterial primers fD1 and rP1 (Weisburg *et al.*, 1991). The DNA sequences of the 16S rRNA genes were determined directly using the PCR-amplified DNA as the sequencing template. The amplified PCR products were purified using the Wizard PCR Preps DNA purification system (Promega) according to the manufacturer's instructions. DNA sequences were determined with automated fluorescent *Taq* cycle sequencing using the ABI Catalyst 800 and ABI 373A Sequencer (Applied Biosystems). Approximately 100 ng of the purified DNA was used for one automated fluorescent sequencing reaction. The primers for sequencing used in this study spanning the *Escherichia coli* 16S rRNA gene positions are fD1 (8–27), f270 (246–261), f519 (519–533), f788 (787–802), f1099 (1099–1144), r350 (357–342), r529 (529–512), r925 (920–906), r1240 (1242–1227) and rP1(1511–1492). Multiple sequence alignment was done with the PILEUP program in the Genetics Computer Group (GCG) software package (Devereux *et al.*, 1984). The phylogenetic analyses were performed with the PHYLIP 3.5 program (Felsenstein, 1989). Kimura's method (Kimura, 1980) was used to calculate evolutionary distances, from which a phylogenetic dendrogram was drawn using neighbour-joining analysis (Saitou & Nei, 1987). The SEQBOOT program was used to obtain confidence levels for neighbour-joining analysis using a 100 bootstrapped data set (Felsenstein, 1985).

DNA–DNA hybridization. The DNA preparations were labelled using the BRL nick-translation kit with ^{3}H-radiolabelled dTTP. DNA–DNA hybridization was performed using the S1 nuclease method as reported in detail previously (Song *et al.*, 1998).

DNA homology value transformation. The construction of a three-dimensional model for the DNA homology values was performed with the 3D program calculating a three-dimensional coordinate using the DNA homology value (HOM) (Hildebrand *et al.*, 1982). The accuracy, limitations and predictability of the program are reported elsewhere (Hildebrand *et al.*, 1984).

PAGE of soluble cell proteins. After the cells were aerobically cultured for 1 d in M-R2A liquid medium at 30 °C, the protein sample was prepared as previously described by Jackman (1984). The amount of protein in the cell suspension was measured by heating the cells in 1 M NaOH for 10 min and assaying by the Bradford method (Bradford, 1976). SDS-PAGE (10%) was performed by a slightly modified Laemmli method (Hood *et al.*, 1988). Electropherograms were developed for 17 h in a Protean II xi vertical electrophoresis cell (Bio-Rad) at 10 mA and 20 °C. The protein banding pattern was revealed by staining with 0·2% Coomassie brilliant blue R-250 and 0·2% Coomassie brilliant blue G-250 in 50 % (v/v) methanol plus 15% acetic acid.

PFGE. PFGE analysis was performed using the method of Rainey *et al.* (1994). The embedded DNAs were treated with restriction endonuclease *Xba*I which yields between 20 and 30 DNA fragments. *Not*I and *Asp*I were also tested, but they are not useful for genomic characterization, since they digested the chromosomal DNA into many small fragments. The digested DNA slivers were loaded on a 1·3% agarose gel and the gels were electrophoresed in a contour-clamped homogeneous electric field DR II PFGE system (Bio-Rad) at 200 V with a ramped pulse time of 1–25 s for 23 h at 14 °C.

RESULTS AND DISCUSSION

Morphological and physiological features

The cells of *A. tolulyticus* strains were reported to be short motile rods (Zhou *et al.*, 1995), and the same appears to be true for cells of the isolates from Moffett Field and Northern Michigan (Fig. 1). Motility is due to the presence of single polar flagella.

The biochemical properties of the isolates are very similar, with minor exceptions. All strains are oxidase-positive and the catalase reaction is positive, except for some strains from Moffett Field (MF7, MF23, MF58, MF62, MF63^T, MF66 and MF441). Poor growth is observed on complex media such as nutrient, brain-heart infusion and trypticase soy agar plates. Strain

