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Evidence for Acetyl Coenzyme A and Cinnamoyl Coenzyme A in the Anaerobic Toluene Mineralization Pathway in *Azoarcus tolulyticus* Tol-4

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A toluene-degrading denitrifier, *Azoarcus tolulyticus* Tol-4, was one of eight similar strains isolated from three petroleum-contaminated aquifer sediments. When the strain was grown anaerobically on toluene, 68% of the carbon from toluene was found as CO_2 and 30% was found as biomass. Strain Tol-4 had a doubling time of 4.3 h, a V_{max} of 50 μmol · min^{-1} · g of protein^{-1}, and a cellular yield of 49.6 g · mol of toluene^{-1}. Benzoate appeared to be an intermediate, since F-benzoates accumulated from F-toluenes and [^{14}C]benzoate was produced from [^{14}C]toluene in the presence of excess benzoate. Two metabolites, *E*-phenylitaconic acid (1 to 2%) and benzylsuccinic acid (<1%), accumulated from anaerobic toluene metabolism. These same products were also produced when cells were grown on hydrocinnamic acid and *trans*-cinnamic acid but were not produced from benzylalcohol, benzaldehyde, benzoate, *p*-cresol, or their hydroxylated analogs. The evidence supports an anaerobic toluene degradation pathway involving an initial acetyl coenzyme A (acetyl-CoA) attack in strain Tol-4, as proposed by Evans and coworkers (P. J. Evans, W. Ling, B. Goldschmidt, E. R. Ritter, and L. Y. Young, Appl. Environ. Microbiol. 58:496–501, 1992) for another toluene-degrading denitrifier, strain T1. Our findings support a modification of the proposed pathway in which cinnamoyl-CoA follows the oxidation of hydrocinnamoyl-CoA, analogous to the presumed oxidation of benzylsuccinic acid to form *E*-phenylitaconic acid. Cinnamic acid was detected in Tol-4 cultures growing in the presence of toluene and [^{14}C]acetate. We further propose a second acetyl-CoA addition to cinnamoyl-CoA as the source of benzylsuccinic acid and *E*-phenylitaconic acid. This pathway is supported by the finding that monofluoroacetate added to toluene-growing cultures resulted in a significant increase in production of benzylsuccinic acid and *E*-phenylitaconic acid and by the finding that [^{14}C]benzylsuccinic acid was detected after incubation of cells with toluene, [^{14}C]acetate, and cinnamic acid. Evidence for anaerobic toluene metabolism by methyl group oxidation was not found, since benzylsuccinic acid and *E*-phenylitaconic acid were not detected after incubation with benzylalcohol and benzaldehyde, nor were benzylalcohol and benzaldehyde detected even in ^{14}C trapping experiments.

Benzene, toluene, ethylbenzene, and xylenes, collectively known as BTEX compounds, are primary contaminants of concern in aquifer water and sediments where petroleum leakages and spills have occurred. The toxicities of these compounds range in severity from causing leukemias to causing minor dermal and central nervous system effects (10). Biological schemes for cleanup of these contaminants have been designed to optimize rates of degradation by providing adequate oxygen to the habitat. Oxygen serves as an electron acceptor and a cosubstrate in the metabolism of these compounds but is usually the limiting factor in aerobic treatment because of its low solubility in water and diffusional constraints in subsurface environments (27, 34, 41). Nitrate is an attractive alternative bacterial electron acceptor because of its high solubility in water, mobility in soil, and potential for rates of degradation comparable to those under aerobic conditions.

In recent years, a number of denitrifying toluene degraders have been isolated (1, 6, 11, 17, 20, 35); however, only a few have been extensively characterized. Furthermore, defining the anaerobic toluene degradation pathway and its biochemical features has proven to be a challenge, resulting in only partial characterization of the pathways in the better-studied strains (2, 7, 17, 19, 36). Most of the denitrifying toluene degraders can also catabolize toluene under aerobic conditions (17, 20, 35). Anaerobic degradation of toluene has also been observed under Fe(III)-reducing (30), methanogenic (13, 23, 24, 40), and sulfidogenic (4, 13) conditions, demonstrating a wide range of electron acceptors that might support the anaerobic biodegradation of BTEX compounds.

The pathways and mechanisms for anaerobic metabolism of aromatic compounds, including toluene, are of considerable interest, since this metabolism must be accomplished without the involvement of oxygenases. Two pathways have been suggested for anaerobic toluene metabolism on the basis of studies with pure cultures. One pathway involves methyl group hydroxylation (2, 36), and the other involves a coenzyme A (CoA) esterification reaction (16), as the postulated first step. Evidence obtained from a mixed methanogenic consortium also indicated toluene degradation via methyl hydroxylation (12). Other pathways that could conceivably occur include hydroxylation of the ring nucleus (39), ring reduction (22), demethylation followed by ring reduction or hydroxylation (18, 24), or carboxylation, similar to phenol metabolism (37, 38). No evidence for any of these last four mechanisms has been observed in pure culture studies. Of the group of BTEX compounds, toluene has been the focus of most studies, and elucidation of its pathway may lead to further understanding of the degradation of other nonoxygenated monoaromatic compounds.

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This paper describes anaerobic toluene metabolism by a new bacterium, strain Tol-4, which was isolated from petroleum-contaminated aquifer sediment obtained from northern Michigan. Besides Tol-4, seven other aquifer strains were isolated in this study. A phylogenetic analysis based on the 16S rRNA sequence and certain physiological characteristics identified strain Tol-4 as a member of a genus of free-living nitrogen fixers, *Azoarcus* (42). It has recently been shown in this laboratory that this strain is one of eight other toluene-degrading denitrifiers (20), isolated from a variety of different sources, that form a new species, *Azoarcus tolulyticus* (42). Strain Tol-4 is able to degrade toluene under anaerobic (denitrifying) conditions and has been proposed as the type strain for this group. In this paper, we detail its physiological characteristics specific to toluene metabolism, including the complete stoichiometry for toluene degradation under denitrifying conditions. We also provide evidence for important modifications in the acetyl-CoA pathway of anaerobic toluene metabolism, namely, the involvement of cinnamoyl-CoA and a second acetyl-CoA addition to form a minor accumulating product, identified as *E*-phenylitaconic acid (32).

MATERIALS AND METHODS

Aquifer sediment sampling and characterization. BTEX-contaminated aquifer sediments collected from three sites (Bear Lake, Wexford, and Kalkaska) in northern Michigan were used for enrichment of denitrifying toluene degraders. All sites contained petroleum from oil well production and processing operations. The sediments were collected from the saturated zone in intact cores (1.5 m by 5 cm) drilled by Hunter/Keck, Inc., Cadillac, Mich., using a hollow stem auger (inner diameter, 4.25 in. [ca. 10.8 cm]). The Kalkaska sediment was sand and gravel, while the other two were primarily sand. Cores were kept sealed at 4°C until use. Sediment (10 g) was dried at 105°C until a constant weight to determine dry weight. BTEX concentrations were measured by gas chromatography with 20 g of sediment (33). Nitrate concentrations were analyzed after extracting 10 g of sediment with 100 ml of 2 M KCl. Slurries were shaken at room temperature for 1 h and filtered through no. 42 Whatman filters, and the supernatant was analyzed for NO_3^- by high-pressure liquid chromatography (HPLC) as described below.

Enrichment and isolation. The procedure used for enrichment and isolation of toluene-degrading denitrifying strains in this study was that described by Fries et al. (20), with some modifications. The toluene concentration in the initial enrichments was 1,000 μM (92 ppm), and the isolates were grown and maintained at 540 μM (50 ppm). Anaerobic growth on toluene by isolate Tol-4 was verified by growth in basal salts (BS) medium plus NO_3^-, with 0.1 mM amorphous FeS added as a reductant and scavenger of oxygen (5). Cultures of Tol-4 were maintained either on anaerobic BS-NO_3^- medium containing toluene or on aerobic M-R2A agar (20).

Degradation of toluene and other substrates. Strain Tol-4 was grown anaerobically on 0.54 mM toluene in BS-NO_3^- medium for 48 h, centrifuged at 10,000 $\times g$ for 15 min, and washed twice with sterile BS. Approximately 10^6 cells per ml were added to 20 ml of anaerobic BS-NO_3^- medium plus 0.54 mM toluene in Balch tubes under an O_2-free N_2 atmosphere. Uninoculated controls and triplicate cultures were used. The optical density at 600 nm was measured with a spectrophotometer (Turner, Amsco Instrument Co.), and the concentrations of toluene, nitrate, and nitrite were measured. Aerobic growth on toluene was determined by using aerobically prepared BS-toluene medium (prepared in serum bottles containing 20 ml of air headspace). The rate of anaerobic toluene consumption was measured with dense stationary-phase cell cultures. Protein was measured colorimetrically after alkaline hydrolysis of cells by the Folin reaction (25). To determine the cell yield, 300-ml cultures of Tol-4 were anaerobically grown under toluene-limiting conditions in BS plus 5 mM nitrate in sealed 500-ml flasks until toluene was completely consumed. Dry weights were determined by filtering cells and then drying them at 105°C for 2 h before weighing.

