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ORIGINAL PAPER

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Phylogenetic and metabolic diversity of bacteria degrading aromatic compounds under denitrifying conditions, and description of *Thauera phenylacetica* sp. nov., *Thauera aminoaromatica* sp. nov., and *Azoarcus buckelii* sp. nov.

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Abstract Six strains of denitrifying bacteria isolated from various oxic and anoxic habitats on different monocyclic aromatic substrates were characterized by sequencing 16S rRNA genes, determining physiological and morphological traits, and DNA-DNA hybridization. According to these criteria, strains S100, SP and LG356 were identified as members of *Thauera aromatica*. Strains B5–1 and B5–2 were tentatively affiliated to the species *Azoarcus tolulyticus*. Strains B4P and S2 were only distantly related to each other and to other described *Thauera* species. These two strains are proposed as the type strains of two new species, *Thauera phenylacetica* sp. nov. and *Thauera aminoaromatica* sp. nov., respectively. By 16S rRNA gene analysis, strain U120 was highly related to the type strains of *Azoarcus evansii* and *Azoarcus anaerobius*, whereas corresponding DNA-DNA reassociation values indicated only a low degree of genomic relatedness. Based upon a low DNA similarity value and the presence of distinguishing physiological properties, strain U120 is proposed as the type strain of a new species, *Azoarcus buckelii* sp. nov. Almost all of the new isolates were obtained with different substrates. The highly varied substrate spectra of the isolates indicates that an even higher diversity of denitrifying bacteria degrading aromatic compounds would be discovered in the different habitats by using a larger spectrum of aromatic substrates for enrichment and isolation.

Keywords *Thauera* · *Azoarcus* · *Thauera phenylacetica* sp. nov. · *Thauera aminoaromatica* sp. nov. · *Azoarcus buckelii* sp. nov. · Nitrate reduction · Aromatic compounds · anaerobic degradation

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Introduction

Aromatic compounds are structurally complex and represent one of the most diverse groups of organic substrates for microorganisms. They are widespread in the environment and are natural products of plants (Harborne, 1980), microorganisms and animals; they are also released as a result of industrial processes. Tarvin and Buswell (1934) were the first to show that monocyclic aromatic compounds were degraded to methane and carbon dioxide under anoxic conditions. Since then, numerous investigations have demonstrated that aromatic compounds can be degraded to CO_2 or volatile fatty acids under anoxic conditions by sulfate-reducing, nitrate-reducing, ferric-iron-reducing, photosynthetic, or fermenting bacteria.

Denitrifyers use a wide variety of aromatic substrates under oxic or denitrifying conditions, such as benzoate and its hydroxylated derivatives, phenolic compounds, aromatic hydrocarbons, and halogenated benzoates, but not polyhalogenated aromatic compounds (Anders et al. 1995; Bakker 1977; Gallus et al. 1997; Khoury et al. 1992; Rabus and Widdel 1995; Rudolphi et al. 1991; Seyfried et al. 1991; Song et al. 1998, 1999, 2000, 2001; Springer et al. 1998; Tschech and Fuchs 1987; Zhou et al. 1995). Rockne et al. (1999) reported a nitrate-dependent anoxic degradation of naphthalene by pure cultures. These bacteria are capable of also using a variety of organic non-aromatic substrates such as alcohols, aliphatic hydrocarbons, amino acids, and organic acids; only few sugars are used. Some of these bacteria exhibit other interesting properties, such as N_2 fixation (Anders et al. 1995) and aerobic denitrification (Scholten et al. 1999).

Most of the aromatic compounds are converted via different peripheral pathways to the central intermediate benzoyl-CoA (Harwood et al. 1999; Heider and Fuchs 1997a, b), which is further reduced to a cyclic non-aromatic compound and cleaved hydrolytically. However, the

anaerobic degradation of α-resorcylate and resorcinol proceeds via a different pathway, which does not involve benzoyl-CoA but hydroxyquinol (Gallus et al. 1997).

Here, we report the isolation of eight strains of denitrifying bacteria that degrade aromatic compounds. The strains were characterized by DNA-DNA hybridization, 16S rRNA gene sequence analysis and physiological, morphological and biochemical characteristics. These bacteria were enriched from different oxic or anoxic soil or sludge samples under denitrifying conditions with aromatic substrates and were isolated using mesophilic conditions and slightly alkaline, phosphate-buffered mineral salt medium. Some of the isolates were used before in studies of anoxic metabolism of phenol and cresols (Rudolphi et al. 1991) and phenylacetic acids (Seyfried et al. 1991), but their taxonomic status and many properties were unknown. As reference strains for comparison we used the β-Proteobacteria *Thauera aromatica* strain K172^T (DSM 6984^T), *Azoarcus evansii* strain KB740^T (DSM 6898^T), and *Azoarcus* strain EbN1 (Anders et al. 1995; Rabus and Widdel 1995).

Materials and methods

Cultivation and growth media

The medium used for enrichment, isolation and routine cultivation contained per liter of distilled water: 1.08 g KH_2PO_4, 5.6 g K_2HPO_4, 0.54 g NH_4Cl, 0.15 g $CaCl_2 \cdot 2H_2O$, 0.2 g $MgSO_4 \cdot 7H_2O$, 1.27 g $NaNO_3$, 1 ml trace element solution SL-10 (Widdel et al. 1983), 1 ml selenite/tungstate solution (Tschech and Pfennig 1984), and 1 ml vitamin solution VL-7 (Pfennig 1978). The medium was dispensed into 150-ml bottles (100 ml) or into 18-ml Hungate tubes (10 ml) (Bellco Glass, Vineland, N.J.) and made anoxic by applying 10 cycles (3 min per cycle) of vacuum and flushing with oxygen-free nitrogen gas at room temperature. The pH of the growth media was varied by adding sterile HCl, Na_2CO_3, or $NaHCO_3$ solution.

