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Toluene 2-Monooxygenase-Dependent Growth of *Burkholderia cepacia* G4/PR1 on Diethyl Ether

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Aerobic bacterial growth on aromatic hydrocarbons typically requires oxygenase enzymes, which are known to fortuitously oxidize nongrowth substrates. In this study, we found that oxidation of diethyl ether by toluene 2-monooxygenase supported more rapid growth of *Burkholderia cepacia* G4/PR1 than did the aromatic substrates *n*-propylbenzene and *o*-xylene. The wild-type *Burkholderia cepacia* G4 failed to grow on diethyl ether. Purified toluene 2-monooxygenase protein components oxidized diethyl ether stoichiometrically to ethanol and acetaldehyde. Butyl methyl ether, diethyl sulfide, and 2-chloroethyl ethyl ether were oxidized by *B. cepacia* G4/PR1.

Growth and nongrowth metabolism are sometimes delineated by calling the former metabolism and the latter cometabolism, but the latter term is an oversimplification (4, 6, 9, 10, 26). The inability of a bacterium to grow on a particular carbon source can have one of several explanations: (i) a failure of any enzyme in a metabolic pathway to transform the starting compound or its intermediate, (ii) a failure to induce the requisite enzymes, (iii) the failure of an organism to recover sufficient carbon and energy from the starting compound, or (iv) the toxicity of the starting compound or an intermediate.

Burkholderia cepacia G4, formerly *Pseudomonas cepacia*, was isolated and extensively studied for its ability to oxidize trichloroethylene (TCE) (5, 13, 16, 17, 22, 23), although it does not grow on TCE (11). TCE oxidation was subsequently shown to be dependent on the expression of an aromatic hydrocarbon oxygenase(s) in vivo (17, 22), and this activity was confirmed to be dependent on toluene 2-monooxygenase by using purified enzyme components (18, 19). TCE oxidation by *B. cepacia* G4 is sometimes referred to as cometabolism, but it remains undefined why the organism fails to grow on TCE, given that glyoxylic acid is the major in vivo oxidation product (19) and exogenously added glyoxylic acid supports bacterial growth (11).

Ethers are prevalent in nature and industry (27). Lignin is a large source of natural product ether linkages. Industrial products and by-products with ether linkages include the phenoxyacetate herbicides, alcohol ethoxylate surfactants, and 2,3,7,8-tetrachlorodibenzodioxin. Most microbial metabolism studies have focused on the generally more toxic aromatic ethers, but there is considerable interest in the biodegradation of solvents and fuel additives, such as diethyl ether and *tert*-butyl methyl ether, respectively (15, 20, 21).

The present study was done to determine if toluene 2-monooxygenase would allow *B. cepacia* G4/PR1 to grow on ether compounds via novel metabolic pathways. *B. cepacia* G4/PR1 is a toluene 2-monooxygenase constitutive derivative of *B. cepacia* G4 (23), and it enabled us to investigate the role of enzyme induction in allowing growth on compounds not resembling toluene or phenol, most significantly, in this case, aliphatic ethers.

Growth of *B. cepacia* G4 and G4/PR1 on ether compounds. It has been previously shown that *B. cepacia* G4 grows on glucose, lactate, succinate, acetate, ethanol, phenol, toluene, benzene, *o*-cresol, *m*-cresol, *p*-cresol, or *o*-xylene (16, 23). To determine if *B. cepacia* G4 and G4/PR1 had the ability to grow on ether compounds, we inoculated each strain into minimal salts buffer (MSB) medium (24) containing diethyl ether, 2-chloroethyl ethyl ether, diethyl sulfide, butyl methyl ether, or *tert*-butyl methyl ether. Strains were maintained on MSB agar plates containing 20 mM lactate. Kanamycin (50 μg/ml) was added to all cultures of *B. cepacia* G4/PR1. *B. cepacia* strains were grown, with shaking at 200 rpm, at 27°C by using 50 ml of

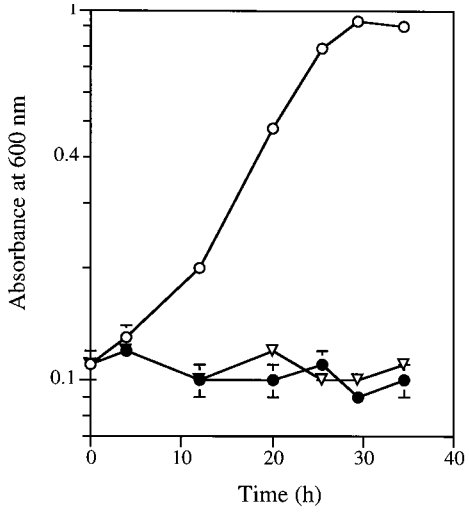

FIG. 1. Growth curves of *B. cepacia* G4 (●) and G4/PR1 (○) on 10 mM diethyl ether and of *B. cepacia* G4/PR1 with no carbon source (▽).