Cells grown in [U-^{14}C]toluene (specific activity, 10.2 mCi/mmol; >98%; Sigma) diluted in cold toluene in 150 ml of BS-NO_3^- medium were used to determine a carbon balance. [^{14}C]toluene was added neat (approximately 0.001 μCi/ml) 24 h prior to inoculation. Subsamples of 1 to 5 ml were removed and treated according to one of the following protocols: (i) whole sample, pH 12; (ii) unfiltered sample, N_2 purged; (iii) unfiltered sample, pH 12, N_2 purged; (iv) unfiltered sample, pH 2, N_2 purged; (v) filtered sample; (vi) filtered sample, N_2 purged; (vii) filtered sample, pH 12, N_2 purged; and (viii) filtered sample, pH 2, N_2 purged. Adjustments in pH were made by addition of either 10 N NaOH (pH 12) or 10 N HCl (pH 2), and filtered samples were collected after filtration through 0.45-μm-pore-size filters. Samples which were purged were done so under a steady stream of N_2 gas for 10 min. The radioactivity in each subsample was measured by scintillation counting (model 1500 Tri-Carb Liquid Scintillation Analyzer; Packard Instrument Co.). Radioactivity associated with cells was measured by using cells trapped on 0.45-μm-pore-size filters and included both purged and unpurged filters. Separate cultures of cells were grown anaerobically on toluene under nitrate-limiting conditions to determine nitrogen and electron balances. Toluene, nitrate, nitrite, nitrous oxide, and dinitrogen gas were measured. All experiments were done in triplicate.

Aromatic substrates for testing utilization by Tol-4 under anaerobic conditions were added at a final concentration of 0.5 mM to 40 ml of cells pregrown on toluene (72 h). Substrate concentrations were monitored periodically for up to 2 weeks. The degradation of substrates was compared with that of controls, which consisted of uninoculated BS-NO_3^- medium along with the corresponding compound. Toluene; ethylbenzene; benzene (>99.9%; Aldrich); *o*-, *m*-, and *p*-xylenes (98 to >99.9%; Aldrich); *o*-, *m*-, and *p*-cresols (99.9%; Aldrich) were added neat. Phenol; catechol; resorcinol; 4-methylcatechol; benzylalcohol (99%; Sigma); benzaldehyde; benzoate; *o*-, *m*-, and *p*-hydroxybenzylalcohols; *o*-, *m*-, and *p*-hydroxybenzaldehydes; and *o*-, *m*-, and *p*-hydroxybenzoates (Sigma) were added from 20 mM stock solutions to obtain a 0.5 mM final concentration. Hydrocinnamic acid, *trans*-cinnamic acid (Sigma), benzoate, and benzyl succinate were prepared in dilute NaOH as 10 mM stocks and added to the same final concentration. *E*-Phenylitaconic acid was synthesized and characterized as described previously (32).

Analog and substrate inhibition studies. Cultures of Tol-4 (150 ml) were pregrown anaerobically on 80 μmol of toluene and respiked with 80 μmol of toluene more plus 15 μmol of either *o*-fluorotoluene, *m*-fluorotoluene, or *p*-fluorotoluene (Sigma). Similar cultures but with the addition of benzylalcohol, benzaldehyde, or benzoate instead of the fluorinated toluenes were prepared. Nitrate was added as needed. Additional toluene and other substrates were added when depletion occurred. Subsamples were removed at regular intervals to analyze by gas and liquid chromatography for substrate disappearance and metabolites. Retention times of peaks were compared with those of authentic compounds.

Tol-4 cells were pregrown anaerobically on toluene as described above before the addition of 10, 100, or 1,000 μM monofluoroacetate (MFA). Subsamples of 1 ml were removed periodically for analysis by HPLC. Each MFA concentration was incubated as triplicate cultures. Control cultures included ones inoculated with cells with toluene added alone and a sterile control containing toluene and 1,000 μM MFA.

Isotope trapping studies using ^{14}C-labeled compounds. Dense anaerobic toluene-grown cultures (150 ml) were spiked with [*methyl*-^{14}C]toluene (500 μM, 3.3 nCi/ml) and 100 μM benzaldehyde or benzoate. Samples were removed at intervals for scintillation counting and also acidified (to pH 2 with HCl) and filtered (0.45-μm pore size) for HPLC analysis. Samples were analyzed immediately to minimize loss due to chemical oxidation. Cultures (100 ml) were also prepared by using cold toluene and [^{14}C]carboxy-acetate (10 μM, 10 nCi/ml) both with and without 100 μM *trans*-cinnamic acid. Samples were removed at intervals from these cultures for scintillation counting and for organic extraction and concentration before HPLC analysis.

Chemical analyses. Toluene and fluorinated toluenes were analyzed by gas chromatography with a flame ionization detector (Varian model 3700). Headspace samples (50 μl) were injected onto a DB-624 column (30 m by 0.543 mm; J & W Scientific), using a helium carrier (1 kg/cm^2). Toluene analysis was made with 90°C column, 200°C injector, and 200°C detector temperatures. Fluorinated toluenes were analyzed similarly but with a 50°C column temperature. Quantitation was based on comparisons with aqueous standards.

To prepare samples for metabolite analysis, culture fluids were centrifuged at 10,000 $\times g$ for 20 min. Cells were discarded, and the supernatant was acidified to pH 1 with H_3PO_4. The samples were solvent extracted three times with 25 ml of diethyl ether. Extracts were pooled and dried with $NaSO_4$, and the solvent was evaporated under a stream of carbon-filtered argon. The residue was dissolved in methanol-water (1:1) and filtered through 0.45-μm-pore-size filters.

Other aromatic substrates and soluble metabolites were separated and analyzed by HPLC with a UV detector (Hewlett-Packard series 1050) and a LiChrosorb RP-18 (10-μm) Hibar RT column (250 by 4 mm) (EM Separations, Gibbstown, N.J.). Culture fluids were directly filtered through 0.45-μm-pore-size filters into vials with Teflon-lined caps. Samples of 40 to 100 μl were analyzed. The solvent system was 0.1% H_3PO_4–methanol (60:40) at a flow rate of 1.5 ml/min. Wavelengths used were 218, 230, and 270 nm. Authentic standards were prepared in aqueous solutions. Retention times (in minutes) are as follows: toluene, 30.0; benzoate, 6.5; benzylalcohol, 4.3; benzaldehyde, 7.2; hydrocinnamic acid, 9.4; *trans*-cinnamic acid, 12.7; *E*-phenylitaconic acid, 9.5; and DL-benzylsuccinic acid, 7.5. ^{14}C-labeled compounds were detected by using a radiotracer detector (IN/US Systems, Inc.) along with the HPLC system for organic compounds described above.

Nitrate and nitrite concentrations were measured at 210 nm by HPLC with a Partisil 10 SAX column (Whatman) and a mobile phase of 50 mM phosphate (pH 3.0). Samples were filtered (0.45-μm pore size) and diluted 100-fold. Nitrous oxide was measured in a gas chromatograph equipped with a ^{63}Ni electron capture detector (Perkin-Elmer 910 gas chromatograph; 95% argon–5% methane carrier; flow rate, 15 ml $\cdot$ min^{-1}). Headspace samples of 0.5 ml were injected onto a stainless steel Porapak Q column (1.8 m by 0.32 cm), and 55°C column,

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TABLE 1. Aquifer sediments used for enrichments and denitrifying toluene-degrading isolates obtained from each sediment

Source[a]	Depth (m)	Description	Initial NO_3^- concn (ppm [dry wt])	Isolate(s)
Bear Lake	24–25	Fine- to medium-grain sand; 5% moisture	0.47	Tol-4, BL-2, BL-3, BL-4, BL-11
Wexford	8.5–10	Fine- to medium-grain sand with small gravel; 10% moisture	0.23	2a-1
Kalkaska	2.4–4	Sand and gravel, silt; 11.5% moisture	0.34	3a-1, 7a-1

[a] BTEX compounds were detected in all samples but were present at less than 10 ppb (sediment basis) each.

