RESEARCH ARTICLE

Highly enriched *Betaproteobacteria* growing anaerobically with *p*-xylene and nitrate

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Abstract

The identity of the microorganisms capable of anaerobic *p*-xylene degradation under denitrifying conditions is hitherto unknown. Here, we report highly enriched cultures of freshwater denitrifying bacteria that grow anaerobically with *p*-xylene as the sole organic carbon source and electron donor. Long curved rods, with 95% 16S rRNA gene sequence identity to *Denitratisoma oestradiolicum*, dominated the enrichment cultures (> 91% of all cells), as detected by phylotype-specific probes. These *Rhodocyclaceae* microorganisms were distantly related to other denitrifying hydrocarbon-degrading *Betaproteobacteria* from the *Azoarcus* –*Thauera* clade. Complete oxidation *p*-xylene to CO_2 coupled to denitrification was suggested by quantitative measurements of substrate consumption. Metabolite analysis identified (4-methylbenzyl)succinate and (4-methylphenyl)itaconate, suggesting addition to fumarate as an initial activation reaction.

Introduction

The aromatic hydrocarbon *p*-xylene (1,4-dimethylbenzene) is a constituent of petroleum (Tissot & Welte, 1984) and raw material for the chemical industry. *p*-Xylene production reaches 2.2×10^6 tons year^{-1} (A.P.P.E, 2006), because it is used to manufacture solvents and precursors, such as terephthalic acid, the precursor of polyethylene terephthalate, a thermoplastic polymer resin of the polyester family.

p-Xylene is a nonpolar compound with a relatively high water solubility (0.18 g L^{-1} at 25 °C) (Shiu & Ma, 2000). Such properties place *p*-xylene among the most mobile and toxic petroleum-derived groundwater contaminants, along with benzene, toluene, ethylbenzene and *o*- and *m*-xylene (Anderson & Lovley, 1997). The fate of *p*-xylene in the environment is determined by chemical and microbial processes (Tuazon *et al.*, 1984; Atkinson *et al.*, 1991; Head *et al.*, 2006). Aerobic microorganisms activate *p*-xylene to 3,6-dimethylcatechol, a process catalyzed by dioxygenases (Wackett, 2006), whereas under anaerobic conditions, which often prevail in underground waters and aquifers, *p*-xylene is removed by microorganisms thriving under nitrate-, sulfate-, iron (III)-reducing or methanogenic conditions (Kuhn *et al.*, 1985; Häner *et al.*, 1995; Morasch & Meckenstock, 2005; Reinhard *et al.*, 2005; Botton & Parsons, 2007; Nakagawa *et al.*, 2008).

p-Xylene is among the least degradable constituents of the BTEX fraction (benzene, toluene, ethylbenzene, xylenes), along with benzene and *o*-xylene (Widdel *et al.*, 2006; Foght, 2008). In contrast to *p*-xylene, the other hydrocarbon constituents of the BTEX fraction could sustain anaerobic growth of pure cultures (Dolfing *et al.*, 1990; Rabus & Widdel, 1995; Harms *et al.*, 1999; Coates *et al.*, 2001; Morasch *et al.*, 2004; Kasai *et al.*, 2006). To date, only three studies have reported stable enrichment cultures and confirmed anaerobic utilization of *p*-xylene under denitrifying and sulfate-reducing conditions (Häner *et al.*, 1995; Morasch & Meckenstock, 2005; Nakagawa *et al.*, 2008). The

phylogeny of sulfate-reducers that might be involved in *p*-xylene-degradation was shown in an enrichment culture by denaturing gradient gel electrophoresis where a single sequence type dominated (Nakagawa *et al*., 2008). This phylotype was related to *Desulfosarcina ovata* strain oXyS1, an *o*-xylene-degrading sulfate-reducing bacterium (Harms *et al*., 1999). Molecular analysis of an enrichment culture capable of *p*-xylene consumption under iron (III)-reducing conditions (Botton & Parsons, 2007) showed the presence of 16S rRNA gene fragments related to *Geobacter* and *Deltaproteobacteria* (Botton *et al*., 2007).

The identity of microorganisms involved in *p*-xylene degradation under denitrifying conditions has not been addressed. Therefore, in this study, we established highly enriched cultures that degrade *p*-xylene under nitrate-reducing conditions, and identified and quantified the community members. The phylotypes of the microbial community were detected by cloning and sequencing of 16S rRNA genes, subsequently used as a template for the design of phylotype-specific oligonucleotide probes. The phylotype-specific probes were used to detect, by FISH, the dominant microorganisms in the enrichment cultures (Amann *et al*., 1995).

