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Transformation of *o*-xylene to *o*-methyl benzoic acid by a denitrifying enrichment culture using toluene as the primary substrate

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Abstract

A highly enriched denitrifying mixed culture transformed *o*-xylene cometabolically along with toluene by methyl group oxidation. *o*-Methyl benzaldehyde and *o*-methyl benzoic acid accumulated transiently as metabolic products of *o*-xylene transformation. Transformation of *o*-methyl benzyl alcohol and *o*-methyl benzaldehyde occurred independently of toluene degradation and resulted in the formation of a compound coeluting with *o*-methyl benzoic acid on a gas chromatograph. The cometabolic relationship between toluene and *o*-xylene could be attributed to a mechanism linked to the initial oxidation of the methyl group.

Introduction

Microbial degradation of aromatic hydrocarbons was until recently thought to be solely an aerobic process. However, substantial evidence now exists that this is not the case for the monoaromatic hydrocarbons, even at very low redox conditions (Grbić-Galić and Vogel 1987; Edwards et al. 1992). Degradation under denitrifying conditions has received special attention, because of the potential role of nitrate as an electron acceptor in *in situ* biological remediation of gasoline-polluted aquifers. Kuhn et al. (1985) were the first to show that *m*- and *p*-xylene were degraded under denitrifying conditions, and they succeeded in isolating a bacterium that was able to grow on toluene as the sole source of carbon and energy (Dolfing et al. 1990). Their results have now been confirmed several times, and it is found that most of the commonly encountered monoaromatic hydrocarbons except benzene can be completely degraded under denitrifying conditions (Major et al. 1988; Hutchins et al. 1991; Evans et al. 1991).

Although the degradation of monoaromatic hydrocarbons by denitrification has been thoroughly investigated, the initial pathway of degradation is not fully understood. Substantial evidence for two pathways for toluene degradation exists: (i) oxidation of the methyl group producing benzoic acid as an intermediate (Kuhn et al. 1988; Schocher et al. 1991; Altenschmidt and Fuchs 1992), and (ii) addition of acetyl-CoA to the methyl group (Evans et al. 1992; Frazer et al. 1993).

Evans et al. 1992 also suggested that *o*-xylene is transformed by the addition of succinyl-CoA. In our work with degradation of toluene and *o*-xylene under denitrifying conditions, we found that *o*-xylene removal only occurred concomitantly with toluene degradation (Jensen et al. 1988), and that two metabolic products appeared when *o*-xylene was transformed. When analyzed on a gas chromatograph, the products coeluted with *o*-methyl benzaldehyde and *o*-methyl benzoic acid. It was hypothesized that the transformation of *o*-xylene under denitrifying conditions is initiated by methyl group oxidation to produce *o*-methyl benzoic acid via *o*-methyl benzyl alcohol and *o*-methyl benzaldehyde.

The aim of this study was to confirm this hypothesis and to investigate the relationship between the degradation of *o*-xylene and toluene in more detail.

Experiments were designed to: (i) show the influence of toluene on the degradation of *o*-xylene, (ii) identify the metabolites of *o*-xylene transformation, and (iii) investigate the degradability of *o*-methyl benzyl alcohol and *o*-methyl benzaldehyde in the absence of toluene, to determine at which step of the pathway the cometabolic relationship between toluene and *o*-xylene is established.

Materials and methods

Chemicals

All chemicals used were purchased from Merck, Darmstad, Germany and were of analytical purity.

Inoculum source

The inoculum used was a mixed denitrifying enrichment culture that originated from sewage treatment plants with alternating nitrification and denitrification. The culture grows anaerobically on toluene as the sole source of carbon and energy, but only in the presence of nitrate or other nitrogen oxides. The culture is described in detail by Jørgensen et al. (1995).

