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ORIGINAL PAPER

Gerda Harms · Ralf Rabus · Friedrich Widdel

Anaerobic oxidation of the aromatic plant hydrocarbon *p*-cymene by newly isolated denitrifying bacteria

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Abstract The capability of nitrate-reducing bacteria to degrade alkyltoluenes in the absence of molecular oxygen was investigated with the three isomers of xylene, ethyltoluene, and isopropyltoluene (cymene) in enrichment cultures inoculated with freshwater mud. Denitrifying enrichment cultures developed most readily (within 4 weeks) with *p*-cymene, a natural aromatic hydrocarbon occurring in plants, and with *m*-xylene (within 6 weeks). Enrichment of denitrifiers that utilized *m*-ethyltoluene and *p*-ethyltoluene was slow (within 8 and 12 weeks, respectively); no enrichment cultures were obtained with the other alkylbenzenes within 6 months. Anaerobic degradation of *p*-cymene, which has not been reported before, was studied in more detail. Two new types of denitrifying bacteria with oval cells, strains pCyN1 and pCyN2, were isolated; they grew on *p*-cymene (diluted in an inert carrier phase) and nitrate with doubling times of 12 and 16 h, respectively. Strain pCyN1, but not strain pCyN2, also utilized *p*-ethyltoluene and toluene. Both strains grew with some alkenoic monoterpenes structurally related to *p*-cymene, e.g., α-terpinene. In addition, the isolates utilized *p*-isopropylbenzoate, and mono- and dicarboxylic aliphatic acids. Determination of the degradation balance of *p*-cymene and growth with acetate and nitrate indicated the capacity for complete oxidation of organic substrates under anoxic conditions. Adaptation studies with cells of strain pCyN1 suggest the existence of at least two enzyme systems for anaerobic alkylbenzene utilization, one metabolizing *p*-cymene and *p*-ethyltoluene, and the other metabolizing toluene. Excretion of *p*-isopropylbenzoate during growth on *p*-cymene indicated that the methyl group is the site of initial enzymatic attack. Although both strains were facultatively aerobic, as revealed by growth on acetate under air, growth on *p*-cymene under oxic conditions was observed only with strain pCyN1. Strains pCyN1 and pCyN2 are closely related to members of the *Azoarcus-Thauera* cluster within the β-subclass of the Proteobacteria, as revealed by 16S rRNA gene sequence analysis. This cluster encompasses several described denitrifiers that oxidize toluene and other alkylbenzenes.

Key words Anaerobic degradation · Denitrifying bacteria · Aromatic hydrocarbons · Alkylbenzenes · *p*-Cymene · Alkylbenzoates · Intermediates · 16S rRNA sequence analysis

Dedicated to Professor Rudolf K. Thauer on the occasion of his 60th birthday

G. Harms · R. Rabus · F. Widdel (✉)
Max-Planck-Institut für Marine Mikrobiologie, Celsiusstrasse 1, D-28359 Bremen, Germany
e-mail: fwiddel@mpi-bremen.de,
Tel.: +49-421-2028-702, Fax: +49-421-2028-790

Present address:
G. Harms, Center for Environmental Biotechnology, University of Tennessee, 676 Dabney Hall, Knoxville, TN 37996–1605, USA

Introduction

Studies on the anaerobic degradation of aromatic hydrocarbons have mainly focused on toluene. Several pure cultures of toluene-degrading denitrifying bacteria (Dolfing et al. 1990; Altenschmidt and Fuchs 1991; Evans et al. 1991; Schocher et al. 1991; Fries et al. 1994; Seyfried et al. 1994; Rabus and Widdel 1995 a; Hess et al. 1997), iron(III)-reducing bacteria (Lovley et al. 1989), or sulfate-reducing bacteria (Rabus et al. 1993; Beller et al. 1996) have been isolated and characterized. It has been recently demonstrated that the initial enzymatic reaction of anaerobic toluene degradation in strains of denitrifying bacteria (Biegert et al. 1996; Beller and Spormann 1997 a; Leuthner et al. 1998; Rabus and Heider 1998) and sulfate-reducing bacteria (Beller and Spormann 1997b; Rabus and Heider 1998) occurs at the methyl group by condensation with fumarate, yielding benzylsuccinate. The enzyme catalyzing this reaction, benzylsuccinate synthase, involves a glycyl radical, as indicated by genetic analysis (Coschigano et al. 1998; Leuthner et al. 1998). It has been shown that the higher homologues of toluene, ethylbenzene, and *n*-propylbenzene are also degraded by pure cultures of

denitrifying bacteria (Rabus and Widdel 1995 a; Ball et al. 1996). Initial biochemical studies with ethylbenzene have indicated formation of 1-phenylethanol as a product of the anaerobic activation (Ball et al. 1996; Rabus and Heider 1998). It has been shown that of the dialkylbenzenes, *o*- and *m*-xylene are degraded by pure cultures of anaerobic bacteria. *m*-Xylene is utilized by isolated strains of denitrifying bacteria (Dolfing et al. 1990; Fries et al. 1994; Seyfried et al. 1994; Rabus and Widdel 1995 a; Hess et al. 1997) and sulfate-reducing bacteria (Harms et al. 1999). Anaerobic degradation of *o*-xylene has been demonstrated in a pure culture of a sulfate-reducing bacterium (Harms et al. 1999) and in enriched bacterial communities (Edwards et al. 1992; Edwards and Grbić-Galić 1994; Rueter et al. 1994; Chen and Taylor 1997). Anaerobic mineralization of *p*-xylene has been documented in enrichment cultures (Edwards et al. 1992; Häner et al. 1995; Chen and Taylor 1997), but not in cultures of isolated strains. In pure cultures of toluene-degrading anaerobes, *o*- and *p*-xylene have been converted to dead-end metabolites (Evans et al. 1991; Biegert and Fuchs 1995; Rabus and Widdel 1995 b; Beller et al. 1996; Beller and Spormann 1997 a). Relatively little is known about the anaerobic degradation of higher homologues of xylenes. *o*-Ethyltoluene, *m*-ethyltoluene, *m*-propyltoluene, and *m*-cymene in crude oil are slowly utilized by a sulfate-reducing (Rabus et al. 1996) and a denitrifying (Rabus et al. 1999) enrichment culture. Furthermore, slow utilization of *p*-ethyltoluene added to an enrichment culture obtained with *p*-xylene has been observed (Häner et al. 1995). The reported slow anaerobic utilization of xylenes other than the *m*-isomer and the limited information about degradability of the higher homologues of xylenes prompted us to carry out enrichment studies with alkyltoluenes of varying chain sizes under conditions of nitrate reduction. Among the alkyltoluenes tested, *p*-cymene was the most readily degradable compound. *p*-Cymene is a natural aromatic hydrocarbon that occurs in the oils of many gymnospermic and angiospermic plants (Gildemeister and Hoffmann 1960; Hegnauer 1966). Furthermore, formation of *p*-cymene as a dead-end metabolite from certain nonaromatic monoterpenes has been observed in anoxic enrichment cultures (Harder and Probian 1995; Harder and Foß 1999). To our knowledge, microbial degradation of *p*-cymene has been demonstrated with aerobic bacteria (DeFrank and Ribbons 1977; Eaton 1997), but to date not with anaerobic bacteria. The present study focuses on the anaerobic degradation of *p*-cymene by the two pure cultures of newly isolated denitrifying bacteria, strains pCyN1 and pCyN2. Strain pCyN1, but not strain pCyN2, also utilized *p*-ethyltoluene and toluene for growth.

