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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 intermediates were identified as 2-hydroxyethoxyacetic acid (HEAA), ethylene glycol, glycolate, and oxalate. Studies with uniformly labeled ^{14}C dioxane showed that over 50% of the dioxane was mineralized to CO_2 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 CO_2. 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 CO_2 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 $= 5 \times 10^{-6}$ atm m^3 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 toluene-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 tetrahydrofuranoxydans* 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 P_{450} 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

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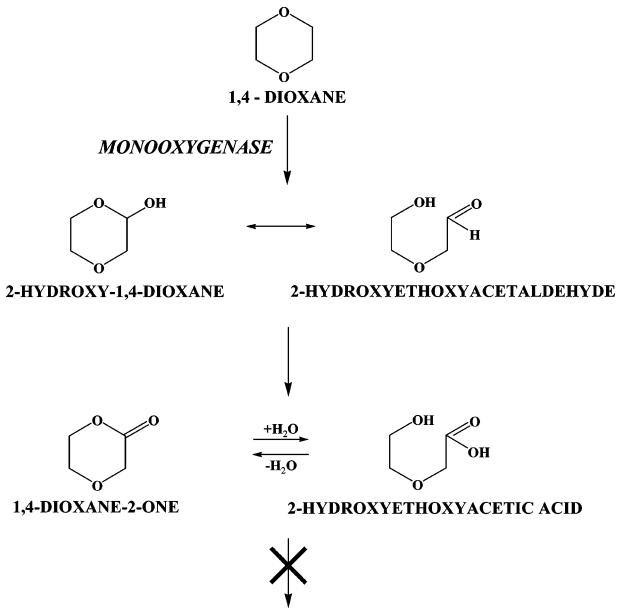

FIGURE 1. Dioxane degradation pathway previously reported for mammalian cells (*21*, *22*) and aerobic bacteria (*14*).

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, glycoaldehyde, 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 Burdick and Jackson (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* JOB5 (*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 nitrate 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 centrifugation 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-monooxygenase-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 quadropole ion trap experiments was carried out using a 1g C^{18} 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-3000 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 TurboIonSpray 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

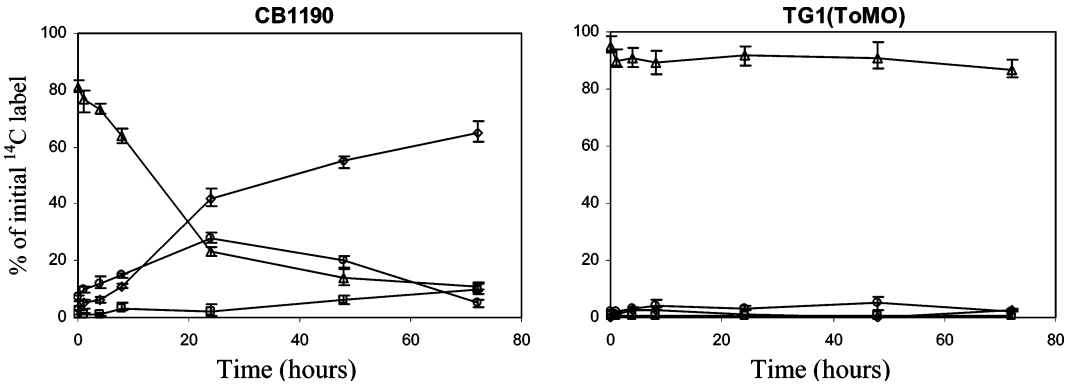

FIGURE 2. Time-course distribution of ^{14}C derived from the degradation of uniformly labeled 1,4-dioxane by strains CB1190 and TG1(ToMO). △ = non-volatile intermediates + dioxane, ◇ = CO_2, ○ = volatile organic acids, and □ = cells associated. Values are expressed as a percent of initial ^{14}C radiolabel, and the error bars represent the range of six replicates.

using the standards of pure compounds. Data acquisition was performed by Analyst 1.4 (Applied Biosystems and MDS Sciex).