The anaerobic degradation of toluene, *o*- and *m*-xylene involves addition of the hydrocarbon to fumarate with the formation of benzylsuccinic acids (Evans *et al*., 1992; Biegert *et al*., 1996; Beller & Spormann, 1997; Krieger *et al*., 1999; Leuthner & Heider, 2000). Hence, benzylsuccinic acid and its methylated analogues have been proposed as indicators of anaerobic toluene and xylene metabolism in contaminated environments (Beller *et al*., 1995; Beller, 2000; Elshahed *et al*., 2001; Gieg & Suflita, 2002). A similar activation mechanism was proposed for *p*-xylene-degrading sulfate-reducing cultures (Morasch & Meckenstock, 2005). In this study, we resolved the activation of *p*-xylene under denitrifying conditions by GC–MS analysis of metabolites.

Materials and methods

Source of organisms and cultivation

A mixed inoculum of 150 mL freshwater pond sediment and 50 mL sludge from a wastewater treatment plant (Osterholz-Schambeck, Germany) was added to 800 mL defined anaerobic freshwater medium containing 5 mM nitrate (after Widdel & Bak, 1992). As an electron donor, we added 1% v/v *p*-xylene in 50 mL 2,2,4,4,6,8,8-heptamethylnonane (HMN). A control culture was prepared with HMN in the absence of *p*-xylene. The subsequent transfers were established with 10% v/v inoculum from the initial enrichment cultures and incubated under similar conditions. Isolation was initiated by four consecutive dilution-to-extinction series established in serum bottles containing 100 mL freshwater medium overlaid with 5% HMN as carrier for 1% v/v *p*-xylene. The second dilution to extinction was performed with a one to two dilution, whereas the remaining series had a one to 10 dilution. Each subsequent series was inoculated from the highest dilution that provided a grown culture. The resulting highly enriched cultures were incubated under moderate shaking (80 r.p.m.) with 10 mM nitrate and 1% *p*-xylene in HMN (v/v).

Isolation was attempted in solid agar, overlaid with 10% HMN as a carrier for 1% *p*-xylene. Colonies (*c*. 30) were selected and inoculated in liquid denitrifying media with 1% *p*-xylene in HMN (v/v).

All cultures were sealed with butyl rubber stoppers that had no contact with the organic phase, and secured with plastic screw caps (Duran bottles) or aluminum crimps (serum flasks). The headspace volume was between 10% and 40% of the bottle volume. Incubations were carried out at 28 °C under an N_2/CO_2 atmosphere (90/10 v/v).

The optimal *p*-xylene concentration for growth was determined in a culture volume of 10 mL with 10% v/v inoculum, 10 mM nitrate and 10% v/v HMN as the carrier phase for the hydrocarbon. The concentrations of *p*-xylene varied between 0.25% and 10% in HMN (v/v).

Growth tests were performed with the following hydrocarbons in HMN (v/v in %): benzene (0.5), toluene (1), ethylbenzene (1), *o*-xylene (1) *m*-xylene (1), 2-methylnaphthalene (2), naphthalene (2), limonene (1), *n*-hexane (0.5), cyclohexane (0.5) and *n*-decane (0.5). In addition, we tested cholesterol (granules), *o*-methylbenzoate (5 mM), *m*-methylbenzoate (5 mM) and *p*-methylbenzoate (5 mM), benzoate (5 mM), fumarate (10 mM) and lactate (10 mM).

The consumption of *p*-xylene and nitrate, and cell density increase were measured in cultures grown in 400 mL media, with 10 mM nitrate and 0.2% *p*-xylene (v/v in 40 mL HMN), inoculated with 5 mL concentrated cell suspension, which corresponds to a 12% v/v inoculum. Inoculated media in the absence of *p*-xylene and sterile media with *p*-xylene served as controls. Cell dry mass was quantified from 150 mL culture. The washed cell suspension was brought to a constant weight as described elsewhere (Harder, 1997), and we established a conversion factor between OD and cell dry weight.

Chemical analysis

Samples for chemical analysis were taken with N_2-flushed hypodermic needles and syringes.

Nitrate and nitrite samples were diluted 1 : 10 before ion chromatography on a Sykam HPLC IBJ A3 anion-exchange column (4 × 60 mm) (Sykam, Munich, Germany). Separation under isothermal conditions (50 °C) was achieved with 20 mM NaCl in 45% ethanol at a flow rate of 1 mL min^{-1}. Nitrate (retention time 3 min) and nitrite (2.1 min) were detected at 220 nm on a LINEAR Spectrophotometer. The signals were processed with DATAAPEX CLARITY HPLC software

(Gamma Analysen Technik GmbH, Bremerhaven, Germany). Fast nitrate and nitrite monitoring was performed using Merckoquant Test Strips (Merck, Darmstadt, Germany). Ammonium and dinitrogen oxide were determined as described elsewhere (Harder & Probian, 1997).

p-Xylene concentrations were measured by head-space GC (reproduced from Musat & Widdel, 2008). Measurements in the gaseous phase were possible because changes in xylene concentrations were rapid in all three phases (aqueous, gaseous and organic phase-HMN). A nearly equilibrated system was obtained by suppressing the formation of diffusive gradients via moderate shaking of cultures. Moreover, the consumption of *p*-xylene was much slower than the phase equilibrium. Gas volumes of 0.1 mL were withdrawn at 28 °C and injected into a Shimadzu GC-14B (Duisburg, Germany) equipped with a Supel-Q PLOT fused silica capillary column (length 30 m, diameter 0.53 mm) and a flame ionization detector. Isothermal separation was performed at 200 °C with N_2 as the carrier gas, the injector at 150 °C and the FID at 280 °C.