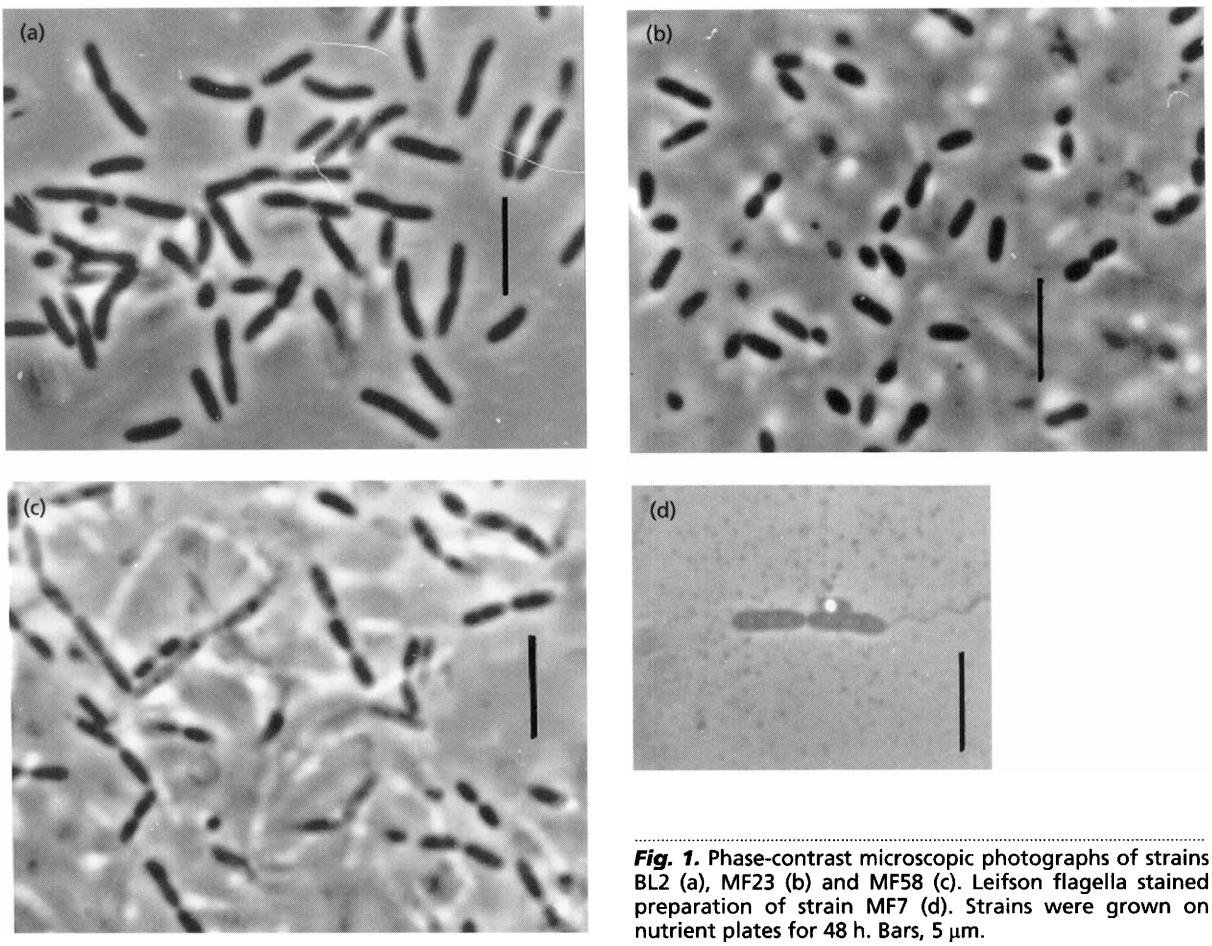

Fig. 1. Phase-contrast microscopic photographs of strains BL2 (a), MF23 (b) and MF58 (c). Leifson flagella stained preparation of strain MF7 (d). Strains were grown on nutrient plates for 48 h. Bars, 5 μm.

MF62 does not grow on brain-heart infusion agar, strain MF63^T does not grow on nutrient agar and strain MF66 does not grow on nutrient or trypticase soy agar.

G+C content determination

The members assigned to the species *A. tolulyticus* have a G+C content of 66–69 mol% (Table 1). This range is approximately the same for the isolates from Moffett Field (66–68 mol%) and for the Northern Michigan strains (67–69 mol%). Strain MF62 is exceptional in having a low G+C content (61·8 mol%). These results are compatible with the allocation of most strains to a single genus (*Azoarcus*). MF62 could be excluded because it differs by more than 5 mol% in G+C content from the other strains (Goodfellow & O'Donnell, 1993).

Cellular fatty acid analysis

After culturing the cells as described in Methods, the predominant fatty acids of all strains were 12:0, 16:0, 10:0 3-OH, 16:1ω7*c* and 18:1ω7*c*/ω9*t*/ω12*t* (Table 2). The identity of these fatty acids was confirmed by GC-MS (data not shown). Cluster analysis of the 21 strains (Fig. 2) showed that they were grouped at a Euclidian distance of less than 9, with the exception of strain MF107, and that they could be differentiated into two groups at a Euclidian distance of 6, with the exception of the strains MF107 and MF62. On the basis of fatty acid analysis, strain MF107 could be located closer to the genus *Thauera* than to *Azoarcus*, considering the greater amount of 18:1ω7*c*/ω9*t*/ω12*t* in its composition (Table 2).

Four main clusters were obtained within the *Azoarcus* group. Strains Tol-4^T, Td1, Td2, Td19, 3a1, BL11 and MF66 grouped together at a Euclidian distance of less than 4. Several strains (MF23, MF58, MF63^T, MF441, 2a1, 7a1 and Td3) were clustered together and were not differentiated further by fatty acid composition. Strains Td17, Td21^T and MF7 form a group at a Euclidian distance of less than 6. Strain MF118 was grouped with strain KB 740^T (*A. evansii*). Thus, approximately 9 units of Euclidian distance could constitute the boundary for the differentiation between the genera *Azoarcus* and *Thauera* (Fig. 2).

Phylogenetic analysis

Strains of *A. tolulyticus* were previously studied for their taxonomic and phylogenetic relationships (Zhou *et al.*, 1995). Partial sequencing studies of the 16S

Table 2. Fatty acid composition of strains grown on succinate under denitrifying conditions

Strain	Total fatty acids (%)							
	10:0	**10:0 3-OH**	**12:0**	**14:0**	**16:1ω7c**	**16:1ω5c**	**16:0**	**18:1ω7c/ ω9t/ω12t**
Tol-4^T	0·6	6·6	7·1	0·8	53·7	1·0	22·1	7·8
Td1	0·5	6·0	7·0	1·0	52·8	0·4	27·5	4·8
Td2	0·7	6·2	7·0	1·0	51·2	0·0	26·8	6·7
Td3	0·5	5·6	6·2	0·7	53·2	1·0	25·7	7·2
Td17	0·4	4·3	3·7	0·5	51·2	0·9	25·8	13.1
Td19	0·7	7·2	6·8	0·7	50·6	0·8	26·2	6·8
Td21^T	0·5	3·1	4·7	0·8	51·0	0·0	29·7	10·3
MF7	0·8	4·9	6·4	0·0	53·8	0·0	23·6	10·7
MF23	0·6	3·5	6·5	0·6	53·9	0·7	25·9	7·6
MF58	0·4	5·0	5·2	0·3	53·0	0·8	26·5	8·7
MF62	0·0	3·0	4·7	3·7	56·4	0·0	28·7	2·5
MF63^T	0·5	5·5	5·6	0·4	51·8	0·8	25·6	9·3
MF66	0·6	6·6	7·1	1·3	52·5	0·0	26·7	5·3
MF107	0·7	5·7	6·3	0·0	48·5	0·0	21·7	17.1
MF118	0·5	5·7	6·5	0·9	49·3	0·0	30·9	6.2
MF441	0·5	5·6	5·7	0·3	53·1	0·9	25·5	8.5
2a1	0·6	6·0	6·7	0·4	52·9	0·0	26·2	7·4
3a1	0·5	6·7	6·8	0·9	50·9	0·4	27·7	5·7
7a1	0·5	6·1	6·4	0·5	53·9	0·0	25·7	6·9
BL2	0·5	6·7	6·5	0·4	51·2	0·6	24·0	8·6
BL11	0·5	5·5	6·3	0·9	52·2	0·5	27·1	6·5
KB 740^T	0·8	8·0	6·9	0·8	46·9	0.5	27·5	8·2