Substrate utilization

To determine the substrate spectrum for all strains, the following compounds were tested at 28 °C as the sole carbon and energy sources, either under denitrifying conditions with 10 mM sodium nitrate or under oxic conditions, at the indicated concentration (in mM): benzoate (2.5), 2-hydroxybenzoate (2.5), 3-hydroxybenzoate (2.5), 4-hydroxybenzoate (2.5), 2-aminobenzoate (2.5), 3-aminobenzoate (2.5), 4-aminobenzoate (2.5), 2-methylbenzoate (1), 3-methylbenzoate (1), 4-methylbenzoate (1), 2-fluorobenzoate (2.5), hydroquinone (1), resorcinol (1), catechol (1), indoleacetate (1), phenol (1), *o*-cresol (1), *p*-cresol (1), *m*-cresol (1), adipate (2.5), pimelate (2.5), cyclohexanecarboxylate (2.5), L-lactate (2.5), D-glucose (2.5), D-fructose (2.5), acetate (2.5), succinate (2.5), gentisate (1), protochatechuate (1), toluene (1), *o*-phthalate (2.5), L-tyrosine (2.5), L-tryptophan (2.5), L-phenylalanine (2.5), benzaldehyde (1), *p*-anisic acid (1), phenylacetate (1), phenylpropionate (1), 4-hydroxyphenylacetate (1), sucrose (2.5), maltose (2.5) and acetone (2.5).

Electron acceptors utilization and nitrogen fixation test

Sulfate, thiosulfate, sulfite, nitrate, nitrite, oxygen and fumarate were tested as electron acceptors at a final concentration of 5 mM with acetate (20 mM). Nitrogen fixation was tested under microoxic conditions using a semi-solid (0.8% agar) growth medium lacking nitrogen. A growth zone of a few millimeters below the surface was taken as a sign for nitrogen fixation. This test was complemented by the acetylene reduction assay with liquid media (Postgate 1972).

Analytical methods

Growth was measured spectrophotometrically at 578 nm (1-cm light path). Aromatic compounds were determined using HPLC equipped with a UV detector set at 280 nm using an Ultrasphere (4.6×250 mm, 5-μm particle size) column (Beckman) maintained at room temperature. The mobile phase comprising a mixture of two solvents, (1) water and (2) 0.01% (v/v) acetic acid in 50% (v/v) acetonitrile, was used at a flow rate of 1 ml min^{-1}. The solvent phase (25% (v/v) acetonitrile) was initially held for 20 min, then the concentration increased to 50% over a period of 5 min and held for 5 min. The column was re-equilibrated with 25% acetonitrile for at least 5 min before the next injection. Quantification was carried out using external standards. Nitrate and nitrite were estimated using the Quantofix test (Macherey-Nagel). N_2O in the gas phase of the Hungate tubes was determined at 26 °C by gas chromatography (Neyra et al. 1977), and the total amount of N_2O produced was calculated taking into account the gas and liquid space and assuming that 24 mM N_2O is dissolved at 26 °C at 1 bar pressure.

Determination of G+C content

The DNA was isolated and purified by chromatography on hydroxyapatite, and the G+C content was determined by HPLC (Mesbah et al. 1989). Nonmethylated lambda phage DNA (Sigma) was used as the standard.

DNA-DNA hybridization

DNA was isolated by the method of Cashion et al. (1977). DNA-DNA hybridization was carried out as described by De Ley et al. (1970), with the modification described by Escara and Hutton (1980) and Huss et al. (1983) using a Gilford System model 2600 equipped with a Gilford model 2527-R thermoprogrammer and plotter. Renaturation rates were computed with the TRANSFER.BAS program of Jahnke and Bahnweg (1986) and Jahnke (1992).

Whole-cell proteins electrophoresis

Cells of all tested strains were grown aerobically in liquid mineral medium supplemented with 10 mM acetate at 30 °C. Cells were harvested in the mid-exponential growth phase and passed through a French pressure cell, followed by centrifugation (100,000×*g*, 40 min). The concentration of soluble protein was measured according to the method of Bradford (1976), and the same amount of protein from all strains was loaded onto an SDS-(12%) polyacrylamide gel and separated by electrophoresis. Proteins were stained with Coomassie blue.

Immunoblotting

Extracts from cells grown on benzoate under anoxic nitrate-reducing conditions were separated by SDS-PAGE, and proteins were blotted onto a nitrocellulose sheet (Scheicher and Schüll) using a multiphor system (Pharmacia). Proteins were detected by immunostaining according to Rao et al. (1983). The antiserum used was prepared against purified native benzoyl-CoA reductase from *T. aromatica*. This antiserum was shown to react specifically with the four subunits αβγδ of the benzoyl-CoA reductase and showed the strongest interaction with the α-subunit (Heider et al. 1998).

Table 1 Characteristics of *Thauera aromatica* strain K172^T (Anders et al. 1995) and the *Thauera* sp. strain LG356 (this study), strain S100 (Tschech and Fuchs 1987, and this study), and strain SP, strain B4P, and strain S2 (Seyfried et al. 1990, and this study). + Positive or present, – negative or absent, *nd* not determined

Properties	Strain S100	Strain SP	Strain LG356	Strain K172^T	Strain B4P	Strain S2
Cell morphology	Straight rods	Straight rods	Straight rods	Coccoid rods	Oval rods to coccoid	Straight rods
Cell size (μm)	0.5–0.6×1.1–2.2	0.5–0.75×1.5–2.5	0.5–0.75×1.5–2	0.5–1.5×1–2.5	0.75–1×1.5–2	0.5–0.75×2-3
Motility	+	+	+	+	+	+
pH optimum	7–7.4	7–7.6	7.2–7.6	7–7.4	7–7.5	7.5–8.6
Temperature optimum (°C)	28	28	28	28	28	28
Growth factor requirement	None	Vitamin B12	None	None	*p*-Amino-benzoate	None
Catalase reaction	+	+	+	+	+	+
Oxidase reaction	+	+	+	+	+	+
Oxidative metabolism	+	+	+	+	+	+
G+C content (mol%)	nd	68	63.2	67	70.6	68.4
Substrate utilization under denitrifying conditions:						
Benzoate	+	+	+	+	+	+
4-OH benzoate	+	+	+	+	+	+
2-OH benzoate	+	–	–	+	–	+
3-OH benzoate	+	+	+	+	+	–
Phenylpropionate	+	+	–	nd	+	–
Phenylacetate	+	+	–	+	+	+
4-OH phenylacetate	+	+	–	+	+	+
Indoleyl 3-acetate	+	+	–	+	–	+
Indole	+	–	–	+	–	+
2-Aminobenzoate	+	+	–	+	–	+
p-Cresol	+	+	–	+	+	+
Phenol	+	+	–	+	–	+
Toluene	–	+	+	+	–	+
Adipate, pimelate	–	–	–	–	–	+

16S rRNA gene sequencing and phylogenetic analysis

Genomic DNA extraction, PCR-mediated amplification of the 16S rRNA gene, and sequencing of PCR products were carried out as described by Rainey et al. (1996). Purified PCR products were sequenced directly using the *Taq* DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions. The Applied Biosystems 310 DNA Genetic Analyzer was used for electrophoresis of the sequence reaction products.