High-resolution mass spectra were acquired on a Bruker Apex-Q Fourier transform ion cyclotron resonance-mass spectrometer (FTICR-MS, Bruker Daltonics, Billerica, MA) equipped with a 9.4 T actively shielded magnet. Processed cell culture supernatants, from SPE described previously, diluted to achieve a 7:1 methanol/H_2O solution were introduced into the mass spectrometer at 2 μL/min using an Apollo (Bruker Daltonics) pneumatically assisted electrospray source in negative ion mode. After ionization, ions were accumulated in an rf-only hexapole for 1 s before being transferred to the ICR cell for mass analysis. 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.

^{14}C Analyses. The mineralization and assimilation of dioxane by cultures and the formation of products were tested by analyzing ^{14}C labeled products in the headspace, cell biomass, and culture medium of cells fed uniformly ^{14}C-labeled dioxane. After removing the growth substrate from cell cultures, ^{14}C-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 $^{14}CO_2$ 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 ^{14}C. The cell-associated ^{14}C 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 N_2 for 10 min. The non-purgeable acid fraction represented the non-volatile compounds, including dioxane. Volatile organic acids were calculated by subtracting the non-purgeable base counts from the total counts, and CO_2 was calculated by subtracting non-purgeable acid from non-purgeable base counts. The identity and amount of radiolabeled CO_2 was confirmed by converting it to carbonate in NaOH traps and precipitating it with $BaSO_4$ in separate experiments. The liquid in each bottle was flushed with 200 mL/min N_2 for 30 min to transfer CO_2 and any volatile organics into a trap containing 20 mL of 10 N NaOH. Total dissolved ^{14}C 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 $BaSO_4$ to precipitate all CO_2 as $BaCO_3$. 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 $BaSO_4$ treatment gave the amount of $^{14}CO_2$. The counts in the traps after $BaSO_4$ treatment represented the volatile degradation products.

Results

Mineralization of Dioxane. Uniformly ^{14}C labeled dioxane was used to test for mineralization to CO_2 by strains capable of degrading dioxane. The distribution of dioxane and its degradation products into cell biomass, CO_2, 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 CO_2 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, CO_2, or volatiles (Figure 2).

The final distribution of ^{14}C 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% ^{14}C recovered as CO_2. 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 $[HEAA-H]^-$ 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

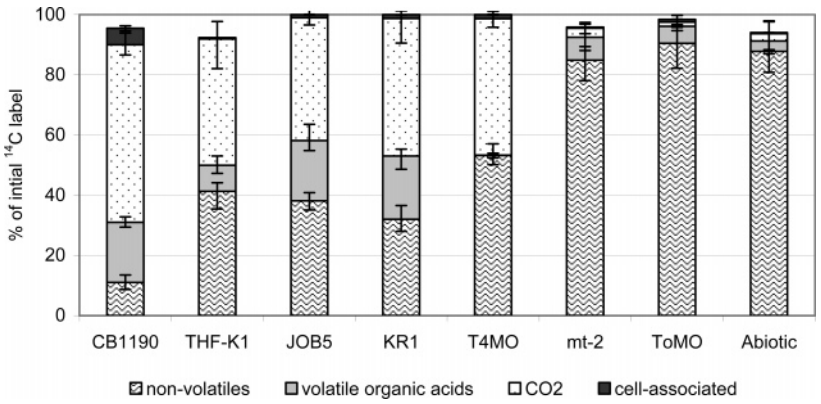

FIGURE 3. Final distribution of ^{14}C derived from the degradation of dioxane after 48 h incubation. Strain metabolizing dioxane: CB1190; strains cometabolizing dioxane: K1, JOB5, KR1, and TG1(T4MO); and strains incapable of degrading dioxane: mt-2 and TG1(ToMO). Values are expressed as a percent of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

