

## Isolation and characterization of a bacterium that mineralizes toluene in the absence of molecular oxygen

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Received January 14, 1990/Accepted May 21, 1990

**Abstract.** A bacterium tentatively identified as a *Pseudomonas* sp. was isolated from a laboratory aquifer column in which toluene was degraded under denitrifying conditions. The organism mineralized toluene in pure culture in the absence of molecular oxygen. In carbon balance studies using [*ring*-UL-<sup>14</sup>C]toluene, more than 50% of the radioactivity was recovered as <sup>14</sup>CO<sub>2</sub>. Nitrate and nitrous oxide served as electron acceptors for toluene mineralization. The organism was also able to degrade *m*-xylene, benzoate, benzaldehyde, *p*-cresol, *p*-hydroxybenzaldehyde, *p*-hydroxybenzoate and cyclohexanecarboxylic acid in the absence of molecular oxygen.

**Key words:** *Pseudomonas* sp. – Toluene – Xylene – Dimethylbenzene – Aromatic compounds – Denitrification – Nitrate reduction – Anaerobic degradation

### Introduction

Molecular oxygen plays a dual role in the aerobic degradation of aromatic compounds such as toluene, benzoate, aniline and phenol. It is a cosubstrate in initial reactions like the oxidation of toluene to benzylalcohol and it also serves as the final electron acceptor for mineralization (Gibson and Subramanian 1984). Under anaerobic conditions, nitrate, sulfate or other reducible species can substitute for oxygen as a final electron acceptor (Zehnder and Svensson 1986; Zehnder and Stumm 1988), however, there is no evidence that they can serve as a cosubstrate for the initial reactions. Nevertheless, a series of aromatic compounds including phenol and benzoate have been shown to be degradable under anaerobic conditions. All these compounds exhibited, however, func-

tional groups (e. g. a hydroxy or a carboxyl group) which may facilitate the initial catabolic step (Berry et al. 1987; Evans and Fuchs 1988; Young 1984).

Anaerobic mineralization of alkylated benzenes, i. e. aromatic hydrocarbons containing no activating groups, has never been clearly demonstrated in pure cultures. Evidence for the occurrence of such processes in mixed microbial cultures has been reported by various groups (Grbić-Galić and Vogel 1987; Vogel and Grbić-Galić 1986; Wilson et al. 1986). Recently, Lovley et al. (1989) reported microbial oxidation of toluene coupled to iron reduction, but the turnover was not quantified and a carbon mass balance using <sup>14</sup>C-labelled substrate was not provided. In previous work, we reported the anaerobic degradation of alkylated benzenes in denitrifying laboratory aquifer columns (Kuhn et al. 1985, 1988; Zeyer et al. 1986). In this paper, we describe the isolation and characterization of a bacterium originating from these aquifer columns that is able to mineralize toluene with nitrate or nitrous oxide as electron acceptor.

### Materials and methods

#### Chemicals

Benzene, benzaldehyde, sodium benzoate and *o*-cresol were purchased from Merck (Darmstadt, FRG). Toluene, *o*-, *m*-, *p*-xylene, *p*-cresol, *p*-hydroxybenzylalcohol, *p*-hydroxybenzaldehyde, *p*-hydroxybenzoate, benzylalcohol, cyclohexanecarboxylic acid, naphthalene, pyruvate, sodium nitrate and sodium nitrite were from Fluka AG (Buchs, Switzerland). All chemicals were of the highest available purity and used as received. [*Ring*-UL-<sup>14</sup>C]toluene (specific activity, 10.9 mCi/mmol) and (*methyl*-<sup>14</sup>C]toluene (specific activity, 8.6 mCi/mmol) were purchased from Pathfinder Laboratories (St. Louis, MO, USA). [*Ring*-UL-<sup>14</sup>C]*m*-xylene (specific activity, 93 mCi/mmol) was from Amersham International (Arlington Heights, IL, USA). The radiochemicals had purities of greater than 98% according to the supplier and were dissolved and stored in ethanol (about 50000 dpm per µl). N<sub>2</sub> (purity: >99.999%), N<sub>2</sub>O (>99.5%) and N<sub>2</sub>O/N<sub>2</sub> (5/95, v/v, >99.5%) were from PanGas (Lucerne, Switzerland).