Analytical methods

The aromatic compounds were analyzed on a DANI 8520 gas chromatograph (Merck, Denmark) equipped with a 30 m J&W DB5 capillary column, i.d. 0.53 mm, film thickness 1.5 μm, and an FI-detector kept at 275 °C. The temperature program of the oven was 40 °C for 1 min, then the temperature was increased by 30 °C per min to 250 °C, and held constant for 0.5 min. The injection port was mounted with a Programmable Temperature Evaporizer. The temperature programme of the injection port was 36 °C for 1 min, then the temperature was increased to 275 °C at a rate of 1300 °C per min. Nitrogen was used as the carrier gas at 10 ml/min. Extraction of the aromatic compounds was carried out by acidifying a 10 ml sample in a volumetric flask with four drops of a 3 M phosphoric acid, and adding 1 ml of diethyl ether containing heptane as an internal standard (4.1 mg/l). The sample was then shaken vigorously by hand for 3 min. As much as possible of the organic phase was transferred to a 0.9 ml vial and 1 μl of the organic phase was injected. At low concentrations of the aromatic compounds (< 5 μM in solution), the organic phase was evaporated to about 25 μl under a stream of nitrogen gas to achieve a better detection limit (0.5 μM). Samples with *o*-methyl benzoic acid had to be analyzed immediately after extraction, because the compound decayed completely in the organic phase within 24 h. Immediate analysis was not always possible. The peak areas were determined by integration on a MAXIMA 820 Chromatographic Workstation (Millipore Corporation, Massachusetts), and the concentration of the aromatic compounds were calculated on the basis of standard curves.

Nitrate and nitrite were measured as described by Jørgensen et al. (1995).

Qualitative determination of metabolic products was carried out on a Carlo Erba QMD 1000 quadropole mass spectrometer in the scanning mode. Scanning times were 0.5 s on 10 to 300 amu, the interscanning time was 0.05 s, and the electron impact ionizing energy was 70 eV. The spectra were compared with the NBS database containing 49469 entries. The injection temperature was 275 °C on a split injector system, which split the sample 1:10. The transfer line temperature was kept at 250 °C, and the source temperature at 200 °C. The oven temperature was set at 50 °C for 2 min, then increased to 250 °C by 15 °C per min, and held constant for 5 min. The column was a J&W DB-5, 30 m x 0.32 mm i.d., 0.25 μm film thickness. Helium was used as the carrier gas at velocities of about 2 ml per min at an oven temperature of 40 °C. Extraction was carried out by vigorously shaking 90 ml of an acidified sample (pH 2) with 10 ml of diethyl ether for 3 minutes by hand. To increase concentrations, the extract (3 ml) was then evaporated under a stream of nitrogen gas to about 50 μl. Then 3 μl of the extract was injected with the split open.

Experimental setup

The experiments were carried out as anaerobic batch experiments in 1.25 l bottles as described in Jørgensen et al. (1994). 50 ml of inoculum were added to each of three bottles, and toluene was added to a concentration of 80 μM. The toluene was completely degraded in less than a week, simultaneously with reduction of nitrate to nitrite. Two weeks after depletion of toluene, toluene and *o*-xylene were added to the first batch to show the influence of toluene on the degradation of *o*-xylene. To investigate the degradability of *o*-methyl benzyl alcohol and *o*-methyl benzaldehyde in the absence of toluene, the second batch was supplemented with *o*-methyl benzyl alcohol, and the third batch with *o*-xylene and *o*-methyl benzaldehyde. The fourth bottle,

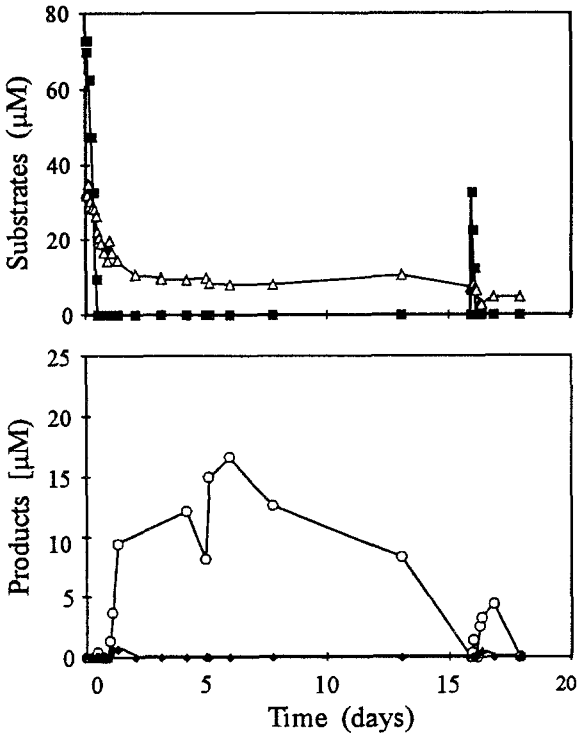


Fig. 1. Degradation of toluene (■) and *o*-xylene (Δ) and the transient accumulation of compound 1 (*o*-methyl benzaldehyde) (♦) and compound 2 (*o*-methyl benzoic acid) (o). Extra toluene was added to the culture on day 16.

which was used as an uninoculated control, received toluene, *o*-xylene, and *o*-methyl benzaldehyde. The control experiment was not poisoned.