Materials and methods

Sources of bacteria

Enrichment of denitrifiers was attempted with a homogenized mixture of freshwater mud samples from ditches in Bremen, Germany. The alkylbenzene-degrading denitrifying strains ToN1, mXyN1, EbN1, and PbN1 were maintained in the laboratory since their isolation on toluene, *m*-xylene, ethylbenzene, and propylbenzene, respectively (Rabus and Widdel 1995 a).

Media, routine cultivation, and quantitative growth experiments

Techniques for preparation of media and cultivation of bacteria under anoxic conditions have been described elsewhere (Widdel and Bak 1992). The defined, bicarbonate-buffered mineral medium with vitamins and trace metals had the same composition as that used for cultivation of other alkylbenzene-degrading denitrifiers (Rabus and Widdel 1995 a). Unless otherwise indicated, the medium contained 10 mM $NaNO_3$. Only pure cultures were grown in the presence of sodium ascorbate (4 mM) as a reductant (Rabus and Widdel 1995 a), which did not serve as a growth substrate for the isolated strains.

Anaerobic cultures were routinely grown in volumes of 15 ml in glass tubes (20 ml) that were anoxically sealed with butyl-rubber stoppers. The head space was N_2/CO_2 (90 : 10, v/v). Filter-sterilized hydrocarbons, aromatic alcohols, aldehydes, and ketones (viz. poorly water-soluble compounds) were diluted (0.5–5%, v/v) in a carrier phase (0.5 or 1 ml per tube) of deaerated 2,2,4,4,6,8,8-heptamethylnonane or mineral oil (high-viscosity, pharmaceutical grade; Lamotte, Bremen) to avoid toxic effects of the pure substances (Rabus et al. 1993). Cultures with a carrier phase were incubated nearly horizontally on a rotary shaker at low speed (65 rpm). The stopper-sealed orifices were always kept below the surface level of the medium to avoid contact between stoppers and carrier phase. Anoxic, sterile crude oil was prepared and added as previously described by Rabus and Widdel (1996). Soluble substrates were added from separately sterilized, aqueous stock solutions (Widdel and Bak 1992).

Utilization of oxygen as electron acceptor was tested in reductant-free medium in closed bottles under a head space of air with 10% (v/v) CO_2 as described by Rabus and Widdel (1995 a).

Quantitative growth experiments to measure the consumption of *p*-cymene and nitrate, and the formation of nitrite, nitrous oxide, and cell dry mass were carried out in flat bottles (500 ml) containing 400 ml medium, 10 ml inoculum, and 10 ml heptamethylnonane as carrier phase for *p*-cymene under an atmosphere of N_2/CO_2 (90 : 10, v/v), as described for growth experiments with other aromatic hydrocarbons (Rabus and Widdel 1995 a). The dry mass of washed cells was determined gravimetrically as described by Rabus and Widdel (1995 a).

Enrichment procedure, isolation, purity control, and maintenance

Initial enrichment studies were carried out in 500-ml glass bottles containing 150 ml mud, 250 ml medium without reductant, and 12 ml heptamethylnonane with 2% (v/v) of the respective alkyltoluene under a gas phase of N_2/CO_2 (90 : 10, v/v). Nitrate was added only at a concentration of 5 mM. Bottles were incubated nearly horizontally while contact of the hydrocarbon phase with the stoppers was avoided. Bottles were shaken only twice per day for a few seconds. Growth of denitrifying bacteria was monitored by releasing and measuring the gas produced during denitrification as described recently (Rabus et al. 1999). When gas production was no longer detected, 5 mM nitrate was again injected from an anoxic, concentrated stock solution. Thereafter, bottles were shaken and incubated nearly horizontally as before.

Strains were isolated via repeated dilution in agar tubes (Widdel and Bak 1992) under conditions of denitrification in the presence of ascorbate (2.7 mM) as a reductant. *p*-Isopropylbenzoate (0.7 mM) and acetate (7 mM) were used as soluble organic substrates instead of *p*-cymene. The homogeneity of cells in cultures on various substrates (Table 1) was routinely checked by phase-contrast microscopy. In addition, cultures were supplied with yeast extract (0.5 g/l) and glucose (5 mM), and were examined microscopically. For maintenance, strains were grown on *p*-cymene [2% (v/v), in heptamethylnonane], stored at 4 °C, and transferred every 4 weeks.

Table 1 Growth tests with denitrifying strains pCyN1 and pCyN2 under anoxic conditions on aromatic and nonaromatic compounds. Each compound was tested twice at the concentrations given *in parentheses*. Unless otherwise noted, concentrations are given in mM. Concentrations in % (v/v) refer to dilutions of hydrophobic compounds in heptamethylnonane as an inert carrier phase. Test media were inoculated with 5% (v/v) of their volume from fresh cultures grown with *p*-cymene. ++ Good growth within approximately 4 days or less; + growth time longer than 4 days; – no growth