The almost complete 16S rRNA gene sequences of the isolates were aligned manually with those of all currently available nucleotide sequences of representatives of β-Proteobacteria retrieved from GenBank and EMBL databases using the ae2 editor (Maidak et al. 1997). The method of Jukes and Cantor (1969) was used to calculate evolutionary distances. Phylogenetic dendrograms were reconstructed according to the method of DeSoete (1983) and the neighbor-joining and maximum-likelihood methods contained in the PHYLIP package (Felsenstein 1993). The 16S rRNA gene sequences have been deposited with EMBL under accession numbers AJ315676 (U120), AJ315678 (BP4), AJ315677 (S2), AJ315681 (S100), AJ315680 (Lg356) and AJ315679 (SP).

Results

Enrichment and isolation of strains

Several strains of denitrifying bacteria were isolated in previous studies of anaerobic aromatic metabolism, but these strains were not further characterized. Strain U120 was enriched and isolated from an oxic soil sample (Ulm, Germany) in the medium described by Tschech and Fuchs (1987), on 1 mM *o*-cresol as carbon source and 5 mM nitrate as electron acceptor (Rudolphi et al. 1991). Strain S100 was enriched and isolated from anoxic sludge (Konstanz, Germany) on 1 mM phenol as carbon source and 5 mM nitrate as electron acceptor (Tschech and Fuchs 1987). Strains SP and S2 were enriched from anoxic ditch sludge (Konstanz, Germany) and B4P from activated sludge from a sewage treatment plant (Ulm, Germany) on nitrate and the aromatic amino acids L-phenylalanine (SP), L-tryptophan (S2), and L-tyrosine (B4P) first, then transferred to new enrichment media containing nitrate and phenylacetate (SP), salicylate (S2), and 4-hydroxyphenylacetate (B4P) (Seyfried et al. 1991).

Furthermore, strain LG356 was enriched and isolated from the activated sludge of a wastewater treatment plant (Ulm, Germany) on 1 mM phenylacetate and 5 mM nitrate; strains B5–1 and B5–2 were enriched and isolated from an oxic soil sample (Ulm, Germany) on 1 mM 3-methylbenzoate and 5 mM nitrate.

Morphological and physiological properties

Different morphological and physiological features of the isolated strains were studied (Tables 1 and 2). All tested strains were gram-negative, oxidase- and catalase-positive, motile, and able to grow with oxygen, nitrate and nitrite as electron acceptor in the presence of acetate. All strains could reduce nitrate to N_2, but only strains B5–1 and B5–2 were capable of nitrogen fixation; this feature was also checked in *A. evansii* strain KB 740^T and was confirmed to be positive. All strains except strain S2 produced large amounts of N_2O, corresponding to 16–53% of the nitrate consumed being transformed to N_2O.

The temperature optimum for growth of all strains was between 28 and 30 °C; no growth was observed below 4 °C or above 40 °C. The pH optimum for growth was between 7 and 7.6 with a pH range for growth from 6.5 to 8.6.

The cell morphology of the various strains is shown in Figure 1; the cell sizes are given in Tables 1 and 2. Cells of strains SP, LG356, S2, and S100 were straight short rods; cells of strain B4P were oval rods to coccoid; cells of strain U120 were coccoid of irregular size, and cells of strains B5–1 and B5–2 were coccoid or short rods. For comparison, cells of *T. aromatica* strain K172^T and *A. evansii* strain KB740^T are also shown in Fig. 1.

Growth in solid media seemed to be affected by the agar source. Strain B4P and *A. evansii* strain KB740^T grew better on a medium made with purified Difco agar, while T. aromatica strain K172^T and strains SP and LG356 grew better on a medium made with purified Oxoid agar. Strains U120, B5–1, B5–2 and S100 are able to grow equally well on both types of agar. The different growth response may be explained by different sensitivity towards impurities in the agar.

Tables 1 and 2 describe some other physiological properties such as catalase and oxidase reaction and substrate utilization under denitrifying conditions.

Comparison of the protein electrophoretic pattern

All tested strains were grown aerobically on acetate, and extracts were prepared from cells of the exponential growth phase. The soluble protein profiles were examined by SDS-PAGE (Fig. 2). The observed protein bands showed two different pattern groups. The first group is constituted by strains phylogenetically related to *T. aromatica*, and the second group by strains related to *A. evansii* (see below). Within the *Thauera* group, the protein pattern of strain K172^T is similar to that of strains S100, SP, and LG356. The protein profiles of strains B4P and S2

Table 2 Characteristics of *Azoarcus evansii* strain KB740^T (Anders et al. 1995), strain EBN1 (Rabus and Widdel 1995), strain strain B5-1 (this study), strain U120 (Rudolphi et al. 1991, and this study), and *Azoarcus anaerobius* DSM 12081^T (Springer et al. 1998). + Positive reaction or present, – negative or absent, *nr* not reported, *nd* not determined

Characteristics	Strain U120	Strain B5-1	Strain EBN1	DSM 12081^T	Strain KB740^T
Cell morphology	Cocci	Short rods	Oval to short rods	Short rods	Rods
Cell size (µm)	0.5 ±0.5	0.5–0.6×1	0.6–0.8×1.5–2.5	2.7–3.3×1.5	0.6–0.8×1.5–3.0
N_2-fixation	–	+	nr	–	+
Electron acceptors	Nitrate, nitrite, and O_2	Nitrate, nitrite and O_2	Nitrate, nitrite and O_2	Nitrate only	Nitrate, nitrite and O_2
G+C content (mol%)	66	nd	65	66.5	67.5
Temperature optimum (°C)	28	nd	31	20–32	28–37
pH optimum	7–7.4	nd	7.1–7.4	7.8	7.2
Substrate utilization under denitrifying conditions:					
Toluene	+	+	+	nr	–
Ethylbenzene	–	–	+	nr	–
Phenol	+	–	–	+	–
Recorcinol	–	–	nr	+	–
m-Cresol	–	–	–	–	–
p-Cresol	+	+	–	+	+
o-Cresol	+	–	–	–	–
L-Tyrosine	–	+	–	+	–
L-Phenylalanine	–	+	–	+	–
L-Tryptophan	–	–	–	nr	–
Benzaldehyde	–	+	+	nr	nr
Phenylacetate	–	+	+	+	+

