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Anaerobic degradation of ethylbenzene and other aromatic hydrocarbons by new denitrifying bacteria

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Abstract Anaerobic degradation of alkylbenzenes with side chains longer than that of toluene was studied in freshwater mud samples in the presence of nitrate. Two new denitrifying strains, EbN1 and PbN1, were isolated on ethylbenzene and *n*-propylbenzene, respectively. For comparison, two further denitrifying strains, ToN1 and mXyN1, were isolated from the same mud with toluene and *m*-xylene, respectively. Sequencing of 16S rDNA revealed a close relationship of the new isolates to *Thauera selenatis*. The strains exhibited different specific capacities for degradation of alkylbenzenes. In addition to ethylbenzene, strain EbN1 utilized toluene, but not propylbenzene. In contrast, propylbenzene-degrading strain PbN1 did not grow on toluene, but was able to utilize ethylbenzene. Strain ToN1 used toluene as the only hydrocarbon substrate, whereas strain mXyN1 utilized both toluene and *m*-xylene. Measurement of the degradation balance demonstrated complete oxidation of ethylbenzene to CO_2 by strain EbN1. Further characteristic substrates of strains EbN1 and PbN1 were 1-phenylethanol and acetophenone. In contrast to the other isolates, strain mXyN1 did not grow on benzyl alcohol. Benzyl alcohol (also *m*-methylbenzyl alcohol) was even a specific inhibitor of toluene and *m*-xylene utilization by strain mXyN1. None of the strains was able to grow on any of the alkylbenzenes with oxygen as electron acceptor. However, polar aromatic compounds such as benzoate were utilized under both oxic and anoxic conditions. All four isolates grew anaerobically on crude oil. Gas chromatographic analysis of crude oil after growth of strain ToN1 revealed specific depletion of toluene.

Key words Anaerobic degradation · Aromatic hydrocarbons · Alkylbenzenes · Ethylbenzene · Crude oil · Denitrifying bacteria · Phylogeny · *Thauera selenatis*

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Introduction

Benzene, toluene, ethylbenzene, and xylene (together known as BTEX) are natural constituents of crude oil (Tissot and Welte 1984) and gasoline (Schmidt and Romey 1981) and are produced as chemicals on a large scale (Koch 1989). Spillage and transport or production accidents may result in the contamination of soil and aquifers by BTEX (US Public Health Service 1989), which are more water-soluble than other hydrocarbons. The frequent occurrence of anoxic conditions in subsurface waters evokes an interest in bacteria that may degrade BTEX without molecular oxygen.

Among aromatic hydrocarbons, toluene has been studied most intensively as a substrate in anaerobic pure cultures of denitrifying (Dolfing et al. 1990; Altenschmidt and Fuchs 1991; Evans et al. 1991; Schocher et al. 1991), ferric iron-reducing (Lovley and Lonergan 1990), or sulfate-reducing (Rabus et al. 1993) bacteria, and in enrichments (Kuhn et al. 1988; Edwards and Grbić-Galić 1994). Anaerobic degradation of the three xylenes was demonstrated in aquifer enrichments under sulfate-reducing conditions (Edwards et al. 1992). Of the three isomers, only *m*-xylene has been used so far as a growth substrate in pure cultures of denitrifying bacteria (Schocher et al. 1991; Fries et al. 1994). A denitrifying pure culture has been reported to transform *o*-xylene to dead-end metabolites in the presence of toluene, although *o*-xylene did not serve as growth substrate (Evans et al. 1992). Anaerobic degradation of benzene in natural bacterial communities has been demonstrated under methanogenic (Grbić-Galić and Vogel 1987), sulfate-reducing (Edwards and Grbić-Galić 1992), or ferric iron-reducing conditions (Lovley et al. 1994). Growth of anaerobic bacteria on alkylbenzenes with side chains longer than a methyl group has neither been demonstrated in enrichments nor in pure cultures. Here we report on two new isolates of denitrifying bacteria, strains EbN1 and PbN1, that were isolated on ethylbenzene and propylbenzene, respectively, as sole electron donor and carbon source. For physiological and phyloge-

netic comparison, two further strains of denitrifying bacteria, ToN1 and mXyN1, were isolated on toluene and *m*-xylene, respectively.

Materials and methods

Sources of bacteria

Enrichments were attempted with a homogenized mixture of mud samples from ditches and the Weser river in Bremen, Germany.

Media and cultivation conditions

Techniques for preparation of media and cultivation of bacteria under anoxic conditions have been described elsewhere (Widdel and Bak 1992). The medium contained in 1 l of distilled water: 0.5 g KH_2PO_4, 0.3 g NH_4Cl, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, and 0.85 g $NaNO_3$ (10 mM). After autoclaving and cooling under an atmosphere of N_2/CO_2 (90/10, v/v), 40 ml $NaHCO_3$ solution (84 g/l, autoclaved under CO_2), vitamins, EDTA-chelated mixture of trace elements, and selenite and tungstate solution (Widdel and Bak 1992) were added. The EDTA-chelated stock solution of trace elements contained an increased concentration of copper (29 mg $CuSO_4 \cdot 5H_2O$ per l). The pH of the medium was adjusted to 7.2. For isolation and growth of pure cultures, the medium was reduced with 4 mM ascorbate added from a 1 M stock solution (ascorbic acid neutralized in an ice bath with NaOH, filter-sterilized, and stored at 4°C in the dark under N_2). Ascorbate did not serve as a growth substrate.

