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Metabolic By-Products of Anaerobic Toluene Degradation by Sulfate-Reducing Enrichment Cultures

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Two dead-end metabolites of anaerobic toluene transformation, benzylsuccinic acid and benzylfumaric acid, accumulated in sulfate-reducing enrichment cultures that were fed toluene as the sole carbon source. Stable isotope-labeled toluene and gas chromatography-mass spectrometry were used to confirm that the compounds resulted from toluene metabolism. The two metabolites constituted less than 10% of the toluene carbon (over 80% was mineralized to carbon dioxide, according to a previous study). This study demonstrates that the novel nonproductive pathway 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 a denitrifying pure culture applies to disparate anaerobic bacteria.

Over the past 5 years, anaerobic degradation of toluene and other alkylbenzenes has been an active area of research. The ability of bacterial pure cultures or consortia to degrade toluene has been demonstrated under a range of electron-accepting conditions (including nitrate-reducing, ferric iron-reducing, sulfate-reducing, and fermentative-methanogenic conditions) and for a variety of inoculum sources. However, efforts to elucidate metabolic pathways of anaerobic toluene degradation have met with very limited success to date; for sulfate-reducing bacteria in particular, no metabolite studies are available. The most direct evidence of anaerobic toluene metabolites has consisted of mass spectrometric identification of transient intermediates in culture medium (for fermentative-methanogenic conditions [5, 9]), enzyme assays (1), and various methods of inducing metabolite accumulation, some of which have resulted in the detection of toluene-derived benzoic acid (6, 8). The two most frequently postulated metabolic pathways, both of which include benzoic acid as an intermediate, proceed either via initial hydroxylation of the aromatic ring to form *p*-cresol or via initial hydroxylation of the methyl group to form benzyl alcohol (e.g., see references 1, 5, and 6 to 9). In a recent paper, Evans and coworkers proposed novel pathways of toluene transformation and mineralization for a denitrifying pure culture that grew on toluene as a sole carbon source (3). In that paper, a nonproductive transformation pathway was described in which a nucleophilic attack of the benzylic (i.e., methyl) carbon of toluene by succinyl coenzyme A (succinyl-CoA) ultimately resulted in two dead-end metabolites, benzylsuccinic acid and benzylfumaric acid (Fig. 1). The identity of these accumulating metabolites was supported by mass spectrometry (MS). An analogous but productive pathway was proposed in which acetyl-CoA, rather than succinyl-CoA, attacked the benzylic carbon of toluene, resulting in β-phenylpropionyl-CoA, followed by benzoyl-CoA (via β-oxidation) and, ultimately, mineralization. No evidence was given for the productive pathway (other than detection of the end product, CO_2).

In this paper, we report that toluene-degrading, sulfate-reducing enrichment cultures produced the same two dead-end metabolites that Evans et al. observed (3), indicating that the novel, nonproductive pathway is not restricted to the pure culture described by Evans and coworkers, or even to denitrifiers in general, and could be carried out by disparate anaerobic bacteria. We also report that benzoic acid is a likely intermediate of productive toluene degradation, which is consistent with findings for denitrifying (1, 6, 8) and fermentative-methanogenic (5) conditions.