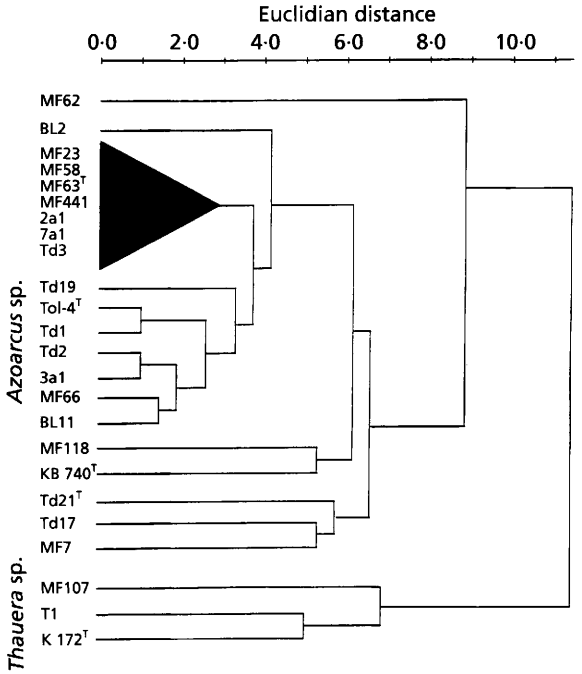

Fig. 2. Dendrogram of strains on the basis of cellular fatty acids analysis. Strains were grown with 10 mM succinate and 30 mM nitrate for 24 h under denitrifying conditions.

rRNA gene were performed on isolates from Moffett Field and Northern Michigan. From the preliminary analysis of the sequences, three strains (BL11, MF7 and MF63^T) were selected for complete sequence analysis of the 16S rRNA gene. These strains share among them, and with strains Tol-4^T and Td1, over 99% 16S rRNA sequence similarity (Table 3), and therefore are closely related to *A. tolulyticus*.

A neighbour-joining tree (Fig. 3) revealed that the species may be subdivided into three clusters. Thus, strains Tol-4^T, Td1 and Td2 cluster with strain BL11, while two additional groups are evident (Td17/Td21^T and Td3/Td19). These last two groups appear to cluster in the neighbourhood of *A. evansii* (Fig. 3). Strains MF7 and MF63^T also clustered between the species *A. tolulyticus* and *A. evansii*.

The newly described species *A. anaerobius* (Springer *et al.*, 1998) does not cluster with the strains included in our study, and it appears to be located outside of the cluster of *A. tolulyticus* and *A. evansii*, in agreement with basic differences in its physiological properties (Fig. 3). In view of the limitations of 16S rRNA sequence studies for the circumscription of taxa at the species level (Stackebrandt & Goebel, 1994), it appears that a revision of the classification of *Azoarcus* species may be required in the near future.

Table 3. Similarity (%) and genetic distance of strains on the basis of 16S rRNA sequence analysis

Strains: 1, *A. tolulyticus* Tol-4^T; 2, *A. tolulyticus* Td1; 3, *A. tolulyticus* Td2; 4, Td3; 5, Td17; 6, Td19; 7, Td21^T; 8, BL11; 9, MF7; 10, MF63^T; 11, *A. evansii* KB 740^T; 12, *A. anaerobius* LuFRes1^T; 13, *A. indigens* VB32^T; 14, *T. aromatica* K 172^T; 15, *T. aromatica* T1; 16, *T. selenatis* AX^T. Top right, similarity; bottom left, genetic distance.

Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1		99·8	99·3	98·2	98·3	97·7	98·1	99·5	99·3	99·4	98·6	97·1	95·0	93·1	93·3	93·4
2	0·07		99·3	98·2	98·3	97·7	98·1	99·5	99·3	99·4	98·6	97·1	95·0	93·1	93·3	93·4
3	0·37	0·30		97·9	97·8	97·3	97·7	99·0	98·8	98·9	98·1	96·8	94·4	92·5	92·7	92·8
4	1·82	1·74	1·82		98·5	98·8	98·9	97·9	97·9	98·0	99·2	96·6	94·8	93·0	93·2	93·6
5	1·66	1·59	1·89	1·51		98·2	99·1	98·0	98·3	98·3	99·2	96·6	94·5	93·5	93·7	93·3
6	2·05	1·97	2·20	0·98	1·59		98·5	97·4	97·4	97·5	98·9	96·1	94·2	92·3	92·4	92·7
7	1·89	1·82	1·97	1·21	0·90	1·28		97·8	97·9	98·0	99·3	96·3	94·3	93·1	93·3	93·2
8	0·45	0·37	0·68	2·12	1·97	2·35	2·20		99·2	99·1	98·3	96·4	94·6	92·8	93·0	93·1
9	0·68	0·60	0·90	2·12	1·66	2·35	2·12	0·83		99·3	98·5	97·4	94·4	93·1	93·3	93·2
10	0·60	0·53	0·83	1·97	1·66	2·20	1·97	0·90	0·68		98·5	97·1	94·7	93·0	93·2	93·4
11	1·36	1·28	1·59	0·75	0·75	0·83	0·68	1·66	1·51	1·51		96·5	94·9	93·5	93·6	93·7
12	3·21	3·13	3·44	3·76	3·68	4·23	4·15	3·52	2·82	3·13	3·68		95·1	94·3	94·4	93·8
13	4·95	4·87	5·19	5·11	5·35	5·43	5·60	5·27	5·52	5·19	5·03	5·60		94·0	94·1	94·2
14	6·81	6·73	7·05	6·90	6·40	7·39	6·82	7·14	6·81	6·89	6·48	6·65	5·91		99·8	97·7
15	6·65	6·56	6·89	6·73	6·24	7·22	6·66	6·98	6·64	6·73	6·32	6·49	5·75	0·15		97·8
16	6·58	6·49	6·82	6·25	6.66	6·91	6·83	6·91	6·82	6·58	6·33	6·34	5·76	2·36	2·20	