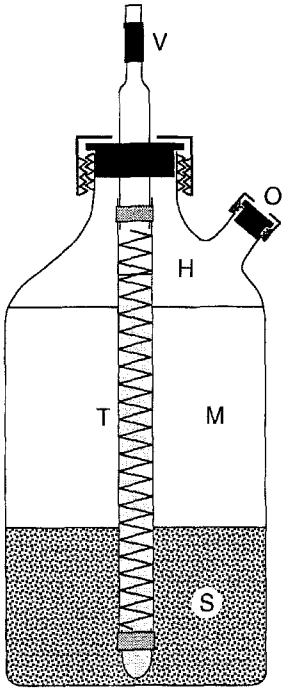

Fig. 1 Special glass bottle used for anoxic enrichment of alkylbenzene-degrading denitrifying bacteria. Bottles were filled with approx. 400 ml freshwater sediment (S) and 600 ml mineral medium (*M*). The headspace (H) contained an anoxic atmosphere of N_2/CO_2 (90/10, v/v). Stoppers were of butyl rubber. Alkylbenzenes were supplied as 2% solutions in viscous mineral oil (30 ml) inside a large (inner diameter, 18 mm) thin-walled (1 mm) silicon tubing (T) sealed at the bottom with a glass cap. Collapse of the silicon tubing was prevented by an inserted spiral of stainless steel. The top of the silicon tubing was connected to a glass orifice sealed with a short piece of Viton tubing (V) and a glass rod. Withdrawal of samples for analyses and release of formed N_2 was achieved via the stopper of the side opening (O) using a syringe

Cultures were routinely cultivated in volumes of 15 ml in glass tubes (20 ml) that were anoxically sealed with butyl rubber stoppers. Filter-sterilized hydrocarbons and other poorly water-soluble organic compounds were added as dilute solutions (0.5–5%, v/v) in 2,2,4,4,6,8,8-heptamethylnonane or mineral oil (high viscosity, pharmaceutical grade, Lamotte, Bremen, Germany) as inert carrier phase to avoid toxic effects (Rabus et al. 1993). Cultures with a carrier phase were incubated horizontally on a rotary shaker at low speed (70 rpm). The stopper-sealed orifices were always kept below the surface level of the medium to avoid contact between stoppers and carrier phase (Aeckersberg et al. 1991; Rabus et al. 1993). Initial enrichments were carried out in special 1-l flasks (Fig. 1). Aromatic hydrocarbons in the enrichment flasks were supplied as solutions in mineral oil via a vertical silicon tubing, allowing permanent diffusion of the substrate into the medium. Crude oil was obtained from a North Sea oil tank at Wilhelmshaven, Germany. Crude oil was deaerated by shaking under N_2 and repeated exchange of the atmosphere in a flask sealed with Teflon-coated rubber. The crude oil was then autoclaved in the same tightly sealed flask under N_2. Medium was overlaid with undiluted crude oil; contact with the stopper was avoided. Soluble substrates were added from separately sterilized, aqueous stock solutions (Widdel and Bak 1992).

Growth experiments in the presence of oxygen were carried out in 50-ml bottles with screw caps fitted with Teflon-coated rubber seals. The bottles were filled with 10 ml of ascorbate-free medium in the absence or presence of nitrate (10 mM). The medium was inoculated from anaerobically grown cultures. Hydrocarbons and other substrates were added in the same way as under anoxic conditions. The gas headspace contained sterile air with 10% (v/v) CO_2. The amount of oxygen in the headspace (40 ml) was sufficient for complete oxidation of the substrates.

Chemicals were of analytical grade.

Isolation

Denitrifiers were isolated via repeated agar dilution series (Widdel and Bak 1992) overlaid with the alkylbenzene of interest in mineral oil (Rabus et al. 1993). Purity of isolates was verified by routine phase-contrast microscopy. In addition, cultures were supplied with yeast extract (0.5 g/l) and glucose or pyruvate (5 mM) and examined microscopically.

For maintenance, strains were grown on the same aromatic hydrocarbons as used for isolation, stored at 4°C, and transferred every 4 weeks.

16S rDNA sequence analysis

Extraction of genomic DNA and PCR-mediated amplification of the 16S rDNA was done as described previously (Rainey et al. 1992). PCR products were directly sequenced using the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Warrington, UK), according to the manufacturer's protocol. The Applied Biosystems 373A DNA sequencer was used for the electrophoresis of the sequence reaction products. The 16S rDNA sequences were manually aligned with sequences currently available from public databases. Evolutionary distances were calculated by the method of Jukes and Cantor (1969) and used in the reconstruction of the phylogenetic dendrogram using the algorithm of DeSoete (1983). Sequence analyses were carried out by F.A. Rainey, Braunschweig.

Nucleotide sequence accession number

Data are deposited under EMBL accession numbers X83531 (strain EbN1), X83532 (strain PbN1), X83534 (strain ToN1), and X83533 (strain mXyN1).

Determination of degradation balances and growth parameters

The degradation balance of the ethylbenzene-degrading denitrifying strain EbN1 was determined in flat glass bottles (500 ml volume). Medium (390 ml) and inoculum (10 ml) were aseptically added to the bottles that were kept under a N_2/CO_2 (90/10, v/v) atmosphere. After anoxically sealing with butyl rubber stoppers and screw caps, the bottles were inverted. Anoxic, sterile heptamethylnonane (10 ml) was then added through the butyl stopper using sterile, N_2-flushed plastic syringes. Thereafter, defined amounts of ethylbenzene were added using sterile, N_2-flushed microliter syringes. This procedure allowed ethylbenzene to dissolve in heptamethylnonane without coming into contact with the stopper. Cultures were incubated on a rotary shaker in the same way as described for routine cultures. Samples for chemical analyses were taken with sterile, N_2-flushed syringes.