Results

Toluene was added to the first batch to attain a concentration of 73 µM and *o*-xylene to a concentration of 35 µM. The results of the experiment are seen in Figure 1. The degradation of toluene commenced immediately and reached a value below the detection limit within 11 h. The transformation of *o*-xylene was also seen to begin within the first hours, and the process continued during the next 2 days. The concentration of *o*-xylene thereafter remained constant until readdition of toluene. After approximately one day, compounds that coeluted on the GC with authentic standards of *o*-methyl benzaldehyde (compound 1) and *o*-methyl benzoic acid (compound 2) appeared in the solution. The first compound disappeared quickly from the solution, but the second compound accumulated and remained in the solution for almost 2 weeks. The scatter of the data is due to the quick decay of the second compound in the organic phase after the extraction. A second addition of toluene resulted in further *o*-xylene removal so that *o*-xylene reached a concentration of 3.6 µM, and in transient accumulation of metabolic products. During degradation, the concentration of nitrate decreased from 1.3 mM to 0.7 mM; nitrite was produced and reached a concentration of 0.8 mM. The amount of nitrite was far below the concentration at which nitrite inhibits the culture (Jørgensen et al. 1991). It was also shown that addition of succinate (2 mM) resulted in decreasing concentrations of *o*-xylene (22 µM–12 µM), but in this case, products of *o*-xylene transformation could not be detected.

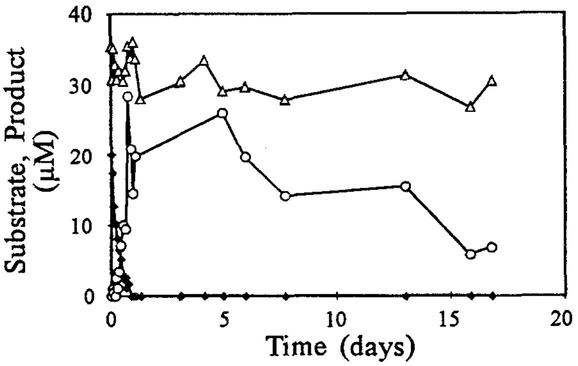


Fig. 2. Oxidation of *o*-methyl benzaldehyde (♦) and accumulation of a compound coeluting with *o*-methyl benzoic acid (o) in the bottle to which *o*-xylene (Δ) and *o*-methyl benzaldehyde had been added. Toluene was not added.

o-Methyl benzyl alcohol was added to the second batch to attain a concentration of about 25 µM. Toluene was not added. After three days of incubation, a compound coeluting with *o*-methyl benzoic acid began to accumulate in the medium and remained there until day 18. This compound could not be detected in samples taken on day 30. *o*-Methyl benzyl alcohol appeared on the gas chromatogram as a wide peak and could not be quantified, but the peak could not be recognized after 7 days.

The third bottle received *o*-xylene at a concentration of about 33 µM and *o*-methyl benzaldehyde at a concentration of 20 µM. Toluene was not added to the culture. The result of this experiment is seen in Figure 2. Transformation of the aldehyde began immediately, and a compound coeluting with *o*-methyl benzoic acid accumulated in the medium. After about

6 days, the concentration of the metabolic product began to decrease and sank to below the detection limit between day 17 and day 30. During the same period, no detectable transformation of *o*-xylene occurred.