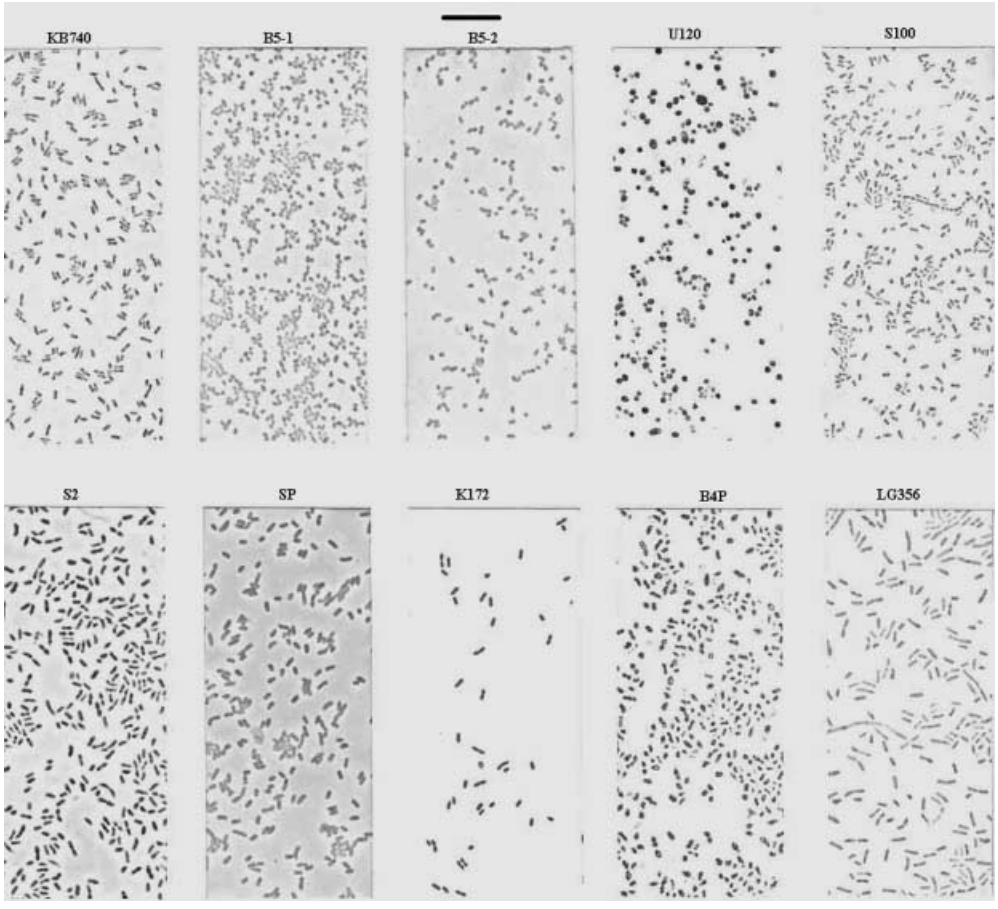

Fig. 1 Phase contrast micrographs of cells in the exponential growth phase of strains: *Upper row (left to right)*: *Azoarcus evansii* strain KB740^T, strain B5–1, strain B5–2, strain U120, strain S100. *Lower row (left to right*: strain S2, strain SP, *Thauera aromatica* strain K172^T, strain B4P, strain LG356. *Bar* 10 μm. Wet mounts for photomicrographs of the microorganisms were made according to Pfennig and Wagener (1986)

differ markedly and also differ from that of all other strains. Three different protein profiles were observed for the strains constituting the *Azoarcus* group (U120, B5–1, and KB740^T). The protein profile of strains B5–1 and B5–2 is more similar to that of strain KB740^T than to that of strain U120. Protein profiles of strains B5–1 and B5–2 are virtually indistinguishable (not shown) suggesting identical strains.

Differentiation of the strains using Western blots and antiserum raised against benzoyl-CoA reductase

An antiserum raised against benzoyl-CoA reductase from *T. aromatica* strain K172^T was used for the immunostaining of proteins of cells grown anaerobically on benzoate and nitrate. The antiserum reacted specifically with the four subunits αβγδ of benzoyl-CoA reductase. Figure 3 shows the Western blot of cell extracts of *T. aromatica* strain K172^T (control), *A. evansii* strain KB740^T, strains S100, SP, S2, B4P, U120, B5–1, and B5–2 separated by SDS-PAGE. The antiserum reacted strongly with four specific protein bands of strains K172^T, S100, SP and LG356 but with only one protein band of the other strains or showed no reaction. This suggests that strains K172^T, S100, SP, and LG356 harbored similar benzoyl-CoA reductases composed of four similar subunits.

Strains KB740^T, S2, B4P, U120, B5–1, and B5–2 contained only one major protein band that cross-reacted with the antiserum. Strain KB740^T contained a small, strongly cross-reacting protein band which might correspond to the smallest (δ-subunit) of the four enzyme subunits; this band also stained the strongest in strain LG 356. Interestingly, strains B5–1 and B5–2 had a strongly staining protein band that might correspond to the second-smallest subunit (γ-subunit) of benzoyl-CoA reductase. Strain U120 did not contain any cross-reacting protein. The differences between *Thauera* and *Azoarcus* species are reflected by considerable differences in the sequences and predicted sizes of the subunits of benzoyl-CoA reductase in *T. aromatica* and *A. evansii* (gene bank accession numbers AJ 224959 and AJ 428529, respectively).