Compound tested[a, b]	Strain	
	pCyN1	pCyN2
Aromatic hydrocarbons		
Toluene (2%)	++	–
p-Xylene (2%)	–	–
p-Ethyltoluene (2%)	++	–
p-Cymene (2, 5%)	++	++
Aromatic compounds with functional groups		
p-Cresol (0.5, 2)	+	–
Benzyl alcohol (0.5, 2)	++	–
3-Phenyl-1-propanol (0.5, 2)	++	–
p-Methylbenzyl alcohol (0.5, 2)	–	–
p-Ethylbenzyl alcohol (0.5, 1%)	–	–
p-Isopropylbenzyl alcohol (0.5, 1%)	+	+
Benzaldehyde (0.5, 2)	+	–
p-Ethylbenzaldehyde (0.5, 1%)	+	+
p-Isopropylbenzaldehyde (0.5, 1%)	++	++
Benzoate (1, 4)	++	–
Phenylacetate (1, 4)	++	–
3-Phenylpropionate (1, 4)	++	–
p-Methylbenzoate (1, 4)	–	–
p-Ethylbenzoate (0.5, 2)	++	+
p-Propylbenzoate (0.5, 2)	+	–
p-Isopropylbenzoate (0.5, 2)	++	++
p-*tert*-Butylbenzoate (0.5, 2)	–	–
Mandelate (0.5, 2)	+	–
Phenylalanine (0.5, 2)	+	–
Tyrosine (0.5, 2)	+	–
Tryptophan (0.5, 2)	+	–
Further compounds		
Ethanol (1, 5)	++	–
1-Propanol (1, 5)	+	–
Isopropanol (5, 10)	++	–
1-Butanol (1, 5)	+	–
2-Butanol (1, 5)	+	–
Acetone (0.5, 2)	+	–
2-Butanone (0.5, 2)	++	–
Acetate (5, 10)	++	++
Propionate (5, 10)	++	++
Butyrate (5, 10)	++	++
Pyruvate (1, 5)	+	+
Cyclohexanecarboxylate (0.5, 2)	+	–
Serine (1, 5)	–	+

[a] Further compounds utilized by both strains as growth substrates: α-phellandrene (1, 2%), α-terpinene (1, 2%), γ-terpinene (1, 2%), limonene (1, 2%), isolimonene (1, 2%), sabinene (1, 2%), α-pinene (1, 2%), *p*-menth-1-ene (1, 2%), valerate (1, 5), caproate (1, 5), caprylate (1, 2), palmitate (0.5, 1), succinate (1, 5), fumarate (1, 4), malate (1, 5), glutarate (1, 4), lactate (5, 10), and glutamate (1, 5)

[b] Further compounds tested but not utilized by both strains: benzene (2%), ethylbenzene (2%), *n*-propylbenzene (2%), isopropylbenzene, (2%), *o*-xylene (2%), *m*-xylene (2%), *o*-ethyltoluene (2%), *m*-ethyltoluene (2%), *o*-cymene (2%), *m*-cymene (2, 5%), *p*-*tert*-butyltoluene (2, 5%), 1,3,5-mesitylene (2, 5%), naphthalene (20 mg/ml heptamethylnonane), 2-carene (1, 2%), 3-carene (1, 2%), *n*-hexane (2, 5%), *n*-decane (5, 10%), cyclohexane (2%), methylcyclohexane (2%), phenol (0.5, 2), *o*-cresol (0.5, 2), *m*-cresol (0.5, 2), acetophenone (1%), *o*-methylbenzoate (1, 4), *m*-methylbenzoate (1, 4), 4-phenylbutyrate (1, 4), benzylsuccinate (0.5, 2), methanol (5, 10), formate (10, 20), citrate (1, 5), glucose (1, 5), fructose (1, 5), mannose (1, 5), glycine (1, 5), alanine (1, 5), aspartate (1, 5), and H_2

Adaptation studies with alkylbenzenes

The adaptation of strain pCyN1 to the utilization of aromatic compounds was studied with freshly grown, resuspended cells. Cells were first grown in flat 500-ml bottles with 400 ml medium and 5 ml carrier phase containing *p*-cymene (5%, v/v), *p*-ethyltoluene (2%, v/v), or toluene (2%, v/v). Catabolic activities of harvested cells (concentrated twofold) toward individual alkylbenzenes were measured under anoxic conditions in 70-ml vials (shaken) with 50 ml medium and 2 ml heptamethylnonane as described elsewhere (Champion et al. 1999).

Chemical analyses

p-Cymene in the heptamethylnonane phase was determined by means of an Autosystem gas chromatograph (Perkin Elmer, Überlingen, Germany) equipped with a PVMS 54 column (length, 50 m; internal diameter, 0.32 mm) and a flame ionization detector. The flow rate of the carrier gas, N_2, was 1.7 ml · min^{-1}. The temperature of the injection port was 150 °C. The temperature gradient for the column was run from 60 °C (2-min isotherm) to 240 °C at a heating rate of 15 °C · min^{-1}. The temperature of the detector was 280 °C. *p*-Cymene in the aqueous phase was quantified with a high-performance liquid chromatography system (Sykam, Gilching/Munich, Germany) on a Spherisorb ODS-2 reversed-phase column (250 × 5 mm) with a 4 : 1 mixture of acetonitrile and distilled water as eluent; the flow rate was 1 ml · min^{-1}. The temperature of the column was 25 °C (Rabus and Widdel 1995 a). *p*-Cymene was detected by UV absorption at 265 nm (fast scanning detector; Linear Instruments, Nev., USA). The detection limit in the aqueous phase was around 5 µM. A search for aromatic metabolites in the aqueous phase was carried out with the same system; the eluent, however, was a 1:1 mixture of acetonitrile and distilled water with 0.75 mM H_3PO_4 (final concentration). For identification, retention times and absorption spectra between 195 and 360 nm were compared to those of standards. Samples and standards were prepared with acetonitrile and aqueous H_3PO_4 so as to obtain the composition of the eluent. To confirm identity, a mixture of the sample and the standard solution was injected. If no additional peak occurred and the absorption spectrum remained unchanged, the detected metabolite was assumed to be identical with the standard compound.

Nitrate and nitrite were quantified by high-performance liquid chromatography according to Rabus and Widdel (1995 a). Nitrous oxide (N_2O) in the gas phase of cultures was measured by gas chromatography according to Rabus and Widdel (1995 a). Nitrous oxide dissolved in the aqueous phase was calculated from the partial pressure in the gas phase by using a solubility coefficient (at 25 °C) of 0.24 mmol l^{-1} kPa^{-1} [calculated from Lide (1998)]. Ammonium was measured using the indophenol formation reaction (Berthelot reaction) according to Marr et al. (1988). Dinitrogen formed by nitrate reduction was quantified in a volumetric device (with CO_2 absorption) as described by Rabus et al. (1999).

