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# Anaerobic degradation of toluene in denitrifying *Pseudomonas* sp.: indication for toluene methylhydroxylation and benzoyl-CoA as central aromatic intermediate

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**Abstract.** The anaerobic degradation of toluene has been studied with whole cells and by measuring enzyme activities. Cultures of *Pseudomonas* strain K 172 were grown in mineral medium up to a cell density of 0.5 g of dry cells per liter in fed-batch culture with toluene and nitrate as the sole carbon and energy sources. A molar growth yield of 57 g of cell dry matter formed per mol toluene totally consumed was determined. The mean generation time was 24 h. The redox balance between toluene consumed (oxidation and cell material synthesis) and nitrate consumed (reduction to nitrogen gas and assimilation as $NH_3$) was 77% of expectation if toluene was completely oxidized; this indicated that the major amount of toluene was mineralized to $CO_2$. It was tested whether the initial reaction in anaerobic toluene degradation was a carboxylation or a dehydrogenation (anaerobic hydroxylation); the hypothetical carboxylated or hydroxylated intermediates were tested with whole cells applying the method of simultanous adaptation: cells pregrown on toluene degraded benzyl alcohol, benzaldehyde, and benzoic acid without lag, 4-hydroxybenzoate and p-cresol with a 90 min lag phase, and phenylacetate after a 200 min lag phase. The cells were not at all adapted to degrade 2-methylbenzoate, 4-methylbenzoate, o-cresol, and m-cresol, nor did these compounds support growth within a few days after inoculation with cells grown on toluene. In extracts of cells anaerobically grown on toluene, benzyl alcohol dehydrogenase, benzaldehyde dehydrogenase, and benzoyl-CoA synthetase (AMP forming) activities were present. The data (1) conclusively show anaerobic growth of a pure culture on toluene; (2) suggest that toluene is anaerobically degraded via benzoyl-CoA; (3) imply that water functions as the source of the hydroxyl group in a toluene methylhydroxylase reaction.

**Key words:** Toluene – Aromatic hydrocarbons – Anaerobic metabolism – *Pseudomonas* – Benzoyl-CoA – Benzyl alcohol – Benzaldehyde – Toluene methylhydroxylation – Dehydrogenases

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Aromatic hydrocarbons such as benzene, toluene, and xylenes are aerobically metabolized; degradation is initiated by oxidative attack using oxygenases (for review see Gibson and Subramanian 1984). The question whether this class of chemically inert aromatic compounds is degraded under anoxic conditions has been disputed (Young 1984; Berry et al. 1987; Evans and Fuchs 1988), but for a long time has only been shown in enrichment cultures or sludge (Kuhn et al. 1985, 1988; Zeyer et al. 1986; Wilson et al. 1986; Vogel and Grbic-Galic 1986; Grbic-Galic and Vogel 1987).

Recently, it has been reported that pure cultures of nitrate reducing (Dolfing et al. 1990) and iron(III) reducing bacteria (Lovley et al. 1989; Lovley and Lonergan 1990) are able to degrade toluene in the absence of molecular oxygen. Zeyer and coworkers determined the turnover of toluene with a denitrifying bacterium and established a carbon mass balance using $^{14}C$-labelled substrate (Dolfing et al. 1990). It was found by B. Seyfried (EAWAG, Switzerland) that the *Pseudomonas* strain K 172, which originally was enriched and isolated under denitrifying conditions with phenol (Tschech and Fuchs 1987), is able to use toluene and nitrate as sole sources of cell carbon and energy. Toluene has been excluded to be growth substrate of this strain (Tschech and Fuchs 1987); the toluene concentration applied when the substrate spectrum was determined was probably too high and therefore was toxic. The possible initial steps in anaerobic toluene degradation have been discussed critically (Kuhn et al. 1988). So far no evidence for the anaerobic toluene pathway is available.

Our work aims at understanding the anoxic metabolism of aromatic compounds. Here we demonstrate that the denitrifying *Pseudomonas* strain K 172 can easily be grown in a large scale on toluene and nitrate under anoxic conditions. Our results support the conclusion that toluene is degraded anaerobically via methylhydroxylation; this reaction implies that oxygen is derived from water. The common aromatic intermediate is suggested to be benzoyl-CoA.