Phylogenetic analysis based on 16S rRNA gene sequence comparison

The almost complete sequences of 16S rRNA gene were aligned with those of related *Thauera* and *Azoarcus* strains. Phylogenetic analysis showed that the strains formed three different clusters (see below) which emerged with any algorithm used. A distance matrix dendrogram is shown in Fig. 4. The first cluster encompasses strains S100, S2, SP, B4P, and LG356 related to *T. aromatica*

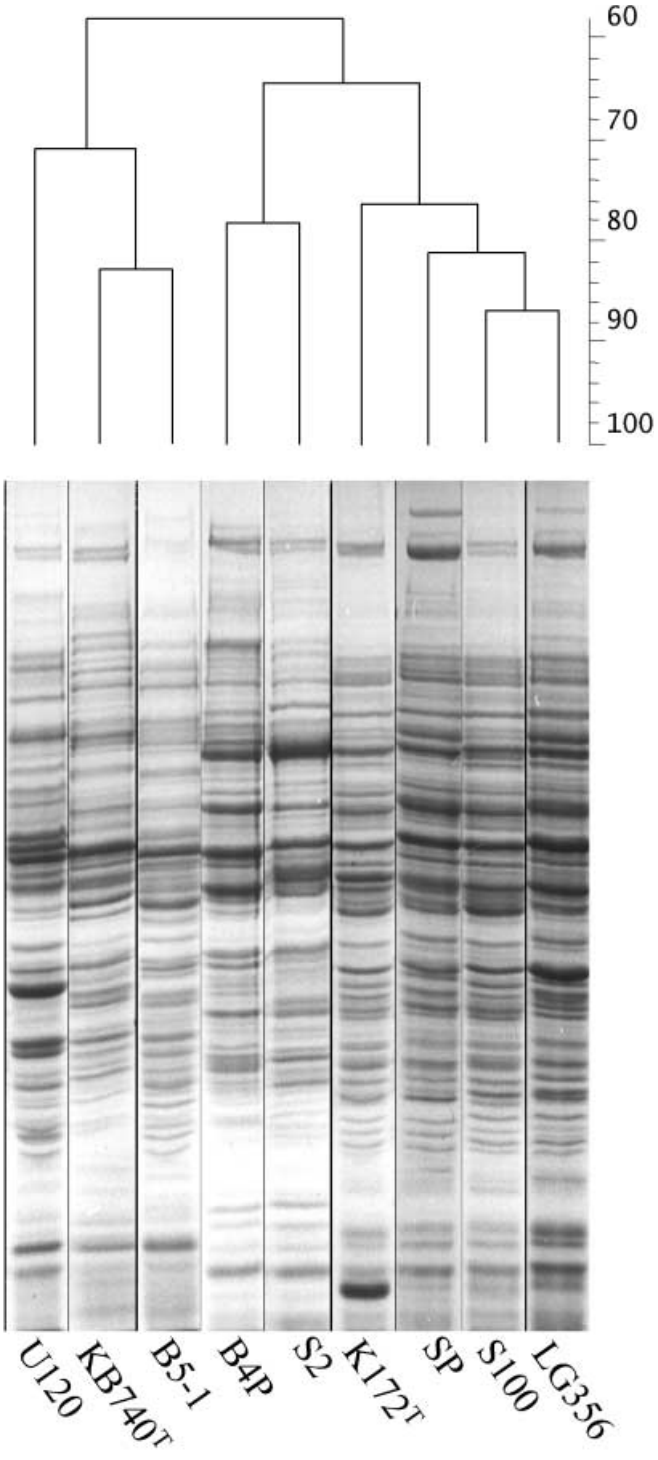

Fig. 2 Electrophoretic pattern of proteins of the cell extract (100,000×*g* supernatant) from the denitrifying strains. The lanes are labeled according to the respective strain designations. Dendrogram parameters: The algorithms used are: Pearson product-moment correlation between densitometric profiles obtained from the patterns, to generate a similarity matrix; unweighted pair-group method with arithmetic averages (UPGMA), to calculate the dendrogram from the similarity matrix. The *scale bar* refers to similarity values. Pearson correlation optimization: 0.21% range (0.0%–100.0%)

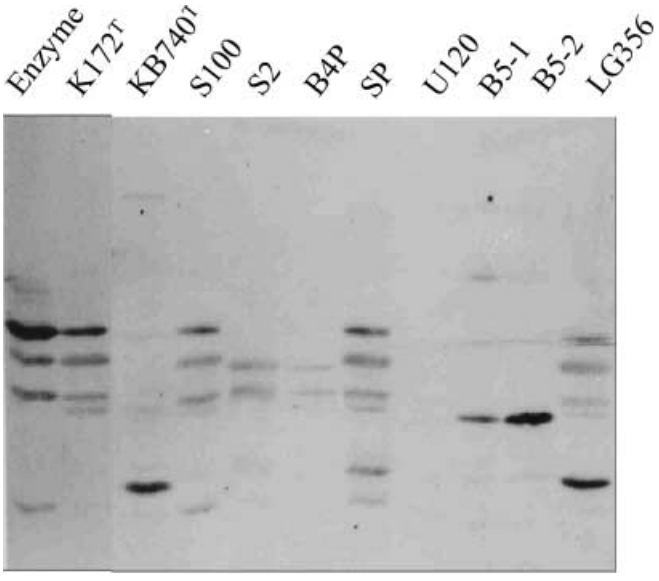

Fig. 3 Western immunoblot analysis of cell extracts from different strains grown on benzoate under anoxic denitrifying conditions. Antiserum raised against purified benzoyl-CoA reductase from *T. aromatica* strain K172^T was used. Enzyme refers to purified benzoyl-CoA reductase (positive control); other lanes contain cell extract from strains as indicated

strain K172^T, with similarity values of 100, 99.1, 99.7, 99.7, and 98.8%, respectively. The second group encompasses strains U120 and EbN1 related to *Azoarcus anaerobius*, with similarity values of 99.1% and 98.5%, respectively, and to A. *evansii* strain KB740^T, with similarity values of 96.6% and 96.5%, respectively. The third cluster encompasses strains B5–1 and B5–2 related to *A. tolulyticus* strain Td1 (not shown), with a similarity value of 99.9%.