The G+C content of the DNA was determined at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braun-

schweig, Germany) as described elsewhere (Rabus and Widdel 1995 a).

Sequence analysis of 16S rRNA genes

Sequence analysis of 16S rRNA genes was carried out at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) as described previously (Rainey et al. 1992; Rabus and Widdel 1995 a). Data are deposited under EMBL accession nos. Y17284 (strain pCyN1) and Y17285 (strain pCyN2).

Results

Enrichment studies with *p*-alkyltoluenes

The capacity of nitrate-reducing bacteria to degrade various alkyltoluenes (alkylmethylbenzenes) was examined in anaerobic enrichment cultures in volumes of 400 ml with the *ortho*-, *meta*-, and *para*-isomers of xylene, ethyltoluene, and isopropyltoluene (cymene); the alkyltoluenes were dissolved in heptamethylnonane. A control contained heptamethylnonane without any aromatic hydrocarbon. All enrichment cultures contained the same amount of homogenized mud. Gas production began within 12 h in each bottle. New nitrate was added in portions of 5 mM whenever gas production ceased. Denitrification during the first few days was vigorous in each bottle including the control (approximately 25 ml gas per day), indicating denitrification with compounds from the mud. Thereafter, gas production in the bottles decreased gradually. However, in the bottles with *p*-cymene and *m*-xylene, denitrification increased again after 3–4 and 5–6 weeks, respectively. When denitrification in these bottles slowed down again, new *p*-cymene and *m*-xylene, respectively, were added. This caused a new increase of gas production (approximately 25 ml gas per day) in the presence of nitrate, indicating that *p*-cymene and *m*-xylene indeed served as organic substrates for denitrification. After 8 and 12 weeks, bottles containing *m*- and *p*-ethyltoluene, respectively, also showed a slight increase of denitrification. Denitrification in these bottles was also accelerated (18 ml gas per day) by the new addition of ethyltoluenes. In all other bottles, no increase of gas production was observed within 6 months, indicating that *o*-xylene, *p*-xylene, *o*-ethyltoluene, *o*-cymene, and *m*-cymene were not utilized during this period by denitrifying bacteria in the added sediment.

Further studies of anaerobic *p*-alkyltoluene degradation were focused on *p*-cymene because it represents a plant hydrocarbon and is therefore expected to be more widespread in natural habitats than the other eight alkyltoluenes tested. The utilization of *p*-cymene was also of interest because an anaerobic degradation of this aromatic compound had not been reported before. In contrast to the initial enrichment culture, subcultures exhibited fast nitrate reduction only if slightly agitated. The high content of mud in the original enrichment culture probably stabilized emulsions of the carrier phase, which allowed good *p*-cymene supply to bacteria even without shaking. With the gradual disappearance of mud, the carrier phase turned into a coherent layer such that molecular diffusion of *p*-cymene into nonagitated medium probably became growth-limiting.

Enrichment studies with *p*-alkylbenzoates

The results of the enrichment studies with various alkyltoluenes under anoxic conditions suggested that there are marked differences in the efficacy of the anaerobic degradation of the three homologues *p*-cymene, *p*-ethyltoluene, and *p*-xylene in denitrifying bacteria. Interestingly, the compound with the largest *p*-alkyl group was degraded most readily. The structure of the *p*-alkyl substituent may be relevant not only for the initial reaction, but also for subsequent reactions of intermediates. Assumed intermediates in the degradation of *p*-alkyltoluenes are the corresponding *p*-alkylbenzoyl-CoA thioesters, in analogy to toluene metabolism via benzoyl-CoA (Heider et al. 1999). We therefore tested in separate enrichment cultures with *p*-alkylbenzoates whether the structure of the *p*-alkyl substituent has an influence on their degradation similar to that of the *p*-alkyltoluenes. Indeed, the most rapid growth of denitrifying bacteria (in initial enrichment culture within 5 days) was observed with *p*-isopropylbenzoate (*p*-cumate); sediment-free subcultures exhibited a doubling time of approximately 7 h. However, results obtained with *p*-methylbenzoate and *p*-ethylbenzoate did not parallel those obtained with the corresponding *p*-alkyltoluenes. The enrichment culture on *p*-methylbenzoate grew almost as rapidly (in initial culture, within 10 days) as that on *p*-cumate; a sediment-free subculture exhibited a doubling time of approximately 8 h. With *p*-ethylbenzoate, distinctive development of denitrifying bacteria from sediment was slower (within approximately 15 days), and growth in subcultures was poor.

Isolation of denitrifying bacteria that utilize *p*-cymene

After the mud particles had disappeared in subcultures with *p*-cymene, isolation of denitrifiers was attempted in agar dilution series. When *p*-cymene was provided as substrate in an overlying carrier phase, colonies developed only in the upper few millimeters below the carrier phase. Under the stereomicroscope, two types of colonies were detected with approximately equal abundance. One type was whitish and opaque; the other type was slightly pinkish and somewhat transparent. However, isolation by means of drawn Pasteur pipettes (Widdel and Bak 1992) was difficult because colonies were very small (diameter, < 0.1 mm). Alternative dilutions were therefore carried out in agar with *p*-isopropylbenzoate (the free form of an assumed intermediate of *p*-cymene degradation) and acetate. These substrates yielded bigger colonies (diameter, ≥ 0.2 mm) of the previously observed types. All isolated strains continued to grow in liquid medium with *p*-cymene

in a carrier phase. One isolate from a whitish colony and one isolate from a pinkish colony were chosen for further studies. The strains are designated as pCyN1 and pCyN2, respectively.

Morphological, physiological, and other characteristics

Both isolates had the same cell shape and cell size (strain pCyN1 is shown in Fig. 1), with dimensions of 0.8–1 × 1.2–2 μm. Motility was observed only with strain pCyN2. The G+C content of the DNA of strains pCyN1 and pCyN2 was 61 and 59.8 mol%, respectively.

Strain pCyN1 showed a temperature range for growth of 5–34 °C, with an optimum around 32 °C. The temperature range for growth of strain pCyN2 was between 15 and 35 °C, with an optimum around 32 °C. The pH range for growth of strain pCyN1 was between pH 6.1 and 9.0, with an optimum around pH 7.4. Strain pCyN2 had a pH range between pH 6.6 and 9.0, with an optimum around pH 8.0.