60°C injector, and 300°C detector temperatures were used. N_2 gas was measured with a gas chromatograph (Carle Gas Chromatograph, Porapak Q column, argon carrier) equipped with a thermal conductivity detector.

RESULTS

Enrichment and isolation of strains. Benzene, toluene, ethylbenzene, and *o*-, *m*-, and *p*-xylenes were detected in all three aquifer sediments, but the concentrations were very low (<10 ppb). Nitrate was also detected in the three sediments (Table 1). Eight strains of denitrifying toluene degraders were isolated from the three sediments (Table 1). The eight strains were differentiated by colony and cell morphologies on M-R2A. All strains were confirmed to grow anaerobically on toluene by observing turbidity increases and toluene consumption in BS-NO_3^- medium plus toluene under a headspace of oxygen-free argon. Strain Tol-4 was selected for further characterization since it had the highest rate of anaerobic toluene degradation among the eight strains. In addition, strain Tol-4 grew aerobically on toluene.

To verify the ability of strain Tol-4 to grow and degrade toluene under strict anaerobiosis with NO_3^- as the electron acceptor, amorphous FeS was added as a reductant to anaerobically prepared BS-NO_3^- medium plus toluene. The presence of the reductant and incubation of the tubes in an anaerobic chamber eliminated any possibility of oxygen in the cultures. Separate cultures grown in the absence of reductant showed that anaerobic consumption of toluene was directly dependent on *N*-oxides, since toluene removal ceased completely when NO_3^-, NO_2^-, and N_2O were depleted and resumed only when more NO_3^- was added (Fig. 1). Dinitrogen gas was the final N-containing product of denitrification. Strain Tol-4 was also able to use NO_2^- and N_2O as sole electron acceptors for its anaerobic growth on toluene, but growth was poor compared with growth with NO_3^- as the electron acceptor (data not shown).

Characteristics of growth on toluene. Additional characteristics of anaerobic growth on toluene, including a stoichiometric balance, were determined for Tol-4. Concentrations of toluene above 0.54 mM (50 ppm) were inhibitory. The maximum cell density under anaerobic conditions as measured by optical density (600 nm) was reached at approximately 45 h, and toluene reached a nondetectable concentration by 40 h (Fig. 2). Strain Tol-4 reduced nitrate to nitrite first; a nearly stoichiometric accumulation of nitrite often occurred under toluene-limiting conditions. The doubling time was 4.3 h.

Strain Tol-4 degraded 50 μmol of toluene per min per g of protein when grown anaerobically on toluene. On the basis of the actual cell yield (49.6 g per mol of toluene) obtained from anaerobic growth on toluene, and assuming half reactions for cell formation and substrate oxidation of $C_5H_7O_2N$ (standard cell formula [26]) + $8H_2O \rightarrow 5CO_2 + NH_3 + 20H^+ + 20e^-$, $C_7H_8 + 14H_2O \rightarrow 7CO_2 + 36H^+ + 36e^-$, and $2NO_3^- + 12H^+ + 10e^- \rightarrow N_2 + 6H_2O$, 24.4% of the electrons from toluene would have been used for biomass synthesis, or the

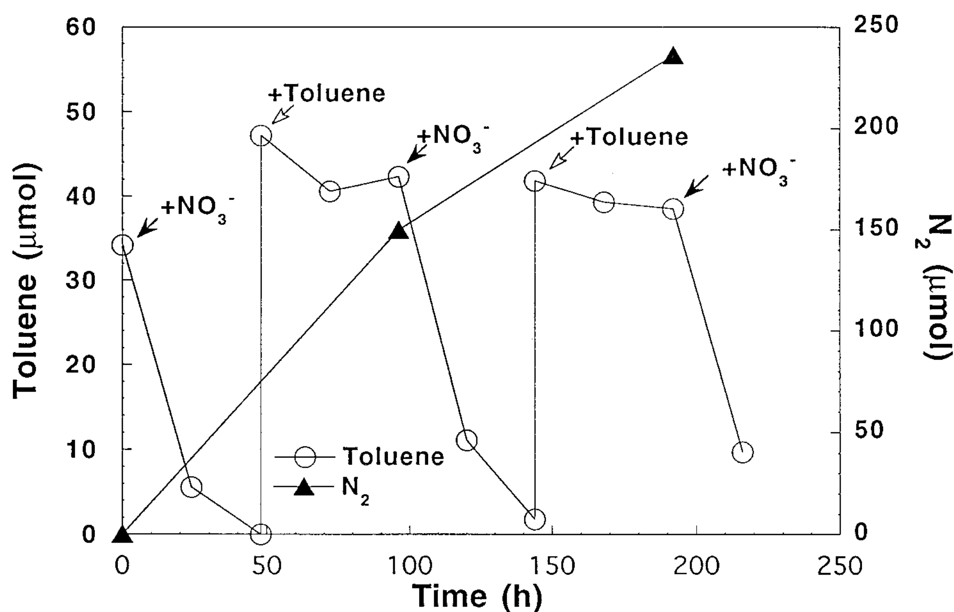

FIG. 1. Nitrate-dependent anaerobic toluene degradation in strain Tol-4 and corresponding N_2 production. Arrows indicate addition of NO_3^- or toluene. NO_3^-, NO_2^-, and N_2O were not detected at times when additional NO_3^- was added.

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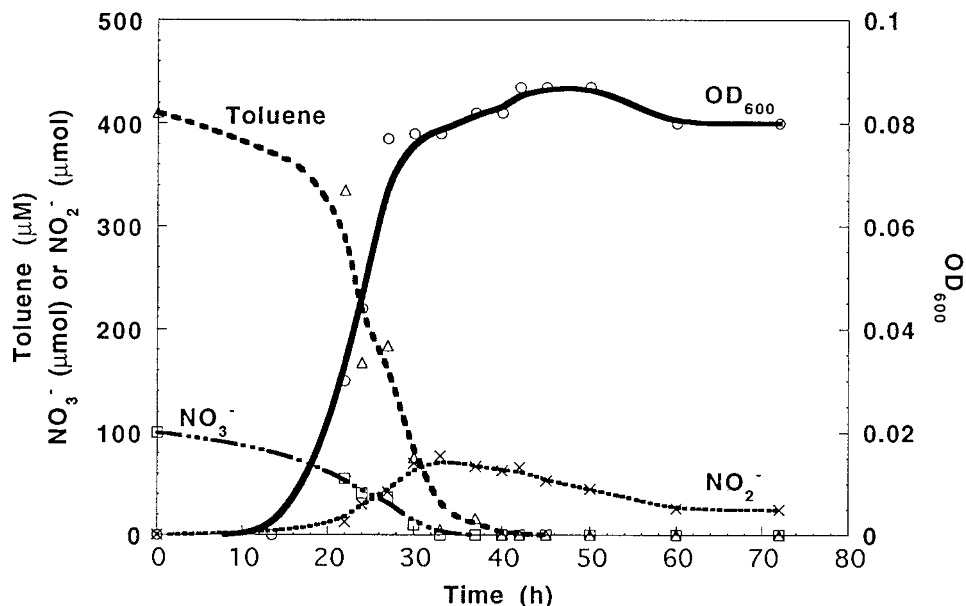

FIG. 2. Pattern of growth of strain Tol-4, toluene consumption, and depletion of nitrate. The doubling time was calculated to be 4.3 h. OD_{600}, optical density at 600 nm.

fraction of electrons used for biomass synthesis (f_s) equals 0.244 (8, 31). The following stoichiometry is obtained: C_7H_8 + 5.43 NO_3^- + 0.44NH_3 + 5.43H^+ → 4.78CO_2 + 2.73N_2 + 5.79H_2O + 0.44$C_5H_7O_2N$.

The stoichiometry was supported by the nitrogen and electron balances obtained when cells were grown anaerobically on toluene under NO_3^--limiting conditions (Table 2). The total N recovered as denitrification products was 96%. The N unaccounted for was probably a small amount of N_2. On the basis of the total amount of electrons from toluene and the electrons accounted for in electron acceptors, the fraction of the electrons transferred to electron acceptors (f_e) is 0.791. The sum of the two experimentally obtained values, f_e (0.791) and f_s (0.244), is 1.035, which matched closely the theoretical sum $f_e + f_s = 1.000$.

Cells grown anaerobically on [^{14}C]toluene demonstrated that carbon from toluene was mineralized to CO_2 and incorporated into biomass (Table 3). The remaining 2.3% of label that was unaccounted for may be present as nonvolatile, soluble metabolites but in amounts too small to quantify. The percent carbon mineralized was 68%, which matched precisely the value predicted from the stoichiometry given above. The percent carbon incorporated into cells was 29%, which also correlated well with the 31% predicted from the same stoichiometric balance. This stoichiometry also confirms that oxygen is not involved in the reaction.

