Current Microbiology Vol. 51 (2005), pp. 127–130
DOI: 10.1007/s00284-005-4518-5

**Current Microbiology**
An International Journal


# Anaerobic Degradation of *p*-Xylene by a Sulfate-Reducing Enrichment Culture

Barbara Morasch,* Rainer U. Meckenstock†

Zentrum für Angewandte Geowissenschaften, Eberhard-Karls-Universität Tübingen, Sigwartstrasse 10, 72076, Tübingen, Germany

Received: 19 November 2004 / Accepted: 13 March 2005

**Abstract.** A strictly anaerobic enrichment culture was obtained with *p*-xylene as organic substrate and sulfate as electron acceptor from an aquifer at a former gasworks plant contaminated with aromatic hydrocarbons. *p*-Xylene was completely oxidized to $CO_2$. The enrichment culture depended on Fe(II) in the medium as a scavenger of the produced sulfide. 4-Methylbenzylsuccinic acid and 4-methylphenylitaconic acid were identified in supernatants of cultures indicating that degradation of *p*-xylene was initiated by fumarate addition to one of the methyl groups. Therefore, *p*-xylene degradation probably proceeds analogously to toluene degradation by *Thauera aromatica* or anaerobic degradation pathways for *o*- and *m*-xylene.

The monoaromatic hydrocarbons benzene, toluene, ethylbenzene, and xylene isomers (BTEX) are mutagenic and putatively carcinogenic substances. Due to their abundance and relatively high solubility in water, aromatics are prominent groundwater contaminants. Among the BTEX compounds, anaerobic degradation pathways of toluene, ethylbenzene, and *m*-xylene have been well investigated [8, 24, 27]. In all cases investigated so far, under anoxic conditions methylated aromatic hydrocarbons were activated by a reaction of the benzylsuccinate synthase type, where fumarate is added to the methyl side chain [3, 27]. A similar reaction takes place in anaerobic degradation of ethylbenzene by sulfate-reducing bacteria where fumarate is added to the benzyl carbon atom of the ethyl side chain producing (1-phenylethyl)succinate [17]. In contrast, the denitrifying organism EBN1 activates ethylbenzene by a direct hydroxylation by the enzyme ethylbenzene dehydrogenase generating 1-phenylethanol [16].

All three isomers of xylene are known to be readily degradable under oxic conditions [8], whereas degradation under anoxic conditions is not as well studied. So far, two anaerobic *o*-xylene-degrading pure bacterial cultures have been documented, both of them sulfate reducers. Strain oXyS1 was enriched from marine sludge [12], whereas strain OX39 was isolated from contaminated sediment and is a freshwater organism [21]. Strain OX39 was also able to grow with *m*-xylene, but did not use the third isomer, *p*-xylene. Isolation efforts with *m*-xylene as organic substrate led to some denitrifying [14, 22] and to one marine sulfate-reducing culture, strain mXyS1 [12]. Several denitrifying strains isolated with toluene were later found to grow as well with *m*-xylene, for example, *Azoarcus* strain T and *Azoarcus tolulyticus* strain Td15 [7, 23]. Under anoxic conditions, *p*-xylene has not yet been found to be degraded by pure cultures. However, removal of *p*-xylene was found in sediment column experiments under anoxic—but not well-defined redox—conditions, and another study recorded degradation of *p*-xylene by bacterial enrichment with nitrate provided as the electron acceptor [11, 15]. Until now, this is the only known enrichment culture that degraded *p*-xylene anaerobically

**Present address:* Centre d' Hydrogéologie, Université de Neuchâtel, Rue Emile-Argand 11, 2007 Neuchâtel, Switzerland
†*Present address:* Institut für Grundwasserökologie, GSF-Forschungszentrum für Umwelt und Gesundheit, Ingolstädter Landstrasse 1, 85764 Neuherberg, Germany
*Correspondence to:* Rainer U. Meckenstock; *email:* rainer.meckenstock@gsf.de

and further attempts to isolate the bacteria responsible for removal of *p*-xylene failed.