Samples for volatile fatty acid analysis were filtered and diluted 1 : 10 before injection onto a Sykam HPLC (Fürstenfeldbruck, Germany) equipped with an Aminex HPX-87 H HPLC column (300 × 7.8 mm). The eluent was 5 mM H_2SO_4. The separation was isothermal at 40 °C with the UV detector at 210 nm. Standards consisted of the following fatty acids: succinate, lactate, formate, acetate, propionate and butyrate. The HPLC detection limit was 100 μM.

Metabolites were extracted from 100 mL cells disrupted by heating at 85 °C for 40 min. Samples were acidified to pH 1 with H_3PO_4 before extraction with dichloromethane. The dichloromethane extract was dried with anhydrous Na_2SO_4 and derivatized before GC–MS analysis using a solution of diazomethane in diethyl ether. GC–MS measurements were performed using a Trace GC–MS (Thermoelectron, Dreieich, Germany) equipped with a temperature-programmable injection system and a BPX5 fused silica capillary column (length 50 m, inner diameter 0.22 mm, film thickness 0.25 μm). Helium was used as the carrier gas. The GC oven temperature was programmed from 50 °C (1-min isothermal) to 310 °C (30-min isothermal) at a rate of 3 °C min^{-1}. The mass spectrometer was operated in the electron-impact mode and at an ion source temperature of 230 °C. Full-scan mass spectra were recorded over the mass range of 50–600 Da at a rate of 2.5 scans s^{-1}.

Molecular analysis

Extracted and purified genomic DNA from highly enriched cultures (Zhou *et al.*, 1996) was used to amplify the almost full 16S rRNA gene sequence with specific bacterial primers, 8F (Hicks *et al.*, 1992) and 1492R (Kane *et al.*, 1993). The PCR products were cleaned using a QIAquick Purification Kit (Qiagen, Hilden, Germany) cloned into pCR4-TOPO vector (Invitrogen) and transformed into TOP10 chemically competent *Escherichia coli* cells (Invitrogen). Positive clones were sequenced using the ABI Prism BigDye Terminator 3.0 cycle sequencing kit and an ABI Prism 3130XL Genetic Analyzer (Applied Biosystems). Sequences were cleaned of vector data with SEQUENCE ANALYSIS 5.2 (Applied Biosystems) and assembled into full-length 16S rRNA gene sequences using SEQUENCHER software (Gene Codes Corporation). 16S rRNA gene sequences were aligned with sequences from the SILVA database version 94 (http://www.arb-silva.de) using the ARB software package (Ludwig *et al.*, 2004; Pruesse *et al.*, 2007). Different phylogenetic trees were calculated with nearly complete sequences (> 1300 nucleotides) using methods such as maximum likelihood, maximum parsimony and neighbor joining.

Two probes for FISH, pxyn-440 and pxyn-644, were specifically designed to target the dominant phylotype from our clone libraries (ARB PROBE DESIGN tool). Probes were tested *in silico* against RDP II database version 9.48 (http://rdp.cme.msu.edu/probematch/search.jsp). As two mismatch control for the specificity of pxyn-440, we used strain 72Chol (Harder & Probian, 1997). Probe pxyn-644 did not match any cultivated microorganism in the database. The specificity of the probes was ensured with a high formamide concentration (40%) during hybridization experiments. Nested FISH was performed with phylum to group-specific probes (Table 1) as described elsewhere (Amann *et al.*, 1995). All probes used were synthesized with a 5′-Cy3 modification.

Nucleotide sequence accession numbers

FM207901–FM207960 are the EMBL-EBI accession numbers of 16S rRNA gene sequences retrieved from two different enrichment cultures thriving on *p*-xylene.