Other aromatic substrate use. Several other aromatic compounds were used by strain Tol-4 as growth substrates under the same conditions used for anaerobic toluene degradation (Table 4). Benzene; ethylbenzene; *o*-, *m*-, and *p*-xylenes; *o*- and *m*-cresols; phenol; resorcinol; and *o*-hydroxybenzoate were not used by inocula pregrown on toluene. Catechol and *p*-cresol were consumed after a lag period of 24 h. Hydrocinnamic acid; *trans*-cinnamic acid; benzylalcohol; benzaldehyde; benzoate; *o*-hydroxybenzylalcohol and -benzaldehyde; *m*-hydroxybenzylalcohol, -benzaldehyde, and -benzoate; and *p*-hydroxybenzylalcohol, -benzaldehyde, and -benzoate were all degraded immediately. Some of these substrates were metabolized more slowly, as they were not completely removed in 2 weeks. Benzylsuccinic acid was not metabolized at the 500 μM concentration tested.

Metabolite production and substrate inhibition of toluene degradation. When cells were grown on toluene alone, no transient metabolites were detected. However, a small amount of an accumulating metabolite consistently appeared in culture fluids when cells were grown anaerobically on toluene. This compound was identified as *E*-phenylitaconic acid (32) and was not detected during aerobic growth on toluene. Culture fluids

TABLE 2. Nitrogen and electron balances for strain Tol-4 grown anaerobically on toluene under nitrate-limiting conditions

Substrate	Product	Amt used or produced (μmol)	Amt of electrons transferred (μmol)
Toluene		42	1,512
NO_3^-		554	
	NO_2^-	470	940
	N_2O	32	256
	N_2	ND[a]	
Total N and electrons recovered		534	1,196

[a] ND, not detected.

TABLE 3. Carbon-14 balance for strain Tol-4 grown anaerobically under toluene-limiting conditions

Location of ^{14}C label	Value	
	dpm (10^3)	%
Initial toluene	360 ± 2.2	100
Remaining toluene	2.55 ± 0.032	0.7
CO_2	243 ± 1.20	67.6
Cells	106 ± 1.45	29.4
Total label recovered	352	97.7

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TABLE 4. Substrate use, as measured by HPLC, under anaerobic denitrifying conditions with a toluene-grown inoculum of strain Tol-4

Substrate[a]	% Loss (time)[b]
Toluene	>99 (72 h)
p-Cresol[c]	>99 (2 wk)
Catechol[c]	37 (2 wk)
Benzylalcohol	>99 (72 h)
Benzaldehyde	>99 (72 h)
Benzoate	>99 (72 h)
o-OH-benzylalcohol	91 (1 wk)
o-OH-benzaldehyde	>99 (2 wk)
m-OH-benzylalcohol	76 (2 wk)
m-OH-benzaldehyde	>99 (1 wk)
m-OH-benzoate	98 (2 wk)
p-OH-benzylalcohol	>99 (2 wk)
p-OH-benzaldehyde	99 (2 wk)
p-OH-benzoate	34 (2 wk)
Hydrocinnamic acid	>99 (5 days)
trans-Cinnamic acid	>99 (5 days)

[a] Substrates were added at a final concentration of 0.5 mM. Benzene, ethylbenzene, *o*-xylene, *m*-xylene, *p*-xylene, *o*-cresol, *m*-cresol, phenol, resorcinol, and *o*-OH-benzoate were not degraded.

[b] Percent losses are maximum values at the time indicated and after subtraction for abiotic losses as determined with the uninoculated controls.

[c] Depletion began after a 24-h lag.

were analyzed for metabolites after growth on a variety of possible intermediates and substrate analogs, some in combination with toluene (Table 5). When cells metabolized benzylalcohol, benzaldehyde, and benzoate in the absence of toluene, no detectable metabolites, including *E*-phenylitaconic acid, were observed. Either benzylalcohol or benzaldehyde added to cultures growing on toluene altered the rate of toluene degradation (Fig. 3). The addition of benzaldehyde in the presence of toluene resulted in the transient appearance of a small amount of benzoate (<20 μM), and toluene metabolism was slowed. The presence of benzylalcohol completely inhibited toluene metabolism, and the metabolism of benzylalcohol was also stopped. In isotope trapping experiments, a small amount of [^{14}C]benzoate (7 μM) appeared transiently after 30 min when cold benzoate was added with [^{14}C]toluene (Table 6). [^{14}C]benzoate was not detected in cultures containing labeled toluene and cold benzaldehyde. ^{14}C-labeled benzylalcohol and benzaldehyde were not detected at any time in these cultures.

Cultures grown on either hydrocinnamic acid or *trans*-cinnamic acid produced *E*-phenylitaconic acid and benzylsuccinic acid (Table 5). In isotope trapping experiments using toluene and [^{14}C]acetate, a small transient peak tentatively identified by HPLC as cinnamic acid (0.1 μM) appeared after 10 min (Table 6). Cultures which contained toluene, cinnamic acid, and [^{14}C]acetate produced [^{14}C]benzylsuccinic acid after 30 min.

o- and *p*-cresols were metabolized in the presence of toluene, with some *o*- and *p*-hydroxybenzoate, respectively, detected. The latter compound accumulated (Table 5). *o*-Hydroxybenzoate was not metabolized when added alone to cultures, but this compound was only transient when cells were grown on *o*-cresol in the presence of toluene. *E*-Phenylitaconic acid was detected only when toluene was metabolized. *m*-Cresol inhibited toluene degradation and was not metabolized in the presence of toluene. Growth on hydrocinnamic acid and *trans*-cinnamic acid resulted in production of *E*-phenylitaconic acid plus a second metabolite that we identified as benzylsuccinate on the basis of it having the HPLC retention time and UV absorption characteristics of the authentic standard.

Fluorinated toluenes were used as structural analogs to toluene. In the presence of toluene and *o*-, *m*-, or *p*-fluorotoluene, *o*-, *m*-, and *p*-fluorobenzoate, respectively, accumulated (Table 5). Only in cultures containing *o*-fluorotoluene did a product accumulate along with the usual (nonfluorinated) *E*-phenylitaconic acid. This product was presumed to be an analogous fluorinated *E*-phenylitaconic acid because its retention time and UV absorption ratios at wavelengths of 218, 230, and 275 nm were similar to those of *E*-phenylitaconic acid. The amount of product was too small to confirm this structure by other methods.

To test the effect of tricarboxylic acid cycle intermediates on anaerobic toluene metabolism, MFA was added to cultures growing on toluene. MFA readily condenses with oxaloacetate to form fluorocitrate, a specific and potent inhibitor of aconitase, which is near the entry point of the tricarboxylic acid cycle (3). In cultures containing 10, 100, and 1,000 μM concentrations of MFA, toluene metabolism was slowed (Fig. 4a). The larger amounts of benzylsuccinic acid and *E*-phenylitaconic acid that accumulated correlated with higher MFA concentrations (Fig. 4b and c).

TABLE 5. Metabolites detected during anaerobic growth on toluene or with other substrates in the presence or absence of toluene

Substrate(s)[a]	Metabolites detected[b]	Time of detection (h)
Toluene	Benzylsuccinic acid	48
	E-Phenylitaconic acid	48
Benzoate	None	
Hydrocinnamic acid	Benzylsuccinic acid	96
	E-Phenylitaconic acid	96
trans-Cinnamic acid	Benzylsuccinate	96
	E-Phenylitaconic acid	96
DL-Benzylsuccinate	None	
Benzylalcohol	None	
Toluene + benzylalcohol	None	
Benzaldehyde	None	
Toluene + benzaldehyde	Benzoate	3 (transient)
	E-Phenylitaconic acid	48
o-Cresol	None	
Toluene + *o*-cresol	*E*-Phenylitaconic acid	48
	o-Hydroxybenzoate	48
m-Cresol	None	
Toluene + *m*-cresol	None	
p-Cresol	None	
Toluene + *p*-cresol	*E*-Phenylitaconic acid	48
	p-Hydroxybenzoate	48
Toluene + *o*-F-toluene	*E*-Phenylitaconic acid	24
	o-F-Benzoate	24
	o-F-Phenylitaconic acid[c]	24
Toluene + *m*-F-toluene	*E*-Phenylitaconic acid	24
	m-F-Benzoate	24
Toluene + *p*-F-toluene	*E*-Phenylitaconic acid	24
	p-F-Benzoate	24

[a] All compounds were tested at 500 μM, with the exception of the fluorinated toluenes, which were tested at 100 μM. The inoculum was grown anaerobically on toluene before testing.