Dry mass of cells was determined after washing with ammonium acetate (20 mM) and drying of the cell pellet at 60°C to constant weight. The optical density was measured at 660 nm. The ratio of the optical density to the cell mass per volume was determined once with a defined culture volume.

Chemical analyses

Ethylbenzene in the aqueous phase was quantified using a high-performance liquid chromatography system (Sykam, Gilching/Munich, Germany) and a Spherisorb OD S2 reverse-phase column (5 × 250 mm) with 80% acetonitrile in distilled H_2O as eluent. The temperature of the column was 25°C and the flow rate was 1 ml/min. Ethylbenzene (5.8 min) was detected at 215 nm. Ethylbenzene in heptamethylnonane from grown cultures was determined by diluting aliquots in pure heptamethylnonane and measuring the absorbance at 254 nm. Calibration was carried out with defined, freshly prepared solutions of ethylbenzene in solvent.

For oil analyses, the aromatic hydrocarbon fraction was separated by medium-pressure liquid chromatography (Radke et al. 1980) and analyzed by gas chromatography as described elsewhere (Rueter et al 1994). Oil analyses were performed by H. Wilkes, Jülich, Germany.

Nitrate and nitrite were measured by high-performance liquid chromatography (Sykam, Gilching/Munich, Germany) on an A09-micro anion exchange column (3 × 125 mm). The eluent was 70 mM NaCl in distilled H_2O. The flow rate was 1 ml/min and the temperature of the column was constant at 65°C. Nitrate (2.5 min) and nitrite (1.8 min) were detected at 220 nm with an S 3200 UV-detector (Sykam, Gilching/Munich, Germany). Diluted samples (1:10) were injected using an autosampler (Jasco, Tokyo, Japan). Data acquisition and processing were done with Pyramid software (Axxiom Chromatography, Moorpark, Calif., USA).

N_2O was determined on a Shimadzu GC-8A gas chromatograph equipped with a thermal conductivity detector. Separation was carried out on a Poraplot Q column (3 mm × 2 m) at 40°C, using N_2 as carrier gas at a flow rate of 32 ml/min.

Ammonium was measured using the indophenol formation reaction according to Marr et al. (1988).

For the determination of the G+C content, DNA was isolated by chromatography on hydroxyapatite using the procedure of Cashion et al. (1977). The G+C content of the DNA was determined by HPLC according to Mesbah et al. (1989). The analyses were carried out by F.A. Rainey, Braunschweig, Germany.

Results

Enrichment and isolation

Enrichment of anaerobic bacteria oxidizing alkylbenzenes with nitrate as electron acceptor was attempted at 28°C with ethylbenzene, *n*-propylbenzene, isopropylbenzene, *n*-butylbenzene, *o*-xylene, and *p*-xylene (each 2% in carrier phase) in 1-l bottles (Fig. 1) containing 400 ml freshwater mud and 600 ml mineral medium. For comparison, enrichments were also carried out with toluene and *m*-xylene, which are known to serve as substrates in pure cultures of anaerobic bacteria (Dolfing et al. 1990; Lovley and Lonergan, 1990; Altenschmidt and Fuchs 1991; Evans et al. 1991; Schocher et al. 1991; Rabus et al. 1993; Fries et al. 1994). Nitrate reduction was monitored by HPLC. Overpressure due to vigorous N_2 production was released two to five times per week by insertion of a hypodermic needle. Enrichments were briefly shaken once per day. Upon depletion of nitrate and nitrite, 10 mM of the electron acceptor was added again. In a control with no addition of substrate, denitrification by endogenous electron donors was initially just as fast as in the presence of aromatic compounds. After 2 weeks, the culture with toluene began to denitrify faster than the control. The denitrification rate in the bottles with ethylbenzene and *m*-xylene increased with respect to the control after 6 weeks, with propylbenzene only after 14 weeks. During 14 weeks, new nitrate was added 12 times. Increase of denitrification was accompanied by development of a dense biofilm of bacteria and protozoa on the silicon tubing in the bottle. Samples from this biofilm and from the sediment were transferred to subcultures (40 ml) in small anoxic bottles (50 ml) with aromatic hydrocarbons dissolved in heptamethylnonane. Loss of enrichments by grazing flagellates was prevented by frequent transfer.

The fourth subcultures served as starting inocula for agar dilution series. Within 1 to 2 weeks ochre colonies developed; the colonies next to the overlying hydrocarbon phase were the largest. Four denitrifiers, strains EbN1, PbN1, ToN1, and mXyN1, were isolated on ethylbenzene, propylbenzene, toluene, and *m*-xylene, respectively. Purity controls with various organic compounds always yielded the same cell-type of an isolate.

No denitrifiers were enriched on isopropylbenzene, butylbenzene, *o*-xylene, and *p*-xylene.

Morphological, physiological, and other characteristics

All isolates had morphologically rather similar, oval to rod-shaped cells, with dimensions of 0.6–0.8 × 1.5–2.5 µm (EbN1; Fig. 2), 1 × 1.5–2.5 µm (PbN1), 0.6–0.8 × 1–2 µm (ToN1), and 0.5–1 × 1.5–3 µm (mXyN1). Cells of all strains were motile. The G+C content of the DNA was 65.0 mol% (EbN1), 66.8 mol% (PbN1), 66.0 mol% (ToN1), and 66.9 mol% (mXyN1).