Results and discussion

Enrichment and cultivation

Four sediment-containing enrichment cultures with 1% *p*-xylene in HMN as an organic energy source consumed their initial 5 mM nitrate in 80 days. Nitrate (5 mM) was resupplied several times until nitrate reduction ceased. The mixed inoculum contained endogenous carbon and energy sources, which supported the reduction of 25 mM nitrate within 450 days in the control enrichment without *p*-xylene. However, the four enrichments supplemented with *p*-xylene continued to reduce nitrate (50 mM) for 200 additional days. In these cultures, *p*-xylene was no longer detected after 650 days. Subsequent subcultures consumed 13–27 mM nitrate within 270 days. The two subcultures with the highest cumulative nitrate reduction were used as inoculum for four successive liquid dilutions-to-extinction series, which resulted in sediment-free highly enriched cultures. Highly enriched cultures showed faster growth

Table 1. Oligonucleotide probes used for FISH of enrichment cultures pXyN1 and pXyN3

Probe name	Sequence (5′–3′)	Target group coverage (%)	rRNA target site (*E. coli* numbering)	References
Eub-338 I*	GCT GCC TCC CGT AGG AGT	94% Domain Bacteria	16S (338–355)	Amann *et al*. (1990a)
Eub-338 II*	GCA GCC CAC CCG TAG GTG T	69% Order *Planctomycetales*	16S (338–355)	Daims *et al*. (1999)
Eub-338 III*	GCT GCC ACC CGT AGG TGT	93% Order *Verucomicrobiales*	16S (338–355)	Daims *et al*. (1999)
Bet-42a†	GCC TTC CCA CTT CGT TT	86% Class *Betaproteobacteria*	23S (1027–1043)	Manz *et al*. (1992)
pxyn-440	ACC ACC GTT TCG TTC CTG CT	*p*-Xylene phylotype-specific	16S (440–460)	This study
pxyn-644	CGT ATT AGG GAC CAC CGT TT	*p*-Xylene phylotype-specific	16S (644–664)	This study

*An equimolar amount of Eub (I, II and III) was used to enumerate Bacteria.

†This probe was used in equimolar amount with an unlabeled Gam 42a competitor probe (5′-GCC TTC CCA CAT CGT TT-3′) to enhance specificity.

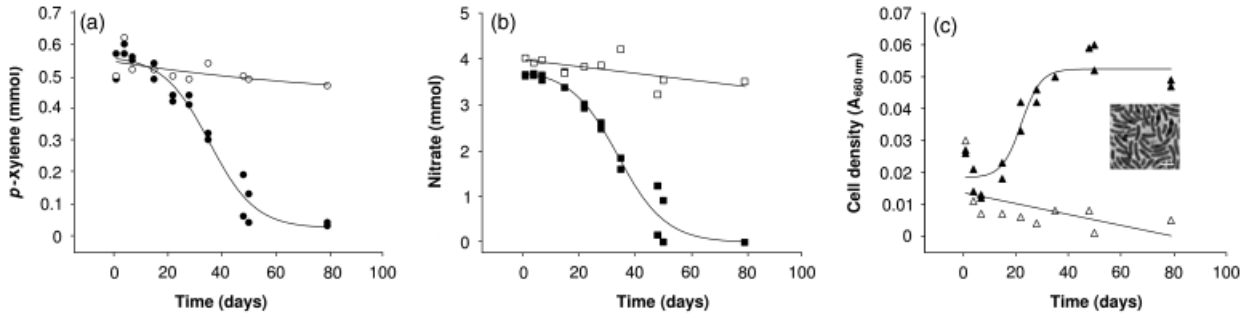

Fig. 1. Degradation of *p*-xylene in two enrichment cultures under denitrifying conditions (filled symbols). (a) *p*-xylene degradation (●), (b) nitrate consumption (■) and (c) the cell density (▲). Filled symbols are from two different enrichment cultures. *p*-Xylene did not decrease significantly in a sterile control (○). A minor nitrate loss (□) without an increase in cell density (△) was detected in an inoculated control incubated in the absence of the hydrocarbon. (c) Inset shows a phase-contrast micrograph of viable cells. Scale bar = 5 μm length.

rates than previously, with doubling times of 4 days at the optimal *p*-xylene concentration of 2% in HMN (v/v). These enrichments were dominated (> 96%) by thin, curved, rod-shaped microorganisms with 0.5 μm diameter and 2 μm length (Fig. 1c, inset). Anaerobic isolation attempts, in solid agar overlaid with 1% *p*-xylene in HMN (v/v) as an organic energy source, resulted in small colonies. Although five of 30 selected colonies grew in denitrifying liquid media with 1% *p*-xylene in HMN (v/v), they did not survive further transfers in liquid media. Some of the colonies were partially sequenced and were unrelated to the dominant phylotype (data not shown). An alternative isolation attempt was aerobic plating on different substrates that generated colonies incapable of utilizing *p*-xylene under denitrifying conditions during later transfers (data not shown).

Although several studies showed *p*-xylene consumption under anaerobic conditions (Edwards *et al*., 1992; Häner *et al*., 1995; Morasch & Meckenstock, 2005; Botton & Parsons, 2007; Nakagawa *et al*., 2008), this is the first highly enriched culture capable of *p*-xylene utilization under denitrifying conditions.