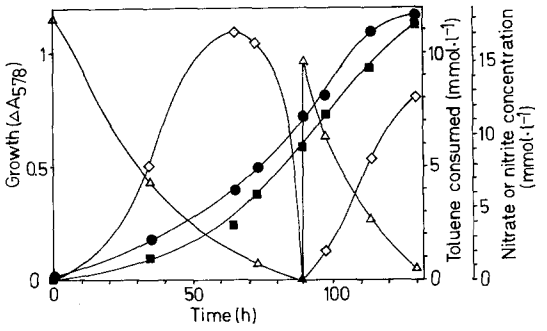


**Fig. 1.** Anaerobic growth of *Pseudomonas* sp. K 172 on toluene under denitrifying conditions. The initial substrate concentrations in the fed-batch culture were 1 mM toluene and 18 mM nitrate. At the different time points the toluene added had just been consumed and 1 mM toluene was repeatedly fed until the culture reached a cell density of $\Delta A_{578\ nm} < 0.3$; later on 1.5 mM toluene each was fed, and at $t = 90$ h 15 mM nitrate was added. A total of 45 ml toluene was consumed by the 47-l culture. For composition of the mineral salt medium see 'Materials and methods'. (●) growth; (■) toluene consumed; (△) nitrate concentration; (◇) nitrite concentration

## Materials and methods

### *Materials*

Chemicals were reagent grade. Biochemicals were from Boehringer (Mannheim, FRG). [$^{14}$C]$Na_2CO_3$ was purchased from Amersham Buchler (Braunschweig, FRG). Gases were from Linde (Höllriegelskreuth, FRG) or Nüssle (Ulm-Söflingen, FRG). The *Pseudomonas* strain K 172 was that described by Tschech and Fuchs (1987). Molecular mass standards were from Pharmacia (Freiburg, FRG).

### *Bacterial growth*

*Pseudomonas* K 172 was grown anaerobically on mineral salts medium at 28° C, with different organic substrates (1 – 5 mM) as carbon and electron sources and with nitrate (generally 15 mM) as the electron acceptor. The growth conditions, the trace element solution, and the vitamin solution used were essentially as described by Tschech and Fuchs (1987). In this investigation nitrate was also the only nitrogen source added. The standard medium contained per liter: 5.6 g $K_2HPO_4$, 1.1 g $KH_2PO_4$, 0.05 g $Na_2SO_4$, 0.004 g $MgCl_2$, 1.3 g $NaNO_3$, 0.027 g $CaCl_2$, 10 ml trace element solution SL 10, 1 ml vitamin solution V7, 0.092 g toluene. The final pH was 7.8. The bacteria were grown on this medium with other aromatic compounds (5 mM) except for toxic substrates (1 mM initial concentration) which were repeatedly fed. Cells were grown in a large scale in a 50-l stainless steel jar with an initial toluene concentration of 1 mM, and toluene or nitrate were fed each time when toluene or nitrite had been consumed.

### *SDS polyacrylamide gel electrophoresis (SDS PAGE)*

Proteins in cell extracts (100,000 × *g* supernatant) were analyzed by SDS PAGE using the method of Laemmli (1970). Proteins were silver stained (Morrissey 1981).

### *Cell extracts and enzyme assays*

Cell extracts and ammonium sulfate precipitated protein fractions were obtained and the enzyme assays were performed essentially as described by Dangel et al. (1991).

**Table 1.** Redox balance and stoichiometry of anaerobic growth on toluene: toluene consumption, carbon requirement for cell material synthesis, nitrate and nitrite consumption in respiration and cell nitrogen assimilation. The culture was grown in a stainless steel jar (≈50 l) which contained only a smaller rubber septum. The experimentally determined dry cell matter content was 378 mg per liter at $\Delta A_{578\ nm} = 1$ (d = 1 cm). The 47 l culture at $\Delta A_{578\ nm} = 0.72$ had consumed 4.8 mM toluene and 18 mM nitrate.

The electron balance in this experiment was 77% of expectation if all toluene degraded was completely oxidized to $CO_2$ and all nitrite was reduced to $N_2$ or assimilated as $NH_3$. Part of the toluene may have escaped with pressurized gases ($N_2$, $CO_2$) during sampling. In addition the culture may have excreted some organic products

| | |
|---|---|
| Toluene consumed | 225.6 mmol |
| Cell material formed | 12.8 g dry cell matter |
| Molar growth yield | 56.7 g dry cell matter formed per mol toluene consumed |
| Toluene assimilated into cell material (6.4 g cell carbon) | 76.2 mmol |
| Reducing equivalents formed during assimilation | 610 mequivalents |
| Toluene dissimilated (assumed to be oxidized to $CO_2$) | 149.4 mmol |
| Reducing equivalents formed during toluene dissimilation if oxidized to $CO_2$ | 5,378 mequivalents |
| Total reducing equivalents formed | 5,988 mequivalents |
| Nitrate consumed | 846 mmol |
| Nitrate assimilated into cell material (1.8 g cell nitrogen) | 128 mmol |
| Reducing equivalents consumed for nitrate reduction to $NH_3$ | 1,014 mequivalents |
| Nitrate dissimilated to $N_2$ | 718 mmol |
| Reducing equivalents consumed for nitrate reduction to $N_2$ | 3,590 mequivalents |
| Total reducing equivalents consumed | 4,604 mequivalents |

### *Synthesis of 4-hydroxybenzoyl-CoA*

4-Hydroxybenzoyl-CoA was synthesized enzymatically and purified as described before (Glöckler et al. 1989).