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## Media

The basal medium had the following composition (g/l):  $\text{KH}_2\text{PO}_4$  (0.33),  $\text{Na}_2\text{HPO}_4$  (1.2),  $\text{NH}_4\text{Cl}$  (0.11),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.10),  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (0.044) and trace elements (1 ml/l) from a solution described previously (Zeyer and Kearney 1982). The pH of the medium was adjusted to 7.5 with NaOH. Oxygen was removed from the medium by boiling, and subsequent cooling under a flux of  $\text{N}_2$  (Hungate 1969). Aliquots (30 ml) of medium were placed into 50 ml serum bottles preflushed with  $\text{N}_2$ , sealed with butyl rubber stoppers (Bellco Glass, Vineland, NJ, USA) and autoclaved. To avoid precipitation,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  was added from a concentrated stock solution aseptically after autoclaving. Unless stated otherwise, the media were supplemented with 10 mM  $\text{NaNO}_3$  as the only electron acceptor and incubations were conducted with a  $\text{N}_2$ -headspace. In some degradation studies,  $\text{NaNO}_3$  was replaced by  $\text{N}_2\text{O}$ . In these cases, the media were purged with  $\text{N}_2\text{O}$  and incubated with a  $\text{N}_2\text{O}$ -headspace.

To exclude the involvement of traces of molecular oxygen in the degradation of toluene, experiments were performed not only in oxygen-free media but also in chemically prerduced media. In these experiments, the media were supplemented with FeS (prepared according to Hanert 1981) to a final concentration of 22 mg/l. Resazurin, which was added as a redox indicator to these media, was decolorized within a few hours, indicating strictly anaerobic conditions (Hungate 1969). FeS was used because addition of  $\text{Na}_2\text{S}$  in concentrations sufficient to decolorize the resazurin proved to inhibit microbial growth (data not shown). Since the solubility product of FeS is  $3.7 \times 10^{-19} \text{ mol}^2/\text{l}^2$  (Weast and Astle 1981), the actual concentration of dissolved sulfide in these media remained very low ( $< 10^{-9} \text{ M}$ ).

In the enrichment cultures, 0.2 mM toluene was supplied by adding aliquots from autoclaved saturated solutions of toluene in distilled water (solubility: 6 mM, Tewari et al. 1982). Sometimes, however, micelles of toluene were formed in the stock solution which precluded accurate dosage of toluene. Therefore, for pure culture experiments, toluene and *m*-xylene were added as pure liquid by microsyringe (Hamilton, Bonaduz, Switzerland) to a final concentration of 0.2 to 0.3 mM. [ $^{14}\text{C}$ ]Toluene and [ $^{14}\text{C}$ ]m-xylene dissolved in ethanol (about 50000 dpm/ $\mu\text{l}$ ) were also added by microsyringe.

## Isolation of strain T and growth conditions

An enrichment culture was established by inoculating oxygen-free medium (basal medium + 0.2 mM toluene + 10 mM  $\text{NaNO}_3$  +  $\text{N}_2$ -headspace) with effluent from an anaerobic, toluene-degrading laboratory aquifer column that had been operated for more than one year under continuous-flow conditions (Kuhn et al. 1988). The culture was incubated and aliquots were periodically transferred to fresh medium. After several transfers, the enriched culture was serially diluted into oxygen-free basal medium supplemented with 0.2 mM toluene and 10 mM  $\text{NaNO}_3$ . The highest dilution showing growth and toluene degradation was again serially diluted. A pure culture was then isolated from the highest dilution of this series that showed growth by repeatedly applying the agar shake culture method as described by Pfennig and Trüper (1981). Purity of cultures was checked microscopically and by plating culture samples on solid basal medium supplemented with pyruvate (40 mM) as the only organic carbon source. These plates were incubated aerobically. The colonies that developed were uniform and consisted of one distinct morphotype, designated strain T. Single colonies could be cultured again in oxygen-free basal medium supplemented with nitrate and toluene, and they mineralized toluene.