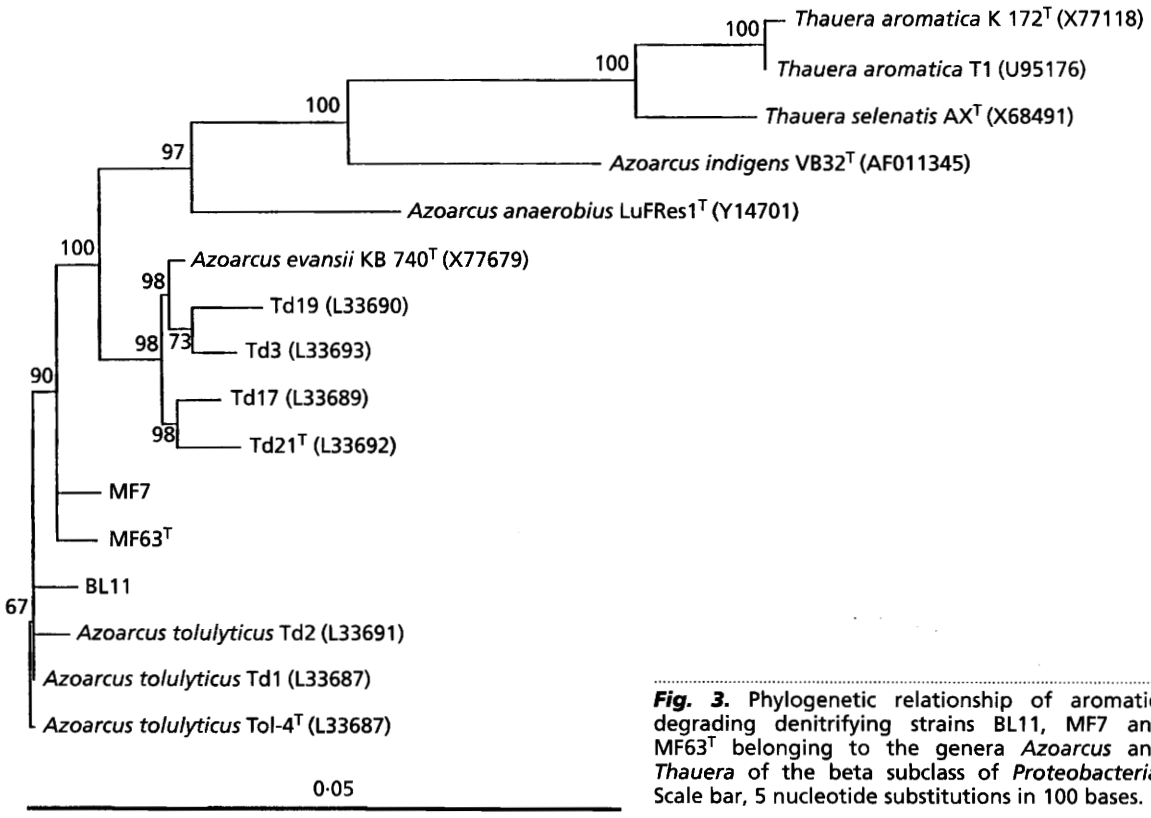

Fig. 3. Phylogenetic relationship of aromatic-degrading denitrifying strains BL11, MF7 and MF63^T belonging to the genera *Azoarcus* and *Thauera* of the beta subclass of *Proteobacteria*. Scale bar, 5 nucleotide substitutions in 100 bases.

DNA–DNA hybridization experiments

As shown in Fig. 4, the strains can be divided into five DNA homology groups. Strain Td1 has about 90% DNA similarity with Td2 and Tol-4^T, which were previously assigned to *A. tolulyticus* (Zhou *et al.*, 1995), and the similarity of MF66 to Td1 is 92%, all of which indicates that these four strains (group 1) should be considered as belonging to the same species. On the other hand, strains Td3, Td17, Td19 and Td21^T have