Concentration analysis in the environment provided indications for *p*-xylene utilization under anoxic conditions in situ [29]. These attempts generally were based on a combined quantification of *m*- and *p*-xylene concentrations as the two isomers coelute in standard gas and liquid chromatographic analysis. Thus, assessment of *m*-/*p*-xylene degradation in the environment by a decrease in concentration does not necessarily account specifically for degradation of *p*-xylene. Furthermore, it has to be considered that physicochemical parameters such as adsorption or dilution can also lead to declining concentrations without sustainable removal of the compounds from the site. Evidence for *p*-xylene degradation in the environment was given by the analysis of putative signature metabolites 4-methylbenzylsuccinic acid and *p*-toluic acid, extracted from a contaminated groundwater site [6]. Recently, Griebler et al. were able to identify *p*-toluic acid in several environmental samples taken from monitoring wells of a BTEX- and PAH-contaminated site [10]. However, it is unclear whether *p*-xylene degradation was initiated by fumarate addition to the methyl group analogously to toluene, *o*- and *m*-xylene degradation.

We report here on *p*-xylene degradation under sulfate-reducing conditions. The bacterial culture presented was enriched from material of a site where Griebler et al. [10] attested bacterial potential of *p*-xylene degradation.

## Materials and Methods

**Purification and culture conditions.** Bacteria were enriched from anoxic mud from the bottom of a monitoring well at a former gasworks site near Stuttgart, Germany. The area was contaminated with mono- and polycyclic aromatic hydrocarbons, as well as with heterocyclic compounds. Bacteria were cultivated at 30°C in bicarbonate-buffered freshwater mineral medium, pH 7.4, with sulfate as electron acceptor (10 mM) [20]. The medium was prepared under an atmosphere of $N_2$/$CO_2$ (80:20 v/v) and reduced with $Na_2S$ (1 mM) [26]. Sterile, anoxic $FeCl_2$ solution was added to a final concentration of 3 mM and led to precipitation of black ferrous sulfide.

For first enrichments, 10 mL of mud per 50 mL of mineral medium were used. The culture was subsequently transferred 1:5. In the meantime, bacteria were growing in the sixth transfer. *p*-Xylene was supplied from anoxic stock solutions with concentrations of 2–3 mM. In order to keep concentrations of *p*-xylene in the water phase low but to simultaneously provide a substrate reservoir, 0.3 g adsorber resin Amberlite-XAD7 (Fluka, Buchs, Switzerland) was added per bottle. As a result, the *p*-xylene concentration stabilized at a level of 30–60 μm as described previously [20].

Growth was indirectly monitored as the increase in sulfide concentrations over time [4]. This method also determines sulfide from precipitated FeS which was completely dissolved by zinc acetate treatment prior to colorimetric analysis. *p*-Xylene concentrations were determined by high-performance liquid chromatography (HPLC; Bischoff Chromatography, Leonberg, Germany) [20].

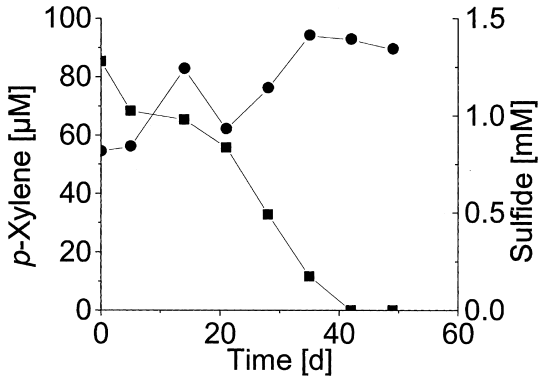


Fig 1. Substrate utilization by strain OX39 in a batch experiment. Degradation of *p*-xylene (■) and concomitant production of sulfide (●) were monitored over time.

To identify putative intermediates of *p*-xylene degradation, cultures were adjusted to pH < 1.5 and subsequently extracted with diethyl ether. Metabolites were converted to methyl esters with a mixture of trimethylchlorosilane (Fluka, Buchs, Switzerland) and methanol (1:8) at 75°C for 1 h, or carboxylic groups were silylated with *N*,*O*-bistrimethylsilyltrifluoro-acetamide (Merck, Darmstadt, Germany) for 6 h at 60°C. For mass spectroscopic analysis, the reaction mixture was dried under a stream of $N_2$ and then dissolved in ethyl acetate (for methylated compounds) or cyclohexane (for silylated compounds). Samples were analyzed by gas chromatography/mass spectrometry (GC/MS) as described previously [20]. Identity of substances was confirmed by published chromatograms or reference compounds.

All chemicals used were of analytical grade (Merck, Darmstadt, Germany, or Fluka, Buchs, Switzerland). Gases were purchased from Messer Griesheim (Krefeld, Germany).