### *Experiments with cell suspensions*

Cells were pregrown under denitrifying conditions in mineral medium containing toluene as sole organic compound. When the cell density had reached an $A_{578\ nm} = 0.5$, the cells were harvested at 4°C by centrifugation and washed in anaerobic mineral medium (nitrogen gas head space) lacking organic substrates. The washed cells were resuspended in mineral medium (5% of the original culture volume) lacking organic substrates. An aliquot of the concentrated cell suspension was anaerobically incubated at 28° C with the indicated aromatic compound. The disappearance of the organic substrate was determined by spectroscopic analysis of the cell-free supernatant; the sample was rapidly cooled and the cells were removed by centrifugation in an Eppendorf centrifuge.

### *Determinations*

$^{14}$C was determined in a liquid scintillation counter using external standardization. Aromatic compounds were quantified spectrophotometrically. Bacterial growth was followed by determining the apparent absorption increase at 578 nm (d = 1 cm), corrected for

the absorption of the medium; samples with $A_{578\ nm} > 0.5$ were diluted with 100 mM phosphate buffer pH 7. The cell dry weight was determined after desiccation at 80° C until constant weight was reached. Protein was determined by the method of Bradford (1976). Nitrate and nitrite were determined by colorimetric tests (Rider and Mellon 1946; Gesellschaft Deutscher Chemiker 1979).

## Results and discussion

### *Anaerobic growth of Pseudomonas sp. K 172 on toluene and nitrate*

*Pseudomonas* strain K 172 was anaerobically grown in large scale in a mineral salt medium containing toluene and nitrate as the sole sources of cell carbon, nitrogen, and energy (Fig. 1). Only 1 mM toluene was initially added since toluene at concentrations $\geq 2$ mM turned out to be toxic. Toluene was repeatedly fed at different times when it had been consumed. The doubling time under fed-batch conditions was approximately 24 h, but the optimal generation time is probably shorter.

In a 47 l fed-batch culture (Fig. 1) a total of 534 mmol toluene and 1627 mmol nitrate were consumed at the end of growth. Toluene served as sole carbon source for the formation of 20.9 g dry cell matter. Nitrate which was the only electron acceptor was first reduced to nitrite before nitrite was reduced to nitrogen gas. In addition nitrate served as sole nitrogen source for approximately 2.9 g cell nitrogen. Since the experiment was conducted in a 50-l stainless steel jar (with a small nitrogen head space and only a small thick rubber septum) any role of molecular oxygen in toluene metabolism can be excluded. Even if the medium would have been air-saturated only approximately 11 mmol molecular oxygen would have been dissolved (compare with 534 mmol toluene consumed). Therefore oxygen must be derived from water.

A carbon and redox balance of this experiment was determined at the mid of growth after 90 h (Fig. 1) when nitrate and nitrite had just been consumed (Table 1). This balance was based on the following assumptions: (1) 50% of dry cell weight is carbon and 14% is nitrogen; (2) the cell carbon has an average oxidation state of $CH_2O$, (3) toluene was either assimilated into cell carbon or completely mineralized to $CO_2$. The redox balance was 77% of the expected value based on the assumption that the toluene degraded had been completely oxidized to $CO_2$. The apparent molar growth yield was 57 g of cell dry matter formed per mol toluene.

Since the redox balance is close to but not identical with theory a small part of toluene had not been completely mineralized to $CO_2$ and/or part of the cells were lysed. Nevertheless the data prove that most of the toluene was oxidized to $CO_2$ without involvement of molecular oxygen.

### *Simultaneous adaptation of toluene-grown cells for degradation of other aromatic compounds*

Since toluene was not attacked by molecular oxygen two other principles are envisable: Toluene is either dehydrogenated (hydroxylated) with water as source of the hydroxyl group, or carboxylated with $CO_2$ derived from toluene degradation (Fig. 2). The first possibility is realized in an analogous reaction, *p*-cresol methylhydroxylation (Hopper 1978; McIntire et al. 1985; Bossert et al. 1989), a model for the second possibility is "phenol carboxylation" (Tschech and Fuchs 1989; Lack et al. 1991). These alternatives were tested applying the simultaneous adaptation techniques (Stanier 1947) and using thick suspensions of cells pregrown anaerobically on toluene and nitrate (Fig. 3).