Liquid cultures of strain T were always incubated at  $30 \pm 1^\circ\text{C}$  on a rotary shaker. Strain T was stored under aerobic conditions on agar slants (basal medium + 20 mM pyruvate + 2% agar) at  $4^\circ\text{C}$ . To maintain viability, the strain had to be restreaked every 2 to 3 weeks.

## Identification and properties of strain T

The identification of strain T was performed in the laboratories of the German Collection of Microorganisms (DSM, Braunschweig, FRG). Cells of strain T are gram-negative rods measuring  $0.5\text{--}0.7 \times 1.5\text{--}3.5 \mu\text{m}$ . They are yellow-pigmented, non-spore forming, motile, denitrifying, and they accumulate poly- $\beta$ -hydroxybutyrate. Electron microscopy revealed the presence of one flagellum. After aerobic growth, cells are oxidase and catalase positive and arginine dihydrolase and  $\beta$ -galactosidase negative. These characteristics allow tentative identification of strain T as a *Pseudomonas* sp. However, the organism forms indole from tryptophan, which is atypical of the known *Pseudomonas* sp.

Strain T is not able to utilize sulfate (3 mM), sulfite (3 mM), thiosulfate (3 mM), or Fe(III) (added as Fe-citrate at a concentration of 15 mM) as electron acceptors (tested with pyruvate as electron donor). Electron donors degraded with nitrate as electron acceptor include acetate, pyruvate, fructose, and glucose. The strain, however, does not grow on ethanol. It should be noted that the growth rate of strain T varied from batch to batch without apparent variation in media composition or other growth conditions. For example, the exponential growth rate on basal medium + pyruvate +  $\text{NaNO}_3$  +  $\text{N}_2$ -headspace varied between 0.06 and  $0.15 \text{ h}^{-1}$ .

## Carbon balance studies using radiolabelled toluene

Oxygen-free or chemically reduced media were supplemented with [ $^{14}\text{C}$ ]toluene and [ $^{14}\text{C}$ ]m-xylene, respectively (approximately 2000–5000 dpm/ml), and inoculated with aliquots of a culture of strain T pregrown on oxygen-free basal medium plus 10 mM nitrate plus 5 mM pyruvate. Non-inoculated media served as controls. At the end of the incubation (usually 2 weeks), the cultures were acidified to a pH of about 2 with HCl, and subsequently purged with air. The air was passed through two traps, each containing 20 ml of isobutanol (to trap any remaining toluene, m-xylene or other volatile organic compounds), and through two traps each containing 20 ml of 0.1 N NaOH (to absorb  $\text{CO}_2$ ). After purging, the radioactivity in the traps and the radioactivity remaining in the acidified culture was quantified. To recover and quantify sorbed [ $^{14}\text{C}$ ]toluene, the butyl rubber stoppers were extracted overnight with ethyl acetate. Quantification of  $^{14}\text{C}$  was performed by adding 1 ml-samples of isobutanol, NaOH solution, ethyl acetate and culture fluid, respectively, to 10 ml of Kontrogel (Kontron, Zürich, Switzerland) and subsequent counting in a Tri-Carb liquid scintillation counter (Packard, Downers Grove, IL, USA).

## Determination of growth and analytical methods

Growth was measured by determining the optical density of cultures at 546 nm ( $\text{OD}_{546}$ ) with a Uvikon 810 spectrophotometer (Kontron, Zürich, Switzerland). To study growth-inhibitory effects of certain compounds (e.g. nitrite or suspected intermediates of toluene degradation) on strain T, the organism was grown on basal medium + 40 mM pyruvate in the presence of  $\text{N}_2\text{O}$  as sole electron acceptor in a 50 ml serum bottle. At the beginning of the exponential growth phase, the test compound was added from a concentrated aqueous stock solution. The exponential growth rate of the spiked culture was then compared to the growth rate of an unspiked culture.