The temperature range for growth of strain EbN1 was between 17 and 40°C, with an optimum at 31°C. The pH range of strain EbN1 was between 6.4 and 8.1, with an optimum between 7.1 and 7.4. The other three strains also grew well at the temperature and pH values that were optimal for strain EbN1. The shortest doubling time of strain EbN1 during growth on ethylbenzene was 11 h. Cultures of strain EbN1 with 5% (v/v) inoculum were fully grown on ethylbenzene within 2 days. Strain ToN1 with the same

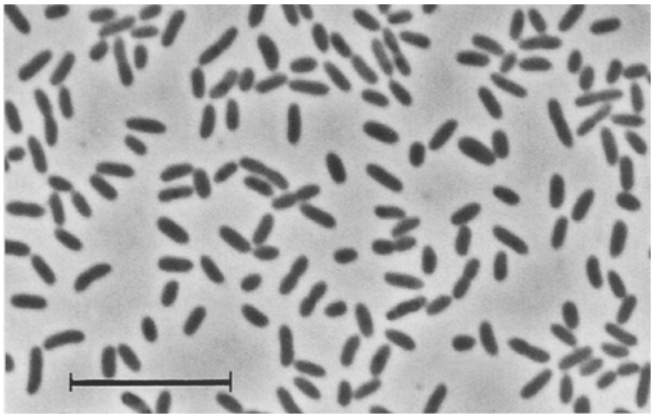

Fig. 2 Phase-contrast photomicrograph of denitrifying strain EbN1, grown on ethylbenzene (*bar* 10 µm)

inoculum size was fully grown on toluene even within 1 day. *m*-Xylene-utilizing strain mXyN1 grew as fast as strain EbN1, whereas strain PbN1 required 3–4 days for full growth on propylbenzene.

Routine cultivation of strain EbN1 was optimal if the concentration of ethylbenzene in heptamethylnonane was increased from 2 to 5% (v/v). The increased concentration in the carrier phase yielded an equilibrium concentration of ethylbenzene in the aqueous phase of 0.29 mM.

All strains were able to grow anaerobically on various aromatic compounds in addition to alkylbenzenes (Table 1). A special nutritional characteristic of strains EbN1 and PbN1 is the ability to grow on aromatic ketones and aromatic secondary alcohols with the OH-group next to the phenyl residue (phenylalkylcarbinols).

During growth on ethylbenzene, strain EbN1 excreted nitrite as an intermediate (see Fig. 4). Formation of N_2O or NH_4^+ was not detected.

Genealogical relationships

Sequencing of 16S rDNA of the four isolates revealed an affiliation with the phylogenetic group comprising *Thauera selenatis*, *Azoarcus* species, and known toluene-degrading denitrifying bacteria (Altenschmidt and Fuchs 1991; Fries et al. 1994) that belong to the β-group of the Proteobacteria (Fig. 3).

Degradation balance and growth yield

The degradation balance was determined in cultures of strain EbN1 with a limiting and an excess amount of ethylbenzene (Table 2) with respect to the available electron acceptor (10 mM NO_3^-). Increase in optical density, consumption of nitrate, and intermediate formation of nitrite were monitored during incubation (Fig. 4). If a limiting amount of ethylbenzene was added, none was detected in the carrier and aqueous phase after growth (Table 2). In the experiment with the excess amount of ethylbenzene,

Table 1 Anaerobic growth tests of denitrifying strains EbN1, PbN1, ToN1, and mXyN1 on aromatic hydrocarbons and polar compounds. Each compound was tested twice at concentrations given in parentheses. Unless otherwise noted concentrations, are given in mM. Concentrations in % (v/v) refer to dilutions of poorly soluble compounds in heptamethylnonane as an inert carrier phase [++ good growth within approximately 4 days or less (5% inoculum); + poor growth, i.e., growth time longer than 4 days; – no growth; *nt* not tested]

	Strain			
Compound tested	EbN1[a]	PbN1	ToN1	mXyN1
Aromatic hydrocarbons				
Benzene (1%)	–	–	–	–
Toluene (2%)	++	–	++	++
Ethylbenzene (2%)	++	++	–	–
Propylbenzene (2%)	–	++	–	–
Butylbenzene (2%)	–	–	–	–
o-Xylene (2%)	–	–	–	–
m-Xylene (2%)	–	–	–	++
p-Xylene (2%)	–	–	–	–
Phenolic compounds				
Phenol (0.5, 2)	–	+	+	–
p-Cresol (0.5, 2)	–	–	+	+
Alcohols, aldehydes and ketones				
Benzyl alcohol (0.5, 2)	++	+	++	–
1-Phenylethanol (0.5, 2)	++	+	–	–
2-Phenylethanol (0.5, 2)	–	nt	nt	nt
1-Phenyl-1-propanol (0.5, 2)	–	+	–	–
1-Phenyl-2-propanol (0.5, 2)	nt	–	nt	nt
3-Phenyl-1-propanol (0.5, 2)	nt	–	nt	nt
m-Methyl-benzyl alcohol (0.5, 2)	–	–	–	–
Benzaldehyde (0.5, 2)	++	+	++	+
Acetophenone (1%)	++	++	–	–
Propiophenone (1%)	–	++	–	–
Acetone (0.5, 2)	++	++	+	–
2-Butanone (0.5, 2)	++	++	+	–
3-Pentanone (0.5, 2)	–	–	–	–
Carboxylic acids				
Benzoate (1, 4)	++	++	++	++
Phenylacetate (1, 4)	++	++	++	++
m-Methylbenzoate (1, 4)	–	–	–	++
Acetate (1, 5)	++	++	++	++
Pyruvate (1, 5)	++	++	++	++