DNA-DNA hybridization

The strains were phylogenetically closely related, and the similarity of their 16S rRNA gene sequence was higher than the threshold value that would allow unambiguous discrimination between species from the same genus. Therefore, DNA-DNA hybridization experiments were done using DNA from various strains and DNA from *T. aromatica* strain K172^T as reference (two measurements). The observed amount of DNA-DNA hybridization between *T. aromatica* strain K172^T and strains SP, LG356 and S100 (72.4–76, 64.4–64.0, and 90.9–88.0%, respectively) indicates membership in the species *T. aromatica*. Further hybridization experiments with strains SP and LG356 showed 75% DNA-DNA hybridization with each other. Strains B4P and S2, on the other hand, are moderately related to *T. aromatica* strains, as the amount of DNA hybridization with DNA of strain K172^T is low, i.e. 31–33.8% and 26.9–41%, respectively. The level of DNA hybridization between strains B4P and S2 is low (39.2%), indicating the presence of separate genomic entities worth of species status.

Strain U120 showed 50–57% DNA similarity values with strain EbN1. These two strains were moderately re-

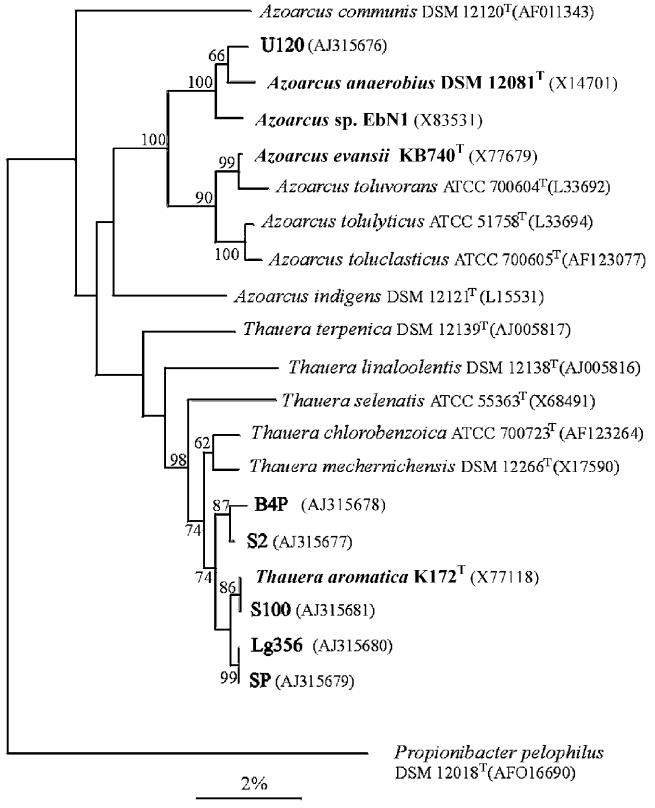

Fig. 4 16S-rRNA gene-based phylogenetic tree showing relationships between the aromatic-compounds-degrading nitrate-reducing strains U120, S2, B4P, S100, SP, and LG356 and their closest relatives from the genera *Azoarcus* and *Thauera*. Numbers at branching points refer to bootstrap values (1,000 resamplings). *Scale bar* 2 nucleotide substitutions in 100 bases. The names in *bold letters* refer to strains used in this study

lated to *A. anaerobius* (DSM 12081^T) (54.2 and 62.9% DNA similarity, respectively), while the similarity values of strains U120 and EbN1 with *A. evansii* strain KB740^T were almost insignificant (10–25%).

Discussion

Diversity of nitrate-reducing, aromatic-compounds-degrading bacteria and their habitats

We have isolated mesophilic, nitrate-reducing bacteria from anoxic and oxic soil, sediment, and sewage treatment sludge samples that differ significantly in their substrate spectrum and other properties. Still, all are members of the β-subclass of the Proteobacteria and belong to the related genera *Thauera* and *Azoarcus*. Obviously they occur in many types of soil and sediment habitats where they profit from their common ability to metabolize a great variety of low-molecular aromatic and non-aromatic substrates both under oxic and anoxic denitrifying conditions. Almost all of the new isolates were obtained with different substrates. The highly varied substrate spectra of the isolates indicates that an even higher diversity of denitrifying bacteria degrading aromatic compounds would be discovered in the different habitats by using an even larger spectrum of aromatic substrates for enrichment and isolation.

Similar nitrate-reducing, aromatic-compounds-degrading facultative bacteria have been obtained from many other natural habitats. Most of the described strains are neutrophilic (pH optimum for growth 7–8), mesophilic (optimum growth temperature 28–40 °C), and motile (motility or flagella observed). Strains that belong to the genus *Thauera* are *T. aromatica* (Anders et al. 1995), *T. mechernichensis* (Scholten et al. 1999) and *T. chlorobenzoica* (Song et al. 2001; Heider and Fuchs 2002). The validated new species of the genus *Azoarcus* are *A. evansii* (Anders et al. 1995), *A. tolulyticus* (Zhou et al. 1995), *A. anaerobius* (Springer et al. 1998), *A. toluvorans* and *A. toluclasticus* (Song et al. 1999).

A number of nitrate-reducing, aromatic-compounds-degrading bacteria have been isolated and characterized, such as strains EbN1, PbN1, ToN1, and mXyN1, but they were not affiliated to any of the known genera or species (Rabus and Widdel 1995). These strains use under denitrifying conditions ethylbenzene, propylbenzene, toluene, and *m*-xylene, respectively. Meanwhile, the 16S rRNA gene sequences of these strains were made available by Widdel and associates; analysis indicates that strains EbN1 and PbN1 are closely related and form a cluster that branches within *Azoarcus* strains. Strain ToN1 is closely related to *A. evansii* (DSM 6898^T), and strain mXyN1 is closely related to *T. aromatica* (DSM 6984^T).

Within the *T. aromatica* species several strains exhibit differences in their metabolic and physiological characteristics or in their morphology (Heider and Fuchs 2002); this also may hold true for some *Azoarcus* species. For example, *T. aromatica* (DSM 6984^T) forms coccoid rods, while strains 3CB-1 and T1 are rod-shaped. Furthermore, in contrast to strains K172^T and T1, strain 3CB-1 uses benzyl alcohol and caproate but not propionate and toluene, while strains K172^T and T1 were able to use toluene and propionate but not caproate and benzyl alcohol. Strain AR-1 differs from all *T. aromatica* strains by its ability to grow optimally at 37 °C and to use α-resorcylate. Several of the strains studied here also belong to *T. aromatica*, which demonstrates its wide occurrence and versatility.