The enrichment cultures used in this study, which were derived from contaminated subsurface soil from an aviation fuel storage facility in Maryland, are described elsewhere (2). As described previously (2), the cultures were maintained under strictly anaerobic conditions in amber glass, 250-ml screw-cap bottles sealed with Mininert valves (Alltech Associates, Inc., Deerfield, Ill.); the combined volume of medium and culture inoculum was 200 ml. Metabolic studies were performed with primary or secondary enrichments spiked with neat unlabeled toluene (>99.9% purity; Aldrich Chemical Co., Inc., Milwaukee, Wis.) or, in most cases, with neat stable isotope-labeled toluene ([*methyl*-^{13}C]-toluene [MSD Isotopes, Pointe Claire-Dorval, Quebec, Canada] or toluene-d_8 [Aldrich]). The isotopic purity of the deuterium- and ^{13}C-labeled toluene was ≥99%; none of the labeled metabolites reported in this study were present as impurities in toluene. Samples of culture (20 or 40 ml) were acidified to pH ≤2 with solvent-cleaned HCl and extracted three times in a separatory funnel with diethyl ether (high purity, distilled in glass; J. T. Baker, Inc., Phillipsburg, N.J.). The extracts were dried with anhydrous sodium sulfate, derivatized with ethereal diazomethane (4), concentrated under a gentle stream of high-purity nitrogen at room temperature, exchanged into dichloromethane (high purity, distilled in glass; J. T. Baker), and analyzed by gas chromatography (GC)-MS. GC-MS analyses were performed with an HP model 5890A GC (Hewlett-Packard Co., Palo Alto, Calif.) with a DB-5 fused silica capillary column (60-m length, 0.32-mm inner diameter, 1-μm film thickness; J & W Scientific, Folsom, Calif.) coupled to an HP 5970 series mass selective detector with an HP 59970C ChemStation used for data analysis. For some samples, GC-MS analyses were conducted both before and after derivatization to confirm that methyl esters found in derivatized extracts were originally present as free carboxylic acids. A DL-benzylsuccinic

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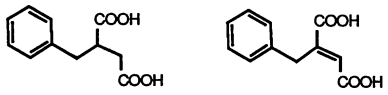

I II

FIG. 1. Structures of benzylsuccinic acid (I) and benzylfumaric acid (II).

acid standard (Sigma Chemical Co., St. Louis, Mo.) was derivatized and used as a GC-MS standard. A standard for benzylfumaric acid was not available. Analyses of aqueous toluene concentrations were performed by a static headspace technique involving GC-photoionization detection, as described elsewhere (2).

Numerous preliminary analyses for metabolites all yielded benzylsuccinic acid (and, after derivatization, dimethyl benzylsuccinate) peaks that predominated the total ion chromatograms of culture extracts. An experiment was performed to quantitatively assess benzylsuccinic acid accumulation. Two secondary enrichment cultures were prepared by using 15% inocula from a primary enrichment culture that had received only unlabeled toluene; one of the secondary enrichment cultures was fed [*methyl*-^{13}C]toluene, and the other was fed toluene-d_8 (C_7D_8). At the beginning of this experiment, no unlabeled toluene was present, but approximately 13 μM unlabeled benzylsuccinic acid was present as a result of transfer from the inoculum. Throughout the experiment, isotope-labeled benzylsuccinic acid was generated from labeled toluene, as illustrated by the mass spectra of benzylsuccinic acid that had accumulated (Fig. 2). Figure 2 displays mass spectra of the benzylsuccinic acid standard, unlabeled benzylsuccinic acid (which derived from unlabeled toluene and was present in the inoculum), ^{13}C-labeled benzylsuccinic acid (which accumulated in the [*methyl*-^{13}C]toluene enrichment culture), and benzylsuccinic acid-d_8 and -d_9 (which accumulated in the toluene-d_8 enrichment culture); all spectra were acquired from derivatized extracts and represent dimethyl esters. The d_8 analog of dimethyl benzylsuccinate (molecular weight, 244; Fig. 2D) was the most abundant deuterated product, although a coeluting d_9 analog was also present. The presence of more than seven deuterium atoms on benzylsuccinic acid indicates that the attacking succinyl group contained deuterium and therefore derived from deuterated toluene (the sole carbon source). If the attacking succinyl group were not deuterated, benzylsuccinic acid-d_7 would result, because toluene-d_8 must lose a deuterium atom to form a new carbon-carbon bond. In GC-MS analyses, deuterated dimethyl benzylsuccinate was partially resolved from the unlabeled analog, but the ^{13}C-labeled and unlabeled analogs coeluted, resulting in composite spectra (Fig. 2C). From Fig. 2C, it appears probable that two or more ^{13}C-labeled analogs of dimethyl benzylsuccinate coeluted and are represented in the mass spectrum; the most abundant of these analogs contained one ^{13}C atom (in the benzylic position), and the less abundant analog(s) contained two ^{13}C atoms (one in the benzylic position and one in the succinyl group, which derived from toluene).