[a] Further substrates utilized by strain EbN1: phenylalanine (0.5, 2), succinate (1, 5), malate (1, 5), propionate (5, 10), butyrate (5, 10), valerate (1, 5), caproate (1, 5), lactate (5, 10), ethanol (1, 5), propanol (1, 5), isopropanol (5, 10), 1-butanol (1, 5), 2-butanol (1, 5)

[b] Further compounds tested but not utilized by strain EbN1: isopropylbenzene (2, 5%), *p*-ethyltoluene (0.5, 2%), styrene (2%), naphthalene (20 mg/ml in mineral oil), 1-methylnaphthalene (2, 5%), pyridine (0.5, 2%), 2-ethylphenol (0.5, 2%), 3-ethylphenol (0.5, 2%), 2-phenyl-2-propanol (0.5, 2%), cyclohexane (2%), methylcyclohexane (2%), ethylcyclohexane (2%), hexane (2, 5%), decane (5, 10%), hexadecane (20%, undiluted), *o*-cresol (0.5, 2), *m*-cresol (0.5, 2), *o*-methylbenzoate (1, 4), *p*-methylbenzoate (1, 4), *p*-ethylbenzoate (0.5, 2), mandelate (0.5, 2), 3-phenylpropionate (1, 4), 4-phenylbutyrate (1, 4), tryptophan (0.5, 2), tyrosine (0.5, 2), methanol (5, 10), formate (10, 20), caprylate (1, 2), palmitate (0.5, 1), glucose (1, 5), fructose (1, 5), mannose (1, 5), citrate (1, 5), glycine (1, 5), serine (1, 5), alanine (1, 5), aspartate (1, 5), glutamate (1, 5)

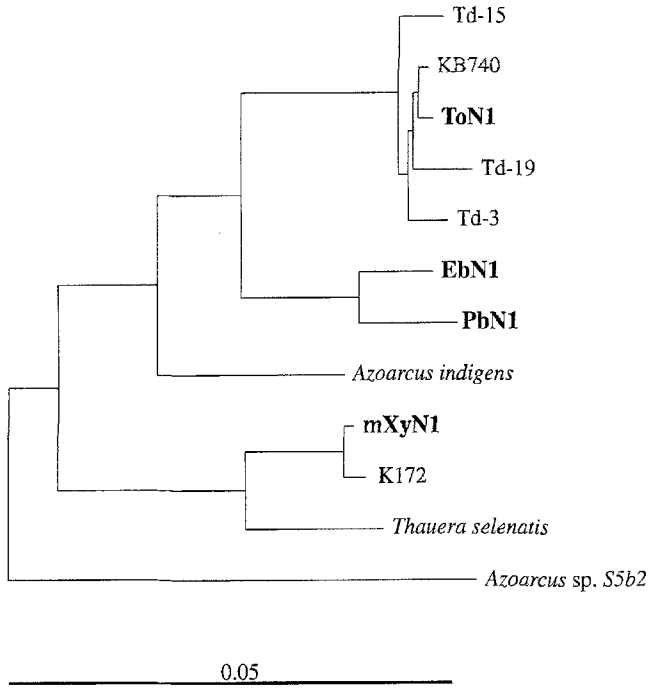

Fig. 3 Phylogenetic relationships of the newly isolated alkylbenzene-degrading denitrifying strains EbN1, PbN1, ToN1 and mXyN1 to *Thauera selenatis*, *Azoarcus* species and known toluene and *m*-xylene degrading denitrifiers. The reconstructed tree is part of the β-group of the Proteobacteria. *Scale bar* represents five inferred nucleotide substitutions per 100 nucleotides

one-third was recovered, while the electron acceptor was completely consumed. The average molar growth yield of strain EbN1 was 114 g of dry mass per mol of ethylbenzene oxidized.

Growth inhibition of strain mXyN1 by aromatic alcohols

Among the newly isolated denitrifiers, strain mXyN1 was the only one not able to grow on aromatic alcohols. Moreover, benzyl alcohol or *m*-methyl-benzyl alcohol at a concentration as low as 0.1 mM specifically inhibited growth of strain mXyN1 on toluene and *m*-xylene. In contrast, growth of strain mXyN1 on benzoate and *m*-methylbenzoate was not even inhibited by 1 mM benzyl alcohol or *m*-methyl-benzyl alcohol.

Aerobic growth experiments

The capability of strains EbN1, PbN1, ToN1, and mXyN1 to grow in the presence of oxygen was tested with aromatic hydrocarbons in heptamethylnonane and with related polar aromatic derivatives (Table 3). None of the new strains grew aerobically (either in the presence or absence of nitrate) on any of the aromatic hydrocarbons that were utilized anaerobically. In contrast, the majority of polar aromatic compounds that were used anaerobically also served as growth substrates under oxic conditions.