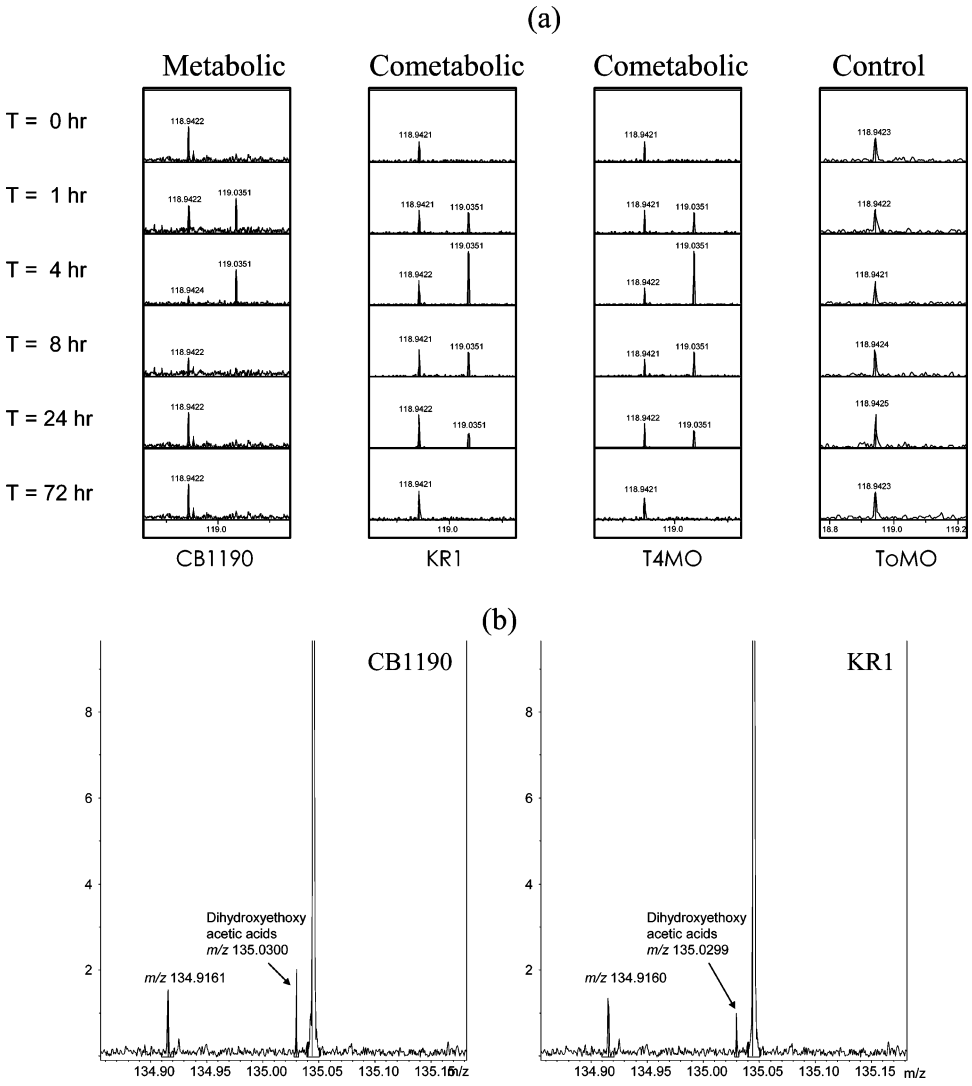

FIGURE 4. Transient detection of HEAA. (a) FTICR mass spectra of HEAA as a metabolite of dioxane degradation by CB1190, KR1, and TG1(T4MO). HEAA peak is at *m/z* 119.0351, and $NaHPO_4^-$ (a component of bacterial growth medium) is at *m/z* 118.9422. Dioxane concentration decreased to below the GC-FID 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 $C_4H_7O_5^-$ (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 $CaPO_4^-$, a component of the medium.