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TABLE 1. Stoichiometry of diethyl ether oxidation by purified toluene 2-monooxygenase

Reaction mixture	Initial diethyl ether concn (μM)	Concn of compound present after 40-min incubation (μM)		
		CH_3CHO	CH_3CH_2OH	$(CH_3CH_2)_2O$
NADH alone	610	<20	<20	412
Heat-killed T2M[a] + NADH	570	<20	<20	452
T2M + NADH	590 ± 61[b]	594 ± 48	567 ± 122	68 ± 6

[a] T2M, toluene 2-monooxygenase.
[b] Values are means of duplicate experiments ± standard errors and are corrected for background levels of acetaldehyde and ethanol.

MSB liquid medium containing 20 mM lactate in 500-ml flasks with toluene in the vapor phase as described (2). Cells were harvested at an optical density at 600 nm (OD_{600}) of 2.0 by centrifugation at 10,000 × *g*, washed twice with 20 mM phosphate buffer (pH 7.0), and resuspended in an equal volume of the same medium. Washed *B. cepacia* cultures were added to 5 ml of MSB medium in 75-ml septum vials to obtain a starting OD_{600} of 0.1. The vials were crimp sealed with Teflon-lined rubber septa. The ether compounds were added, with a gastight syringe, to a final concentration of 10 mM, assuming that all of the compound was present in the liquid phase. All chemicals were reagent grade or better and were obtained from Aldrich Co. (Milwaukee, Wis.), except for 2-chloroethyl ethyl ether, which was obtained from Pfaltz & Bauer, Inc. (Waterbury, Conn.). Diethyl ether was high-pressure liquid chromatography grade (99.9%). Cell growth was determined spectrophotometrically at 600 nm, and an absorbance of <0.2 after 72 h was considered to indicate no growth.

B. cepacia G4/PR1 grew relatively rapidly on diethyl ether (Fig. 1). With a starting concentration of 10 mM diethyl ether, the OD_{600} was 0.9 after 30 h of growth. The doubling time of *B. cepacia* G4/PR1 on diethyl ether was about 6 h. By comparison, toluene-grown cells had a doubling time of 4 h. *B. cepacia* G4 failed to grow on diethyl ether, and neither strain grew on 2-chloroethyl ethyl ether, diethyl sulfide, butyl methyl ether, or *tert*-butyl methyl ether as the sole carbon source. The significant extent of growth of *B. cepacia* G4/PR1, the failure of strain G4 to grow, and the reported purity of diethyl ether (99.9%) suggested that the observations were likely not due to substrate impurities.

Oxidation of diethyl ether and 2-chloroethyl ethyl ether by purified toluene 2-monooxygenase. Purified toluene 2-monooxygenase (18) was used to determine specific activity and the products of diethyl ether oxidation. Reaction mixtures contained 7 μM hydroxylase, 14 μM small component, 10 μM reductase, and 5 mM ethanol-free NADH in 25 mM MOPS (morpholinepropanesulfonic acid) buffer (pH 7.5) in 1.8-ml crimp-sealed septum vials with Teflon-lined rubber septa. Diethyl ether or 2-chloroethyl ethyl ether was added to a final concentration of 500 μM. The reaction was initiated by the addition of NADH, and vials were incubated, with shaking at 200 rpm, at 23°C. Reaction mixtures were incubated for 40 min and used to determine the reaction products and stoichiometries. After incubation, 1% trichloroacetic acid was added to the reaction mixtures, the vials were immediately shaken at 4°C, and the reaction mixtures were centrifuged at 14,000 rpm in an Eppendorf microcentrifuge (Brinkmann Instruments, Inc., Westbury, N.Y.) for 15 min at 4°C. Supernatant fractions were analyzed as described below. A control reaction with toluene 2-monooxygenase and NADH but without diethyl ether gave 32 μM acetaldehyde and 417 μM ethanol as background. Protein concentrations were determined by using a protein assay kit (Bio-Rad Laboratories, Hercules, Calif.) with bovine serum albumin as the standard (1).