Concentrations of aromatic substrates and of cyclohexanecarboxylic acid were assayed by gas chromatography or high-pressure liquid chromatography as described previously (Kuhn et al. 1988). Pyruvate was quantified enzymatically with a lactate dehydrogenase assay (Sigma Diagnostics, St. Louis, USA). Nitrate was determined by a salicylate based colorimetric method (Legler 1971) and nitrite by a diazotization-based colorimetric method (Montgomery and Dymock 1961).

**Table 1.** Degradation of [<sup>14</sup>C]toluene by strain T with nitrate as sole electron acceptor. The data represent average values of at least two independent determinations. Media and analytical procedures are described in Methods

Growth conditions and substrate	<sup>14</sup> C Balance after an incubation time of 2 weeks [%]				
	[ <sup>14</sup> C]Toluene recovered from culture	<sup>14</sup> CO <sub>2</sub> evolved	<sup>14</sup> C-labeled residues remaining in culture	[ <sup>14</sup> C]Toluene sorbed to stopper	Total <sup>14</sup> C recovery
Oxygen free medium <sup>a</sup>					
[Ring-UL- <sup>14</sup> C]toluene		51	17	22	91
[Methyl- <sup>14</sup> C]toluene		52	9	18	81
Medium reduced with FeS <sup>b</sup>					
[Ring-UL- <sup>14</sup> C]toluene	<1	51	24	9	>84
Sterile control <sup>a</sup>					
[Ring-UL- <sup>14</sup> C]toluene	13	<1	<1	69	83

<sup>a</sup> Basal medium + 0.3 mM toluene + [<sup>14</sup>C]toluene (2000 dpm/ml) + 10 mM nitrate + N<sub>2</sub>-headspace

<sup>b</sup> As in a, but supplemented with FeS

## Results and discussion

### Degradation of toluene by strain T

Strain T was able to mineralize toluene in an oxygen-free medium supplemented with nitrate as sole electron acceptor. A carbon mass balance was established using [<sup>14</sup>C]toluene as substrate (Table 1). After a 2-week incubation, 2% of the applied radioactivity was recovered as non-degraded toluene, and more than 50% was evolved as <sup>14</sup>CO<sub>2</sub>. Up to 17% remained in the culture as <sup>14</sup>C-labeled non-volatile residues. Analysis of these residues by high-pressure liquid chromatography did not reveal any identifiable metabolites. Mineralization of toluene took also place in cultures growing on chemically reduced medium (Table 1) which demonstrates that molecular oxygen is not involved in the degradation of toluene. At the beginning of the incubation, a large part (usually two thirds) of the applied toluene was sorbed by the butyl rubber stoppers. This sorption process was largely reversible and equilibrium conditions were established within a few hours. During incubation with strain T, toluene was slowly desorbed and converted to CO<sub>2</sub>. At the end of the incubation, the amount of toluene extractable from the butyl rubber stoppers was below 22% in all cultures (Table 1). Only in the sterile control was a large part of toluene associated with the stopper. It is possible that the poor overall recovery of radioactivity (81–91%) in the cultures is due to an incomplete desorption and extraction of toluene from the butyl rubber stoppers.

### Substrate specificity of strain T

In addition to toluene, strain T was also able to mineralize *m*-xylene in the presence of nitrate. A carbon balance study using 0.3 mM [ring-UL-<sup>14</sup>C]*m*-xylene as sole source of energy and 10 mM nitrate as sole electron acceptor revealed that more than 50% of the radioactivity was evolved as <sup>14</sup>CO<sub>2</sub> within 16 days. Strain T, however, was unable to degrade *o*-xylene, *p*-xylene, benzene or naphthalene in the absence of molecular oxygen. It is

noteworthy that the laboratory aquifer column from which strain T was isolated initially degraded *m*-xylene and *p*-xylene, in the presence of nitrate (Kuhn et al. 1985). Upon extended enrichment with *m*-xylene, the microbial population in the column lost the ability to degrade *p*-xylene in the absence of molecular oxygen (Zeyer et al. 1986; Kuhn et al. 1988).