Bakker (1977) and Khoury et al. (1992) reported the presence of spiral bacteria in a mixed culture degrading phenol under denitrifying conditions, but they did not characterize these bacteria. Recently, Shinoda et al. (2000) reported the isolation of a new spiral bacterial strain of nitrate-reducing bacteria that degrades aromatic compounds and belongs to the genus *Magnetospirillum*, a member of the α-subclass of the Proteobacteria. Furthermore, the naphthalene degrader described by Rockne et al. (1999) is phylogenetically related to *Pseudomonas stutzeri* or to *Vibrio pelagius*.

Comparison of strains B4P, S2, S100, LG356, and SP with *T. aromatica* strain K172^T

Strains characterized here belonging to the genus *Thauera* shared many phenotypic traits. However, several differ-

ences were noticed in this group, as reported in Table 1. While strain S100 is phenotypically close to *T. aromatica*, strains SP and LG356 show some differences. Nevertheless, high DNA similarity values and similar protein profiles support the affiliation of strains S100, SP (DNA similarity value >70%) and strain LG356 (DNA similarity value slightly below 70%) to *T. aromatica*.

Despite the high levels of similarity of their 16S rRNA gene sequences, DNA-DNA hybridization tests and protein profiles indicate that strains B4P and S2 are genotypically different from *T. aromatica*. The DNA similarity value of strains B4P and S2 of 39% and values ranging between 27 and 41% with the type strain of *T. aromatica* indicated the presence of two separate genomic entities. Strain B4P differs from the other strains by its cell shape (Fig. 1); the organism is an oval rod or coccus while cells from other strains are straight rods. Strain B4P is the only organism that requires *p*-aminobenzoate as growth factor; it has the highest G+C content and it differs from the other *Thauera* species also by its substrate spectrum (Table 1) and its protein profile (Fig. 2).

Interestingly, the G+C content (mol%) of DNA of the *Thauera* strains differed significantly, up to 7%, whereas the rRNA gene sequences differed by maximally 1.2%. The large deviation in G+C content may be caused by a shift in nucleotide usage in the non-coding intergenic regions for reasons that are not understood. In the related *A. evansii* it was observed that the intergenic regions of two operons which are present as duplicates on the chromosome differed drastically, whereas the genes varied only slightly, mostly in the wobble position (Schühle et al. 2001).

Strains S2 differs from strain B4P significantly in the substrate spectrum, e.g. it grows well under denitrifying conditions on the dicarboxylic acids pimelate and adipate, and on phenol and toluene. Strain S2 is more similar in its morphology and substrate spectrum to strains S100 and K172^T than to strains B4P, SP, and LG356. However it differs considerably in its protein electrophoretic pattern from strains S100 and K172^T. DNA-DNA hybridization of strain S2 with the type strain *T. aromatica* K172^T and with strain B4P indicated that the three strains were genomically significantly different. We therefore propose strain S2 and B4P as the type strains of new species of the genus *Thauera*, *Thauera aminoaromatica* and *Thauera phenylacetica*.

Comparison of strains U120 and B5 with species belonging to the genus *Azoarcus*

Strain B5–1 is obviously identical with strain B5–2 and shows 99.9% rRNA gene sequence identity with *A. tolulyticus*. Based upon the high 16S rRNA gene sequence similarity with *A. tolulyticus* we tentatively assign the two isolates to this species. The strains are also able to grow under anoxic conditions with toluene. Strain U120 is closely related to members of the genus *Azoarcus* (Fig. 4); the closest species were *A. anaerobius* (DSM 12081^T) (Springer et al. 1998), strain EbN1 (Rabus and Widdel 1995) and *A. evansii* (DSM 6898^T) (Anders et al. 1995). Strain U120 clusters together with *A. anaerobius* DSM 12081^T and strain EbN1, while *A. evansii* (DSM 6898^T) and the toluene-degrading *Azoarcus* species *A. toluvorans*, *A. toluclasticus*, and *A. tolulyticus* form a separate cluster. The intra-cluster rRNA gene similarities range between 98.1 and 99.7% similarity, while members of this cluster show only between 94.5 and 95.2% similarity with the type strains of species branching outside this cluster. This branching pattern is consistent with the dendrogram of relationship shown by Song et al. (2001). Despite the relatively high similarities in the 16S rRNA gene sequences between the *Azoarcus* strains, they showed many physiological and morphological differences. *A. anaerobius* (DSM 12081^T) is an obligately denitrifying bacterium, in contrast to all other *Azoarcus* strains, which can use also nitrite and oxygen as electron acceptors. In addition, *A. anaerobius* strain DSM 12081^T differs from strain U120 by its cells shape and its ability to use resorcinol, phenylalanine, tyrosine and phenylacetate under denitrifying conditions. Strain U120 was differentiated by DNA similarity value from the *Azoarcus* strain EbN1 (50–57%) and from *A. anaerobius* (DSM 12081^T) (54.2%) as well as by protein pattern. Considering the differences between strain U120 and the related *Azoarcus* strains, we propose to describe strain U120 as the type strain of a new *Azoarcus* species, *Azoarcus buckelii*. It should be noted, however, that the denitrifying aromatic-compounds-degrading *Azoarcus* species (some of which also fix nitrogen) cluster separately from the nitrogen-fixing *Azoarcus* species.

Description of *Thauera phenylacetica* sp. nov.

Thauera phenylacetica (phe.nyl.a.ce'ti.ca) M.L. acidum phenylaceticum pertaining to 4-OH-phenylacetate, the substrate used for isolation.

Cells are oval, short rods to coccoid (0.75–1×1.5–2 μm), gram-negative, catalase- and oxidase-positive, and motile. Growth between pH 6.9 and 8.9 with an optimal growth between pH 7 and 7.5. The temperature growth range is from 4 to 40 °C with an optimum at 28 °C. Growth requires 4-aminobenzoate. Nitrate, nitrite and oxygen are used as electron acceptor; nitrogen is not fixed. Under oxic conditions, benzoate, 3-hydroxybenzoate, 4-hydroxybenzoate, L-phenylalanine, acetate, glutarate, succinate, and L-glutamate are utilized. Slow growth on 2-aminobenzoate, catechol, phenylacetate, phenylpropionate, pimelate, toluene, phenol, L-aspartate, D-glucose, and D-fructose. No growth on gentisate, D-ribose, and hexadecane.

