Identification of the Intermediates of in Vivo Oxidation of 1,4-Dioxane by Monooxygenase-Containing Bacteria

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1,4-Dioxane is a probable human carcinogen and an emerging water contaminant. Monooxygenase-expressing bacteria have been shown to degrade dioxane via growth-supporting as well as cometabolic mechanisms. In this study, the intermediates of dioxane degradation by monooxygenase-expressing bacteria were determined by triple quadrupole-mass spectrometry and Fourier transform ion cyclotron resonance-mass spectrometry. The major products emerging from dioxane degradation were 2-hydroxy-1,4-dioxane, which is spontaneously oxidized to glycolic acid, ethylene glycol, glycolate, and oxalate. Studies with uniformly labeled 14C dioxane showed that over 50% of the dioxane was mineralized to CO2 by CB1190, while 5% became biomass-associated after 48 h. Volatile organic acids and non-volatiles, respectively, accounted for 20 and 11% of the radiolabeled carbon. Although strains cometabolizing dioxane exhibited limited transformation capacities, nearly half of the initial dioxane was recovered as CO2. On the basis of these analytical results, we propose a pathway for dioxane oxidation by monooxygenase-expressing cells in which dioxane is first converted to 2-hydroxy-1,4-dioxane, which is spontaneously oxidized to HEAA. During a second monooxygenation step, HEAA is further hydroxylated, resulting in a mixture of dihydroxyethoxyacetic acids with a hydroxyl group at the ortho or para position. After cleavage of the second ether bond, small organic molecules such as ethylene glycol, glycolate, glyoxalate, and oxalate are progressively formed, which are then mineralized to CO2 via common cellular metabolic pathways. Bioremediation of dioxane via this pathway is not expected to cause an accumulation of toxic compounds in the environment.

Introduction

1,4-Dioxane (hereafter referred to as dioxane) is a probable human carcinogen (1) that has been detected as an emerging contaminant in the environment and in drinking water supplies (2). Because of its low volatility (Henry’s Law constant \(= 5 \times 10^{-6} \text{ atm m}^3 \text{ mol}^{-1} \) at 20 °C) and hydrophilic nature (log \(K_{ow} = -0.27\)), dioxane is not easily removed from water by air stripping or carbon adsorption. Currently available treatment options for water contaminated with dioxane include oxidation by combinations of UV light, sonication, hydrogen peroxide, ozone, and chemical oxidants (3–8). However, these processes are expensive and require ex situ treatment of groundwater. Biodegradation of dioxane has been reported for several pure and mixed cultures of bacteria (9–16) and fungi (17, 18). While some isolates cometabolically degraded dioxane after using compounds such as propane (10) or tetrahydrofuran (15, 16) as primary growth substrates, other isolates were able to utilize dioxane as the sole carbon and energy source (9, 11, 12, 17). However, none of the studies described the full biochemical pathway for complete mineralization of dioxane.

Monooxygenases are enzymes involved in catalyzing the initial step of dioxane degradation in several bacteria (14, 19). Mahendra and Alvarez-Cohen (19) confirmed the role of monooxygenases in degrading dioxane using several independent lines of evidence and described the kinetics of the reaction. Although strain CB1190 metabolically degrades dioxane using a putative dioxane monooxygenase that is inducible by dioxane or THF, CB1190 cells grown on formate, pyruvate, R2A, or Luria–Bertani (LB) broth were not immediately capable of dioxane degradation (19). Strains K1, JOB5, and KR1 cometabolically degrade dioxane after growth on THF, propane, and toluene, respectively, and this activity is inhibited by exposure to acetylene, confirming the involvement of monooxygenases. Escherichia coli TG1 (T4MO), which contains a constitutively expressed toluene-4-monooxygenase derived from KR1, also degrades dioxane after growth on LB broth, whereas E. coli TG1 (ToMO) expressing toluene/o-xylene monooxygenase derived from Pseudomonas stutzeri OX1 and Pseudomonas putida mt-2 expressing a tolune-side chain-monooxygenase are unable to degrade dioxane (19).