## Results and Discussion

A *p*-xylene-degrading bacterial culture was enriched from contaminated aquifer material with *p*-xylene as organic substrate and sulfate as electron acceptor. Adsorber resin Amberlite-XAD7 was added to the culture bottles to keep the concentration of the aromatic substrate constantly low and to avoid growth inhibition or poisoning. After several 1:5 transfer steps, sediment particles with hydrocarbons that might act as additional carbon sources were diluted out. Also, bacterial growth with *p*-xylene became more reproducible and significantly faster. Degradation of *p*-xylene was stoichiometrically coupled to sulfate reduction. The recovery of electrons from the substrate in the sulfide produced was 95.2%, and verified that the enrichment culture mineralized *p*-xylene completely to $CO_2$ (Fig. 1). The maximum toluene degradation rate obtained was 3.2 μM/day.

In order to elucidate the pathway of *p*-xylene degradation under sulfate-reducing conditions, metabolites were extracted from the enrichment cultures with

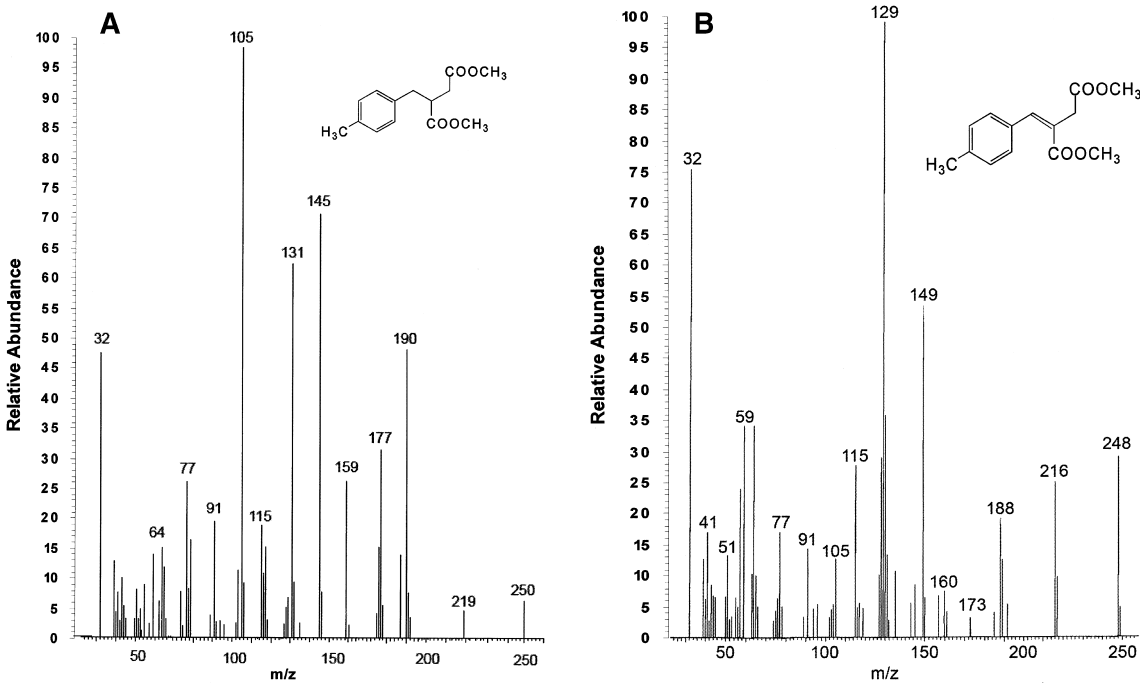


Fig 2. GC-MS fragmentation patterns of tentatively identified intermediates. (A) 4-Methylbenzylsuccinic acid methyl ester and (B) 4-methylphenylitaconic acid methyl ester both extracted from cultures grown with *p*-xylene.