Growth tests on different hydrocarbons

Of all the tested hydrocarbons, besides *p*-xylene, only toluene sustained growth and denitrification. Cultures did not utilize the following hydrocarbons: benzene, ethylbenzene, *o*-xylene, *m*-xylene, naphthalene, 2-methylnaphthalene, limonene, *n*-hexane, cyclohexane or *n*-decane. Other substrates utilized by the denitrifying enrichment cultures were *p*-toluic acid, benzoate, fumarate and lactate. Cultures did not thrive on cholesterol, *o*- and *m*-toluic acid.

In cultures grown on *p*-toluic acid or on toluene, we observed the dominance of a morphotype similar to the one dominating the *p*-xylene-grown cultures. Different cell morphologies were observed in cultures grown on benzoate, fumarate and lactate.

In this study, highly enriched cultures utilized exclusively the *para* isomers of xylene and toluic acids and not the *ortho* or *meta* isomers. Previous studies on different strains capable of *o*- and *m*-xylene degradation showed a similar regiospecificity (Rabus & Widdel, 1995; Harms *et al*., 1999). Solely one strain, OX39, was capable of growing with two different isomers: *o*- and *m*-xylene (Morasch *et al*., 2004). The reasons for isomer-specific growth are hitherto unknown, although one could speculate that the substrate-binding capacity of the activating enzyme might play a role.

Quantification of *p*-xylene degradation

Initial experiments on enrichment cultures showed gas production (traces of methane, N_2O or O_2 were not detected), the consumption of xylene, nitrate and

ammonium and an OD increase (data not shown). This suggested that *p*-xylene degradation is coupled to denitrification.

To quantify the catabolism, cultures were grown with a small amount of hydrocarbon and monitored for 80 days (Table 2). Enrichment cultures utilized *p*-xylene (Fig. 1a) and consumed the nitrate added (Fig. 1b), and at the same time, the cell density increased (Fig. 1c). The concentrations of nitrite did not change during incubation. The nitrate consumed by *p*-xylene degradation (3.12 ± 0.01 mmol) in two enrichment cultures (Table 2) could accept 15.6 ± 0.4 mmol electrons if completely reduced to dinitrogen gas.

The total amount of *p*-xylene consumed by the two cultures was 0.5 ± 0.05 mmol. However, there is a physical loss of *p*-xylene of 6% from the initial amount added, as observed in a sterile control (Table 2). Moreover, a part of the electrons donated by *p*-xylene are used to generate biomass during anabolic processes. The amount of *p*-xylene that is incorporated into biomass (2.35 μmol) was estimated from cell dry weight measurements and the assimilatory reaction: $17C_8H_{10}+32HCO_3^-+32H^++30H_2O \rightarrow 42C_4H_7O_3$. By excluding the physical loss of *p*-xylene and the amount incorporated into biomass, we determined the amount of *p*-xylene (0.46 ± 0.06 mmol) fueling catabolism, which could donate 19.4 ± 2.57 mmol electrons if completely oxidized to CO_2 according to the dissimilatory reaction: $C_8H_{10}+8.4NO_3^-+8.4H^+ \rightarrow 8CO_2+4.2N_2+9.2H_2O$ ($\Delta G'_0$ is -4202.6 kJ mol^{-1} *p*-xylene). Besides the electron balance, additional evidence for the complete oxidation of *p*-xylene was the absence of fatty acid production in the culture media during incubation.

The electrons accepted by nitrate (15.6 ± 0.4 mmol electrons) from the electron donor (19.4 ± 2.57 mmol electrons) showed an electron recovery of $81 \pm 1.4\%$, which indicates stoichiometric coupling of *p*-xylene oxidation and nitrate reduction. An irrefutable conclusion on stoichiometry could be drawn by incubations with isotopically labeled substrates. However, our results on the quantitative turnover of substrates argue in favor of a complete oxidation of *p*-xylene under denitrifying conditions.

One other study proposed complete oxidation of *p*-xylene under denitrifying conditions based on comparable rates of electron transfer from the electron donor to the electron acceptor during exponential growth (Häner *et al.*, 1995). Few previous studies on enrichment cultures have shown that *p*-xylene oxidation is stoichiometrically coupled to reduction of sulfate (Morasch & Meckenstock, 2005;