Although a benzylfumaric acid standard was not available, labeled metabolites accumulated that had mass spectral fragmentation patterns analogous to the spectrum presented by Evans et al. (3) as dimethyl benzylfumarate (data not shown). The (presumed) dimethyl benzylfumarate peak found in this study was consistently much smaller than the dimethyl benzylsuccinate peak (the former compound had less than 10% of the peak area of the latter based on GC-MS total ion chromatograms and on GC-flame ionization detection chromatograms); thus, this paper focuses on the accumulation of benzylsuccinic acid rather than benzylfumaric acid.

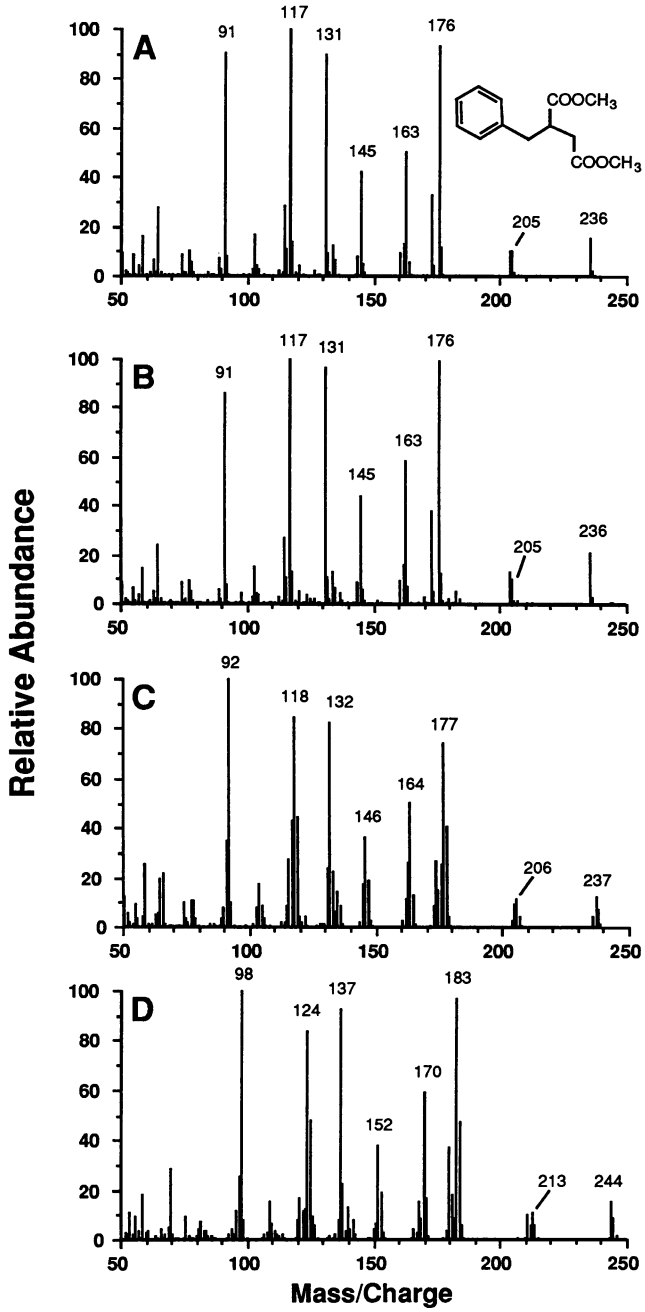

FIG. 2. Mass spectra of the dimethyl esters of (A) DL-benzylsuccinic acid, (B) the predominant metabolite present in an enrichment culture that was fed unlabeled toluene, (C) the predominant metabolite (an isotopic mixture) present in an enrichment culture that was fed [*methyl*-^{13}C]toluene, and (D) the predominant metabolite (an isotopic mixture) present in an enrichment culture that was fed toluene-d_8. The retention times of the metabolites were in close agreement with that of the standard (A).