[b] Metabolites were identified by finding HPLC retention times the same as those of authentic standards. Benzylsuccinate was found in concentrations ranging from 2.4 to 3.0 μM, and *E*-phenylitaconic was found in concentrations ranging from 3.8 to 6.0 μM at the times indicated. Benzoate compounds were not quantified.

[c] Identity based on similarities to *E*-phenylitaconic acid in accumulation and retention time.

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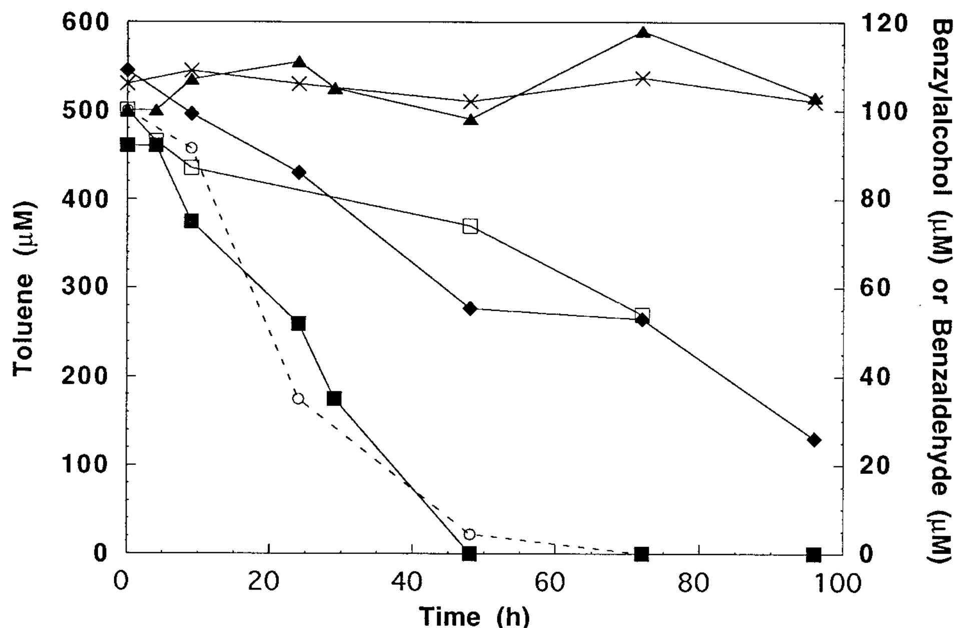

FIG. 3. Benzylalcohol consumption in the presence of toluene (▲) and its effect on toluene degradation (×), benzaldehyde consumption in the presence of toluene (■) and its effect on toluene degradation (◆) under anaerobic conditions, benzylalcohol degradation in the absence of toluene (□), and toluene degradation in a control culture containing toluene alone (○).

DISCUSSION

Denitrifiers capable of anaerobic toluene metabolism may be common in BTEX-exposed aquifers, since they were isolated from all three sites studied. All sources used in our enrichments were primarily sand taken from 2- to 25-m depths and were low in organic matter content and microbial density, yet all yielded denitrifying toluene degraders. Five of eight isolates, including Tol-4, were enriched from the deepest sediment core drilled. We have isolated other denitrifying toluene degraders, but they have been isolated from a variety of surface environments which were more carbon rich and supported a larger microbial population (20). Finding these organisms in a variety of surface and subsurface environments suggests that these organisms and this catabolic process must be widespread.

The absence of oxygen involvement in anaerobic toluene degradation was confirmed by growth of Tol-4 in FeS-reduced BS-NO_3^- medium and routine incubation of cultures in an anaerobic chamber. Our experiments have consistently shown that toluene degradation proceeded only when *N*-oxides were present as electron acceptors.

Toluene was completely mineralized to CO_2 and converted into biomass by strain Tol-4. The carbon, electron, and nitrogen balance predicts well the actual amount of carbon mineralized and cells produced from toluene degradation under denitrifying conditions. In the ^{14}C-labeling study, 98% of the label could be recovered as CO_2 and cell material. By difference, the 2% of label that was unaccounted for was thought to include the water-soluble metabolite *E*-phenylitaconic acid. Direct analysis showed that strain Tol-4 converted between 1 and 2% of the toluene carbon to *E*-phenylitaconic acid (32). Benzylsuccinic acid accumulated in even smaller amounts than *E*-phenylitaconic acid; it was detected in growth medium after several feedings of toluene and concentration of solvent extracts of large volumes of culture fluid. In contrast, strain T1, another toluene-degrading denitrifier, was reported to convert 17% of the carbon from toluene to benzylsuccinic acid and benzylfumaric acid (16, 19). The difference between our strains and strain T1 in the quantities of these dioic acids produced may be due to differences in growth conditions or factors affecting the flux of intermediates leading to the formation of the accumulating products.

The accumulation of benzylsuccinic acid and benzylfumaric acid from toluene by strain T1 was the key evidence which led Evans and coworkers to propose an anaerobic toluene pathway involving an initial acetyl-CoA attack on toluene to form hydrocinnamoyl-CoA (also known as phenylpropionyl-CoA) as the first intermediate (16). They further proposed that an analogous reaction between toluene and succinyl-CoA would be followed by hydrolysis of the CoA ester to form benzylsuccinic acid, which could then be further oxidized to the dead-end metabolite benzylfumaric acid. The identification of *E*-phenylitaconic acid as the accumulating metabolite produced by strain Tol-4 during anaerobic toluene metabolism (32) and the position of the double bond in this metabolite relative to the position of this double bond in benzylfumaric acid lead to several possible modifications to the previously proposed pathway for toluene mineralization. The production of benzylsuccinic acid along with *E*-phenylitaconic acid by strain Tol-4 reasonably suggests an oxidation reaction whereby benzylsuccinic acid (Fig. 5, structure III) is directly oxidized to form

TABLE 6. Metabolites produced by cells from ^{14}C-isotope trapping

Substrates[a]	Metabolite	Amt (μM)
[^{14}C]toluene + benzaldehyde	None	
[^{14}C]toluene + benzoate	[^{14}C]benzoate	7
Toluene + [^{14}C]acetate	Cinnamic acid	0.1
Toluene + [^{14}C]acetate + cinnamic acid	[^{14}C]benzylsuccinic acid	ND[b]

[a] The concentrations of substrates used were as follows: toluene, 500 μM; benzaldehyde, 100 μM; benzoate, 100 μM; acetate, 10 μM; and cinnamic acid, 100 μM.

[b] ND, not determined.

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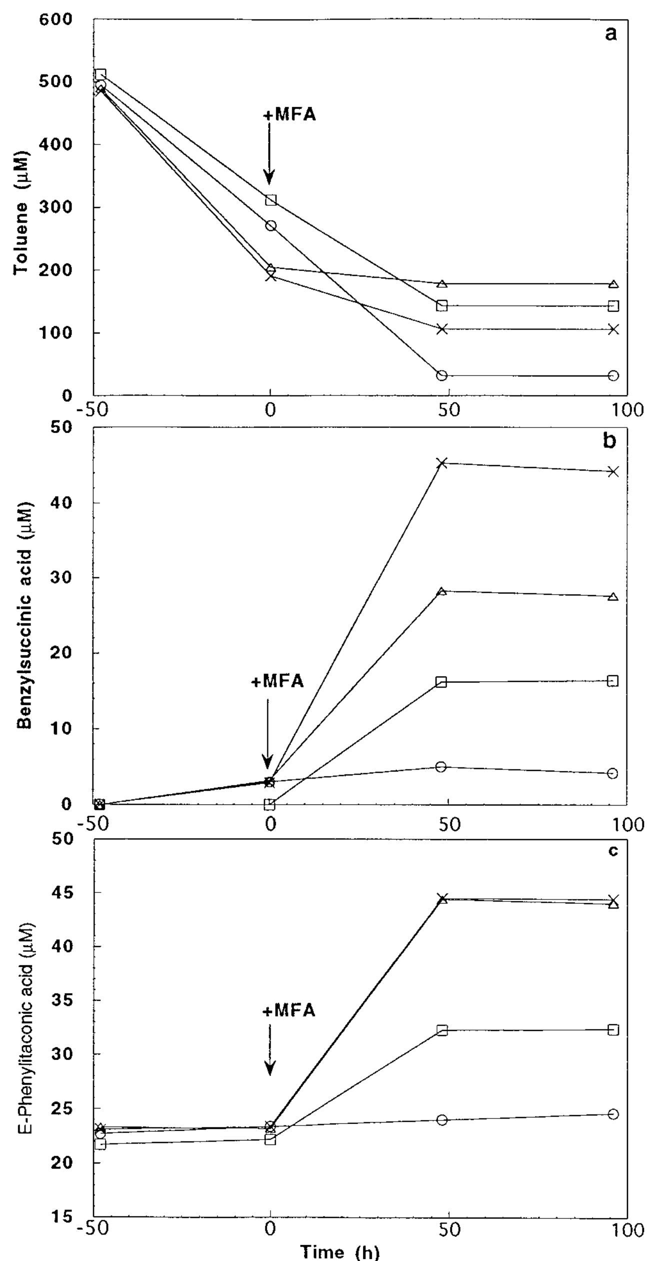

FIG. 4. Effects of 10 μM (□), 100 μM (△), and 1,000 μM (×) MFA on anaerobic toluene-growing cultures. ○, toluene only. (a) toluene consumption; (b) benzylsuccinate production; (c) *E*-phenylitaconic acid production.