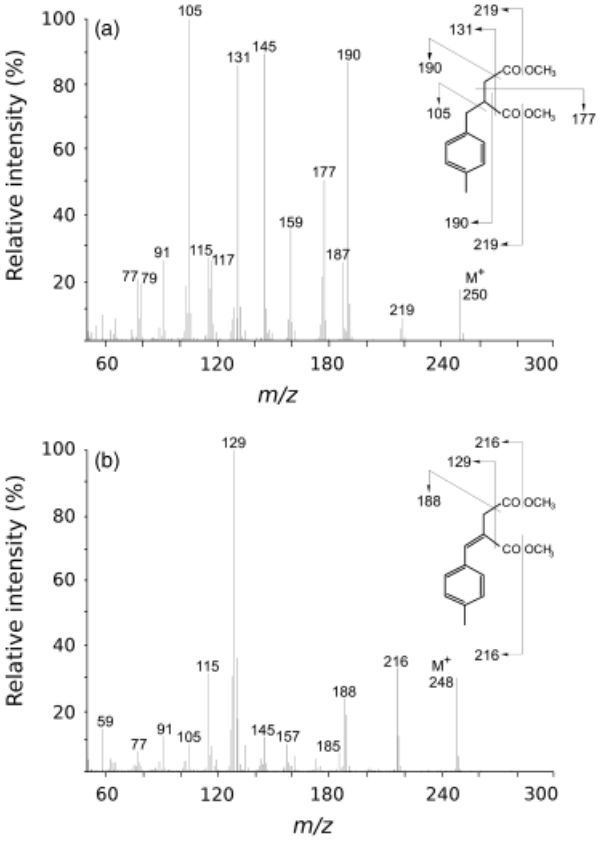

Fig. 2. Mass spectra of two methylated intermediary metabolites obtained from cultures grown with *p*-xylene as the sole organic energy source: (a) (4-Methylbenzyl)succinic acid dimethyl ester and (b) (4-methylphenyl)itaconic acid dimethyl ester.

Table 2. Quantification of *p*-xylene consumption and nitrate reduction in highly enriched cultures

Batch culture	NO_3^- initial (mmol)	NO_3^- final (mmol)	*p*-Xylene initial (mmol)	*p*-Xylene final (mmol)	Dry cell mass (mg)	Electrons accepted (mmol)	Electrons donated (mmol)	Electrons recovered* (%)
Cells with *p*-xylene (culture A)	3.62 ± 0.01	0	0.49 ± 0.05	0.03 ± 0.05	0.59	18.09 ± 0.04	19.33 ± 3.22	87 ± 2
Cells with *p*-xylene (culture B)	3.65 ± 0.00	0	0.57 ± 0.04	0.04 ± 0.06	0.62	18.23 ± 0.02	22.33 ± 2.48	75 ± 2
Sterile control with *p*-xylene	3.58 ± 0.07	3.40 ± 0.01	0.50 ± 0.05	0.47 ± 0.00	–	–	1.34 ± 2.21	–
Cells without *p*-xylene	4.01 ± 0.01	3.50 ± 0.00	0	0	0.12	2.55 ± 0.04	–	–

*The recovery represents the ratio of electrons accepted and electrons donated during catabolism after the following corrections: (1) the amount of electrons accepted by nitrate was corrected by subtracting the electrons gained from endogenous sources in an inoculated control culture without *p*-xylene; (2) similarly, we corrected the electrons donated by subtracting the xylene loss in a sterile control; and (3) the amount of electrons donated during dissimilation of *p*-xylene was calculated by excluding the amount assimilated into biomass from the total *p*-xylene consumed. We assumed that 2.1 mg cell dry mass requires 0.0039 mmol *p*-xylene considering the assimilation reaction (see text).