Growth of strains pCyN1 and pCyN2 occurred with *p*-cymene concentrations of up to 10% (v/v) in the carrier phase. Higher concentrations inhibited growth. Optimal growth was observed with 5% *p*-cymene. Approximate doubling times determined (via semilogarithmic plot) from the initial exponential part of the growth curves obtained at this concentration were 12 and 16 h, respectively.

Nutritionally, strain pCyN1 was more versatile than strain pCyN2. Whereas the latter utilized only *p*-cymene in growth tests with various aromatic hydrocarbons, strain pCyN1 also grew on toluene and *p*-ethyltoluene. Growth on these two compounds was somewhat slower than it was on *p*-cymene. Strain pCyN2 did not use benzoate, which to our knowledge is a common substrate of all alkylbenzene-degrading denitrifiers described to date (Dolfing et al. 1990; Altenschmidt and Fuchs 1991; Evans et al. 1991; Schocher et al. 1991; Rabus and Widdel 1995 a; Ball et al. 1996; Beller and Spormann 1997 a). Both strains were able to utilize a number of nonaromatic monoterpene hydrocarbons containing double bonds (Table 1). Strain pCyN1 grew anaerobically with crude oil; with 1.5 ml oil per 15 ml medium containing 10 mM nitrate, the strain reached an optical density (at 660 nm) of approximately 0.3 within 3 days. Strain pCyN2 grew only poorly on crude oil. The higher cell density reached by strain pCyN1 on oil is probably due the ability of this isolate to utilize toluene (Table 1), a major aromatic oil constituent.

Strain pCyN1 grew equally fast with nitrate concentrations of 5 and 10 mM. In contrast, when 10 mM nitrate was added to cultures of strain pCyN2, proliferation during the late growth phase was somewhat impeded. This effect was probably due to higher nitrite concentrations formed by strain pCyN2 in comparison to strain pCyN1 (6 versus 3 mM, respectively, from 10 mM nitrate). In separate growth tests with potential electron acceptors, both strains utilized nitrite (5 mM) and nitrous oxide (7 ml injected into 20-ml tubes containing 10 ml head space) besides nitrate, but no sulfate (6 mM), sulfite (2 mM), thiosulfate (6 mM), or fumarate (25 mM). Although both strains were facultatively aerobic, as shown by growth on acetate and butyrate under air, only strain pCyN1 was able to grow aerobically with *p*-cymene.

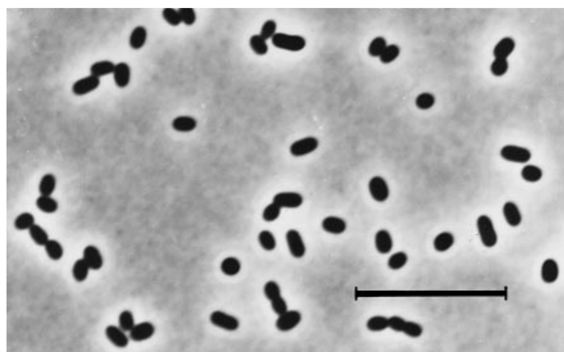


Fig. 1 Phase-contrast photomicrograph of denitrifying strain pCyN1 grown on *p*-cymene and nitrate (*bar* 10 μm)

Growth tests with described alkylbenzene-degrading denitrifying bacteria on *p*-cymene

Growth of denitrifying strains ToN1, mXyN1, EbN1, and PbN1, which had been isolated previously with toluene, *m*-xylene, ethylbenzene, and propylbenzene, respectively (Rabus and Widdel 1995 a), was also tested in the presence of *p*-cymene as sole organic compound. However, none of these strains grew with *p*-cymene.

Degradation balance and growth yield

The balance of anaerobic *p*-cymene degradation by strain pCyN1 was determined in two separate experiments with a small and a large amount of *p*-cymene (0.26 and 1.28 mmol, respectively, per 410-ml culture volume; dissolved in carrier phase) in the presence of the same nitrate concentration (nearly 10 mM). In the experiment with the large amount of *p*-cymene, the disappearance of nitrate, formation and consumption of nitrite, and increase in the cell density (optical density) over time were also monitored (Fig. 2). At the end of growth, remaining *p*-cymene and formed cell dry mass were determined in both experiments, and the electron balance was calculated. Results are shown in Table 2. The amount of electrons that can be theoretically derived from the measured *p*-cymene consumption was somewhat higher than the amount of electrons consumed by nitrate reduction. Similar results were obtained with strain pCyN2 (data not shown in Table 2). Growth of the latter with 0.77 mmol *p*-cymene and 2.4 mmol nitrate (in 410 ml medium) yielded a total consumption of 0.37 mmol of the organic substrate and complete disappearance of the electron acceptor. With 20 mg cell dry mass formed, the dissimilated amount of *p*-

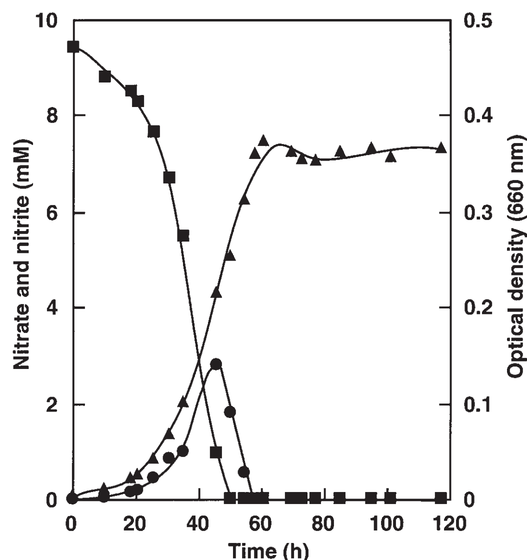


Fig. 2 Anaerobic growth of strain pCyN1 with *p*-cymene and nitrate. The experiment was carried out in 410 ml medium and 0.2 ml (1.28 mmol) *p*-cymene in 10 ml anoxic heptamethylnonane as inert carrier phase. Optical density (▲), nitrate (■), and nitrite (●) were measured in samples taken by means of N_2-flushed syringes. The initial concentration of nitrate was 9.76 mM when calculated from the added amount, and 9.5 mM when measured subsequently

cymene was 0.31 mmol (for calculation, see footnote in Table 2); hence, 16.7 mmol electrons can be derived from the consumed *p*-cymene, while only 12.0 mmol electrons were required for nitrate reduction to dinitrogen. The growth yields calculated as cell dry mass formed per amount of *p*-cymene dissimilated were 68 or 74 g mol^{-1} (strain pCyN1, two experiments) and 65 g mol^{-1} (strain pCyN2). Reduction of nitrate to dinitrogen, as already concluded from the gas formation in the enrichment culture, was confirmed. Measurement of the volume of produced gas (including CO_2 absorption with NaOH solution) after complete consumption of the electron acceptor in two experiments yielded 11.8 and 13.3 ml (at 28 °C) per mmol nitrate reduced, which is 0.48 and 0.54 mmol gas per mmol nitrate. Formation of ammonium ions was not observed; rather, the ammonium concentration in the medium decreased due to assimilation. All these results are in good agreement with the formation of N_2 as the final product of nitrate reduction.