The pathway of toluene degradation in the absence of molecular oxygen is unknown. Vogel and Grbić-Galić (1986) suggested a transformation of toluene to *p*-cresol which can be converted to *p*-hydroxybenzoate and benzoate under anaerobic conditions (Evans and Fuchs 1988; Bossert and Young 1986; Taylor et al. 1970). Alternatively, degradation of toluene through benzyl alcohol and benzaldehyde to benzoate is very common under aerobic conditions (Gibson and Subramanian 1984), but there is only very weak evidence for the occurrence of such a pathway in the absence of molecular oxygen (Grbić-Galić and Vogel 1987). The anaerobic mineralization of benzoate (e.g. through cyclohexanecarboxylic acid) is well documented (Evans and Fuchs 1988; Young 1984). The most intriguing question about the anaerobic toluene degradation is the mechanism of the initial catabolic step, i.e. the activation of the toluene molecule in the absence of molecular oxygen.

Based on literature data and results from studies with a laboratory aquifer column, we postulated a number of hypothetical intermediates of anaerobic toluene degradation (Kuhn et al. 1988). As a follow-up to these suggestions, we determined whether strain T was able to grow on these compounds using nitrate as sole electron acceptor (Table 2). To avoid toxic effects of these hypothetical intermediates on strain T, the growth studies were performed at substrate concentrations of 1 mM. Benzaldehyde exhibited a severe growth-inhibitory effect on strain T (90% growth inhibition at a concentration of 2 mM, Table 2) and therefore this substrate was provided at a concentration of 0.5 mM. Among the substrates tested, only benzylalcohol and *o*-cresol did not serve as growth substrates for strain T. Therefore the pattern of compounds degradable by strain T does not suggest a particular pathway for anaerobic toluene metabolism.

**Table 2.** Degradation of various substrates by strain T

Substrate	Growth inhibition by substrate [%] <sup>a</sup>	Growth on substrate <sup>b</sup>	Substrate recovered after growth [% of initial]
<i>p</i> -Cresol	28		< 1
<i>p</i> -Hydroxybenzyl-alcohol	86	+	< 1
<i>p</i> -Hydroxybenzaldehyde	28	+	< 1
<i>p</i> -Hydroxybenzoate	26	+	< 1
<i>o</i> -Cresol	43	-	> 95
Benzylalcohol	39	-	> 95
Benzaldehyde	90	+	< 1
Benzoate	26		< 1
Cyclohexanecarboxylic acid	26		

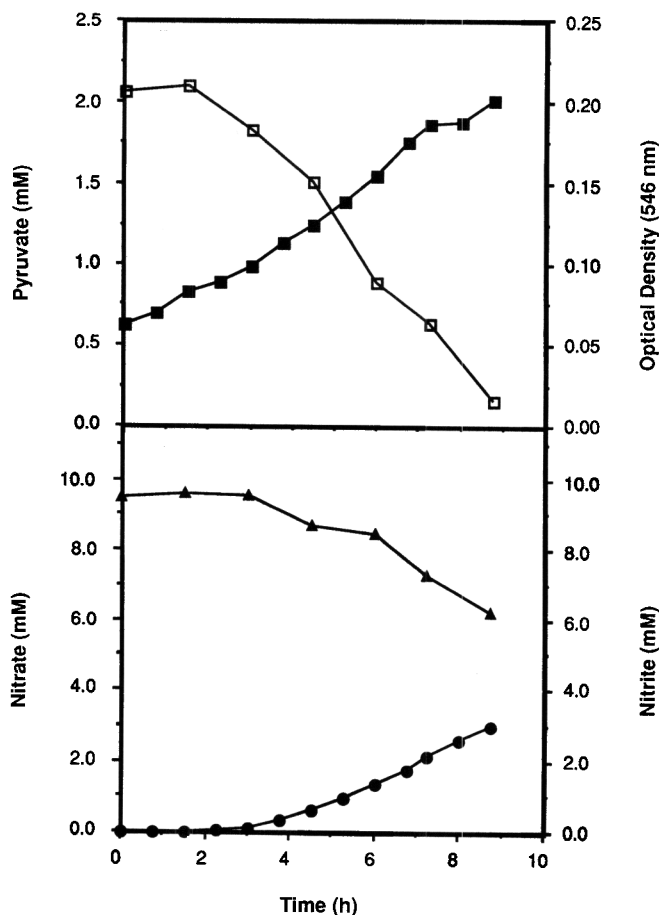
<sup>a</sup> Growth inhibition was measured as described in Methods (substrate concentration: 2 mM)