In a study of the pathway of bacterial dioxane degradation, Vainberg et al. (14) detected 2-hydroxyethoxyacetic acid (HEAA) as the terminal product of dioxane degradation by Pseudonocardia strain ENV478. They suggested that the degradation was carried out by a monooxygenase similar to putative tetrahydrofuran monooxygenase of Pseudonocardia tetrahydrofuranoxylans K1 (20). Neither dioxane mineralization nor its assimilation into biomass was observed. Similarly, HEAA has been reported as the major metabolite of dioxane via a cytochrome P450 monooxygenase reaction in mammalian cells (21, 22). An incomplete dioxane degradation pathway has been reported for mammals and aerobic bacteria (Figure 1). In contrast, HEAA was not detected during dioxane degradation by the fungus Cordyceps sinensis, although it was suggested that etherases or oxidases were involved in the degradation (17). The only intermediates of dioxane degradation identified in that study were ethylene glycol, glycolic acid, and oxalic acid.

This study is the first report of the pathway for complete mineralization of dioxane by aerobic bacteria. Here, we have identified the degradation intermediates previously reported in a terminal pathway, as well as those involved in the
complete mineralization to carbon dioxide. The pathway was confirmed for both metabolic and cometabolic degradation processes by monooxygenase-expressing cells.

Materials and Methods

Chemicals. All chemicals used in medium preparation were of ACS reagent grade or better. Dioxane (99.8%), ethylene glycol, glycolaldehyde, glyoxal, glycolic acid, glyoxylic acid, and oxalic acid were obtained from Aldrich, Milwaukee, WI. Toluene (99.5%) was purchased from JT Baker (Phillipsburg, NJ). THF (99.5%) was purchased from Acros Organics (Morris Plains, NJ). R2A medium was obtained from BBL, Inc. (Cockeysville, MD). High-purity propane (>99.9%) was purchased from Matheson Gas products (Twinsburg, OH). HPLC grade methanol and water were from Honeywell (Morristown, NJ). 1,4-Dioxane-2-one was prepared by CanSyn Chemical Corporation, Toronto, ON, using a previously described method involving the treatment of trans-2,3-dichloro-1,4-dioxane (TCI America, Portland, OR) with anhydrous formic acid (23). At a pH below 12, 1,4-dioxane-2-one was converted to HEAA by hydrolysis (22). Uniformly labeled dioxane (1 mCi/mL) was a gift from the Pall Corporation, Ann Arbor, MI. Deionized water from a Barnstead Nanopure II system (Dubuque, IA) was used for all experiments.

Laboratory Strains. Pseudonocardia dioxanivorans CB1190 (11, 12) was a gift from Dr. Rebecca Parales, University of California, Davis, and Mycobacterium vaccae JOBS (10) was a gift from Dr. Daniel Arp, Oregon State University. Pseudomonas mendocina KR1 (24) and Pseudomonas putida mt-2 (25) were provided by Dr. Jerome Kukor, Rutgers University. Recombinant E. coli strains TG1/pBS(Kan) containing constitutively expressed toluene-4-monooxygenase (T4MO) (26) or toluene/o-xylene monooxygenase (ToMO) (27) were generously supplied by Dr. Thomas Wood, Texas A&M University. P. tetrahydrofuranoxydans K1 (28) was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ).

Culture Conditions. All strains were grown in a nitrile mineral salts (NMS) medium (29) with a monooxygenase-inducing growth substrate. Growth substrates were added to the medium to achieve total aqueous concentrations of approximately 100 mg/L for dioxane, THF, and toluene. Propane was supplied by injecting 25% (vol/vol) of the gas into the sealed liquid culture flasks. The recombinant E. coli strains were grown in LB broth (Becton Dickinson, Sparks, MD) containing 100 mg/L kanamycin. All cultures were incubated at 30 °C while being shaken at 150 rpm, and excess oxygen was present in the headspace assuring aerobic conditions throughout growth. The culture medium was always less than 20% of the total flask volume to prevent mass transfer limitations of oxygen and other gases. Aseptic conditions were maintained to avoid contamination. Cultures were also maintained on Petri plates on NMS medium containing dioxane, THF or toluene and 1.5% Bacto Agar, or a rich medium such as LB Agar or R2A agar.

Experimental Approach. The cells were grown in the presence of monooxygenase-inducing compounds. These growth substrates were removed to prevent competition with dioxane during dioxane degradation experiments. The cell cultures were first purged for 5 min with 300 mL/min nitrogen followed by two rounds of 0.2% oxygenation at 15 000 g for 5 min and resuspended in fresh NMS medium in new bottles. Biodegradation assays were performed at 30 °C in 250 mL clear glass bottles capped with Teflon-lined mininert valves and containing 50 mL of NMS medium to which 100 or 500 mg/L dioxane was added. Duplicate samples were collected at various time intervals to analyze for residual dioxane and degradation products. All experiments included abiotic controls, dioxane-free controls, acetylene-exposed cells, or cells grown on non-monoxygenase-inducing substrates such as R2A or formate.