The disappearance of substrates in supernatant fractions was monitored by using a Varian Aerograph Series 1400 gas chromatograph equipped with a flame ionization detector and a 0.1% AT-1000 Graphpac GC 80/100 column (6 ft by 1/8 in.) (Alltech Associates, Inc., Deerfield, Ill.). The column temperature was 200°C, and injector and detector temperatures were 250°C. Product confirmation and stoichiometric analyses for the oxidation of diethyl ether and 2-chloroethyl ethyl ether by purified toluene 2-monooxygenase were carried out by injecting the liquid portion of reaction mixtures into a Hewlett-Packard gas chromatograph fitted with a flame ionization detector and a DB-624 fused silica capillary column (film thickness, 1.8 μm; inside diameter, 0.32 mm; length, 30 m) (J & W Scientific Co., Folsom, Calif.). The flow rate was 1.5 ml/min, the column temperature was 80°C, and the injector and detector temperatures were 230 and 220°C, respectively.

Samples were also analyzed by using a Kratos MS-25 gas chromatograph-mass spectrometer equipped with a flame ionization detector and a DB-WAX fused silica capillary column (film thickness, 0.5 μm; inside diameter, 0.32 mm; length, 30 m) (J & W Scientific Co.). The column temperature used was 50°C for 3 min, followed by a linear gradient to 150°C at 25°C/min. The flow rate was 1.5 ml/min, and the injector and detector temperatures were 200°C. The mass ionization energy for gas chromatography-mass spectrometry was 70 eV. Product identification was based on gas chromatograph retention times and mass spectra (*m*/*z*) compared to data obtained with authentic compounds. Data for determination of stoichiometry were obtained by peak height analysis of gas chromatograms for substrate disappearance and from peak areas for product formation.

Diethyl ether was oxidized in vitro by the purified and reconstituted toluene 2-monooxygenase enzyme system when it was incubated with NADH under aerobic conditions (Table 1). The specific activity with 590 μM diethyl ether was 28 nmol/min per mg of hydroxylase. A similar specific activity, 27 nmol/min per mg of hydroxylase component, had previously been reported in in vitro assays with toluene as the substrate (18). One mole of diethyl ether was oxidized to 1 mole of acetaldehyde and 1 mole of ethanol (Table 1). Previously, *B. cepacia* G4 was shown to grow readily on ethanol and acetate, suggesting that both C_2 fragments are further oxidized in vivo.

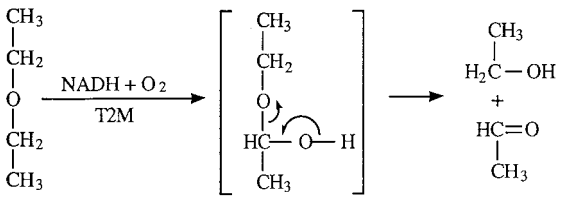

FIG. 2. Proposed metabolic pathway of diethyl ether oxidation by *B. cepacia* G4/PR1.

TABLE 2. Degradation of aliphatic ethers and diethyl sulfide by resting cell suspensions of lactate-grown *B. cepacia* G4 and G4/PR1

Substrate[a]	Structure	% Removed after 10-h incubation by:		
		Lactate-grown *B. cepacia* G4	*B. cepacia* G4/PR1	
			Lactate grown[b]	Heat killed
Diethyl ether	$CH_3CH_2—O—CH_2CH_3$	<5	97 ± 1	<5
Diethyl sulfide	$CH_3CH_2—S—CH_2CH_3$	<5	71 ± 10	6 ± 1
2-Chloroethyl ethyl ether	$ClCH_2CH_2—O—CH_2CH_3$	<5	80 ± 4	<5
Butyl methyl ether	$CH_3(CH_2)_3—O—CH_3$	<5	39 ± 2	<5
tert-Butyl methyl ether	$(CH_3)_3C—O—CH_3$	<5	<5	<5

[a] Initial concentration of substrates, 500 μM.
[b] Values are means of duplicate experiments ± standard errors.