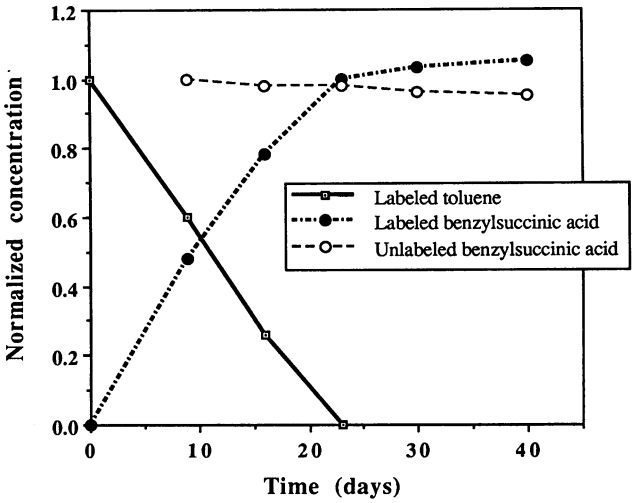

FIG. 3. Normalized plot of [*methyl*-^{13}C]toluene disappearance, ^{13}C-labeled benzylsuccinic acid accumulation, and unlabeled benzylsuccinic acid concentration in a secondary enrichment. Toluene concentration is normalized to the total consumed (approximately 700 μM over three feedings), labeled benzylsuccinic acid is normalized to the amount present when toluene was depleted (day 23), and unlabeled benzylsuccinic acid is normalized to the amount originally transferred with the inoculum. Relative concentrations of toluene, labeled benzylsuccinic acid, and unlabeled benzylsuccinic acid cannot be determined from this figure; however, the relationship between the first two compounds is discussed in the text.

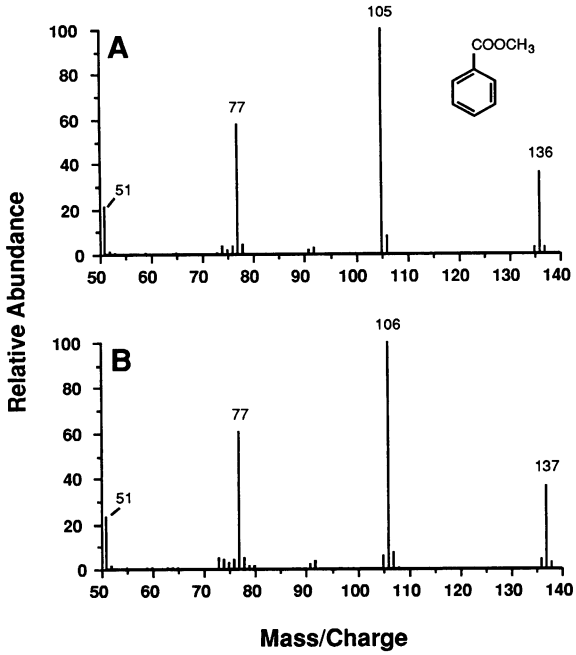

FIG. 4. Mass spectra of (A) a methyl benzoate standard and (B) a methylated transient intermediate present in an enrichment culture that was fed [*methyl*-^{13}C]toluene. The intermediate (B) is assumed to be the methyl ester of [*carboxy*-^{13}C]benzoic acid.

The accumulation of benzylsuccinic acid is illustrated in Fig. 3 for the [*methyl*-^{13}C]toluene enrichment; the toluene-d_8 enrichment yielded similar results. Labeled benzylsuccinic acid accumulated in close correspondence with toluene disappearance (Fig. 3). When toluene was depleted, the labeled benzylsuccinic acid concentration remained constant. As further evidence that benzylsuccinic acid was not degraded in these cultures, the concentration of unlabeled benzylsuccinic acid that was originally transferred with the inoculum also remained constant (Fig. 3). The enrichment cultures remained active through day 40, as confirmed by subsequent feedings with toluene.

The yield of benzylsuccinic acid formed as a percentage of the moles of toluene consumed was estimated to be approximately 4.3%, based on linear regression fits of benzylsuccinic acid accumulation versus cumulative toluene consumption ($r^2 > 0.94$). On a carbon basis, this indicates that approximately 6.7% of the carbon of toluene (C_7H_8) was converted to benzylsuccinic acid ($C_{11}H_{12}O_4$), since toluene was the sole carbon source in these experiments. Accounting for the analytical recovery of benzylsuccinic acid determined in spiking tests (>90%), it can be estimated that approximately 7 to 8% of toluene carbon was converted to benzylsuccinic acid. Previous studies with [*ring*-^{14}C]toluene (2) revealed that these sulfate-reducing enrichment cultures mineralize 83.6% of toluene carbon and convert 11.4% to nonvolatile compounds (either nonvolatile intermediates or biomass). Thus, of the 11.4% nonvolatile carbon generated from toluene, benzylsuccinic and benzylfumaric acids may account for approximately 7 to 9%, with the remainder presumably contributing to biomass. In comparison, Evans et al. (3) estimated that 10 to 17% of the toluene carbon in their denitrifying pure culture was converted to benzylfumaric and benzylsuccinic acids, 29% was converted to biomass, and 51% was mineralized to carbon dioxide.