organic solvents. 4-Methylbenzylsuccinate could be tentatively identified by GC-MS analysis as the mass fragmentation pattern was in agreement with published spectra (Fig. 2A) [18]. Another compound extracted from supernatants of our enrichment culture was 4-methylphenylitaconic acid (Fig. 2B). *p*-Toluic acid or benzoic acid could not be detected as metabolites, although we tried two different derivatization techniques. 4-Methylbenzylsuccinate and 4-methylphenylitaconic acid detected in the cultures are both probably specific for a degradation pathway initiated by fumarate addition to one of the methyl groups [25]. Therefore, we concluded that *p*-xylene is degraded analogously to the well-investigated benzylsuccinate synthase pathway of toluene [3]. Other authors showed that fumarate adducts of *o*-xylene were detected in experiments with permeabilized cells of the denitrifying strain T grown on toluene [2]. In these experiments, *o*-xylene was probably converted because the toluene-activating enzyme benzylsuccinate synthase has rather broad substrate specificity [25]. The benzylsuccinate synthase gene was found to encode for the initial enzyme of toluene and also *m*-xylene degradation in the denitrifying bacterium *Azoarcus* sp. strain T, indicating that both compounds are activated by the same enzyme [1, 5, 19]. Metabolites of *o*- and *m*-xylene degradation were also investigated for the sulfate-reducing strain OX39 [21]. However, in strain OX39, toluene, *o*-, and *m*-xylene were not activated by the identical benzylsuccinate synthase, but by an individual enzyme degrading each substrate [21]. Induction times lasted up to several weeks when strain OX39 was transferred from one of the three compounds to another. This implies that a specific activating enzyme exists for each of the three compounds. However, although it is very unlikely, one can not exclude that one of the consecutive enzymes in the pathway exhibits the substrate specificity and toluene and the xylenes are activated by the same benzylsuccinate synthase type enzyme. The concept of specific degradation pathways for methylated benzenes under sulfate-reducing conditions is supported by the fact that the isolates strain OxyS1 and strain MxyS1 were capable of degrading either *o*-isomers or *m*-isomers of methylated aromatic compounds but not both [12].

Summarizing all these findings, the upper pathway of *o*-, *m*-, and also *p*-xylene degradation under anoxic conditions very likely starts with activation by fumarate addition and further proceeds via stepwise β-oxidation to methyl-benzoyl-CoA, as is known for toluene.

It has to be mentioned that so far nothing is known about the lower pathway and the further degradation of the 4-methylbenzoyl-CoA. 4-Methylbenzoyl-CoA might be demethylated and the following steps could be identical to the metabolization of benzoyl-CoA [9, 13]. Also, in the course of β-oxidation, the methyl group at position 4 of the aromatic ring might be rearranged into

meta-position, because then the methyl group would not interfere with regular β-oxidation [27]. Comparable rearrangement reactions have been found for stepwise oxidation of *n*-hexane by a denitrifying strain [28]. Another possibility might be a hydroxylation of methylbenzoyl-CoA prior to ring reduction facilitating the following reduction steps. Respective substances were discovered in supernatants of sulfate-reducing strain OX39 grown either with *m*-xylene or with toluene [21].

**ACKNOWLEDGMENTS**

The authors thank Bernhard Schink and Stefan Haderlein for continuous support. Christian Griebler is acknowledged for help with the GC-MS analysis and Patrick Höhener for carefully reading the manuscript. This work was in part supported by the Deutsche Forschungsgemeinschaft (grant Schi 180/7).