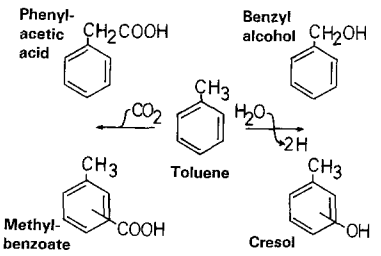


**Fig. 2.** Conceivable initial steps in anaerobic toluene degradation. Since oxygen is excluded toluene may become carboxylated or dehydrogenated (hydroxylated) with water as the source of the hydroxyl oxygen. A direct reductive attack of toluene seems unlikely. The chemical attack may occur at the methyl carbon or at any of the ring carbon positions; however, according to chemical experience not all positions are good candidates. Correspondingly, the hypothetical first intermediates in the case of toluene carboxylation would be phenylacetate or methylbenzoates; in the case of toluene hydroxylation benzyl alcohol or methylphenols (cresols) would be formed

These cell suspensions metabolized 1 mM toluene within 90 min without noticeable lag phase. Also benzyl alcohol, benzaldehyde, and benzoate were immediately metabolized. A lag phase of approximately 90 min was observed with 4-hydroxybenzoate and *p*-cresol; after this lag period the compounds were gradually metabolized. Phenylacetate started to be degraded only after 200 min; 2- and 4-methylbenzoate and o- and m-cresol were not at all metabolized within a period of several hours. A comparison of the specific toluene consumption rate of the growing culture (17 nmol $min^{-1}$ $mg^{-1}$ protein) and of the washed suspended cells (8 nmol $min^{-1}$ $mg^{-1}$ protein) indicates that the cell suspensions were viable (for calculation see legend to Fig. 3).

A comparison of the experimental results with the principal possibilities of anoxic toluene degradation (Fig. 2) indicates that all compounds except benzyl alcohol, benzaldehyde, and benzoic acid are unlikely to function as intermediates of anaerobic toluene degradation. This argument may not necessarily be valid for the aromatic acids which may not be transported unless specific transport systems are induced; all other compounds tested are thought to be membrane permeable. However, the apparent lack of coenzyme A ligases activating aromatic acids other than benzoate (see Table 2) argues against the possibility that e.g. methylbenzoates or phenylacetate are intermediates.

Some of the hypothetical intermediates cannot be used as growth substrates (Fig. 4). Different fed-batch cultures were set up using mineral salt medium containing

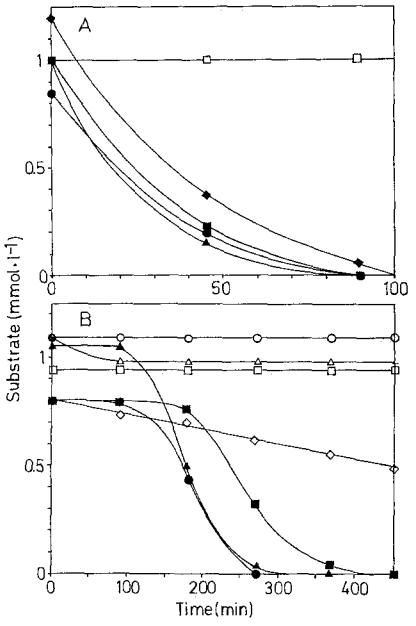


**Fig. 3A, B.** Simultaneous adaptation study with *Pseudomonas* sp. K 172 pregrown under denitrifying conditions on toluene as the sole carbon and electron source. The consumption of the following aromatic compounds ($\approx$1 mM) was studied with thick cell suspensions ($\Delta A_{578\ nm} = 10$) in mineral salt medium (20 mM nitrate) at 28°C. **A** (●) toluene; (■) benzaldehyde; (▲) benzyl alcohol; (◆) benzoic acid. In a control experiment (□) cells were omitted in order to estimate the loss of toluene due to diffusion. **B** (◇) o-cresol; (□) m-cresol; (○) 4-methyl benzoic acid; (△) 2-methyl benzoic acid; (●) *p*-cresol; (▲) 4-hydroxybenzoic acid; (■) phenylacetic acid. 3-Methylbenzoate was not available for testing. Note the different time scales in **A** and **B**. The specific rate of toluene consumption in washed cell suspensions (approximately 8 nmol toluene consumed $\cdot$ min$^{-1}$ $\cdot$ mg$^{-1}$ protein) was estimated from (1) the cell content; 1 ml suspension of an $A_{578\ nm} = 10$ (d = 1 cm) corresponded to 3.8 mg dry cell matter $\cdot$ ml$^{-1}$ or 1.9 mg protein $\cdot$ ml$^{-1}$ (assumed protein content 50% of dry cell matter); (2) the decrease in toluene concentration of approximately 0.65 mM within 45 min. The toluene consumption in this experiment was close to the specific toluene consumption rate of growing cultures (17 nmol $\cdot$ min$^{-1}$ $\cdot$ mg$^{-1}$ protein). The specific rate of substrate S (toluene) consumption by growing cultures was calculated according to $-dS/dt = (\mu/Y) \cdot X$. The specific growth rate $\mu$ was $4.8 \cdot 10^{-4}$ min$^{-1}$ (generation time 24 h), the molar growth yield Y was 57 g dry cell matter formed per mol toluene consumed; X represents gram cells (dry weight) or an equivalent of 500 mg protein per gram dry weight

