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# Benzylsuccinate Formation as a Means of Anaerobic Toluene Activation by Sulfate-Reducing Strain PRTOL1

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**Permeabilized cells of toluene-mineralizing, sulfate-reducing strain PRTOL1 catalyzed the addition of toluene to fumarate to form benzylsuccinate under anaerobic conditions. Recent in vitro studies with two toluene-mineralizing, denitrifying bacteria demonstrated the same fumarate addition reaction and indicated that it may be the first step of anaerobic toluene degradation. This study with strain PRTOL1 shows that anaerobic toluene activation by fumarate addition occurs in bacteria as disparate as sulfate-reducing and denitrifying species (members of the delta and beta subclasses of the *Proteobacteria*, respectively).**

Recent research on anaerobic toluene degradation has resulted in the isolation of novel bacteria and the discovery of a novel biochemical reaction for hydrocarbon activation. The anaerobic, toluene-degrading isolates range phylogenetically from denitrifiers clustering in the *Azoarcus*/*Thauera* branch of the beta *Proteobacteria* (1, 6, 10–12) to several species belonging to the delta *Proteobacteria*, including two sulfate reducers (*Desulfobacula toluolica* and strain PRTOL1) (4, 9) and one ferric iron reducer (*Geobacter metallireducens*) (7). In vitro studies performed with two denitrifying bacteria, *Thauera aromatica* (5) and strain T (3), suggest that the first step of anaerobic toluene degradation in these species is the addition of the methyl carbon of toluene to the double bond of fumarate to form benzylsuccinate (Fig. 1). This reaction is of considerable biochemical interest not only as a novel means of aromatic hydrocarbon activation but also as a novel means of enzymatic carbon-carbon bond formation. In this article, we present evidence that permeabilized, toluene-grown cells of sulfate-reducing strain PRTOL1 also catalyze the addition of toluene to fumarate to form benzylsuccinate, indicating that this reaction is not limited to denitrifiers belonging to the beta *Proteobacteria.*

Strain PRTOL1 was cultivated anaerobically with toluene and sulfate as the sole electron donor and acceptor, respectively, in a bicarbonate-buffered growth medium described previously (4). This medium contains $FeSO_4$ as the sulfate source, which precludes sulfide toxicity to this sulfide-sensitive bacterium by effecting FeS precipitation (4). Incubation and permeabilized-cell assays were carried out at 35°C in an anaerobic glove box (Coy Laboratory Products, Inc., Grass Lake, Mich.) with a gas composition of 80% $N_2$, 10% $CO_2$, and 10% $H_2$. Permeabilized-cell assays were conducted in a manner similar to that described previously (3). Briefly, strain PRTOL1 cells (200 ml) in stationary phase ($\sim 2 \times 10^7$ cells/ml) were harvested anaerobically by centrifugation (10,000 × *g*, 4°C, 20 min) in sealed polycarbonate bottles (Nalge Co., Rochester, N.Y.), washed once in degassed morpholinepropanesulfonic acid (MOPS) buffer (20 mM MOPS, 5 mM $MgCl_2$ [pH 7.2]), suspended in a final volume of 3 to 3.5 ml of MOPS buffer, and then permeabilized with Triton X-100 (2% [vol/vol] final concentration). The assay mixtures (total volume, 1.6 ml) contained toluene-$\alpha,\alpha,\alpha$-$d_3$ (0.4 μmol), fumarate (1 μmol), $TiCl_3$ (as a reductant; 0.2 mM), and 0.9 ml of permeabilized cells. The reaction was halted at selected times by rapid cooling on ice. After incubation, assay mixtures were treated with DNase I, acidified with concentrated HCl, and extracted three times with high-purity diethyl ether. The ether extracts were dried with anhydrous sodium sulfate, derivatized with ethereal diazomethane to convert carboxylic acids into methyl esters, exchanged into high-purity $CH_2Cl_2$, and analyzed by capillary gas chromatography-mass spectrometry in electron ionization mode (3, 4).

Permeabilized cells of strain PRTOL1 catalyzed the addition of toluene to fumarate to form benzylsuccinate. A kinetic study of this reaction is depicted in Fig. 2A. A linear regression of the data ($r^2 = 0.995$) indicated an approximate rate of 0.5 nmol · $min^{-1}$. Based on a rough estimation of protein content, the specific rate of benzylsuccinate formation was on the order of 2.5 nmol · $min^{-1}$ · mg of $protein^{-1}$. The protein content of the permeabilized cell preparation was estimated by using (i) the measured cell density based on microscopic cell counts and (ii) estimated values for cell mass and composition taken from Neidhardt (8); the protein content could not be measured directly because of spectroscopic interferences caused by the presence of FeS. A comparable specific rate of benzylsuccinate formation (within a factor of 2) was determined from a preliminary kinetic study.