## Literature Cited

1. Achong GR, Rodriguez AM, Spormann AM (2001) Benzylsuccinate synthase of *Azoarcus* sp. strain T: Cloning, sequencing, transcriptional organization, and its role in anaerobic toluene and *m*-xylene mineralization. J Bacteriol 183:6763–6770
2. Beller HR, Spormann AM (1997) Anaerobic activation of toluene and *o*-xylene by addition to fumarate in denitrifying strain T. J Bacteriol 179:670–676
3. Biegert T, Fuchs G, Heider J (1996) Evidence that anaerobic oxidation of toluene in the denitrifying bacterium *Thauera aromatica* is initiated by formation of benzylsuccinate from toluene and fumarate. Eur J Biochem 238:661–668
4. Cline JD (1969) Spectrophotometric determination of hydrogen sulfide in natural waters. Limnol Oceanogr 14:454–458
5. Coschigano PW, Wehrman TS, Young LY (1998) Identification and analysis of genes involved in anaerobic toluene metabolism by strain T1: Putative role of a glycine free radical. Appl Environ Microbiol 64:1650–1656
6. Elshahed MS, Gieg LM, McInerney MJ, Suflita JM (2001) Signature metabolites attesting to the in situ attenuation of alkylbenzenes in anaerobic environments. Environ Sci Technol 35:682–689
7. 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
8. Gibson DT, Subramanian V (1984) Microbial degradation of aromatic hydrocarbons. In: Gibson DT (ed) Microbial degradation of organic compounds. Dekker microbiology series, vol 13. New York: Dekker, pp 181–252
9. Gibson J, Harwood CS (2002) Metabolic diversity in aromatic compounds utilization by anaerobic microbes. Annu Rev Microbiol 56:345–369
10. Griebler C, Safinowski M, Vieth A, Richnow HH, Meckenstock RU (2004) Combined application of stable carbon isotope analysis and specific metabolites determination for assessing in situ degradation of aromatic hydrocarbons in a tar oil-contaminated aquifer. Environ Sci Technol 38:617–631
11. Häner A, Höhener P, Zeyer J (1995) Degradation of *p*-xylene by a denitrifying enrichment culture. Appl Environ Microbiol 61:3185–3188
12. Harms G, Zengler K, Rabus R, Aeckersberg F, Minz D, Rossello-Mora R, et al. (1999) Anaerobic oxidation of *o*-xylene, *m*-xylene, and homologous alkylbenzenes by new types of sulfate-reducing bacteria. Appl Environ Microbiol 65:999–1004
13. Harwood CS, Burchhardt G, Herrmann H, Fuchs G (1998) Anaerobic metabolism of aromatic compounds via the benzoyl-CoA pathway. FEMS Microbiol Rev 22:439–458
14. Hess A, Zarda B, Hahn D, Haner A, Stax D, Hohener P, et al. (1997) In situ analysis of denitrifying toluene- and *m*-xylene-degrading bacteria in a diesel fuel-contaminated laboratory aquifer column. Appl Environ Microbiol 63:2136–2141
15. Hunkeler D, Jörger D, Häberli K, Höhener P, Zeyer J (1998) Petroleum hydrocarbon mineralization in anaerobic laboratory aquifer columns. J Cont Hydrol 32:41–61
16. Kniemeyer O, Heider J (2001) Ethylbenzene dehydrogenase, a novel hydrocarbon-oxidizing molybdenum/iron-sulfur/heme enzyme. J Biol Chem 276:21381–21386
17. Kniemeyer O, Fischer T, Wilkes H, Glöckner FO, Widdel F (2003) Anaerobic degradation of ethylbenzene by a new type of marine sulfate-reducing bacterium. Appl Environ Microbiol 69:760–768
18. Krieger CJ, Beller HR, Reinhard M, Spormann AM (1999) Initial reactions in anaerobic oxidation of *m*-xylene by the denitrifying bacterium *Azoarcus* sp. strain T. J Bacteriol 181:6403–6410
19. Leuthner B, Leutwein C, Schulz H, Horth P, Haehnel W, Schiltz E, et al. (1998) Biochemical and genetic characterization of benzylsuccinate synthase from *Thauera aromatica*: A new glycyl radical enzyme catalysing the first step in anaerobic toluene metabolism. Mol Microbiol 28:615–628
20. Morasch B, Annweiler E, Warthmann RJ, Meckenstock RU (2001) The use of a solid adsorber resin for enrichment of bacteria with toxic substrates and to identify metabolites: Degradation of naphthalene, *o*-, and *m*-xylene by sulfate-reducing bacteria. J Microbiol Methods 44:183–191
21. Morasch B, Schink B, Tebbe CC, Meckenstock RU (2004) Degradation of *o*-xylene and *m*-xylene by a novel sulfate-reducer belonging to the genus *Desulfotomaculum*. Arch Microbiol 181:407–417
22. Rabus R, Widdel F (1995) Anaerobic degradation of ethylbenzene and other aromatic hydrocarbons by new denitrifying bacteria. Arch Microbiol 163:96–103
23. Seyfried B, Glod G, Schocher R, Tschech A, Zeyer J (1994) Initial reactions in the anaerobic oxidation of toluene and *m*-xylene by denitrifying bacteria. Appl Environ Microbiol 60:4047–4052
24. Spormann AM, Widdel F (2000) Metabolism of alkylbenzenes, alkanes, and other hydrocarbons in anaerobic bacteria. Biodegradation 11:85–105
25. Verfürth K, Pierik AJ, Leutwein C, Zorn S, Heider J (2004) Substrate specificities and electron paramagnetic resonance properties of benzylsuccinate synthases in anaerobic toluene and *m*-xylene metabolism. Arch Microbiol 181:155–162
26. 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, vol 4. New York: Springer, pp 3352–3378
27. Widdel F, Rabus R (2001) Anaerobic biodegradation of saturated and aromatic hydrocarbons. Curr Opin Biotechnol 12:259–276
28. Wilkes H, Rabus R, Fischer T, Armstroff A, Behrends A, Widdel F (2002) Anaerobic degradation of n-hexane in a denitrifying bacterium: further degradation of the initial intermediate (1-methylpentyl)succinate via C-skeleton rearrangement. Arch Microbiol 177:235–243
29. Zamfirescu D, Grathwohl P (2001) Occurrence and attenuation of specific organic compounds in the groundwater plume at a former gasworks site. J Contam Hydrol 53:407–427