Adaptation experiments with various alkylbenzenes

Strain pCyN1 was able to degrade three different alkylbenzenes (*p*-cymene, *p*-ethyltoluene, and toluene) anaerobically. We therefore tested whether cells growing on one of these compounds were simultaneously adapted to also utilize the other two compounds, or whether adaptation was specific for each alkylbenzene. Cells harvested from cultures on *p*-cymene and *p*-ethyltoluene were resuspended and incubated with each of the three *p*-alkyltoluenes, and the consumption of nitrate was followed. As a control, cells were incubated without added electron donor in the presence of nitrate. Cells from each of these two cultures oxidized *p*-cymene and *p*-ethyltoluene without any lag phase, as was obvious from the time course of nitrate reduction (Fig. 3 A, B). Toluene was utilized only after a pronounced lag phase of >35 h. Vice versa, cells pregrown on toluene exhibited a lag phase of >18 h when incubated with *p*-cymene. When toluene-grown cells were supplied with *p*-ethyltoluene, nitrate reduction did not sig-

Table 2 Quantification of *p*-cymene consumption, nitrate reduction, and formation of cell mass by strain pCyN1. Experiments were carried out in anoxic flat bottles with a culture volume of 410 ml. The total amount of nitrate added to each bottle was 4.0 mmol (concentration, 9.76 mM). The medium was overlaid with 10 ml heptamethylnonane as carrier phase for *p*-cymene

Experiment	*p*-Cymene added (mmol)	*p*-Cymene disappeared[a] (mmol)	Nitrate remaining after growth (mmol)	Nitrite remaining after growth (mmol)	Nitrous oxide remaining after growth[b] (mmol)	Cell dry mass formed[c] (mg)	*p*-Cymene dissimilated[d] (mmol)	Electrons from *p*-cymene dissimilated[e] (mmol)	Electrons consumed by NO_3^- reduction[f] (mmol)
Cells with small amount of *p*-cymene	0.26	0.23	1.93	0.38	0.2	13	0.19	10.3	8.8
Cells with large amount of *p*-cymene	1.28	0.68	0.00	0.00	0.0	41	0.55	30.0	20.0
Cells without *p*-cymene (control)	0.00	0.00	4.00	0.00	0.0	0			0.0
Sterile medium without cells (control)	1.28	0.00	4.00	0.00	0.0				0.0

[a] Difference between *p*-cymene added and *p*-cymene recovered at the end of incubation in the carrier and aqueous phase; the latter contained less than 4 µM *p*-cymene (1.6 µmol total)
[b] Sum of N_2O in gas phase and aqueous phase
[c] Amount of cell dry mass added with the inoculum has been subtracted
[d] Difference between *p*-cymene disappeared and *p*-cymene assimilated. The assimilated amount of *p*-cymene was calculated assuming the equation $17\ C_{10}H_{14} + 46\ HCO_3^- + 46\ H^+ + 24\ H_2O \rightarrow 54\ C_4H_7O_3$; thus, 1 mg of cell dry mass requires 0.00305 mmol of *p*-cymene
[e] 54 mol of electrons are derived from 1 mol of *p*-cymene if completely oxidized
[f] Electrons consumed = 5 · (nitrate added–nitrate remaining) – 3 · (nitrite remaining) – 2 · (nitrous oxide remaining). Nitrate reduction to ammonium was not observed

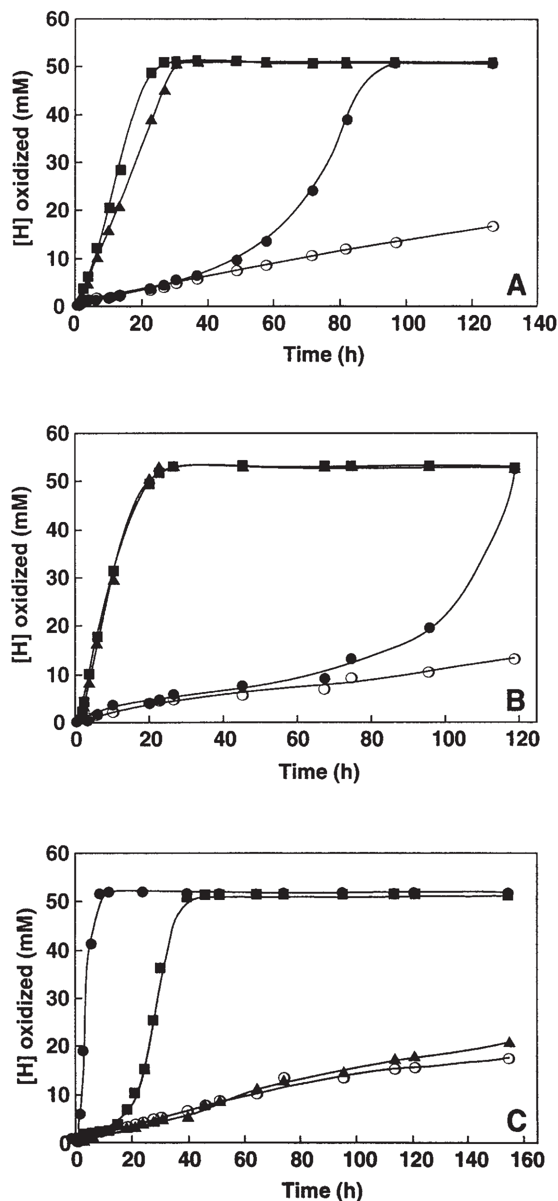


Fig. 3 Anaerobic oxidation of alkylbenzenes by resuspended cells of strain pCyN1 pregrown on **A** *p*-cymene, **B** *p*-ethyltoluene, or **C** toluene. Resuspended cells (in 50 ml medium) were incubated for 1.5 h without organic substrate in the presence of nitrate. Then *p*-alkyltoluenes were added (to 2 ml heptamethylnonane as carrier phase). Curves show denitrification with ■ *p*-cymene, ▲ *p*-ethyltoluene, or ● toluene. Controls (○) did not contain an organic substrate. For comparison of alkylbenzene-degrading activities, oxidized reducing equivalents (calculation given in footnote of Table 2) are shown rather than consumption or formation of nitrogen species

nificantly deviate from that in the substrate-free control within 155 h (Fig. 3C).