On day 45, toluene was added to the second batch to attain a concentration of 73 μM and *o*-xylene to a concentration of 23 μM. Thirty-six hours later, a sample was withdrawn and used for identification of metabolic products on GC/MS. Figure 3 compares the mass spectra of compounds 1 and 2 respectively with the spectra of authentic standards of *o*-methyl benzaldehyde and *o*-methyl benzoic acid. The spectra of compound 2 and *o*-methyl benzoic acid are almost identical, while minor differences exist between the spectra of compound 1 and *o*-methyl benzaldehyde. When comparing the spectra with the entries of the NSB database, the best fit of compound 1 was *o*-methyl benzaldehyde, and the best fit of compound 2 was *o*-methyl benzoic acid. It was therefore concluded that compound 1 was *o*-methyl benzaldehyde and compound 2 was *o*-methyl benzoic acid.

No removal of toluene *o*-xylene or *o*-methyl benzaldehyde was observed in the uninoculated control experiment (data not shown).

Discussion

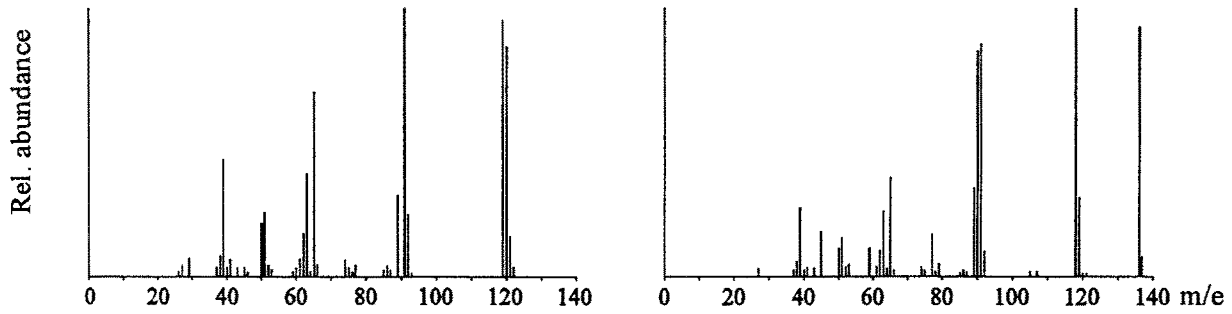
The data presented in Figure 1 show that the culture is able to transform *o*-xylene under denitrifying conditions, but the transformation depended on the simultaneous degradation of toluene. The cometabolic relationship between *o*-xylene and toluene has been observed in denitrification experiments with inocula from a range of different sources, i.e., with aquifer core material (Hutchins et al. 1991), river sediments, anaerobic digester sludge, and soils (Evans et al. 1991). In addition, *o*-xylene can be transformed cometabolically under aerobic conditions (Raymond et al. 1967; Jørgensen and Aamand 1991). Thus, degradation of *o*-xylene by cometabolism may be a widespread process at contaminated sites where *o*-xylene is present in mixtures with compounds that can act as primary substrates.

Two compounds, identified as *o*-methyl benzaldehyde and *o*-methyl benzoic acid by GC/MS, accumulated transiently as a result of *o*-xylene transformation. On a molar basis, about 70% of the transformed *o*-xylene could be recovered as a metabolic product (Fig. 1). In addition, it was observed that the culture was able to transform *o*-methyl benzyl alcohol and *o*-methyl benzaldehyde to a metabolite which coeluted with *o*-methyl benzoic acid. These observations show that the oxidation of *o*-xylene proceeded through *o*-methyl benzyl alcohol, *o*-methyl benzaldehyde, and *o*-methyl benzoic acid.

Evans et al. (1992) also found that toluene induced the transformation of *o*-xylene in a culture of a denitrifying bacterium designated strain T1. They did not find *o*-methyl benzaldehyde or *o*-methyl benzoic acid as metabolites when toluene was the primary substrate, but identified *o*-methyl benzyl succinic acid and *o*-methyl benzyl fumaric acid as dead-end metabolites in the medium. Therefore they suggested that the methyl group of *o*-xylene was attacked by succinyl-CoA, i.e, the methyl group oxidation was not the initial step in the transformation. This study and the work of Evans et al. (1992) therefore present data which prove the existence of two different pathways of transformation of *o*-xylene. These pathways are analogous to the degradation pathways suggested for toluene, namely oxidation of the methyl group (Altenschmidt and Fuchs 1992) and addition of acetyl-CoA to the methyl group (Frazer et al. 1993), and are connected to the degradation of toluene.