Under anoxic conditions, benzoate, 3-hydroxybenzoate, 4-hydroxybenzoate, 2-fluorobenzoate, L-phenylalanine, *p*-cresol, acetate, succinate, L-lactate, and glutarate are utilized. Only poor growth on 2-aminobenzoate, gentisate, protocatechuate, phenylacetate, indole, phenol, *o*-cresol, *m*-cresol, toluene, and 3-chlorobenzoate. No growth on 2-hydroxybenzoate, 4-aminobenzoate, 2-methylbenzoate, *o*-phthalate, resorcinol, hydroquinone, phenylpropionate,

cinnamate, L-tyrosine, L-tryptophan, indolyl 3-acetate, ethylbenzene, benzene, hexadecane, adipate, pimelate, cyclohexanecarboxylate, D-glucose, D-fructose, D-ribose, and lactose.

G+C content of DNA is 70.6 mol%.

Habitat and country of origin: activated sludge from a municipal sewage plant in Ulm, Germany

Type strain is strain B4P^T (=DSMZ 14743^T)

Description of *Thauera aminoaromatica* sp. nov.

Thauera aminoaromatica (a.mi.no.ar.o.ma'ti.ca). M.L. adj. *aminoaromatica* referring to ability to grow with aminoaromatic compounds such as 2-aminobenzoate or aromatic amino acids.

Cells are short rods (0.5–0.75×2-3 μm), gram-negative, catalase- and oxidase-positive, and motile. Growth between pH 7.2 and 9 with optimal pH for growth between 7.5 and 8.6. The temperature growth range is from 4 to 40 °C with an optimum at 28 °C. No growth factor required. Nitrate, nitrite, and oxygen are used as electron acceptor; nitrogen is not fixed.

Under oxic conditions, benzoate, L-phenylalanine, acetate, glutarate, D-ribose, succinate, L-glutamate, toluene, and phenol are utilized. Grows poorly on 4-hydroxybenzoate, phenylacetate, phenylpropionate, pimelate, D-glucose, D-fructose and L-aspartate. No growth on 3-hydroxybenzoate, 2-aminobenzoate, catechol, gentisate, and hexadecane. Under anoxic conditions, benzoate, 2-hydroxybenzoate, 3-hydroxybenzoate, 2-aminobenzoate, 2-fluorobenzaote, *p*-cresol, toluene, acetate, succinate, L-lactate, and L-glutarate are utilized; poor growth on 2-methylbenzoate, catechol, L-phenylalanine, L-tyrosine, indole, and phenol; no growth on 3-hydroxybenzoate, 3-aminobenzoate, 4-aminobenzoate, 2-methylbenzoate, 4-methylbenzoate, *o*-phthalate, resorcinol, hydroquinone, protocatechuate, gentisate, phenylacetate, phenylpropionate, cinnamate, *p*-anisate, L-tryptophan, indole 3-acetate, *o*-cresol, *m*-cresol, ethylbenzene, benzene, hexadecane, 3-chlorobenzoate, adipate, pimelate, cyclohexane carboxylate, D-fructose, D-ribose and lactose.

G+C content of DNA is 68.4 mol%.

Habitat and country of origin: anoxic ditch sludge from Konstanz, Germany.

Type strain is strain S2^T (=DSMZ 14742^T).

Description of *Azoarcus buckelii* sp. nov.

Azoarcus buckelii (buck'e.li.i.) M.L. gen. n. *buckelii* of Buckel, in honor of Professor Wolfgang Buckel, a bacterial biochemist and physiologist.

Cells are coccoid (1.5±0.5 μm in diameter), gram-negative, catalase- and oxidase-positive. Growth between pH 6 and 8.4 with an optimal growth between pH 7 and 7.4. The temperature growth range is from 4 to 40 °C with an optimum at 28 °C. Growth requires vitamin B_{12}. The metabolism is strictly oxidative. Nitrate, nitrite, and oxygen are used as electron acceptors, but not sulfate, thiosulfate, sulfite, or fumarate. Nitrogen is not fixed. Poly-β-hydroxybutyrate is accumulated. Under oxic conditions, benzoate, 3-hydroxybenzoate, L-phenylalanine, acetate, methanol, ethanol, glycerol, L-lactate, L-malate, succinate, pyruvate, and glutarate are utilized; slow growth on 4-hydroxybenzoate, phenylacetate, L-aspartate, L-glutamate; no growth on formate, citrate, 2-aminobenzoate, catechol, gentisate, phenylpropionate, pimelate, D-glucose, D-fructose, hexadecane, *o*-cresol, *p*-cresol, and phenol. Under anoxic denitrifying conditions, benzoate, 3-hydroxybenzoate, 4-hydroxybenzoate, 2-aminobenzoate, *o*-phthalate, protochatechuate, phenol, *o*-cresol, *p*-cresol, cyclohexanecarboxylate, 4-hydroxyphenylacetate, heptanoate, caproate, 2-hydroxybenzaldehyde, 3-hydroxybenzaldehyde, 4-hydroxybenzaldehyde, 2-hydroxybenzoate, 3-hydroxybenzoate, 4-hydroxybenzoate, 2,4-dimethylphenol, and 4-hydroxy-3-methylbenzoate are utilized; slow growth with 3-methylbenzoate, 2-fluorobenzoate, gentisate, phenylacetate, phenylpropionate, L-tyrosine, L-phenylalanine, and indole 3-acetate; no growth on *m*-cresol, 2-hydroxybenzoate, 3-aminobenzoate, 4-aminobenzoate, 2-methylbenzoate, 4-methylbenzoate, catechol, resorcinol, hydroquinone, cinnamate, *p*-anisate, L-tryptophan, indole, ethylbenzene, benzene, 3-chlorobenzoate, adipate, pimelate, D-glucose, D-fructose, D-ribose, lactose, cyclohexanol, cyclohexanone, 1,3-cyclohexanedione, adipate, pimelate, D,L-mandelate, D,L-4-hydroxymandelate, and 2-hydroxybenzyl alcohol.

G+C content of DNA is 66 mol%.

Habitat and country of origin: oxic soil from Ulm, Germany

Type strain is strain U120^T (=DSMZ 14744^T)

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