Possible intermediates and dead-end metabolites

Already before the recognition of toluene activation by condensation with fumarate (Biegert et al. 1996; Beller and Spormann 1997 a,b), benzoate and *p*-methylbenzoate had been detected as a metabolite formed from toluene or as a dead-end metabolite formed from *p*-xylene, respectively (Schocher et al. 1991; Seyfried et al. 1994; Biegert and Fuchs 1995; Rabus and Widdel 1995 b). These findings had formerly supported the hypothesis of an initial attack at the methyl group rather than at the ring. We therefore examined cultures of strains pCyN1 and pCyN2 for possible excretion of metabolites. During growth with *p*-cymene as sole organic substrate, both strains excreted up to 11 μM *p*-isopropylbenzoate. Furthermore, strain pCyN1 was incubated with *p*-xylene, *p*-ethyltoluene, or *p*-*tert*-butyltoluene in a mixture with *p*-cymene [each 1% (v/v) in carrier phase]. During growth, excretion of up to 40 μM *p*-methylbenzoate, 5 μM *p*-ethylbenzoate, and 30 μM *p*-*tert*-butylbenzoate, respectively, was detected. *p*-Methylbenzoate and *p*-*tert*-butylbenzoate were not further degradable, as was revealed by growth tests (Table 1), and hence present dead-end metabolites for strain pCyN1.

Phylogenetic relationships

Sequencing of 16S rRNA genes of the two new isolates revealed affiliation with the *Azoarcus*-*Thauera* cluster

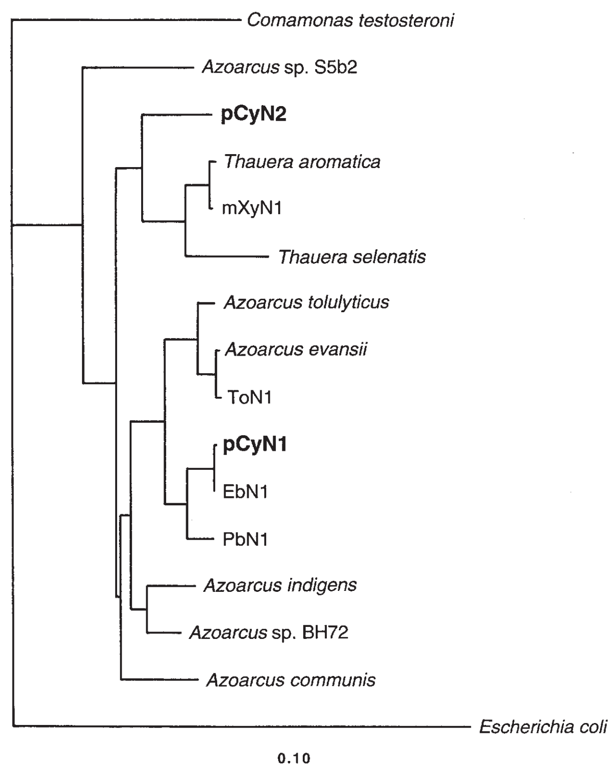


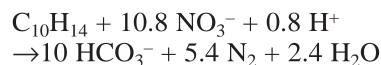
Fig. 4 Relationships based on 16S rRNA gene sequences of the newly isolated denitrifying *p*-cymene-degrading strains pCyN1 and pCyN2. The shown cluster of the β-subclass of the Proteobacteria includes various alkylbenzene-degrading denitrifying bacteria. Strains ToN1, EbN1, PbN1, and mXyN1 are unnamed strains previously isolated with nitrate and toluene, ethylbenzene, propylbenzene, and *m*-xylene, respectively (Rabus and Widdel 1995 a) (*bar* ten inferred nucleotide substitutions per 100 nucleotides)

within the β-subclass of the Proteobacteria (Fig. 4). Strains pCyN1 and pCyN2 are related to each other with a sequence similarity of 93.9%. The closest relatives of strains pCyN1 and pCyN2 are the ethylbenzene-degrading denitrifier strain EbN1 and the *m*-xylene-degrading denitrifier strain mXyN1, respectively. The respective sequence similarity values were 100% (pCyN1/EbN1) and 95.5% (pCyN2/mXyN1). Hence, on the basis of 16S rRNA sequences, strains pCyN1 and EbN1 were not distinguishable despite certain nutritional differences.

Discussion

The newly isolated denitrifying strains pCyN1 and pCyN2 are, to our knowledge, the first pure cultures of anaerobic bacteria to grow with *p*-alkyltoluenes as sole electron donors and carbon sources. Slow anaerobic utilization of *p*-xylene and *p*-ethyltoluene has been previously observed in a denitrifying enrichment culture (Häner et al. 1995). Anaerobic degradation of *p*-isopropyltoluene (*p*-cymene) has not even been shown in enriched communities. Disappearance of *m*-cymene has been observed in an anaerobic enrichment culture of sulfate-reducing bacteria on crude oil (Rabus et al. 1996). In addition to *p*-cymene, which belongs to the monoterpenes, strains pCyN1 and pCyN2 also utilized nonaromatic, alkenoic compounds of this class. Anaerobic utilization of alkenoic monoterpenes by denitrifying bacteria has been demonstrated previously (Harder and Probian 1995; Foß et al. 1998). However, the denitrifying strains isolated from enrichment cultures with nonaromatic monoterpenes do not oxidize *p*-cymene. Rather, some of the enrichment cultures on monoterpenes form *p*-cymene as a dead-end metabolite from the more reduced monoterpenes (Harder and Probian 1995; Harder and Foß 1999).