Analytical Methods. Gas Chromatography. Duplicate cell culture samples were filtered with 0.2 μm syringe filters, and 5 μL of filtrate was injected into a Varian 3400 gas chromatograph equipped with a flame ionization detector (FID) and a GraphPac-GB column (J&W Scientific, Folsom, CA). The injector, oven, and detector temperatures were set at 220, 120, and 250 °C, respectively. Calibration curves were prepared from dioxane standards in NMS medium. The detection limit of GC-FID was 1 mg/L for dioxane.

Solid-Phase Extraction (SPE). Liquid culture samples were filtered with 0.2 μm syringe filters. SPE for quadrupole ion trap experiments was carried out using a 1g C18 Varian cartridge. The cartridges were conditioned by passing 6 mL of methanol followed by 6 mL of HPLC grade water (Milli Q, Billerica, MA). Then, 6 mL of filtered culture sample was introduced into the cartridge followed by passing 6 mL of water through the column for salt elution. Finally, the cartridge was extracted with 6 mL of methanol, and the product was amended with 24 mL of water, subsequently frozen with liquid nitrogen, and lyophilized. The lyophilized product was reconstituted with 500 μL of water and subjected to ultrafiltration using a centrifuge (Beckman, Fullerton, CA), and Vivaspin Concentrator-300 MW (Vivascience, Gloucestershire, U.K.) at 5000 g for 10 min.

Mass Spectrometry. The sample pellet obtained from the preparation described previously was reconstituted in 20% acetonitrile and 80% water containing 5 mM ammonium acetate and infused through an electrospray ionization quadrupole ion-trap mass spectrometer (ESI-Q-Trap, Applied Biosystems, Foster City, CA; MDS Sciex, South San Francisco, CA). Samples were introduced into the Q-Trap MS via an integrated syringe pump (flow rate = 10 μL/min) and a TurbolonSpray source operating in negative ion mode. Negative ions were generated through the application of −3500 V (capillary voltage) to the tip of the probe, with a source cone voltage of 30 V. Desolvation was aided by nebulizing (10 L/h), drying (300 L/h), and curtain gases (20 L/h). The source and probe temperatures were set at 150 and 400 °C, respectively. Mass spectra were acquired in the full scan mode (45–220 m/z) of the deprotonated analyte molecules. Product ion mass spectra were acquired for select peaks in the samples and compared with those acquired.
cocktail. Subsequently, 5 mL of 10 N HCl was added to acidify the liquid added to Whatman glass/microfiber GF/C filters (Whatman, Middlesex, U.K.) and counting the filter in a 10 mL scintillation cocktail equipped with a 9.4 T actively shielded magnet. Processed cell culture supernatants, from SPE described previously, were diluted to achieve a 7:1 methanol/H2O solution were typically 32 scans were acquired of spectra composed of 512 000 data points supplemented with one zero-fill. The spectra were acquired on the FTICR data station, operating ApexControl 1.0 (Bruker Daltonics). External calibration was accomplished with amino acid standards just prior to sample analysis. 14C Analyses. The mineralization and assimilation of dioxane by cultures and the formation of products were tested by analyzing 14C labeled products in the headspace, cell biomass, and culture medium of cells fed uniformly 14C-labeled dioxane. After removing the growth substrate from cell cultures, 14C-labeled dioxane was added from aqueous stock solutions to achieve a final concentration of 100 mg/L and a final radioactivity of 5 μCi in each bottle. Experiments with radiolabeled dioxane were conducted in parallel with unlabeled experiments to facilitate the measurement of the dioxane disappearance and identification of intermediates. Bottles were sacrificed at various time intervals for the analysis of radioactivity in the fractions. Bottles were first basified by adding 2 mL of 10 N NaOH to dissolve all 14CO2 in the aqueous medium, and then 1 mL of the basified liquid was added to 9 mL of the scintiverse BD scintillation cocktail (Fisher Scientific, Fair Lawn, NJ) and analyzed using a Packard Tri-Carb 1900TR liquid scintillation counter (PerkinElmer, Downers Grove, IL) to measure total 14C. The cell-associated 14C label was measured by filtering 5 mL of basified liquid through Whatman glass/microfiber GF/C filters (Whatman, Middlesex, U.K.) and counting the filter in a 10 mL scintillation cocktail. Subsequently, 5 mL of 10 N HCl was added to acidify the culture medium. Non-purgeable base and acid fractions were collected after flushing with 200 mL/min N2 for 30 min to transfer CO2 and any volatile organics into a trap containing 20 mL of 10 N NaOH. Total dissolved 14C was measured by counting 1 mL of trap liquid added to 9 mL of scintillation fluid. Subsequently, 1 mL of trap liquid was added to 9 mL of 0.2M BaSO4 to precipitate all CO2 as BaCO3. This mixture was stored at 4 °C for 24 h, filtered through a 0.2 μm filter, and analyzed for residual radioactivity. The counts in the trap minus the counts after BaSO4 treatment gave the amount of 14CO2. The counts in the traps after BaSO4 treatment represented the volatile degradation products.