20 mM nitrate and 1 mM aromatic substrate. Cells pregrown on toluene and nitrate were used as inoculum. Only cultures with toluene, benzyl alcohol, benzaldehyde, benzoate, 4-hydroxybenzoate, *p*-cresol, and phenylacetate showed immediate growth response. Due to the long generation times and the toxicity of most of the substrates the fed-batch experiment did not allow to detect short lag phases as seen with thick cell suspensions. Within the time period of the experiment no growth was detectable with *o*- and *m*-cresol, 2- and 4-methylbenzoate.

Growth on or consumption of toluene were not stimulated by $CO_2$ as expected if toluene was carboxylated (data not shown). A growth requirement for $CO_2$ has been observed with this strain when anaerobically grown on phenol (Tschech and Fuchs 1987). The $CO_2$ requirement is explained by the fact that phenol metabolism is initiated by carboxylation to 4-hydroxybenzoate (Tschech and Fuchs 1989).

**Table 2.** Specific enzyme activities in extracts of *Pseudomonas* sp. K 172 cells which were anaerobically grown under denitrifying conditions either with toluene or with benzoate as the sole carbon and electron source. As controls cells anaerobically grown on other aromatic compounds were analyzed, as indicated. n.d. = not determined; DCPIP = dichlorophenol indophenol; PMS = phenazine methosulfate; 0 = below detection limit (generally $\leq$ 1 nmol $\cdot$ min$^{-1}$ $\cdot$ mg$^{-1}$ protein)

| Enzyme activity | Specific activity (nmol $\cdot$ min$^{-1}$ protein) in extracts from cells grown on | | |
|---|---|---|---|
| | Toluene | Benzoate | Control (growth substrate) |
| *p*-Cresol methylhydroxylase (DCPIP/PMS) | 8 | 0 | 150 (*p*-Cresol) |
| Benzyl alcohol dehydrogenase ($NAD^+$) | 120 | 34 | n.d. |
| Benzaldehyde dehydrogenase ($NADP^+$) | 74 | 41 | n.d. |
| Benzoyl-CoA synthetase (AMP forming) | 45 | 81 | n.d. |
| 2-Hydroxybenzoyl-CoA synthetase | 0 | 0 | n.d. |
| 3-Hydroxybenzoyl-CoA synthetase | 0 | 0 | n.d. |
| 4-Hydroxybenzoyl-CoA synthetase | 0 | 0 | 120 (4-Hydroxybenzoate) |
| Phenylacetyl-CoA synthetase | 0 | 0 | 46 (Phenylacetate) |
| 2-Methylbenzoyl-CoA synthetase | 0 | 0 | n.d. |
| 4-Methylbenzoyl-CoA synthetase | 0 | 0 | n.d. |
| "Phenol carboxylase" ($^{14}CO_2$:4-OH-benzoate isotope exchange) | 0 | 0 | 80 (Phenol) |
| 4-Hydroxybenzoyl-CoA reductase (dehydroxylating) | 1 | 3 | 48 (4-Hydroxybenzoate) |

*Comparative study of enzyme activities in vitro*

Enzymes possibly involved in anaerobic degradation of toluene were searched for in extracts of cells anaerobically grown on toluene (Table 2). Under the conditions of *p*-cresol methylhydroxylase assay no toluene methylhydroxylase activity could be detected so far even when different electron acceptors were tested. Cells anaerobically grown on *p*-cresol contained high p-cresol methylhydroxylase activity, in contrast to cells grown on toluene. This demonstrates that toluene methylhydroxylase, if existing, must be different from *p*-cresol methylhydroxylase. Benzyl alcohol dehydrogenase ($NAD^+$-dependent), benzaldehyde dehydrogenase ($NADP^+$-dependent), and benzoyl-CoA synthetase (AMP forming) ac-