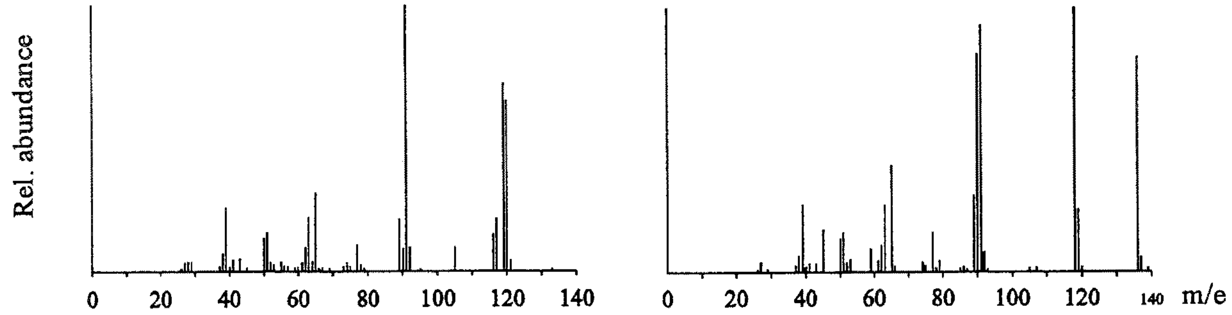
Our culture was able to transform *o*-methyl benzyl alcohol and *o*-methyl benzaldehyde. Approximately 70% of the *o*-methyl benzyl alcohol (data not shown) and 100% of the *o*-methyl benzaldehyde (Fig. 2) could be recovered as a metabolite coeluting with *o*-methyl benzoic acid on GC. In contrast to the transformation of *o*-xylene, the transformation of these substrates was not dependent on the simultaneous degradation of toluene. The relationship between toluene and *o*-xylene is therefore established by a mechanism that is linked to the initial oxidation of the methyl group of *o*-xylene. The transformation of *o*-methyl benzaldehyde proceeded at a high rate and without an apparent lag. This occurred even though the culture had not been exposed to the compound (or to *o*-xylene) during the preceding 2.5 years and indicates that the transformation was due to the action of enzymes already present in the culture.

In general little is known about the mechanisms which are involved in cometabolism, which is often explained by a lower specificity (i.e., ability to catalyze the transformation of a broader range of substrates) of the initial enzymes, than of the subsequent enzymes in a degradation pathway, as well as by a need for reducing equivalents for the initial enzymes (Dalton and Stirling 1982; Stirling and Dalton 1979). This mechanism can also explain the results obtained in this study,



o-methylbenzaldehyde

o-methylbenzoic acid



Compound 1

Compound 2

Fig. 3. Comparison between the mass spectra of authentic standards of *o*-methyl benzaldehyde and *o*-methyl benzoic acid with the mass spectra of the two metabolites produced by transformation of *o*-xylene.

if toluene was degraded by methyl group oxidation, if the toluene-degrading enzymes have a substrate specificity that allow them to transform *o*-xylene, and if the degradation of toluene made the initial oxidation of the methyl group possible. As mentioned earlier, Evans et al. (1992) suggested that the mechanism which connected the degradation of toluene to *o*-xylene transformation was the production of succinyl-CoA. This model cannot explain the results obtained in this study, since the transformation proceeded through oxidation of the methyl group. They also observed that addition of succinate, which can be transformed to succinyl-CoA, induced transformation of *o*-xylene. Addition of succinate to our culture also induced *o*-xylene removal, and interestingly, neither *o*-methyl benzoic acid nor *o*-methyl benzaldehyde accumulated in the medium. This means that our culture may be able to transform *o*-xylene by both mechanisms depending on the presence or absence of toluene and succinate.

In conclusion, the microbial tranformation of *o*-xylene under denitrifying conditions was found to be dependent on the degradation of toluene. The initial step of the transformation was methyl group oxidation, resulting in a transient accumulation of *o*-methyl benzaldehyde and *o*-methyl benzoic acid. The fate of *o*-methyl benzoic acid is unknown. *o*-Xylene transformation appears to be linked to toluene degradation through the initial transformation of *o*-xylene, not through the transformation of *o*-methyl benzyl alcohol or *o*-methyl benzaldehyde.

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