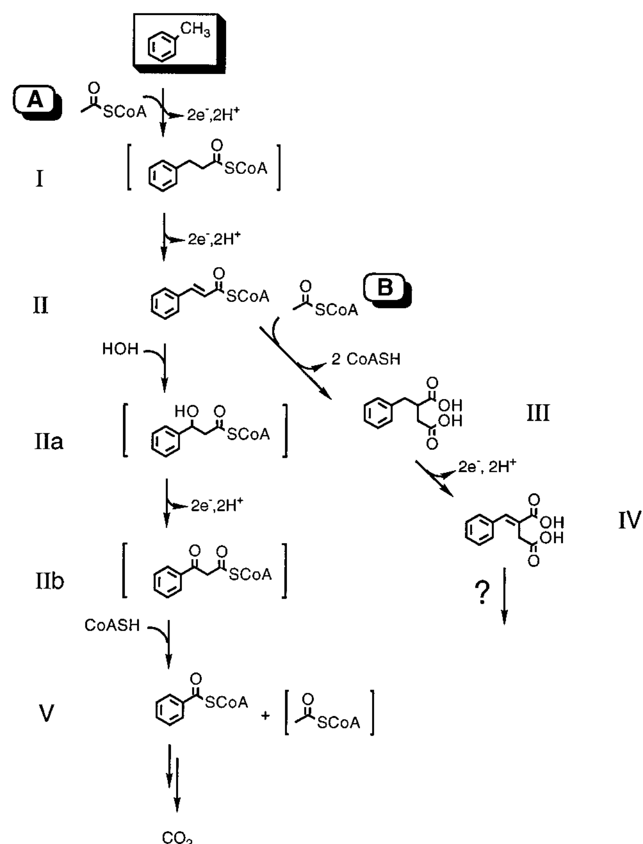

FIG. 5. Proposed mineralization pathway (A) and formation of *E*-phenylitaconic acid (B) from the anaerobic degradation of toluene by strain Tol-4. I, hydrocinnamoyl-CoA; II, cinnamoyl-CoA; IIa, β-hydroxycinnamoyl-CoA; IIb, β-ketocinnamoyl-CoA; III, benzylsuccinic acid; IV, *E*-phenylitaconic acid; V, benzoyl-CoA. Brackets indicate hypothetical intermediates. Cinnamic acid and benzoic acid, rather than the corresponding CoA thioesters, were detected.

E-phenylitaconic acid (Fig. 5, structure IV). Strain Tol-4 did not appear to metabolize benzylsuccinic acid; however, this may be due to the absence of transport systems to take up the compound or to the lack of a ligase to form the corresponding CoA ester.

The oxidation step occurring between benzylsuccinic acid and *E*-phenylitaconic acid could provide a lead on how the two-carbon analog hydrocinnamic acid is metabolized. The same dehydrogenase mechanism, and even perhaps the same enzyme, could oxidize hydrocinnamoyl-CoA (Fig. 5, structure I) to cinnamoyl-CoA (Fig. 5, structure II). To evaluate our pathway hypothesis, we fed *trans*-cinnamic acid as well as hydrocinnamic acid to Tol-4. The cells grew on both substrates and produced both benzylsuccinic acid and *E*-phenylitaconic acid from both substrates, consistent with the hypothesis. The larger amount of benzylsuccinic acid detected, compared with that formed from toluene, might be due to a larger pool of both proposed reactants, acetyl-CoA and cinnamic acid (Fig. 5, path B), when hydrocinnamic acid and cinnamic acid were used than would be the case when toluene is the substrate (Fig. 5, path A). Further evidence to suggest the involvement of cinnamic acid (or its CoA ester) was its detection in cultures incubated with toluene and [^{14}C]acetate. Also consistent with the hypothesis of a branch pathway forming *E*-phenylitaconic acid was the detection of [^{14}C]benzylsuccinic acid when cells were incubated with toluene, [^{14}C]acetate, and *trans*-cinnamic acid.

Cultures grown in the presence of [^{14}C]toluene and benzoate resulted in the transient appearance of [^{14}C]benzoate. Labeled benzoate was not detected when benzaldehyde was added with [^{14}C]toluene in parallel studies. The evidence for benzoate as an intermediate was further supported by the production of F-benzoates from F-toluenes and hydroxylated benzoates from cresol metabolism. The appearance of benzoate may still be consistent with our proposed pathway, since it is not yet clear whether benzoate is formed directly as an intermediate in the pathway prior to CoA esterification or whether it is formed in equilibrium with its CoA ester during toluene metabolism. A small amount of benzoic acid was re-

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ported to be excreted transiently during *trans*-cinnamic acid metabolism by a photosynthetic bacterium, *Rhodopseudomonas palustris* (15). This excretion and subsequent uptake were suggested to be linked to cell regulation and the photometabolism of aromatic acids in these cells, which involve aromatic CoA ligase activities (14). Similar CoA ligase reactions may also occur in Tol-4 as part of the mechanism for its aromatic acid degradation. The small amounts of benzoate and cinnamic acid detected in Tol-4 studies may suggest a similar regulatory mechanism.

Altenschmidt and Fuchs proposed that strain K172 degraded toluene anaerobically via methyl group oxidation to form benzylalcohol followed by oxidation to benzaldehyde (2). Seyfried and coworkers supported this scheme by detecting benzaldehyde and benzoate and, in addition, reported the appearance of benzylsuccinic acid and benzylfumaric acid from anaerobic toluene metabolism by strain K172 and another bacterium, strain T (36). The amount of benzylsuccinic acid and benzylfumaric acid produced by strains K172 and T was reported to be 0.5%. Our studies showed that benzylalcohol and benzaldehyde were used as substrates by Tol-4; however, no detectable metabolites, including *E*-phenylitaconic acid and benzylsuccinic acid, were ever seen. In fact, benzylalcohol was not metabolized in the presence of toluene and, furthermore, inhibited toluene metabolism in Tol-4. In addition, incubating [^{14}C]toluene with benzaldehyde did not produce any labeled intermediates, including benzylalcohol and benzaldehyde. These results and the absence of benzylsuccinic acid and *E*-phenylitaconic acid as products of benzylalcohol or benzaldehyde degradation do not lend support to a methyl hydroxylation pathway for toluene metabolism in strain Tol-4. However, we cannot completely rule out reactions involving methyl group oxidation because of the difficulty of making negative conclusions based on the use of exogenous substrates, especially when toxicity might have been involved, such as appeared to be the case with benzylalcohol.

Succinyl-CoA was proposed to be the cosubstrate with toluene in the formation of benzylsuccinic acid in strain T1 (16). The addition of MFA to strain T1 resulted in the inhibition of both toluene utilization and formation of benzylsuccinic acid and benzylfumaric acid (19). These results were consistent with a hypothesis of an MFA-induced decline in succinyl-CoA availability. We found that MFA, when added to Tol-4 cultures, resulted in the inhibition of toluene degradation but a stimulation in benzylsuccinic acid and *E*-phenylitaconic acid production. The increased production of benzylsuccinic acid and *E*-phenylitaconic acid may be due to the increased availability of acetyl-CoA to act as a substrate in both pathways A and B, since its use in the tricarboxylic acid cycle was blocked (Fig. 5). This is in contrast to what might be expected if succinyl-CoA was key to the formation of the accumulating metabolites. A reaction between monofluoroacetyl-CoA and either toluene or cinnamic acid could proceed through steps leading to the formation of both fluorinated benzylsuccinyl-CoA and *E*-phenylitaconyl-CoA, both of which may be blocked from further reactions. Our analysis would be unable to distinguish between the combined fluorinated and nonfluorinated analogs of benzylsuccinic acid and *E*-phenylitaconic acid if the fluorinated analogs were indeed produced. We also did not observe an accumulation of benzoate in the presence of MFA as reported for strain T1.