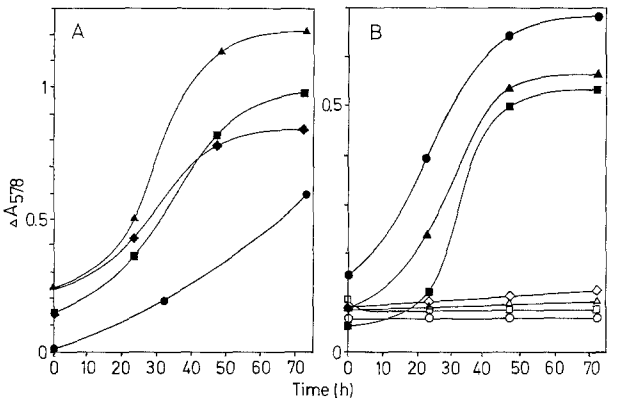


**Fig. 4A, B.** Anaerobic fed-batch cultures of *Pseudomonas* sp. K 172 which contained putative intermediates of anaerobic toluene metabolism as sole carbon and electron sources under denitrifying conditions (20 mM nitrate). The inoculum was pregrown anaerobically on toluene plus nitrate. The initial concentration of aromatic compounds was 1 mM. At the different time points the aromatic compounds had been consumed; they were repeatedly fed at 1 mM concentration. **A** (●) toluene; (▲) benzyl alcohol; (■) benzaldehyde; (◆) benzoic acid. **B** (□) *o*-cresol; (◇) *m*-cresol; (▲) *p*-cresol; (■) phenylacetic acid; (●) 4-hydroxybenzoic acid; (○) 2-methylbenzoic acid; (△) 4-methylbenzoic acid

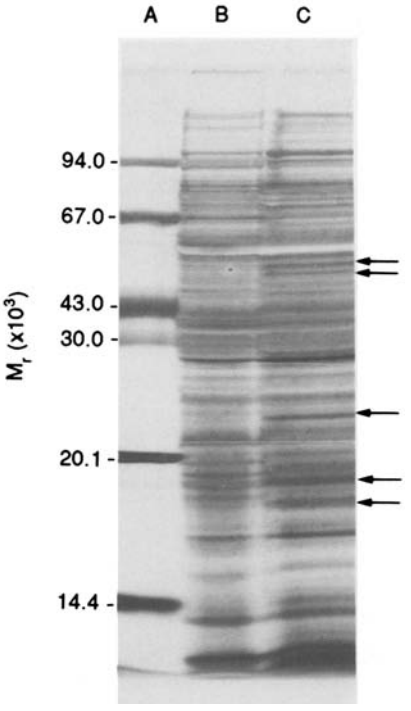


**Fig. 5.** Comparative SDS-PAGE of proteins in extracts (100,000 × *g* supernatant) of *Pseudomonas* sp. K 172 cells after anaerobic growth on (*lane B*) benzoate, (*lane C*) toluene. In lane A molecular mass standard proteins were run. The *arrows* indicate protein bands in toluene-grown cells which are absent in benzoate-grown cells

tivities were present. The stoichiometry of the dehydrogenase reactions was demonstrated to be one mol NADH oxidized per mol benzaldehyde added (benzyl alcohol dehydrogenase), and one mol NADPH formed per mol benzaldehyde added (benzaldehyde dehydrogenase). The product of benzoyl-CoA synthetase reaction was benzoyl-CoA, AMP, and pyrophosphate; the benzoate-, coenzyme A-, and ATP-dependent AMP formation was determined in a coupled assay (Ziegler et al. 1989) in which two mol NADH were oxidized per mol AMP formed in the synthetase reaction. The specific activity of the dehydrogenase was ≥ twice as high in toluene-grown cells compared to benzoate-grown cells. The relative difference is even more pronounced if one takes into account that growth with benzoate was approximately three times faster than with toluene. In any case all three specific enzyme activities can account for the observed growth rate on toluene. Also the kinetic constants were in the physiological order (for similar dehydrogenase see Chalmers et al. 1990; Shaw and Harayama 1990; for similar synthetases see Hutber and Ribbons 1983; Geissler et al. 1988; Nozawa and Maruyama 1988; Ziegler et al. 1989).

The reactions followed Michaelis-Menten kinetics, and the substrate dependence of each individual enzyme was strict. The apparent $K_m$-values were 0.35 mM benzyl alcohol and 0.05 mM $NAD^+$ for benzyl alcohol dehydrogenase, 0.1 mM benzaldehyde and 0.13 mM $NADP^+$ for benzaldehyde dehydrogenase, and 0.015 mM benzoate, 0.3 mM ATP, and 0.2 mM coenzyme A for benzoyl-CoA synthetase. The optimal pH was 9 for benzyl alcohol dehydrogenase, 8.7 for benzaldehyde dehydrogenase, and 8.5 for benzoyl-CoA synthetase. The characteristics of the three enzyme activities were virtually the same independent whether they were studied in benzoate- or toluene-grown cells.