<sup>b</sup> Growth was determined on basal medium + 1 mM substrate (for benzaldehyde: 0.5 mM only) + 10 mM nitrate + N<sub>2</sub>-headspace. (+) significant turbidity. (-) no growth

Moreover, these data have to be interpreted with great caution because growth studies may be impeded both by uptake and induction effects. Tschech and Fuchs (1987) recently reported the isolation of a phenol-degrading denitrifying *Pseudomonas* sp. which grew on most of the hypothetical intermediates tested in Table 2, but which was unable to degrade toluene.

#### Electron acceptors used by strain T

The toluene-degrading laboratory aquifer columns from which strain T was isolated reduced nitrate and accumulated nitrite. Only a fraction of the nitrite was further reduced to gaseous nitrogen species (Zeyer et al. 1986). The reduction of nitrate to nitrite in the presence of a degradable carbon source (e.g. toluene or pyruvate) could also be demonstrated in a pure culture of strain T. The strain was grown on 2 mM pyruvate in the presence of 10 mM nitrate as sole electron acceptor (Fig. 1). After a lag phase of about 4 h, pyruvate was degraded at an apparently constant rate of 0.3 mM/h and this oxidation was coupled to a reduction of nitrate to nitrite at a rate of about 0.5 mM/h. Nitrite reduction only started after a lag phase of several days. Further experiments confirmed that cells of strain T which were pregrown in the presence of nitrate were able to resume growth in the presence of nitrite only after an adaption period of several days. Similarly, in the laboratory aquifer column from which strain T was isolated, toluene degradation was severely reduced upon replacement of nitrate by nitrite (Kuhn et al. 1988). It is noteworthy that two *Pseudomonas* sp. able to grow with nitrate on phenol or anthranilic acid, respectively, showed a behaviour similar to strain T, i.e. nitrite was stoichiometrically accumulated and further reduced only after nitrate depletion (Tschech and Fuchs 1987; Braun and Gibson 1984). In addition, Körner and Zumft (1989) demonstrated that reduction of nitrite to



**Fig. 1.** Coupling of pyruvate oxidation with nitrate reduction. Strain T was grown on basal medium + 2 mM pyruvate + 10 mM nitrate + N<sub>2</sub>-headspace. Samples were periodically withdrawn and analyzed for ■ turbidity, □ pyruvate, ▲ nitrate and ● nitrite

molecular nitrogen by a *Pseudomonas stutzeri* was completely inhibited in the presence of nitrate even though the nitrite reductase was induced.

Nitrite exhibited a severe growth-inhibitory effect on strain T. Addition of 5 mM nitrite to a growing culture of strain T reduced the exponential growth rate by 50% and addition of 10 mM nitrite inhibited growth totally. A slow adaption was recognizable only after several hours. Theoretically, total oxidation of 1 mM toluene (C<sub>7</sub>H<sub>8</sub>) to 7 mM CO<sub>2</sub> and 4 mM H<sub>2</sub>O must be coupled to the reduction of 18 mM nitrate to nitrite. Since up to 0.3 mM toluene was routinely used in the previous degradation studies and more than 50% was mineralized to CO<sub>2</sub> (Table 1), an accumulation of about 3 mM nitrite and hence a growth-inhibition is expected.