Measurement of the degradation balance of *p*-cymene by strains pCyN1 and pCyN2 revealed a certain surplus of the calculated amount of electrons from the consumed hydrocarbon in comparison to the amount of electrons needed for reduction of nitrate. This may be explained by incomplete oxidation of *p*-cymene to currently unknown organic intermediates that were not detected by the applied HPLC analysis. The concentration of excreted *p*-cumate (range of 10 μM) was too low to account for the missing electrons. Furthermore, the assimilation equation (Table 2, footnote) used for balance calculations is only theoretical and may differ from the real stoichiometry of substrate assimilation. Nevertheless, the balance calculated from the growth experiment with the small amount of *p*-cymene is close to the following catabolic equation:

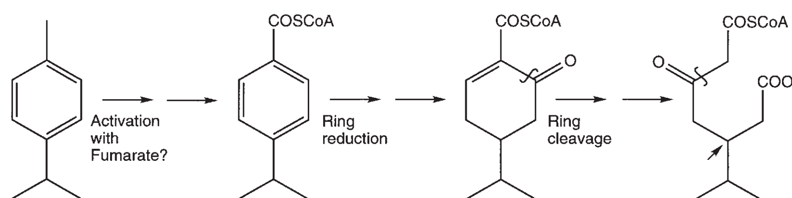


$$C_{10}H_{14} + 10.8\ NO_3^- + 0.8\ H^+ \rightarrow 10\ HCO_3^- + 5.4\ N_2 + 2.4\ H_2O$$

The capacity for complete substrate oxidation is also obvious from growth on acetate.

The observation of an anaerobic utilization of *p*-alkyltoluenes evokes intriguing questions as to the metabolism of these aromatic compounds. In analogy to anaerobic oxidation of toluene (Biegert et al. 1996; Beller and Spormann 1997a; Coschigano et al. 1998; Leuthner et al. 1998; Rabus and Heider 1998; Heider et al. 1999) and from a chemical point of view, the methyl groups of the *p*-alkyltoluenes are likely to be the sites for the initial enzymatic attack; the cosubstrate may also be fumarate, as in the case of anaerobic toluene activation. The assumption of an activation and oxidation at the methyl group is supported by the present detection of *p*-isopropylbenzoate (*p*-cumate) in cultures of strains pCyN1 and pCyN2 growing on *p*-cymene; similarly, excretion of other *p*-alkylbenzoates (including dead-end metabolites) in the presence of the corresponding *p*-alkyltoluenes during growth of strain pCyN1 with *p*-cymene supports the assumption of a methyl group activation. The actual intermediates may be the CoA-thioesters of the metabolizable *p*-alkylbenzoates, in analogy to the formation of benzoyl-CoA from toluene. However, anaerobic enzymatic reactions of benzoate (or benzoyl-CoA) derivatives with alkyl groups have not been studied to date. Hence, another principal question arising from the present study is whether the metabolism of the assumed *p*-alkylbenzoyl-CoA involves ring reduction and ring cleavage mechanisms analogous to those of the known benzoyl-CoA metabolism (Harwood et al. 1999). Such reactions would lead to an aliphatic CoA-thioester (shown in Fig. 5 for the homologue with isopropyl group) that could be subject to only one round of β-oxidation. A second round of β-oxidation would not be possible because of hindrance by the tertiary carbon (marked in Fig. 5). The observed good growth of enrichment cultures and pure cultures (Table 1), in particular with *p*-cymene and *p*-cumate, suggests that the mechanism for circumventing the hindrance by the alkyl side chain is most effective in case of an isopropyl group. This may be explained by the widespread occurrence of

Fig. 5 Hypothetical anaerobic degradation pathway of *p*-isopropyltoluene (*p*-cymene) via *p*-isopropylbenzoyl-CoA, in analogy to the anaerobic degradation of toluene via benzoyl-CoA (Heider et al. 1999; Harwood et al. 1999). Sites of ring cleavage and thioclastic reaction during subsequent β-oxidation are marked by *swung dashes*. The *small arrow* marks the tertiary carbon atom that prevents progression of β-oxidation



p-cymene as a natural product of plants (Gildemeister and Hoffmann 1960; Hegnauer 1966) and as a dead-end metabolite of anaerobic bacteria that grow with nonaromatic monoterpenes (Harder and Probian 1995; Harder and Foß 1999). *p*-Xylene and *p*-ethyltoluene as typical petroleum constituents are probably less widespread in natural habitats than *p*-cymene. Aerobic bacteria degrade *p*-cymene via free *p*-cumate (DeFrank and Ribbons 1977; Eaton 1997), which could, therefore, be a trace metabolite in various habitats.

Although anaerobic bacteria may employ the same principal mechanism for the activation of toluene and *p*-alkyltoluenes, the involved enzymes are expected to be substrate-specific. Adaptation studies with cell suspensions of strain pCyN1 pregrown on different alkylbenzenes suggest that there is one enzyme system for the initial metabolism of toluene and at least one other enzyme system for the initial metabolism of *p*-cymene and *p*-ethyltoluene. The present results do not allow further differentiation of whether *p*-ethyltoluene and *p*-cymene are metabolized by the same enzyme system or by two different, simultaneously synthesized enzyme systems. One may conclude from the inability of toluene-grown cells of strain pCyN1 to adapt to *p*-ethyltoluene during 155 h that this substrate is a poor inducer of the capacity for its own degradation. Adaptation studies with another denitrifying bacterium, strain EbN1, has also revealed distinct, inducible enzyme systems for two other hydrocarbons, toluene and ethylbenzene (Rabus and Heider 1998; Champion et al. 1999).

Both strains isolated with *p*-cymene are members of the *Azoarcus-Thauera* cluster of the β-subclass of Proteobacteria. Due to presently known rRNA sequence data, all other alkylbenzene-degrading denitrifiers described to date also belong to this cluster (Fries et al. 1994; Anders et al. 1995; Rabus and Widdel 1995 a; Zhou et al. 1995; Hess et al. 1997; Song et al. 1999). Strain pCyN1 is very closely related to the denitrifying strain EbN1 isolated on ethylbenzene (Rabus and Widdel 1995 a) according to 16S rRNA gene sequence analysis and the G+C contents of the DNA (61 vs 65 mol% G+C in strain pCyN1 and EbN1, respectively). A more refined and definite resolution of the relationship would require DNA-DNA hybridization. The strains differ mainly in their capacities for degradation of some aromatic compounds. Ethylbenzene, 1-phenylethanol, and acetophenone, which are substrates of strain EbN1, are not utilized by strain pCyN1. Vice versa, *p*-cymene, *p*-ethyltoluene, and *p*-cumate are not utilized by strain EbN1. Both strains share the capacity for utilization of fatty and dicarboxylic acids (Table 1; Rabus and Widdel 1995 a).

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