No enzymatic evidence for degradation of toluene via cresols (Rudolphi et al. 1991) could be found nor for degradation via methylbenzoates or phenylacetate (Seyfried et al. 1991; Dangel et al. 1991). The enzymes to be expected could not be detected; control experiments with cells grown on *p*-cresol, phenylacetate, phenol, or 4-hydroxybenzoate were performed. Especially the fact that no methylbenzoate:coenzyme A ligase or phenylacetate:coenzyme A ligase activity could be detected argues against toluene degradation via ring carboxylation; these enzymes were probably not overlooked since aromatic acid:coenzyme A ligases are normally oxygen-insensitive and more or less stable. So far all benzoic acid analogues were found to be anaerobically metabolized via their coenzyme A thioesters.

### *Comparison of protein pattern of cells anaerobically grown on toluene and benzoate*

The protein pattern of cells anaerobically grown on toluene and benzoate, respectively, was compared by SDS PAGE of cell extracts (100,000 × *g* supernatant) (Fig. 5). Cells grown anaerobically on toluene contained additional protein bands corresponding to molecular weights of approximately 53,000, 51,000, 25,000, 19,000, and 17,000 which were absent in benzoate-grown cells. The other protein pattern was virtually identical.

## Conclusion

The data indicate that toluene is anaerobically attacked by methylhydroxylation to benzyl alcohol. The alcohol is further oxidized to benzaldehyde and benzoate, and benzoate is activated to benzoyl-CoA. Benzoyl-CoA has been shown to be one of the central metabolites in anaerobic oxidation of aromatic compounds (for a scheme of anaerobic metabolism of aromatic compounds in this strain see Dangel et al. 1991). It has been postulated that the further attack of benzoyl-CoA is reductive (Evans 1977) yielding a coenzyme A-activated alicyclic acid. The benzoyl-CoA reducing enzyme and the toluene methylhydroxylating enzyme remain to be studied in vitro.

The hypothetical toluene methylhydroxylation at first sight seems to be similar to *p*-cresol methylhydroxylation (McIntire et al. 1985); however, the enzymes and the mechanisms of both processes may be unrelated. Whereas a quinone methide intermediate is assumed and water is added by a ionic mechanism in p-cresol methylhydroxylation, a substrate radical mechanism appears more plausible in toluene methylhydroxylation. A similarly challenging reaction was recently found in sulfate-reducing bacteria which are able to use saturated hydrocarbons as sole carbon and electron source (Aeckersberg et al. 1991).

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## References

Aeckersberg F, Bak F, Widdel F (1991) Anaerobic oxidation of saturated hydrocarbons to $CO_2$ by a new type of sulfate reducing bacteria. Arch Microbiol (in press)

Berry DF, Francis AJ, Bollag J-M (1987) Microbial metabolism of homocyclic and heterocyclic aromatic compounds under anaerobic conditions. Microbiol Rev 51:43–59

Bossert ID, Whited G, Gibson DT, Young LY (1989) Anaerobic oxidation of *p*-cresol mediated by a partially purified methylhydroxylase from a denitrifying bacterium. J Bacteriol 171:2956–2962

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Chalmers RM, Scott AJ, Fewson CA (1990) Purification of the benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase encoded by the TOL plasmid pWW53 of *Pseudomonas putida* MT53 and their preliminary comparison with benzyl alcohol dehydrogenase and benzaldehyde dehydrogenases I and II from *Acinetobacter calcoaceticus*. J Gen Microbiol 136:637–643

Dangel W, Brackmann R, Lack A, Mohamed M, Koch J, Oswald B, Seyfried B, Tschech A, Fuchs G (1991) Differential expression of enzyme activities initiating anoxic metabolism of various aromatic compounds via benzoyl-CoA. Arch Microbiol 155:256–262

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

Evans WC (1977) Biochemistry of the bacterial catabolism of aromatic compounds in anaerobic environments. Nature 270:17–22

Evans WC, Fuchs G (1988) Anaerobic degradation of aromatic compounds. Annu Rev Microbiol 42:289–317

Geissler JF, Harwood CS, Gibson J (1988) Purification and properties of benzoate-coenzyme A ligase, a *Rhodopseudomonas palustris* enzyme involved in the anaerobic degradation of benzoate. J Bacteriol 170:1709–1714

Gesellschaft Deutscher Chemiker (1979) Deutsche Einheitsverfahren zur Wasser-, Abwasser- und Schlammuntersuchung, 8. Lieferung, D9, pp 1–8