Results

Mineralization of Dioxane. Uniformly 14C labeled dioxane was used to test for mineralization to CO2 by strains capable of degrading dioxane. The distribution of dioxane and its degradation products into cell biomass, CO2, volatile, and non-volatile fractions over time is presented in Figure 2. For the strains capable of dioxane degradation, decreases in the non-volatile fraction, which includes residual dioxane, were accompanied by increases in CO2 production and transient accumulation of volatile organic acids. Dioxane carbon was incorporated into the cell biomass by strain CB1190 but not by strains that cometabolize dioxane. As strains KR1 and TG1(T4MO) exhibited limited transformation capacities for dioxane, the degradation rate slowed down, and the concentration of non-volatiles remained constant after approximately 24 h (data not shown). Strain TG1(ToMO) does not degrade dioxane, and as expected, the non-volatile fraction consisting of undegraded dioxane remained unchanged throughout the incubation, with no increase in labeled biomass, CO2, or volatiles (Figure 2).

The final distribution of 14C in the products derived from dioxane degradation after 48 h by seven strains is shown in Figure 3. All dioxane-degrading strains mineralized dioxane with at least 40% 14C recovered as CO2. Approximately 5% of dioxane carbon was recovered in the CB1190 biomass. Greater than 85% of the radiolabel was recovered as a non-volatile fraction (unaltered dioxane) for the two strains incapable of dioxane degradation (mt-2 and TG1(ToMO)) similar to the abiotic control.

Identification of Dioxane Degradation Intermediates. In experiments designed to detect degradation intermediates, HEAA (measured mass 119.0350 m/z within 1 ppm of HEAA-30 exact mass) was positively identified by FTICR-MS for all strains degrading dioxane via monooxygenase catalyzed reactions (Figure 4a). The HEAA peak was absent in samples containing bacteria unable to degrade dioxane (e.g., mt-2 and ToMO), in bacteria exposed to acetylene (a known inhibitor of monooxygenases (30)), and in dioxane-free and abiotic controls. In strains CB1190, B5, K1, JOB5, KR1, and
T4MO, HEAA was transiently formed as a result of dioxane degradation, but HEAA did not accumulate (Figure 4a). HEAA was subsequently degraded into a mixture of linear dihydroxy-substituted ethoxyacetic acids (Figure 4b).

While FTICR-MS was used for high-resolution and high-mass accuracy measurements, metabolites with molecular weights lower than 90 were also detected using direct infusion MS-MS. Ethylene glycol, glycolic acid, glyoxylic

FIGURE 3. Final distribution of 14C derived from the degradation of dioxane after 48 h incubation. Strain metabolizing dioxane: CB1190; strains cometabolizing dioxane: K1, JOB5, KR1, and TG1(T4MO); and strains incapable of degrading dioxane: mt-2 and TG1(TOMO). Values are expressed as a percent of initial 14C from uniformly labeled dioxane, and error bars represent the range of six replicates.

FIGURE 4. Transient detection of HEAA. (a) FTICR mass spectra of HEAA as a metabolite of dioxane degradation by CB1190, KR1, and TG1(T4MO). HEAA peak is at m/z 119.0351, and NaHPO4 (a component of bacterial growth medium) is at m/z 118.9422. Dioxane concentration decreased to below the GC-FID detection limit in 4 h by CB1190, 10 h by KR1, and 12.5 h by T4MO. (b) FTICR mass spectra highlighting m/z 135.0300 in CB1190 and KR1 after degradation of dioxane. This m/z corresponds to a molecular formula of C4H7O5 (8.8 ppm error) consisting of either 1,2-dihydroxyethoxyethanoic acid or 2-hydroxyethoxy-2-hydroxyethanoic acid or a mixture of both. The peak m/z 134.9161 is CaPO4, a component of the medium.
Acid, and oxalic acid were identified as degradation intermediates by comparison of collision-induced dissociation spectra of sample peaks with standards (data not shown).