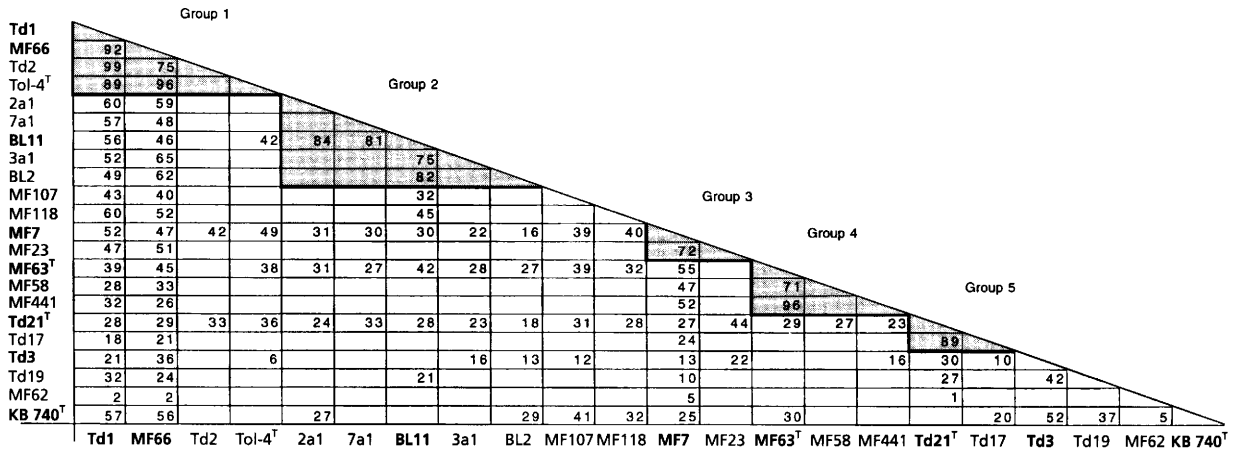

Fig. 4. Triangle matrix representation of pairwise DNA homology values. The heavy line and shaded regions indicate the strains within each one of the groups. Strains used as references in the DNA–DNA hybridization experiments are shown in bold.

less than 30% similarity with strain Td1. Of these four strains, strain Td21^T has 89 % similarity with strain Td17, and therefore the two are considered to be members of another DNA homology group (group 5), whereas strains Td3 and Td19 have DNA of low similarity (about 30%) with that of strain Td21^T, and strain Td3 has 42% similarity with strain Td19, although these two strains form a cluster in the 16S rRNA phylogenetic tree (Fig. 3).

Among strains from Moffett Field, strains MF7, MF63^T and MF66 were chosen as reference strains. As shown in Fig. 4, strain MF7 has 72% DNA similarity with strain MF23 (not represented in Fig. 3), thus generating a new species group (group 3). This group has about 45% DNA similarity with the members of group 1 and with some Moffett Field isolates (strains MF58, MF63^T, MF107, MF118 and MF441). On the other hand, 16S rRNA studies have shown the relatedness of strains MF7 and MF63^T; the latter has, in addition, 71 and 96% DNA similarity with strains MF58 and MF441, respectively, all of which indicates that strains MF63^T, MF58 and MF441 constitute a new DNA homology group, which is named here as group 4.

Of strains from Northern Michigan, strain BL11, used as a reference strain, has more than 70% DNA similarity with the five isolates from the same origin (Table 1) and they are considered to constitute a new group, group 2. Members of this group have a mean DNA similarity of about 55% with strains of group 1 and about 30% with groups 3, 4 and 5, which is in agreement with the data of 16S rRNA sequence analysis.

In summary, five groups are revealed in the collection under study on the basis of the DNA–DNA hybridization experiments (Fig. 4). The remaining strains (Td3, Td19, MF62, MF107 and MF118) have insignificant DNA similarity values among each other and with strains of the species groups defined in our work and they seem to represent separate taxa whose circumscription is better attempted when more strains are added to the collection.

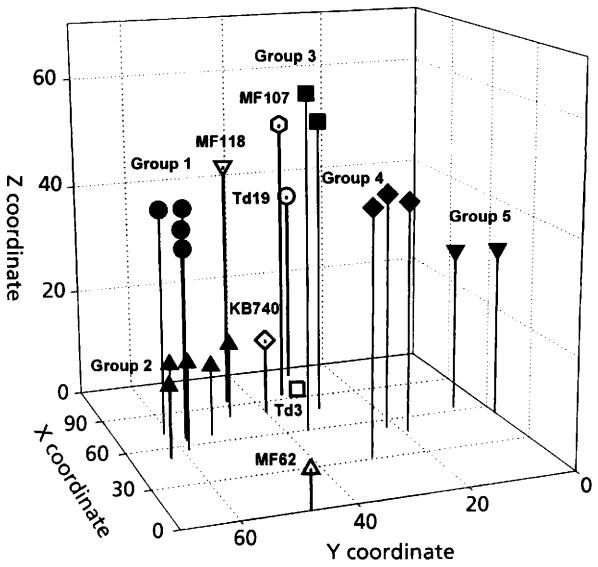

Fig. 5. Three-dimensional representation of species division of the genus *Azoarcus* based on DNA homology values.

Three-dimensional models

This representation, based on the results of DNA–DNA hybridization experiments, locates the various groups in a three-dimensional space (Fig. 5). The advantage of such a model is to emphasize the spatial relationships more clearly than the conventional bi-

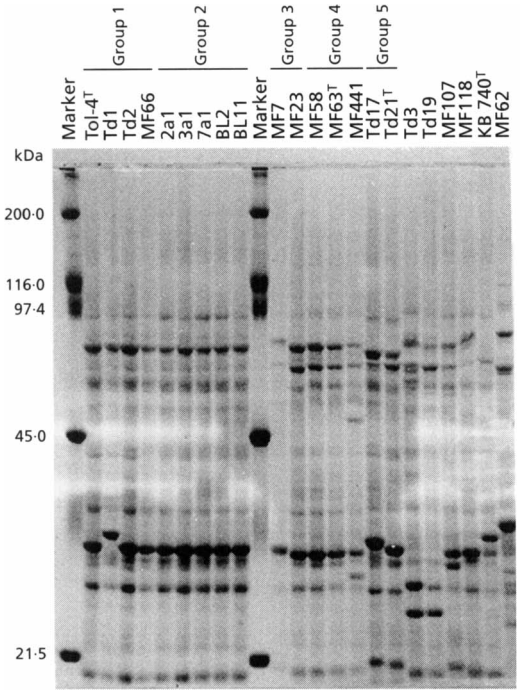

Fig. 6. Protein fingerprinting patterns of strains included in this study. Molecular masses of the marker proteins are indicated on the left-hand side.

dimensional dendrograms and to serve as a guide for future experiments to attain a further degree of refinement.