Although the enrichment culture in this study and the pure culture in the study of Evans et al. (3) both utilized nonproductive pathways involving benzylsuccinic and benzylfumaric acids, there appears to be a notable distinction between these pathways. Evans et al. (3) reported considerably more benzylfumaric acid than benzylsuccinic acid (with a benzylfumaric acid/benzylsuccinic acid ratio of 2.3 to 4.7), whereas this ratio in the present study was 1 to 2 orders of magnitude lower (<0.07, based on peak areas of GC-MS total ion chromatograms). If Evans et al. (3) were correct in asserting that benzylsuccinic acid precedes benzylfumaric acid in the metabolic pathway, then the sulfate-reducing enrichment cultures in this study appear to perform a much slower (and possibly less complete) dehydrogenation of benzylsuccinic acid to benzylfumaric acid.

To date, metabolite studies in our laboratory with sulfate-reducing enrichment cultures have yielded limited information on intermediates of the productive pathway for toluene. One trial with [*methyl*-^{13}C]toluene yielded [*carboxy*-^{13}C]benzoic acid (identified as the free acid before derivatization and as the methyl ester after derivatization). The mass spectra of the methylated metabolite and an unlabeled methyl benzoate standard are shown in Fig. 4. The metabolite agreed well with the unlabeled standard in terms of retention time and mass spectral fragmentation (the ^{13}C label is apparent in the *m/z* 106 and 137 ions of the metabolite in Fig. 4). The metabolite constituted on the order of 2 mol% of the [*methyl*-^{13}C]toluene that had been degraded in the enrichment culture. Unlike benzylsuccinic acid, benzoic acid was a transient intermediate; it was barely detectable in a later extraction of the same enrichment culture and was very seldom detected in other metabolite studies in our laboratory (e.g., benzoic acid was not detected in the study depicted in Fig. 3). As further evidence that benzoic acid is a transient intermediate of toluene degradation, no lag period was

observed when toluene-degrading enrichment cultures were fed benzoic acid as a sole carbon source (data not shown).

The occurrence of benzoic acid as a transient intermediate yields virtually no information on the productive pathway of toluene degradation in these sulfate-reducing enrichment cultures, as several postulated pathways converge on benzoic acid or benzoyl-CoA as an intermediate (e.g., initial hydroxylation of the aromatic ring, initial hydroxylation of the methyl group, or initial attack of the methyl group by acetyl-CoA [e.g., see references 1, 3, 5, and 7]). Further attempts will be made to identify the initial metabolic step in the mineralization of toluene.

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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. **Beller, H. R., D. Grbić-Galić, 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.
3. **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.
4. **Fales, H. M., T. M. Jaouni, and J. F. Babashak.** 1973. Simple device for preparing ethereal diazomethane without resorting to codistillation. Anal. Chem. **45:**2302–2303.
5. **Grbić-Galić, D., and T. M. Vogel.** 1987. Transformation of toluene and benzene by mixed methanogenic cultures. Appl. Environ. Microbiol. **53:**254–260.
6. **Kuhn, E. P., J. Zeyer, P. Eicher, and R. P. Schwarzenbach.** 1988. Anaerobic degradation of alkylated benzenes in denitrifying laboratory aquifer columns. Appl. Environ. Microbiol. **54:**490–496.
7. **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.
8. **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.
9. **Vogel, T. M., and D. Grbić-Galić.** 1986. Incorporation of oxygen from water into toluene and benzene during anaerobic fermentative transformation. Appl. Environ. Microbiol. **52:**200–202.