Table 2 Quantification of ethylbenzene consumption and nitrate reduction by strain EbN1. Incubation experiments were carried out in anoxic flat bottles with a culture volume of 400 ml. The total amount of nitrate added to each bottle was 4.0 mmol (concentration, 10 mM). The medium was overlaid with 10 ml of heptamethylnonane as carrier phase for ethylbenzene

Experiment	Ethylbenzene added (mmol)	Ethylbenzene disappeared[a] (mmol)	NO_3^- remaining after growth (mmol)	NO_2^- remaining after growth (mmol)	Cell dry mass formed[b] (mg)	Ethylbenzene dissimilated[c] (mmol)	Electrons from ethylbenzene dissimilated[d] (mmol)	Electrons consumed by NO_3^- reduction[e] (mmol)
Cells with limiting amount of ethylbenzene	0.25	0.25	2.5	0.4	22	0.17	6.9	6.3
Cells with excess amount of ethylbenzene	1.23	0.80	0.0	0.0	57	0.58	24.3	20.0
Cells without ethylbenzene (control)	0.00	0.00	4.0	0.0	0			0.0
Sterile medium without cells (control)	1.23	0.00	4.0	0.0				0.0

[a] Difference between ethylbenzene added and ethylbenzene recovered at the end of incubation in the carrier and aqueous phase
[b] Amount of cell dry mass added with the inoculum has been subtracted
[c] Difference between ethylbenzene disappeared and ethylbenzene assimilated. The assimilated amount of ethylbenzene was calculated assuming the equation $17\ C_8H_{10} + 32\ HCO_3^- + 32\ H^+ + 30\ H_2O \rightarrow 42\ C_4H_7O_3$; thus, 1 mg of cell dry mass requires 0.00393 mmol of ethylbenzene
[d] 42 mol of electrons are derived from 1 mol of ethylbenzene if oxidized to CO_2
[e] Electrons consumed = 5 · [nitrate added – (nitrate remaining + nitrite remaining)] + 2 · [nitrite remaining]

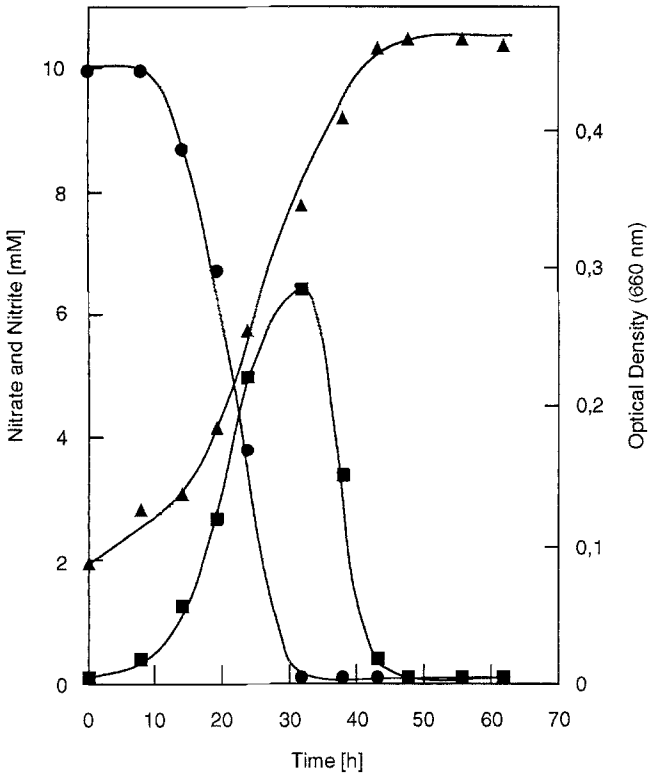

Fig. 4 Anaerobic growth of strain EbN1 with ethylbenzene and nitrate (10 mM). The experiment (Table 2, experiment with excess amount of ethylbenzene) was carried out in a flat, horizontally incubated bottle sealed with butyl rubber: 400 ml ascorbate-reduced medium was overlaid with 10 ml anoxic heptamethylnonane as inert carrier phase; 1.23 mmol (0.150 ml) ethylbenzene was added to the carrier phase by means of a microliter syringe; contact of the hydrocarbon with the stopper was avoided. Samples for determination of the optical density (▲) and analysis of nitrate (●) and nitrite (■) were withdrawn using N_2-flushed syringes

Aerobic growth on benzoate was not affected by a layer of heptamethylnonane, demonstrating that the carrier phase did not prevent diffusion of oxygen from the gas headspace into the aqueous phase.

Growth on crude oil

All four isolates grew anaerobically on crude oil as the only organic substrate with nitrate as electron acceptor. Fastest growth was observed with strain ToN1. With an inoculum of 5% (v/v), strain ToN1 was fully grown on crude oil and 10 mM nitrate within 3 days. Complete reduction of 10 mM nitrate required 13 ml of oil per liter. Gas chromatographic analysis of the aromatic oil fraction revealed that toluene was specifically and completely consumed from the crude oil.

Discussion

The new denitrifying strains EbN1 and PbN1 were able to grow anaerobically on alkylbenzenes with side chains longer than the methyl group of toluene. To our knowledge, such a capacity has not been reported before. Furthermore, the four new isolates are the first pure cultures of denitrifying bacteria shown to grow anaerobically on crude oil as the only organic substrate.