Our results also argue against other hypothesized anaerobic toluene degradation pathways. The mechanism involving hydroxylation of the ring nucleus to form cresols is unlikely, since simultaneous adaptation and trapping studies using cresols and toluene-induced cells either did not show use of the compound, showed a lag phase before onset of degradation, or failed to produce transient or accumulating products. These results suggested that ring hydroxylation reactions with toluene were doubtful as first-step reactions. Carboxylation of the ring, similar to the *para*-carboxylation mechanism in phenol degradation, is also not likely, since Tol-4 does not catabolize phenol nor was toluene degradation stimulated when CO_2 was provided in a bicarbonate-carbonate-buffered system (data not shown). Since benzene was not detected as a transient metabolite and was not utilized for growth, it is also unlikely that demethylation of toluene occurred.

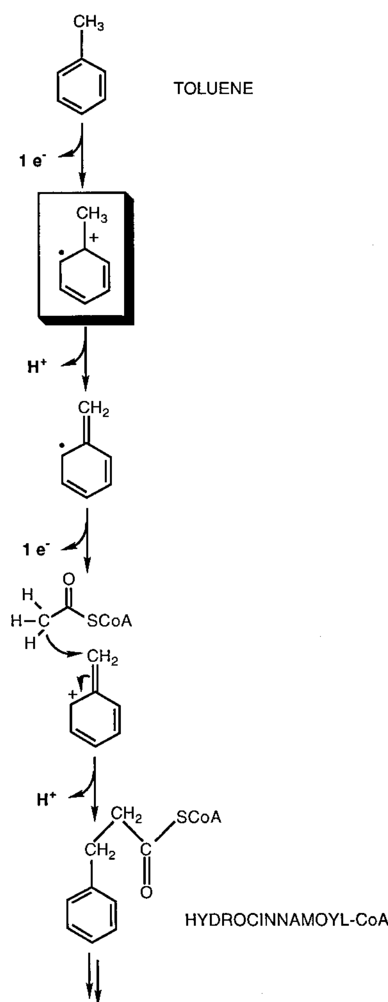

FIG. 6. Proposed mechanism of aryl cation (boxed structure) formation represented by the first step of the reaction between toluene and acetyl-CoA to form hydrocinnamoyl-CoA.

The results obtained from our studies suggest that the pathway illustrated in Fig. 5 is a reasonable one. In addition, this pathway gains further support on the basis of its chemical feasibility. One chemical mechanism suggested for toluene oxidation under anaerobic conditions involves the generation of an aryl cation radical (22, 28). This single electron transfer reaction is then followed by reaction with a nucleophile, as in the first step of the proposed pathway involving acetyl-CoA (Fig. 6). Cation radicals could also, in theory, be generated during the oxidation of hydrocinnamoyl-CoA to form cinnamoyl-CoA, as well as in the analogous oxidation of benzylsuccinyl-CoA to form *E*-phenylitaconyl-CoA. Rather than

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speculate on the exact nature of these enzyme-mediated reactions, we arbitrarily assign such oxidations as involving the release of two electrons and two protons from a metabolite. Further oxidation of cinnamoyl-CoA (Fig. 5, structure II) may follow a mechanism analogous to fatty acid β-oxidation, which was suggested by Evans and coworkers to be involved in the further mineralization of hydrocinnamoyl-CoA (phenylpropionyl-CoA) (16). 1,4-Addition of water to cinnamoyl-CoA would form β-hydroxycinnamoyl-CoA (Fig. 5, structure IIa). Oxidation of the alcohol and reaction of the resulting β-ketocinnamoyl-CoA (Fig. 5, structure IIb) with CoASH would generate benzoyl-CoA and acetyl-CoA. Studies performed with *R. palustris* suggested an analogous β-oxidation mechanism in its metabolism of hydrocinnamic acid and *trans*-cinnamic acid (15). The intermediates formed in anaerobic toluene degradation prior to ring reduction steps may also be central in the metabolism of other aromatic compounds. Such channelling strategies may be important in anaerobic metabolism of aromatic compounds, notably in pathways leading to benzoyl-CoA (1, 9, 21, 29).

Our proposed pathway (Fig. 5) places the branch point leading to the formation of the dioic acid products at cinnamoyl-CoA (structure II). An alternative branch point at hydrocinnamoyl-CoA could also be chemically feasible; however, this was ruled out, in large part because of resulting products that would be isomeric to but chemically distinct from benzylsuccinic acid and *E*-phenylitaconic acid. Additionally, since significant percentages of toluene are apparently converted to dioic acid by-products by a variety of anaerobic toluene degraders, attributing the formation of these compounds to dead-end metabolism may be premature. Benzylsuccinic acid and *E*-phenylitaconic acid, in the form of their CoA esters, may actually occupy key positions along the main pathway for anaerobic toluene mineralization. Such a pathway would be essentially similar to the one we propose for toluene mineralization except that the CoA adduct generated along with benzoyl-CoA from further oxidation of *E*-phenylitaconic acid (or its CoA ester) would be succinyl-CoA rather than acetyl-CoA.

The challenge presented to microorganisms with compounds like toluene is in the ability of the organisms to mediate reactions that begin with destabilizing the highly conjugated, stable aromatic structure in order to facilitate further catabolism and a potential gain in energy. Coenzymes are widely known to provide the requisite chemical reactivities in many enzyme-mediated reactions. A reaction between acetyl-CoA and toluene in the first step of mineralization would be one way to activate the structure for further oxidation while possibly precluding the cell from having to use a high-energy phosphate bond (e.g., that in ATP hydrolysis). The chemical mechanism in Fig. 6 shows the release of two electrons in the steps leading to the formation of hydrocinnamoyl-CoA, which may potentially be used in energy-gaining reactions by the cell. The release of one molecule of acetyl-CoA in the oxidation step leading to benzoyl-CoA formation (Fig. 5) would also allow the cells to recycle this substrate for further toluene catabolism. The nature of the first enzyme involved in an addition of acetyl-CoA is intriguing but yet unknown, and there is not yet direct evidence that hydrocinnamoyl-CoA is produced from this reaction. Also intriguing is the possibility that benzylsuccinic acid and *E*-phenylitaconic acid, seemingly common products among the anaerobic toluene degraders, play major roles as intermediates in toluene mineralization.