To provide a means to compare the rate of toluene transformation by permeabilized cells to that by whole cells, toluene consumption was monitored in PRTOL1 whole cells before they were harvested for permeabilized-cell assays. The cells (200 ml) were maintained with toluene and sulfate in a 250-ml amber glass, screw-cap bottle sealed with a polytetrafluoroethylene Mininert valve (Alltech Associates, Inc., Deerfield, Ill.). Toluene was monitored by a static headspace technique involving capillary gas chromatography and photoionization detection, as described elsewhere (4). Cumulative toluene consumption by whole PRTOL1 cells in the presence of sulfate is shown in Fig. 2B. A linear regression of the data ($r^2 = 0.999$) indicated an approximate rate of 25 nmol · $min^{-1}$, or an estimated specific rate on the order of 35 to 40 nmol · $min^{-1}$ · mg of $protein^{-1}$ (based on the same protein estimation method described previously). Thus, the in vitro rate of benzylsuccinate formation from toluene and fumarate was approximately 7% of the in vivo rate of toluene consumption in the presence of sulfate.

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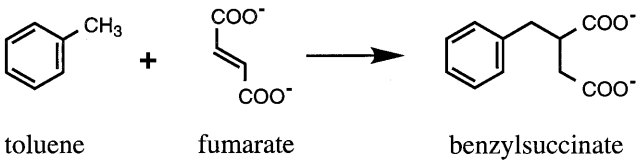


FIG. 1. Proposed anaerobic activation of toluene by addition to fumarate, based on Biegert et al. (5) and Beller and Spormann (3).

Mass spectral data show that deuterium-labeled toluene was converted to deuterium-labeled benzylsuccinate by permeabilized cells of strain PRTOL1 (Fig. 3). Figure 3 presents mass spectra of the dimethyl esters of a DL-benzylsuccinate standard (A) and benzylsuccinate-$d_3$ produced during assays with toluene-$d_3$ and fumarate (B). Based on such mass-spectral evidence, it is apparent that the H atom abstracted from the toluene methyl group during addition to fumarate is retained in the succinyl moiety of benzylsuccinate. To illustrate, comparison of the molecular ions in Fig. 3A and B demonstrates that a total of three deuterium atoms were associated with benzylsuccinate produced from toluene-$d_3$ (the molecular ion in Fig. 3B is at *m/z* 239 as compared to 236 in Fig. 3A). Furthermore, based on analysis of the tropylium ions ($C_7H_7^+$) in Fig. 3, only two of the three deuterium atoms in benzylsuccinate-$d_3$ are associated with the original seven carbon atoms of toluene. Specifically, the tropylium ion of labeled benzylsuccinate (*m/z* 93; Fig. 3B) has two more deuterium atoms than the analogous ion of unlabeled benzylsuccinate (*m/z* 91; Fig. 3A). Thus, the one deuterium atom on labeled benzylsuccinate that is not associated with the original carbon atoms from toluene must be associated with the succinyl moiety. Notably, analogous deuterium-labeling patterns (e.g., benzylsuccinate-$d_8$ formed from toluene-$d_8$) have been observed in PRTOL1 cultures grown anaerobically with toluene (references 2 and 4 and unpublished data), as discussed elsewhere (3). Similarly, suspensions of toluene-grown strain PRTOL1 cells produce (2-methylbenzyl)succinate-$d_{10}$ from *o*-xylene-$d_{10}$ (4).