Growth-inhibitory effects by accumulated nitrite, however, could be avoided by replacing nitrate with nitrous oxide. Growing on 40 mM pyruvate, strain T was able to use not only nitrate but also nitrous oxide as electron acceptor (Table 3). The exponential growth rate in the presence of nitrate, 5% nitrous oxide and 100% nitrous oxide, respectively, was within a narrow range. The cell yield (expressed as OD<sub>546, max</sub>), however, was

**Table 4.** Degradation of [<sup>14</sup>C]toluene by strain T with nitrous oxide as sole electron acceptor. The data represent average values of at least 2 independent determinations. Media and analytical procedures are described in Methods

Growth conditions and substrate	<sup>14</sup> C Balance after an incubation time of 2 weeks [%]				
	[ <sup>14</sup> C]Toluene recovered from culture	<sup>14</sup> CO <sub>2</sub> evolved	<sup>14</sup> C-labeled residues remaining in culture	[ <sup>14</sup> C]Toluene sorbed to stopper	Total <sup>14</sup> C recovery
Oxygen free medium <sup>a</sup>					
[Ring-UL- <sup>14</sup> C]toluene	<1	58	21	13	> 92
[Methyl- <sup>14</sup> C]toluene	<1	61	13	11	> 85
Medium reduced with FeS <sup>b</sup>					
[Ring-UL- <sup>14</sup> C]toluene	14		6	57	79
Sterile control <sup>a</sup>					
[Ring-UL- <sup>14</sup> C]toluene	16	<1	<1	69	> 85

<sup>a</sup> Basal medium + 0.3 mM toluene + [<sup>14</sup>C] toluene (2000 dpm/ml) + N<sub>2</sub>O-headspace

<sup>b</sup> As in a, but supplemented with FeS

**Table 3.** Growth of strain T in the presence of various electron acceptors. Strain T was grown on basal medium + 20 mM pyruvate in the presence of the indicated nitrogen species as sole electron acceptor. The preculture used to inoculate the test cultures contained nitrous oxide as sole electron acceptor

Electron acceptor	μ [h <sup>-1</sup> ]	OD <sub>546, max</sub>
Nitrate [10 mM]	0.26	0.8
N <sub>2</sub> O [100%]	0.20	1.4
N <sub>2</sub> O/N <sub>2</sub> [5/95, v/v]	0.28	1.6
N <sub>2</sub>	—	<0.2

considerably lower with nitrate than with nitrous oxide. This is probably due to nitrite toxicity.

A carbon mass balance for degradation of [<sup>14</sup>C]toluene in the presence of nitrous oxide as sole electron acceptor is presented in Table 4. Within a 2-week incubation, about 60% of the initially applied radioactivity was evolved as <sup>14</sup>CO<sub>2</sub>. Toluene degradation, however, was severely inhibited by FeS, which is in obvious contrast to the results listed in Table 1. This apparent contradiction is probably due to the fact that sulfide inhibits the nitrous oxide reductase but not the nitrate reductase (Sørensen et al. 1980), i.e., in the presence of sulfide, toluene oxidation cannot be coupled to nitrous oxide reduction. It is assumed that the nitrous oxide reductase requires copper as a cofactor (Knowles 1982) and this metal is probably not available in the presence of sulfide due to the low solubility produced of CuS ( $8.5 \times 10^{-45} \text{ mol}^2/\text{l}^2$ , Weast and Astle 1981).

**Acknowledgements.** We thank R. Schocher, M. Glaus and D. Macalady for stimulating discussions and D. Studer for providing the electron micrographs. This project was supported by grants from Ciba-Geigy AG and the European Communities (Project COST 641).

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