Gibson DT, Subramanian V (1984) Microbial degradation of aromatic hydrocarbons. In: Gibson DT (ed) Microbial degradation of organic compounds. Dekker, New York, pp 181–252

Glöckler R, Tschech A, Fuchs G (1989) Reductive dehydroxylation of 4-hydroxybenzoyl-CoA to benzoyl-CoA in a denitrifying, phenol degrading *Pseudomonas* species. FEBS Lett 251:237–240

Grbic-Galic D, Vogel TM (1987) Transformation of toluene and benzene by mixed methanogenic cultures. Appl Environ Microbiol 53:254–260

Hopper DJ (1978) Incorporation of [$^{18}$O]water in the formation of *p*-hydroxybenzyl alcohol by the *p*-cresol methylhydroxylase from *Pseudomonas putida*. Biochem J 175:345–347

Hutber GN, Ribbons DW (1983) Involvement of coenzyme A esters in metabolism of benzoate and cyclohexanecarboxylate by *Rhodopseudomonas palustris*. J Gen Microbiol 129:2413–2420

Kuhn EP, Colberg PJ, Schnoor JL, Wanner O, Zehnder AJB, Schwarzenbach RP (1985) Microbial transformations of substituted benzenes during infiltration of river water to groundwater: laboratory column studies. Environ Sci Technol 19:961–968

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

Lack A, Tommasi I, Aresta M, Fuchs G (1991) Catalytic properties of "phenol carboxylase": in vitro study of $CO_2$:4-hydroxybenzoate isotope exchange reaction. Eur J Biochem 197:473–479

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Lovley DR, Lonergan DJ (1990) Anaerobic oxidation of toluene, phenol, and *p*-cresol by the dissimilatory iron-reducing organism, GS-15. Appl Environ Microbiol 56:1858–1864

Lovely DR, Baedecker MJ, Lonergan DJ, Cozzarelli IM, Philips EJP, Siegel DJ (1989) Oxidation of aromatic contaminants coupled to microbial iron reduction. Nature 339:297–300

McIntire W, Hopper DJ, Singer TP (1985) p-Cresol methylhydroxylase. Assay and general properties. Biochem J 228:325–335

Morrissey JH (1981) Silver stain for proteins in polyacrylamid gels: a modified procedure with enhanced uniform sensitivity. Anal Biochem 117:307–310

Nozawa T, Maruyama Y (1988) Anaerobic metabolism of phthalate and other aromatic compounds by a denitrifying bacterium. J Bacteriol 170:5778–5784

Rider BF, Mellon MG (1946) Colorimetric determination of nitrites. Ind Eng Chem 18:96–98

Rudolphi A, Tschech A, Fuchs G (1991) Anaerobic degradation of cresols by denitrifying bacteria. Arch Microbiol 155:238–248

Seyfried B, Tschech A, Fuchs G (1991) Anaerobic degradation of phenylacetate and 4-hydroxyphenylacetate by denitrifying bacteria. Arch Microbiol 155:249–255

Shaw JP, Harayama S (1990) Purification and characterisation of TOL plasmid-encoded benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase of *Pseudomonas putida*. Eur J Biochem 191:705–714

Stanier RY (1947) Simultaneous adaptation: a new technique for the study of metabolic pathways. J Bacteriol 54:339–348

Tschech A, Fuchs G (1987) Anaerobic degradation of phenol by pure cultures of newly isolated denitrifying pseudomonads. Arch Microbiol 148:213–217

Tschech A, Fuchs G (1989) Anaerobic degradation of phenol via carboxylation to 4-hydroxybenzoate: in vitro study of isotope

exchange between $^{14}CO_2$ and 4-hydroxybenzoate. Arch Microbiol 152:594–599

Vogel TM, Grbic-Galic D (1986) Incorporation of oxygen from water into toluene and benzene during anaerobic fermentative transformation. Appl Environ Microbiol 52:200–202

Wilson BH, Smith GB, Rees JF (1986) Biotransformations of selected alkylbenzenes and halogenated aliphatic hydrocarbons in methanogenic aquifer material: a microcosm study. Environ Sci Technol 20:997–1002

Young LY (1984) Anaerobic degradation of aromatic compounds. In: Gibson DT (ed) Microbial degradation of organic compounds. Dekker, New York, pp 487–523

Zeyer J, Kuhn EP, Schwarzenbach RP (1986) Rapid microbial mineralization of toluene and 1,3-dimethylbenzene in the absence of molecular oxygen. Appl Environ Microbiol 52:944–947

Ziegler K, Buder R, Winter J, Fuchs G (1989) Activation of aromatic acids and aerobic 2-aminobenzoate metabolism in a denitrifying *Pseudomonas* strain. Arch Microbiol 151:176–176