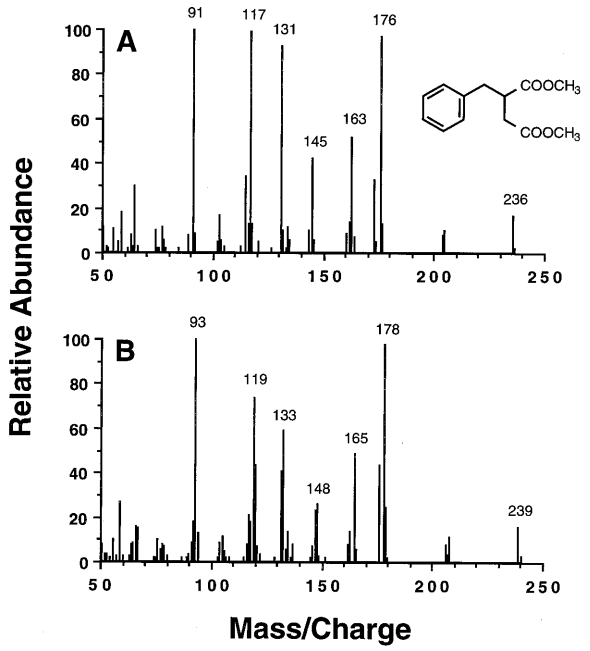


FIG. 3. Mass spectra of dimethyl esters of benzylsuccinic acid. (A) A DL-benzylsuccinate standard; (B) benzylsuccinate-$d_3$ produced from toluene-$\alpha,\alpha,\alpha$-$d_3$ and fumarate by permeabilized cells of strain PRTOL1.

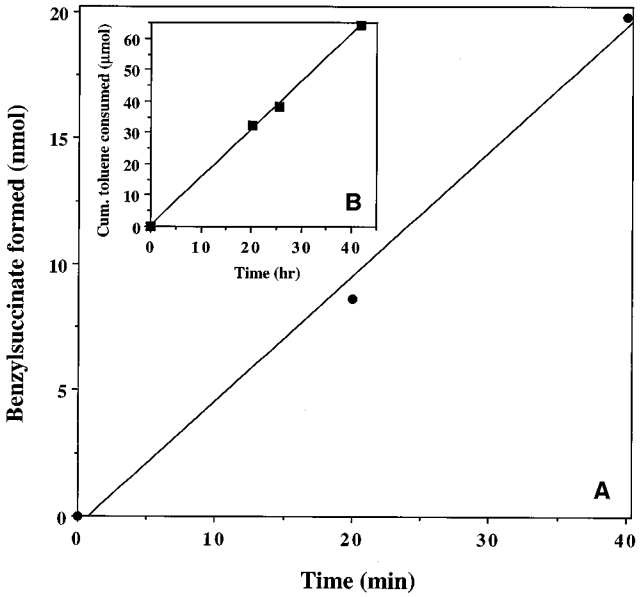


FIG. 2. In vitro (A) and in vivo (B) toluene metabolism by strain PRTOL1. (A) Kinetics of benzylsuccinate formation from toluene and fumarate by permeabilized cells of strain PRTOL1. The estimated protein content of the assay was 0.2 mg (see text). (B) Cumulative toluene consumption by whole cells of strain PRTOL1 in the presence of sulfate. The estimated protein content was 0.7 mg (see text). The 200-ml culture was amended with toluene at 0 and 26 h. The linear regressions used to calculate in vitro and in vivo rates are shown.

Several fundamental features of anaerobic toluene metabolism observed for sulfate-reducing strain PRTOL1 correspond well with observations made for denitrifying *T. aromatica*, strain T, and *Azoarcus tolulyticus* Tol-4: (i) anaerobic, in vitro assays demonstrated that toluene-grown cells catalyzed benzylsuccinate formation from toluene and fumarate (references 3 and 5 and unpublished data regarding strain Tol-4); (ii) assays including toluene-$d_3$ resulted in the formation of benzylsuccinate-$d_3$, suggesting that the H atom abstracted from the toluene methyl group during addition to fumarate is retained in the succinyl moiety of benzylsuccinate (3); and (iii) the in vitro and in vivo specific rates of toluene metabolism observed for strain PRTOL1, although rough estimates, were within a factor of 3 of the corresponding rates reported for strain T (3). Thus, diverse bacteria capable of anaerobic toluene mineralization appear to activate toluene similarly. Furthermore, it is plausible that all anaerobes that initiate toluene degradation by a similar enzymatic reaction, namely benzylsuccinate formation, also catalyze similar subsequent reactions of anaerobic toluene degradation. Such reactions have been postulated to include benzylsuccinate oxidation to benzoyl-coenzyme A via phenylitaconyl-coenzyme A, based on studies of strain T and *T. aromatica* (3, 5). Further research will be required to determine the similarity of toluene metabolic pathways among phylogenetically diverse anaerobes, and in particular, the similarity of enzymes catalyzing anaerobic toluene activation.

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