As can be seen in Fig. 5, the strains constituting groups 1 through 5 appear to form a large cluster on the basis of their DNA similarities, while one of the strains that could not be assigned to any group (strain MF62) is located far from the main cluster. The independent strains Td3, Td19, MF107, MF118 and KB 740^T are found scattered within the main cluster.

Comparison of protein electropherograms

The protein profiles of the aromatic-compound-degrading denitrifiers were also examined (Fig. 6). Strains of group 1 (Tol-4^T, Td2 and MF66) have very similar protein patterns except for strain Td1. Strains of group 2 (2a1, 3a1, 7a1, BL2 and BL11) have identical protein patterns and very similar profiles to those of group 1. Strains of group 3 and group 4 (MF23, MF58 and MF63^T) have similar protein patterns except for strains MF7 and MF441. Identical protein patterns are observed for the strains of the group 5 with the exception of one band. The remaining strains not included in any groups have a variety of protein patterns.

DNA fragmentation analysis

Visual evaluation of DNA fragmentation (Fig. 7) showed that strains in groups 1 and 3 have somewhat different DNA fragmentation patterns within their groups, whereas the strains in groups 2 and 4 have identical patterns within their groups. Strain Tol-4^T in group 1 has identical patterns of DNA fragmentation with the strains in group 2. Groups 3 and 4 have very different patterns although they share 45% DNA similarity. Each of the remaining strains (Td3, Td19, MF62, MF107 and MF118) has a distinctive DNA fragment pattern.

Growth substrates

Growth on various carbon substrates was tested under aerobic and denitrifying conditions. All the strains can grow both under aerobic and denitrifying conditions

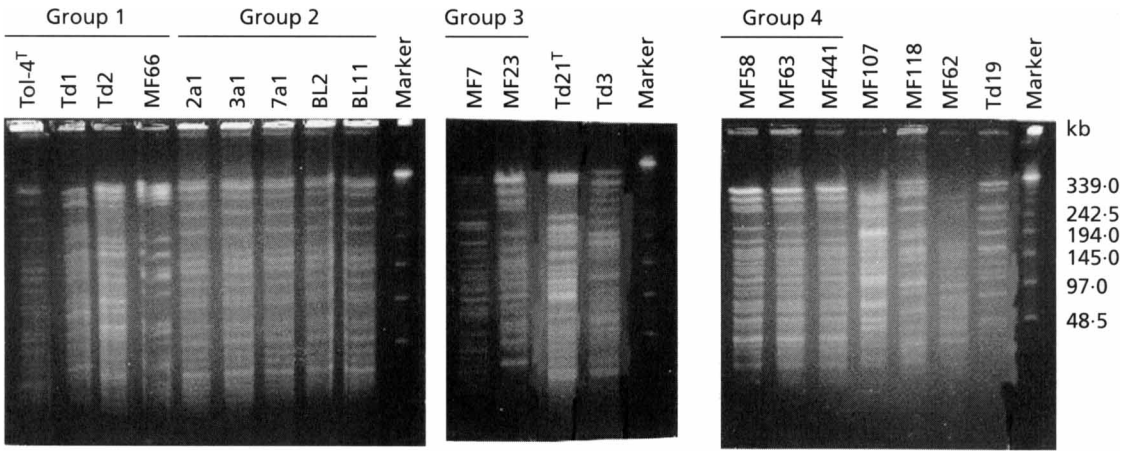

Fig. 7. Fragment separation of *Xba*I digests of chromosomes from aromatic-compound-degrading denitrifying bacteria using contour-clamped homogeneous electric field gel electrophoresis with a ramped pulse time of 1–25 s for 23 h (1·3% agarose).

Table 4. Growth of strains belonging to the species groups on various carbon sources under aerobic and denitrifying conditions

+, Good growth; ±, moderate growth; −, no growth; ND, not determined.

	Group 1								Group 2									
	Tol-4^T		Td1		Td2		MF66		2a1		3a1		7a1		BL2		BL11	
	O_2	NO_3^-	O_2	NO_3^-	O_2	NO_3^-	O_2	NO_3^-	O_2	NO_3^-	O_2	NO_3^-	O_2	NO_3^-	O_2	NO_3^-	O_2	NO_3^-
Caproate	+	+	+	+	−	−	−	−	+	+	−	+	+	−	+	+	+	+
D-Fructose	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+
Fumarate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	±
3-Hydroxybenzoate	+	+	−	+	−	−	−	−	+	+	+	+	−	−	−	−	−	−
4-Hydroxyphenylacetate	−	+	+	+	−	−	−	−	+	+	+	+	−	−	−	−	−	−
Phenol	ND	±	ND	±	ND	±	+	+	ND	±	ND	±	ND	±	ND	−	ND	+
Phenylacetate	+	+	+	+	−	−	−	−	+	+	+	+	−	−	+	+	+	+
Propionate	+	+	−	+	−	−	−	−	+	−	−	−	−	−	+	+	−	−
Toluene	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Valerate	−	−	+	+	−	+	−	−	+	+	−	+	+	+	−	+	+	−
Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Phenylalanine	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−