**Proposed Biodegradation Pathway.** On the basis of the intermediates identified during dioxane degradation by monooxygenase-expressing bacteria, a complete biodegradation pathway is proposed (Figure 5). In this pathway, 2-hydroxy-1,4-dioxane, 2-hydroxyethoxyacetic acid (HEAA), ethylene glycol, glycolate, and oxalate are major intermediates of dioxane degradation, while carbon dioxide is the expected major end product. The first intermediate, 2-hydroxy-1,4-dioxane, is likely produced by hydroxylation of an ether-bond-associated carbon atom by monooxygenases, resulting in ether bond cleavage to form 2-hydroxyethoxyacetaldehyde, which spontaneously oxidizes to HEAA. Monooxygenases then further hydroxylate HEAA, resulting in dihydroxy-substituted ethoxyacetic acids. From mass spectrometric analyses alone, it is not possible to distinguish whether the hydroxyl group was located at either the ortho or the para position with respect to the carboxylic acid group or whether a mixture of both compounds was present. The identification of ethylene glycol as a subsequent degradation product suggests that 2-hydroxyethoxy-2-hydroxyacetic acid is cer-

![Figure 5](image-url)
TABLE 1. Products of 1,4-Dioxane Degradation by Monoxygenase-Expressing Bacteria

<table>
<thead>
<tr>
<th>compound</th>
<th>accurate mass</th>
<th>C oxidation state</th>
<th>detection</th>
<th>growth of CB1190&lt;br&gt;</th>
</tr>
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<tr>
<td>1,4-dioxane</td>
<td>88.0524</td>
<td>−1</td>
<td>GC-FID</td>
<td>yes</td>
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<td>2-hydroxy-1,4-dioxane</td>
<td>104.0401</td>
<td>−0.5</td>
<td>FTICR-MS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>n.t.&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2-hydroxyethoxyacetaldehyde</td>
<td>104.0401</td>
<td>−0.5</td>
<td>FTICR-MS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>n.t.&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1,4-dioxane-2-one (dioxannone)</td>
<td>102.0246</td>
<td>0</td>
<td>FTICR-MS&lt;sup&gt;b&lt;/sup&gt;</td>
<td>n.t.&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>2-hydroxyethoxycetic acid (HEAA)</td>
<td>120.0351</td>
<td>+0.5</td>
<td>FTICR-MS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>n.t.&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>1,2-dihydroxyethoxycetic acid</td>
<td>136.0300</td>
<td>+0.5</td>
<td>FTICR-MS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>n.t.&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2-hydroxyethoxy-2-hydroxyacetic acid</td>
<td>136.0300</td>
<td>+0.5</td>
<td>FTICR-MS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>n.t.&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>1,2-ethanediol (ethylene glycol)</td>
<td>62.04</td>
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<td>2-hydroxyethanoic acid (glycolic acid)</td>
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<td>carbon dioxide</td>
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<td>+4</td>
<td>&lt;sup&gt;14&lt;/sup&gt;C LSC</td>
<td>n.t.</td>
</tr>
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</table>

* Growth of CB1190 using these compounds as sole carbon or energy sources. b Growth was not tested (n.t.) because pure standards for these compounds were not available. c Growth could not be confirmed because these compounds polymerized within 48 h of synthesis.
initiated reaction in *Mycobacterium vaccae* JOB5 (33) and *Nocardia* sp. ENV425 (34). TBA is oxidized to 2-hydroxybutyric acid and is subsequently metabolized via the tricarboxylic acid cycle. Morpholine is degraded by morpholine monoxygenase in *Mycobacterium* strains MO1 and HE5 (31, 32). The first step of the degradative pathway is cleavage of the C–N bond to form an intermediary amino acid, which is followed by deamination and oxidation of this amino acid into diglycolic acid. The diacid is analogous to dihydroxy-substituted ethyoxycetic acids produced during dioxane degradation.

This is the first report of the intermediates generated during growth-supporting degradation of dioxane by *P. dioxanivorans* CBI190, as well as during cometabolic degradation of dioxane by several monooxygenase-expressing strains. A pathway for mineralization of dioxane to CO$_2$ via monooxygenase-mediated inactivation of 1,4-dioxane using hydroperoxide and ozone. *Mycobacterium* sp. JOB5 (33) and *Nocardia* sp. strain K1.

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**Literature Cited**