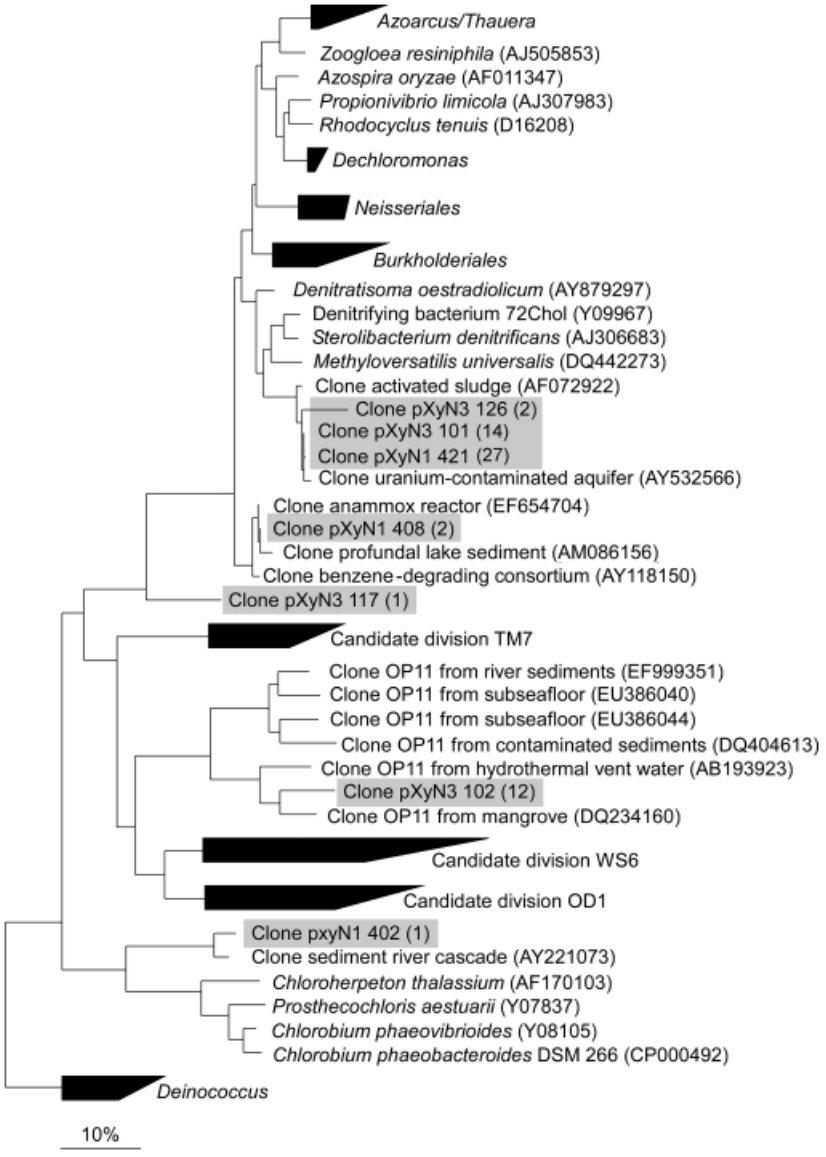

Fig. 3. Maximum-likelihood tree of 16S rRNA gene sequences from two enrichment cultures (pXyN1 pXyN3, 30 and 29 clones, respectively) grown on *p*-xylene as the sole carbon and energy source. Gray boxes indicate different sequence types encountered in the two clone libraries. The number of clone representatives for a phylotype in each clone library is mentioned in parentheses. The arrow points to *Deinococcus* species used as the outgroup. Scale bar = 10% sequence divergence.

Nakagawa *et al.*, 2008). Under iron-reducing (Botton & Parsons, 2007) and methanogenic conditions (Reinhard *et al.*, 2005), the process has not been studied quantitatively, although the consumption of *p*-xylene was observed.

Mechanism of *p*-xylene activation

In methylated extracts of acidified cultures, previously grown with 2% *p*-xylene in HMN (v/v) and 5 mM nitrate, two metabolites were detected as dimethyl esters (Fig. 2). Based on relative retention times, mass spectra and comparison with published spectra, we identified the two compounds in our denitrifying cultures as dimethyl esters of (4-methylbenzyl)succinate and (4-methylphenyl)itaconate. The mass spectra of (4-methylbenzyl)succinic acid dimethyl ester showed the molecular ion at *m*/*z* 250, the base peak at *m*/*z* 105 and further key fragment ions at *m*/*z* 131, 145, 177 and 190 (Fig. 2a). The mass spectrum of (4-methylphenyl) itaconic acid dimethyl ester showed the molecular ion at *m*/*z* 248, the base peak at *m*/*z* 129 and further key fragments at *m*/*z* 115, 188 and 216 (Fig. 2b). Their fragment patterns were similar to those of compounds found in sulfate-reducing enrichments degrading *p*-xylene (Elshahed *et al.*, 2001; Morasch *et al.*, 2004; Morasch & Meckenstock, 2005). The presence of these metabolites suggests a similar degradation pathway as described for toluene and *m*-xylene in the denitrifiers *Thauera aromatica* and *Azoarcus* sp. strain T, respectively (Biegert *et al.*, 1996; Krieger *et al.*, 1999; Leuthner & Heider, 2000). Nevertheless, after ring reduction, the mineralization pathway of *p*-xylene has one