	Group 3				Group 4				Group 5					
	MF7		MF23		MF58		MF63^T		MF441		Td17		Td21^T	
	O_2	NO_3^-	O_2	NO_3^-	O_2	NO_3^-	O_2	NO_3^-	O_2	NO_3^-	O_2	NO_3^-	O_2	NO_3^-
Caproate	−	−	−	−	−	−	−	−	−	−	−	−	+	+
D-Fructose	−	−	−	−	+	−	+	+	+	+	−	−	−	+
Fumarate	−	−	−	−	−	−	−	+	+	+	+	+	+	+
D-Glucose	+	+	−	−	−	−	+	±	+	±	−	−	−	−
3-Hydroxybenzoate	−	−	−	−	−	−	−	−	−	−	−	−	−	−
4-Hydroxyphenylacetate	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Phenol	−	−	−	−	+	+	−	−	ND	+	ND	+	ND	+
Phenylacetate	−	−	−	−	−	−	−	+	−	+	+	+	+	+
Propionate	−	−	−	−	−	−	−	−	−	−	−	−	+	−
Toluene	+	+	−	−	+	+	−	+	−	+	+	+	+	+
Valerate	−	−	−	−	−	−	−	−	−	+	−	+	+	−
Lactose	+	+	+	+	+	+	+	+	+	+	+	−	−	+
Phenylalanine	−	−	+	+	+	+	−	−	+	+	±	±	±	+

on acetate, adipate, arginine, L-arabinose, aspartate, benzoate, D-galactose, lactate, maltose, mannitol, proline, pyruvate, D-ribose, succinate, sucrose or D-xylose. Under denitrifying conditions, 4-hydroxybenzoate was used by all strains. None of them grew on acetamide, histamine, 2-hydroxybenzoate, starch or tartrate under either condition. Only strain MF62 utilized phthalate under denitrifying conditions. Butyrate and toluene were used by all strains except strain MF23, under denitrifying conditions. The variations in the utilization of substrates by strains of different groups are given in Table 4. Although it is true that strains of groups 1, 2 and 5 can grow at the expense of a larger number of different substrates than the strains of groups 3 and 4, from the available data, it is impossible to define characteristic nutritional phenotypes.

Conclusions

The strains in this study can be classified into three species and five separate taxa on the basis of the combination of all the analyses performed. Group 1 consists of strains Td1, Td2, MF66 and Tol-4^T, the latter being the type strain of the species *A. tolulyticus* (Zhou *et al.*, 1995). Thus, group 1 should conserve this species name. Group 2 includes isolates from Northern Michigan and it is closely related to group 1 on the basis of DNA similarity, which is shown graphically in

the three-dimensional representation, in the protein banding pattern and DNA fragmentations, and in the phylogenetic tree of 16S rRNA. The results of the fatty acid analyses and the nutritional screening do not further support the results of the above-mentioned approaches. Thus, group 2 could be considered to be a genomovar of *A. tolulyticus* on the basis of criteria defined by Ursing *et al.* (1995).

Groups 3 (strains MF7 and MF23) and 4 (strains MF58, MF63^T and MF441), which consist of isolates from Moffett Field, have 47–55% DNA similarity with each other and low similarities with other groups. They form a tight group, as suggested by the fatty acid composition, and show very similar protein profiles and DNA fragment patterns (except for strain MF7) and less nutritional versatility than the other groups. In addition, strains MF7 and MF63^T from group 3 and group 4, respectively, form a cluster on the basis of 16S rRNA gene sequence analysis. Thus, groups 3 and 4 should be considered as a distinct species and a genomovar, respectively, of the genus *Azoarcus*.

Group 5 (strains Td17 and Td21^T), previously considered to be *A. tolulyticus* (Zhou *et al.*, 1995), has low DNA similarity and different protein and DNA fragment patterns to group 1 (*A. tolulyticus*) and is well separated from group 1 in the phylogenetic tree and dendrogram of fatty acids analysis. Thus, this group should be considered as a new species of the genus *Azoarcus*.

The remaining strains (Td3, Td19, MF62, MF107 and MF118) do not cluster with any of the main groups 1 through 5. Each of them could represent an individual species. Strains MF107 and MF118 have 50% DNA similarity with group 1 and 40% DNA similarity with group 3 as well as similar protein patterns to groups 1 and 3. Thus, they may be included in the cluster of the genus *Azoarcus* as individual new species related to either group 1 or group 3. Strains Td3 and Td19, previously reported as the species *A. tolulyticus*, have about 30% DNA similarity with groups 1 and 2, which constitute the species *A. tolulyticus*, and low DNA similarity with any of the other groups. 16S rRNA gene sequence analysis of these strains shows a difference with the species *A. tolulyticus*, but they still remain in the cluster of the genus *Azoarcus*. This observation correlates with the analysis of fatty acid profiles. Thus, these separate strains might be considered as individual species in the genus *Azoarcus* and named in the future. Strain MF62 has no relatedness with any of the strains within the genus *Azoarcus*.

In addition to the experiments reported above, *A. evansii* strain KB 740^T was used as a reference strain in the DNA–DNA hybridization experiments. These were done to test its relationship to strains MF118, Td3 and *A. tolulyticus* (strains Td1 and MF66). Strains KB 740^T and MF118 are very similar in terms of cellular fatty acid composition, but the protein profiles are different and the DNA similarity is low (31%). On the other hand, strain Td3 has high 16S rRNA sequence similarity with strain KB 740^T (Fig. 3; Table 3) and the DNA similarity value is relatively high (52%), but both the protein profiles and the fatty acid composition are quite different. A similar value for DNA similarity (57%) has been obtained between KB 740^T and *A. tolulyticus* (strains Td1 and MF66), although there are clear differences both in protein profiles and fatty acid composition. These facts suggest the possibility that *A. evansii* may actually be a sibling and not a genomovar of both strain Td3 and *A. tolulyticus*.