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REFERENCES

1. **Altenschmidt, U., and G. Fuchs.** 1991. Anaerobic degradation of toluene in denitrifying *Pseudomonas* sp.: indication for toluene methylhydroxylation and benzoyl-CoA as central aromatic intermediate. Arch. Microbiol. **156:** 152–158.
2. **Altenschmidt, U., and G. Fuchs.** 1992. Anaerobic toluene oxidation to benzylalcohol and benzaldehyde in a denitrifying *Pseudomonas* strain. J. Bacteriol. **174:**4860–4862.
3. **Becker, W. M.** 1983. Aerobic production of ATP: the TCA cycle, p. 323–358. *In* G. Zubay (ed.), Biochemistry. Addison-Wesley Publishing Co., Inc., Reading, Mass.
4. **Beller, H. R., D. Grbic-Galic, and M. Reinhard.** 1992. Microbial degradation of toluene under sulfate-reducing conditions and the influence of iron on the process. Appl. Environ. Microbiol. **58:**786–793.
5. **Brock, T. D., and K. O'Dea.** 1977. Amorphous ferrous sulfide as a reducing agent for culture of anaerobes. Appl. Environ. Microbiol. **33:**254–256.
6. **Chee-Sanford, J. C., M. Fries, and J. M. Tiedje.** 1992. Anaerobic degradation of toluene under denitrifying conditions in bacterial isolate Tol-4, p. 374. *In* Abstracts of the 92nd General Meeting of the American Society for Microbiology 1992. American Society for Microbiology, Washington, D.C.
7. **Chee-Sanford, J. C., and J. M. Tiedje.** 1994. Detection and identification of metabolites produced by the denitrifying isolate *Azoarcus* sp. (Tol-4), p. 447. *In* Abstracts of the 94th General Meeting of the American Society for Microbiology 1994. American Society for Microbiology, Washington, D.C.
8. **Criddle, C. S., L. M. Alvarez, and P. L. McCarty.** 1991. Microbial processes in porous media, p. 641–691. *In* J. Bear and M. Y. Corapcioglu (ed.), Transport processes in porous media. Kluwer Academic Publishers, Dordrecht, The Netherlands.
9. **Dangel, W., R. Brackman, A. Lack, M. Mohamed, J. Koch, B. Oswald, B. Seyfried, A. Tschech, and G. Fuchs.** 1991. Differential expression of enzyme activities initiating anoxic metabolism of various aromatic compounds via benzoyl-CoA. Arch. Microbiol. **155:**256–262.
10. **Dean, B. J.** 1985. Recent findings on the genetic toxicology of benzene, toluene, xylenes and phenols. Mutat. Res. **154:**153–181.
11. **Dolfing, J., J. Zeyer, P. Binder-Eicher, and R. P. Schwarzenbach.** 1990. Isolation and characterization of a bacterium that mineralizes toluene in the absence of molecular oxygen. Arch. Microbiol. **154:**336–341.
12. **Edwards, E. A., A. M. Edwards, and D. Grbic-Galic.** 1994. A method for detection of aromatic metabolites at very low concentrations: application to detection of metabolites of anaerobic toluene degradation. Appl. Environ. Microbiol. **60:**323–327.
13. **Edwards, E. A., and D. Grbic-Galic.** 1994. Anaerobic degradation of toluene and *o*-xylene by a methanogenic consortium. Appl. Environ. Microbiol. **60:** 313–322.
14. **Elder, D. J. E., and D. J. Kelly.** 1994. The bacterial degradation of benzoic acid and benzenoid compounds under anaerobic conditions: unifying trends and new perspectives. FEMS Microbiol. Rev. **13:**441–468.
15. **Elder, D. J. E., P. Morgan, and D. J. Kelly.** 1992. Anaerobic degradation of *trans*-cinnamate and ω-phenylalkanecarboxylic acids by the photosynthetic bacterium *Rhodopseudomonas palustris*: evidence for a β-oxidation mechanism. Arch. Microbiol. **157:**148–154.
16. **Evans, P. J., W. Ling, B. Goldschmidt, E. R. Ritter, and L. Y. Young.** 1992. Metabolites formed during anaerobic transformation of toluene and *o*-xylene and their proposed relationship to the initial steps of toluene mineralization. Appl. Environ. Microbiol. **58:**496–501.
17. **Evans, P. J., D. T. Mang, K. S. Kim, and L. Y. Young.** 1991. Anaerobic degradation of toluene by a denitrifying bacterium. Appl. Environ. Microbiol. **57:**1139–1145.
18. **Evans, W. C., and G. Fuchs.** 1988. Anaerobic degradation of aromatic compounds. Annu. Rev. Microbiol. **42:**289–317.
19. **Frazer, A. C., W. Ling, and L. Y. Young.** 1993. Substrate induction and metabolite accumulation during anaerobic toluene utilization by the denitrifying strain T1. Appl. Environ. Microbiol. **59:**3157–3160.
20. **Fries, M. R., J.-Z. Zhou, J. C. Chee-Sanford, and J. M. Tiedje.** 1994. Isolation, characterization, and distribution of denitrifying toluene degraders from a variety of habitats. Appl. Environ. Microbiol. **60:**2802–2810.
21. **Fuchs, G., M. E. S. Mohamed, U. Altenschmidt, J. Koch, A. Lack, R. Brackmann, C. Lochmeyer, and B. Oswald.** 1994. Biochemistry of anaerobic biodegradation of aromatic compounds, p. 513–553. *In* C. Ratledge (ed.), Biochemistry of microbial degradation. Kluwer Academic Publishers, Dordrecht, The Netherlands.
22. **Grbic-Galic, D.** 1990. Anaerobic microbial transformation of nonoxygenated aromatic and alicyclic compounds in soil, subsurface, and freshwater sedi-

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ments, p. 117–189. *In* J. M. Bollag and G. Stotzky (ed.), Soil biochemistry, vol. 6. Marcel Dekker, Inc., New York.
23. **Grbic-Galic, D.** 1990. Methanogenic transformation of aromatic hydrocarbons and phenols in groundwater aquifers. Geomicrobiol. J. **8:**167–200.
24. **Grbic-Galic, D., and T. M. Vogel.** 1987. Transformation of toluene and benzene by mixed methanogenic cultures. Appl. Environ. Microbiol. **53:**254–260.
25. **Hanson, R. S., and J. A. Phillips.** 1981. Chemical composition, p. 328–364. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
26. **Hoover, R., and N. Porges.** 1952. Assimilation of dairy wastes by activated sludge. Sew. Ind. Waste **24:**306–312.
27. **Hutchins, S. R., W. C. Downs, J. T. Wilson, G. B. Smith, D. A. Kovacs, D. D. Fine, R. H. Douglass, and D. J. Hendrix.** 1991. Effect of nitrate addition on biorestoration of fuel-contaminated aquifer: field demonstration. Groundwater **29:**571–580.
28. **Kochi, J. K., R. T. Tang, and T. B. Bernath.** 1973. Mechanisms of aromatic substitution. Role of cation-radicals in the oxidative substitution of arenes by cobalt(III). J. Am. Chem. Soc. **95:**7114–7123.
29. **Lochmeyer, C., J. Koch, and G. Fuchs.** 1992. Anaerobic degradation of 2-aminobenzoic acid (anthranilic acid) via benzoyl-coenzyme A (CoA) and cyclohex-1-enecarboxyl-CoA in a denitrifying bacterium. J. Bacteriol. **174:** 3621–3628.
30. **Lovley, D. R., and D. J. Lonergan.** 1990. Anaerobic oxidation of toluene, phenol, and *p*-cresol by the dissimilatory iron-reducing organism, GS-15. Appl. Environ. Microbiol. **56:**1858–1864.
31. **McCarty, P. L.** 1975. Stoichiometry of biological reactions. Prog. Water Technol. **7:**157–172.
32. **Migaud, M. E., J. C. Chee-Sanford, J. M. Tiedje, and J. W. Frost.** 1996. Benzylfumaric, benzylmaleic, and *Z*- and *E*-phenylitaconic acids: synthesis, characterization, and correlation with a metabolite generated by *Azoarcus tolulyticus* Tol-4 during anaerobic toluene degradation. Appl. Environ. Microbiol. **62:**974–978.
33. **Roe, V. D., M. J. Lacy, and J. D. Stuart.** 1989. Manual headspace method to analyze for the volatile aromatics of gasoline in groundwater and soil samples. Anal. Chem. **61:**2584–2585.
34. **Schink, B.** 1988. Principles and limits of anaerobic degradation: environmental and technological aspects, p. 771–846. *In* A. J. B. Zehnder (ed.), Biology of anaerobic microorganisms. John Wiley & Sons, New York.
35. **Schocher, R. J., B. Seyfried, F. Vazquez, and J. Zeyer.** 1991. Anaerobic degradation of toluene by pure cultures of denitrifying bacteria. Arch. Microbiol. **157:**7–12.
36. **Seyfried, B., G. Glod, R. Schocher, A. Tschech, and J. Zeyer.** 1994. Initial reactions in the anaerobic oxidation of toluene and *m*-xylene by denitrifying bacteria. Appl. Environ. Microbiol. **60:**4047–4052.
37. **Tschech, A., and G. Fuchs.** 1987. Anaerobic degradation of phenol by pure cultures of newly isolated denitrifying pseudomonads. Arch. Microbiol. **148:** 213–217.
38. **Tschech, A., and G. Fuchs.** 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.
39. **Vogel, T. M., and D. Grbic-Galic.** 1986. Incorporation of oxygen from water into toluene and benzene during anaerobic fermentative transformation. Appl. Environ. Microbiol. **52:**200–202.
40. **Wilson, B. H., G. B. Smith, and J. F. Rees.** 1986. Biotransformations of selected alkylbenzenes and halogenated aliphatic hydrocarbons in methanogenic aquifer material: a microcosm study. Environ. Sci. Technol. **20:**997–1002.
41. **Zehnder, A. J. B. and W. Stumm.** 1988. Geochemistry and biogeochemistry of anaerobic habitats, p. 1–38. *In* A. J. B. Zehnder (ed.), Biology of anaerobic microorganisms. John Wiley & Sons, New York.
42. **Zhou, J.-Z., M. R. Fries, J. C. Chee-Sanford, and J. M. Tiedje.** 1995. Phylogenetic analyses of a new group of denitrifiers capable of anaerobic growth on toluene: description of *Azoarcus tolulyticus* sp. nov. Int. J. Syst. Bacteriol. **45:**500–506.

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