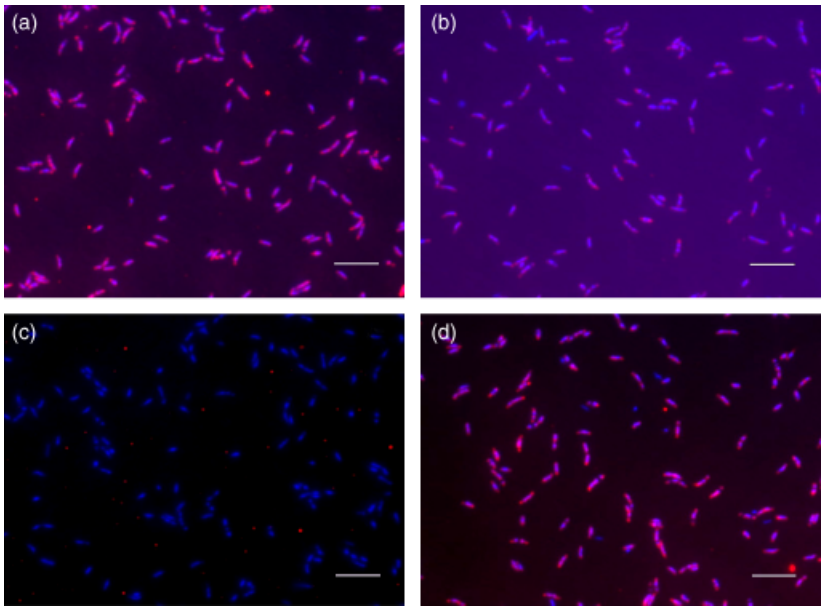

Fig. 4. Epifluorescence micrographs of cells from a denitrifying enrichment culture thriving on *p*-xylene. Images show the result of superimposed cells signals of DNA (DAPI in blue) and 16S rRNA gene (Cy3-labeled probe in red). (a) Cells stained with DAPI and hybridized with a Bacteria probe mix (Eub 338 I, II and III). (b) Cells stained with DAPI and hybridized with Bet-42a in the presence of a Gam 42a competitor. (c) The negative control where DAPI-stained cells were hybridized with a nonsense probe (Non 338). (d) DAPI-stained cells hybridized with a phylotype-specific probe designed during this study (pxyn-440). Scale bar = 5 μm.

additional 'obstacle': the methyl group on the ring system. So far, it is unknown to which extent this modification might influence the degradation. Somehow, these microorganisms succeed in performing complete oxidation, as suggested by the electron balance and the absence of fatty acid accumulation.

Phylogeny and cell hybridization

To identify the phylogeny of microorganisms in two highly enriched *p*-xylene-denitrifying cultures, pXyN1 and pXyN3, we constructed 16S rRNA gene libraries. Both clone libraries were dominated by one phylotype (41/59 clones) (Fig. 3). This phylotype was closely related (95% sequence identity) to the 17β-estradiol degrader, *Denitratisoma oestradiolicum* (Fahrbach *et al.*, 2006), and two cholesterol-degrading bacteria: strain 72Chol (94.4%) and *Sterolibacterium denitrificans* (94.2%) (Harder & Probian, 1997; Tarlera & Denner, 2003). However, the phylotype was distantly related ($< 92\%$) to *Azoarcus* and *Thauera* members, such as the cyclohexane-1,2-diol-degrading strain Lin22 (Harder, 1997), the propylbenzene-degrading strain PbN1 (Rabus & Widdel, 1995) and the toluene degrader *T. aromatica* K172 (Anders *et al.*, 1995).

The clone libraries contained representatives of five other phylotypes: three (5/59 clones) related to *D. oestradiolicum* (84.4–94%), one (1/59 clones) to *Chlorobium phaeobacteroides* DSM 266 (80%) and one (12/59 clones) to candidate division OP11 sequences (89.8%) from a river estuary mangrove (Fig. 3).

To resolve the relative dominance of the organisms in the enrichments, we applied FISH with phylum- to group-specific oligonucleotide probes (Fig. 4). A DNA stain, 4′,6-diamidino-2-phenylindole (DAPI), was used to detect all bacteria. Specific probes targeting different groups at the 16S rRNA gene level were used in hybridization experiments. The relative percentage of probe-targeted cells was related to the number of DAPI-stained cells (Fig. 4). The general Bacteria probe (Eub-338 I–III) hybridized to 97% of the total cells in two enrichment cultures. The nonsense probe, Non 338, hybridized ~1 cell of 500 DAPI-stained cells. The class-specific *Betaproteobacteria* probe (Bet-42a) hybridized to $> 93\%$ cells in both enrichments (93% pXyN1 and 95% pXyN3), whereas the newly designed probes (pxyn-440 and pxyn-644) specific for the *Denitratisoma*-related phylotype targeted $> 91\%$ of cells in both enrichment cultures (92% and 93% in pXyN1; 91% and 95% in pXyN3).

Surprisingly, the dominant microorganisms in our denitrifying cultures were only distantly related to *Azoarcus* and *Thauera* species. So far, these genera were thought to comprise all denitrifiers capable of alkylbenzene degradation (Widdel & Rabus, 2001). In contrast, the next 16S rRNA gene relatives of the dominant phylotype in these *p*-xylene-degrading enrichment cultures were denitrifying microorganisms that degrade sterols. Likewise, the dominant phylotype in denitrifying benzene-degrading enrichment cultures was identified to be related to a steroid degrader, *S. denitrificans* (Ulrich & Edwards, 2003). Presently, it is unknown, whether sterol-degrading bacteria are able to use monocyclic aromatics.

In conclusion, highly enriched cultures dominated by one *Denitratisoma*-related phylotype were capable of growing on two alkylbenzenes (toluene, *p*-xylene) and two polar monoaromatics (benzoate, *p*-toluic acid); however, they did not

utilize cholesterol. These physiologic and phylogenetic differences promote the classification of the dominant phylotype within a new genus of the family *Rhodocyclaceae*.

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