Table 3 Aerobic growth tests of strains EbN1, PbN1, ToN1, and mXyN1 on aromatic hydrocarbons and polar aromatic compounds. With each strain only compounds were tested that served as growth substrates under anoxic conditions (Table 1). Each compound was tested twice at concentrations given in parentheses. Unless otherwise noted, concentrations are in mM. Concentrations in % (v/v) refer to dilutions of insoluble compounds in heptamethylnonane as an inert carrier phase (++ good growth within 2 days; + poor growth, i.e., growth time 2–3 weeks; – no growth; *nt* not tested)

	Strain			
Compound tested	EbN1	PbN1	ToN1	mXyN1
Aromatic hydrocarbons				
Toluene (2%)	–	nt	–	–
Ethylbenzene (2%)	–	–	nt	nt
Propylbenzene (2%)	nt	–	nt	nt
m-Xylene (2%)	nt	nt	nt	–
Aromatic alcohols, aldehydes and ketones				
Benzyl alcohol (1)	++	nt	++	nt
1-Phenylethanol (1)	++	–	nt	nt
1-Phenylpropanol (1)	nt	–	nt	nt
Benzaldehyde (1)	++	nt	++	+
Acetophenone (0.5%)	++	+	nt	nt
Propiophenone (0.5%)	nt	–	nt	nt
Aromatic carboxylic acids				
Benzoate (2)	++	++	++	++
Phenylacetate (1)	++	++	++	++
m-Methylbenzoate (1)	nt	nt	nt	–

Utilization of alkylbenzenes by the new isolates was an obligately anaerobic process (Table 3), although growth on polar aromatic compounds was facultatively anaerobic.

The balance of ethylbenzene degradation by strain EbN1 demonstrated that the amount of reducing equivalents (electrons) that can be derived from the dissimilated substrate is close to the amount of reducing equivalents required for nitrate reduction to nitrite and N_2, or to only N_2 (Table 2). Hence, the principal degradation equation of ethylbenzene by strain EbN1 is as follows:

$$C_8H_{10}+8.4\,NO_3^-+0.4\,H^+ \rightarrow 8\,HCO_3^-+4.2\,N_2+1.2\,H_2O \quad (1)$$
$$\Delta G^{\circ\prime} = -4{,}148\ \text{kJ/mol ethylbenzene}$$

The free energy is calculated from standard values (Thauer et al. 1977; Synowietz 1983). In addition, the capacity for a terminal oxidation under denitrifying conditions was confirmed by the ability to grow on acetate (Table 1). With an excess of ethylbenzene, the oxidized amount was about 20% higher than required for nitrate reduction according to Eq. 1. This may be explained by a conversion of the substrate to currently unidentified organic compounds.

All new alkylbenzene-degrading isolates cluster within a distinctive phylogenetic branch containing *Thauera se-*

lenatis (Macy et al. 1993) and *Azoarcus* species (Reinhold-Hurek et al. 1993) as described organisms (Fig. 3). This finding is in agreement with recent phylogenetic analyses demonstrating that several denitrifiers using toluene (and in one case also *m*-xylene) are members of this branch (Fries et al. 1994; H.-J. Anders and G. Fuchs, personal communication). One may assume that the metabolic capacity for degradation of alkylbenzenes under denitrifying conditions is of monophyletic origin and has been conserved or modified in its specificity in several lines of descent within this group.

The mechanism of the initial reaction during aromatic hydrocarbon degradation in the absence of molecular oxygen is still unknown. Presently favored mechanisms of toluene degradation in denitrifying bacteria are an anaerobic methyl hydroxylation to benzyl alcohol (Altenschmidt and Fuchs 1992) or an oxidative condensation with acetyl-CoA to phenylpropionyl-CoA (Evans et al. 1992). A hydroxylation of ethylbenzene and propylbenzene analogous to the proposed methyl hydroxylation of toluene would lead to the secondary alcohols, 1-phenylethanol and 1-phenylpropanol, respectively. These alcohols could be further oxidized to acetophenone and propiophenone, respectively. Such a pathway would be in complete accordance with the ability of strains EbN1 and PbN1 to grow with these alcohols and ketones. Acetophenone and propiophenone as C-H-acidic compounds could be further metabolized via carboxylation to β-ketoacids. These could then be activated to CoA-esters and successively cleaved by β-oxidation to benzoyl-CoA, and acetyl-CoA or propionyl-CoA, respectively. In the case of aliphatic ketones used by previously described denitrifiers, carboxylation is the most probable mechanism of activation (Platen and Schink 1989). Strains EbN1 and PbN1 were also able to use aliphatic ketones. In the case of strain mXyN1, introduction of a hydroxyl function into the methyl side chain appears unlikely because the alcohols that would result cannot be metabolized and are even actually specific inhibitors of toluene and *m*-xylene utilization. A similar inhibitory effect of benzyl alcohol on toluene degradation has been observed in a sulfate-reducing bacterium (Rabus et al. 1993). Hence, if introduction of a hydroxyl function proximal to the aromatic ring is an activation mechanism in certain anaerobic alkylbenzene-degrading strains, a second mechanism not involving an alcohol as a free intermediate must be postulated in other strains.

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References

Aeckersberg F, Bak F, Widdel F (1991) Anaerobic oxidation of saturated hydrocarbons to CO_2 by a new type of sulfate-reducing bacterium. Arch Microbiol 156:5–14

Altenschmidt U, Fuchs G (1991) Anaerobic degradation of toluene in denitrifying *Pseudomonas* sp.: indication of toluene methylhydroxylation and benzoyl-CoA as central aromatic intermediate. Arch Microbiol 156:152–158

Altenschmidt U, Fuchs G (1992) Anaerobic toluene oxidation to benzyl alcohol and benzaldehyde in a denitrifying *Pseudomonas* strain. J Bacteriol 174:4860–4862

Cashion P, Holder-Franklin MA, McCully J, Franklin M (1977) A rapid method for the base ratio determination of bacterial DNA. Anal Biochem 81:461–466

DeSoete G (1983) A least squares algorithm for fitting additive trees to proximity data. Psychometrika 48:621–626

Dolfing J, Zeyer P, Binder-Eicher P, Schwarzenbach RP (1990) Isolation and characterization of a bacterium that mineralizes toluene in the absence of molecular oxygen. Arch Microbiol 154:336–341