It is clear that the various *Azoarcus* species groups show considerable internal diversity and a wide geographical and ecological distribution (Table 1). They were isolated from soils as well as from freshwater and marine sediments in Michigan, Washington, California and Brazil. For the moment, the identification of additional strains isolated after similar enrichment procedures may require the application of molecular methods, particularly DNA–DNA reassociation studies, supplemented if possible with whole-cell protein and DNA restriction fragmentation studies, using as standards preparations obtained from members of the taxa described here.

New strains assigned to the species *Azoarcus tolulyticus*

One of the main physiological characteristics of the species *A. tolulyticus* is its capacity for toluene degradation under denitrifying conditions. The description of species *A. tolulyticus* was reported previously (Zhou *et al.*, 1995). The cells of strain MF66 of *Azoarcus tolulyticus* are short motile rods with monopolar flagellation. This strain can grow under aerobic and denitrifying conditions with acetate, benzoate, butyrate, D-fructose, fumarate, D-glucose, phenol, pyruvate, succinate, toluene, D-xylose, L-arabinose, D-ribose, D-galactose, sucrose, lactose, maltose, adipate, lactate, mannitol, aspartate, proline, phenylalanine or arginine. Under denitrifying conditions, 4-hydroxybenzoate is used as a growth substrate. The cells are oxidase-positive and catalase-negative and grow on brain-heart infusion plates, but do not grow on nutrient and trypticase soy agar plates. The predominant fatty acids are 16:0 and 16:1ω7*c*, similar to the other members of the species *A. tolulyticus*. Therefore, it belongs to the species *A. tolulyticus* based on 16S rRNA gene sequence analysis, DNA–DNA hybridization, similar patterns of whole-cell proteins and genomic DNA analysis.

The isolates from Northern Michigan (strains 2a1, 3a1, 7a1, BL2 and BL11) are short motile rods with monopolar flagellation They can grow on acetate, benzoate, butyrate, D-fructose, fumarate, D-glucose, pyruvate, succinate, D-xylose, L-arabinose, D-ribose, D-galactose, sucrose, lactose, maltose, adipate, lactate, mannitol, aspartate, proline or arginine under aerobic and denitrifying conditions and use 4-hydroxybenzoate and toluene for growth under denitrifying

conditions. They grow on brain-heart infusion, nutrient and trypticase soy agar plates and give positive reactions in the catalase and oxidase tests. The different colony morphology of these strains on half-strength trypticase soy agar plus nitrate at 30 °C for 48 h has been reported (Chee-Sanford *et al.*, 1996). The predominant fatty acids are 16:0 and 16:1ω7*c*. Whole-cell protein and genomic DNA fragmentation analyses show identical patterns to the other members of *A. tolulyticus* and 16S rRNA sequence analysis of one strain (BL11) shows a close relationship with the members of *A. tolulyticus*. Thus, we conclude that they belong to the species *A. tolulyticus*, although the DNA homologies between these isolates and the strains of *A. tolulyticus* are about 55%; therefore, they constitute a genomovar of this species.

Description of *Azoarcus toluvorans* sp. nov.

Azoarcus toluvorans (to.lu.vo'rans. N.L. n. Fr. Sp. *tolu* balsam from Santiago de Tolu, toluene; L. part. adj. *vorans* devouring; M.L. part. adj. *toluvorans* toluene-devouring).

The species is represented in our collection by strains Td17 and Td21^T, which were previously classified as *A. tolulyticus*. The new designation is justified on the basis of 16S rRNA gene sequence analysis, DNA–DNA hybridization results and characteristic patterns of whole-cell proteins. Both strains grow on brain-heart infusion, nutrient and trypticase soy agar. They can use as sole sources of carbon and energy acetate, benzoate, butyrate, fumarate, phenylacetate, pyruvate, succinate, toluene, D-xylose, L-arabinose, D-ribose, D-galactose, sucrose, maltose, adipate, lactate, mannitol, aspartate, proline, phenylalanine or arginine under aerobic and denitrifying conditions. Both strains can use benzene or ethylbenzene under aerobic conditions only and 4-hydroxybenzoate or phenol under denitrifying conditions. The G+C content of the DNA is about 67·8 mol%. Other characteristics are the same as described previously (Fries *et al.*, 1994). The type strain is Td21^T (= ATCC 700604^T).

Description of *Azoarcus toluclasticus* sp. nov.

Azoarcus toluclasticus (to.lu.clas'ti.cus. N.L. n. Fr. Sp. *tolu* balsam from Santiago de Tolu, toluene; Gr. adj. *clasticus* breaking; M.L. adj. *toluclasticus* toluene-breaking).

The members of groups 3 and 4 (strains MF7, MF23, MF58, MF63^T and MF441) among the isolates from Moffett Field are assigned to this new species. The strains are short motile rods with polar monotrichous flagellation and can grow on acetate, benzoate, pyruvate, succinate, D-xylose, L-arabinose, D-ribose, D-galactose, sucrose, lactose, maltose, adipate, lactate, mannitol, aspartate, proline or arginine under aerobic and denitrifying conditions. All strains except strain MF23 can use toluene as a growth substrate and strains MF58 and MF63^T can also grow on phenol under denitrifying conditions. The strains grow on brain-heart infusion, nutrient and trypticase soy agar, except for strain MF63^T, which does not grow on nutrient agar. The oxidase test is positive and the catalase test is negative. The predominant fatty acids are 16:0 and 16:1ω7*c*. The G+C content of the DNA is about 67·3 mol%. Phylogenetic analyses of 16S rRNA and DNA–DNA hybridization show the relatedness of strains to the genus *Azoarcus* and the differences from the species *A. tolulyticus*. Thus, strains MF58, MF63^T and MF441 are considered to be members of a new species, *Azoarcus toluclasticus*, and strains MF7 and MF23 represent a genomovar of this species. The type strain is MF63^T (= ATCC 700605^T).

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