The identity and stoichiometry of the products indicate that oxidation of diethyl ether by toluene 2-monooxygenase proceeds via hydroxylation at the subterminal carbon to yield an unstable intermediate that decomposes to acetaldehyde and ethanol (Fig. 2). A similar pathway has been proposed by Heydeman (8) for several bacteria belonging to the *Corynebacterium-Mycobacterium-Nocardia* group, which grow on diethyl ether as the sole source of carbon and energy via oxidation to acetaldehyde and ethanol. Colby et al. (3) also reported that methane monooxygenase from *Methylococcus capsulatus* (Bath) oxidizes diethyl ether to acetaldehyde and ethanol. However, diethyl ether did not support growth of *M. capsulatus* (Bath). *Methylomonas albus* BG8 and *Methylosinus trichosporium* OB3B oxidize diethyl ether at a terminal methyl group to yield 2-ethoxyethanol, which is further metabolized to 2-ethoxyacetate via 2-ethoxyethanal (28). Recently, it has been shown that *Nitrosomonas europaea* oxidizes dimethyl, diethyl, *n*-dipropyl, and *n*-dibutyl ethers through the activity of ammonia monooxygenase (12, 29). Dimethyl and diethyl ethers were oxidized to the corresponding aldehydes and alcohols, and *n*-dipropyl and *n*-dibutyl ethers were oxidized to C_6 and C_8 hydroxylated compounds, respectively. However, *N. europaea* was not reported to use diethyl ether as the sole source of carbon and energy.

2-Chloroethyl ethyl ether was also oxidized by purified toluene 2-monooxygenase to yield four products: acetaldehyde, ethanol, chloroacetaldehyde, and 2-chloroethanol. All of the metabolites showed gas chromatograph retention times and mass spectra comparable to those of authentic compounds run in parallel (data not shown). Although two of the products, acetaldehyde and ethanol, could support growth of *B. cepacia* G4/PR1, 2-chloroethyl ethyl ether was not utilized as a growth substrate, perhaps due to the toxicity of chloroacetaldehyde (25).

Degradation of ethers by resting cell suspensions of *B. cepacia* G4 and G4/PR1. Diethyl ether, 2-chloroethyl ethyl ether, diethyl sulfide, butyl methyl ether, and *tert*-butyl methyl ether were tested as in vivo substrates with lactate-grown cells of *B. cepacia* G4 and G4/PR1. Cultures were harvested by centrifugation and washed in phosphate buffer as described above. Cell cultures were resuspended in 20 mM phosphate buffer (pH 7) to a final OD_{600} of 2.0, and 1-ml aliquots were dispensed into 10-ml crimp-sealed septum vials with Teflon-lined rubber septa. Substrates were added to a final concentration of 500 μM from 10 mM stock solutions made up in *N*,*N*-dimethylformamide. Substrate disappearance was analyzed by headspace gas chromatography with a Varian Aerograph Series 1400 instrument as described above.

Resting cell suspensions of *B. cepacia* G4/PR1 degraded diethyl ether, diethyl sulfide, 2-chloroethyl ethyl ether, and butyl methyl ether (Table 2). They did not, however, degrade the oxygenated-fuel additive *tert*-butyl methyl ether. Lactate-grown *B. cepacia* G4 did not significantly degrade any of the compounds tested. The most asymmetric ether, butyl methyl ether, was degraded to the least extent. The most symmetrical oxygen ether, diethyl ether, was degraded most completely.

In summary, toluene 2-monooxygenase oxidized diethyl ether at a significant rate and with the proper regiospecificity to yield growth-supporting intermediates. While microbial growth on diethyl ether has been previously shown only for some actinomycete strains (8, 20) and a filamentous fungus, *Graphium* sp. (29), the enzymes responsible for growth are presently uncharacterized. In the present study, the metabolic disposition of diethyl ether was established by using purified toluene 2-monooxygenase. The difference in growth or no growth is dependent solely on the genetic regulation of toluene 2-monooxygenase. In this context, it is of interest that TCE induces the expression of toluene dioxygenase in *Pseudomonas putida* UV4 (7) and of toluene 4-monooxygenase in *Pseudomonas mendocina* KR1 (14). These results and those of the present study highlight the importance of matching enzyme and induction specificities for optimizing the metabolic capabilities of organisms.

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