Edwards EA, Grbić-Galić D (1992) Complete mineralization of benzene by aquifer microorganisms under strictly anaerobic conditions. Appl Environ Microbiol 58:2663–2666

Edwards EA, Grbić-Galić D (1994) Anaerobic degradation of toluene and *o*-xylene by a methanogenic consortium. Appl Environ Microbiol 60:313–322

Edwards EA, Wills LE, Reinhard M, Grbić-Galić D (1992) Anaerobic degradation of toluene and xylene by aquifer microorganisms under sulfate-reducing conditions. Appl Environ Microbiol 58:794–800

Evans PJ, Mang DT, Kim KS, Young LY (1991) Anaerobic degradation of toluene by a denitrifying bacterium. Appl Environ Microbiol 57:1139–1145

Evans PJ, Ling W, Goldschmidt B, Ritter ER, Young LY (1992) Metabolites formed during anaerobic transformation of toluene and *o*-xylene and their proposed relationship to the toluene mineralization. Appl Environ Microbiol 58:496–501

Fries MR, Zhou J, Chee-Sanford J, Tiedje JM (1994) Isolation, characterization, and distribution of denitrifying toluene-degraders from a variety of habitats. Appl Environ Microbiol 60:2802–2810

Grbić-Galić D, Vogel TM (1987) Transformation of toluene and benzene by mixed methanogenic cultures. Appl Environ Microbiol 53:264–260

Jukes TH, Cantor CR (1969) Evolution of protein molecules. In: Munro HN (ed) Mammalian protein metabolism. Academic Press, New York London, pp 21–132

Koch R (1989) Umweltchemikalien. VCH, Weinheim

Kuhn EP, Zeyer J, Eicher P, Schwarzenbach RP (1988) Anaerobic degradation of alkylated benzenes in denitrifying laboratory columns. Appl Environ Microbiol 54:490–496

Lovley DR, Lonergan DJ (1990) Anaerobic oxidation of toluene, phenol and *p*-cresol by the dissimilatory iron-reducing organism GS15. Appl Environ Microbiol 56:1858–1864

Lovley DR, Woodward JC, Chapelle FH (1994) Stimulated anoxic biodegradation of aromatic hydrocarbons using Fe(III) ligands. Nature 370:128–131

Macy JM, Rech S, Auling G, Dorsch M, Stackebrandt E, Sly LI (1993) *Thauera selenatis* gen. nov., sp. nov., a member of the beta subclass of Proteobacteria with a novel type of anaerobic respiration. Int J Syst Bacteriol 43:135–142

Marr L, Cresser MS, Ottendorfer LJ (1988) Umweltanalytik. Thieme, Stuttgart New York

Mesbah M, Premachandran U, Whitman WB (1989) Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. Int J Syst Bacteriol 39:159–167

Platen H, Schink B (1989) Anaerobic degradation of acetone and higher ketones via carboxylation by newly isolated denitrifying bacteria. J Gen Bacteriol 135:883–891

Rabus R, Nordhaus R, Ludwig W, Widdel F (1993) Complete oxidation of toluene under strictly anoxic conditions by a new sulfate-reducing bacterium. Appl Environ Microbiol 59:1444–1451

Radke M, Willsch H, Welte DH (1980) Preparative hydrocarbon group type determination by automated medium pressure liquid chromatography. Anal Chem 52:406–411

Rainey FA, Dorsch M, Morgan HW, Stackebrandt E (1992) 16S rDNA analysis of *Spirochaeta thermophila*: position and implications for the systematics of the order Spirochaetales. Syst Appl Microbiol 16:224–226

Reinhold-Hurek B, Hurek T, Gillis M, Hoste B, Vancanneyt M, Kersters K, De Ley J (1993) *Azoarcus* gen. nov., nitrogen-fixing *Proteobacteria* associated with roots of Kallar grass (*Leptochloa fusca* (L.) *Kunth*), and description of two species, *Azoarcus indigens* sp. nov. and *Azoarcus communis* sp. nov. Int J Syst Bacteriol 43:574–584

Rueter P, Rabus R, Wilkes H, Aeckersberg F, Rainey FA, Jannasch HW, Widdel F (1994) Anaerobic oxidation of hydrocarbons in crude oil by new types of sulfate-reducing bacteria. Nature 372:455–458

Schmidt K-H, Romey I (1981) Kohle, Erdöl, Erdgas. Vogel, Würzburg

Schocher RJ, Seyfried B, Vazquez F, Zeyer J (1991) Anaerobic degradation of toluene by pure cultures of denitrifying bacteria. Arch Microbiol 157:7–12

Synowietz C (1983) Taschenbuch für Chemiker und Physiker, vol 2. Organische Verbindungen. Springer, Heidelberg Berlin New York, p 1031

Thauer RK, Jungermann K, Decker K (1977) Energy conservation in chemotrophic anaerobic bacteria. Bacteriol Rev 41:100–180

Tissot BP, Welte DH (1984) Petroleum formation and occurrence. 2nd edn. Springer, Berlin Heidelberg New York

US Public Health Service (1989) Toxicological profile for toluene. Publication ATSDR/TP-89/23. Agency for toxic substances and disease registry, US Public Health Service, Atlanta

Widdel F, Bak F (1992) Gram-negative mesophilic sulfate-reducing bacteria. In: Balows A, Trüper HG, Dworkin M, Harder W, Schleifer KH (eds), The prokaryotes, 2nd edn. vol 4. Springer, Berlin Heidelberg New York, pp 3352–3378