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

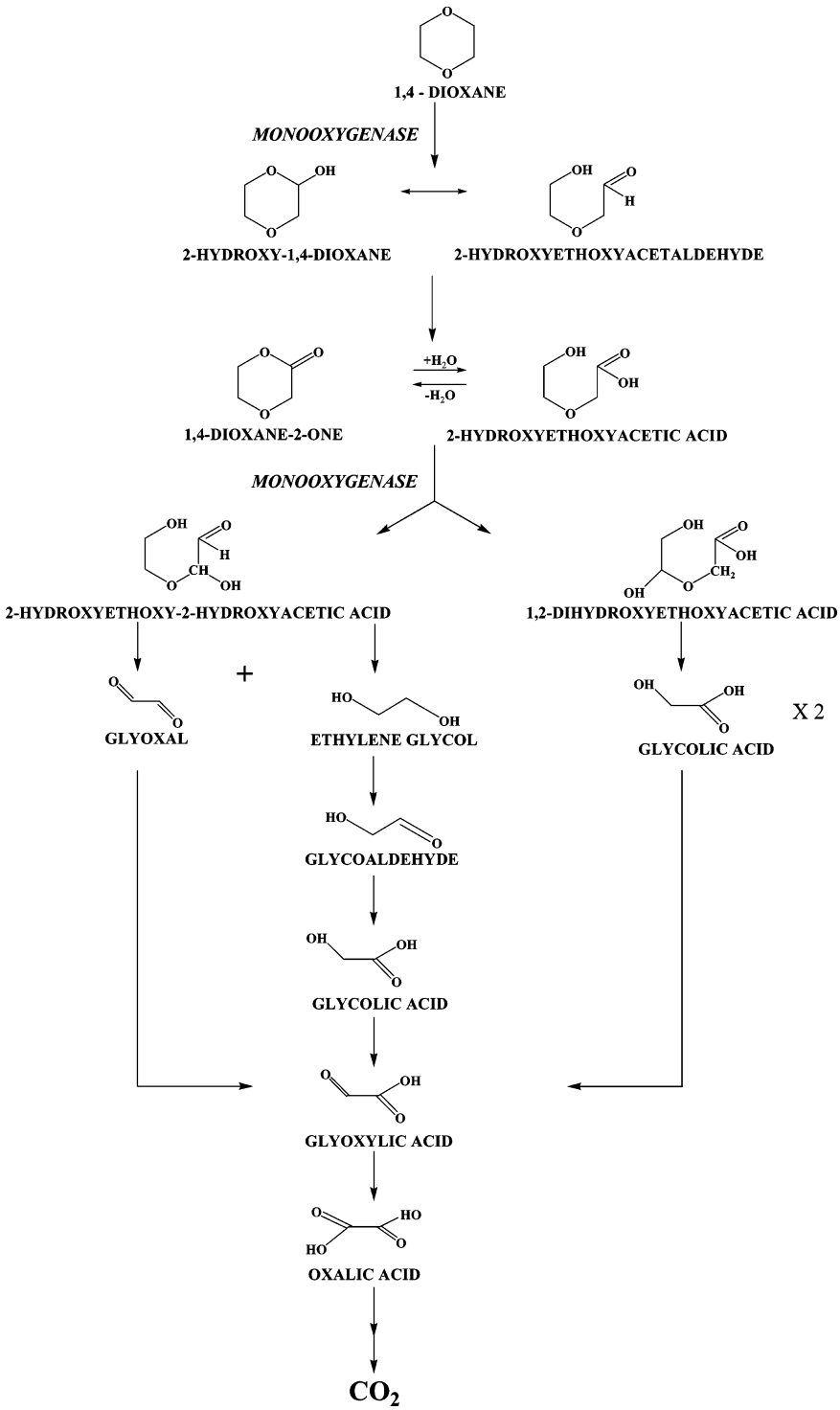

FIGURE 5. Observed biodegradation pathway of 1,4-dioxane by monooxygenase-expressing bacteria in this study. All degradation products shown in this pathway were identified using a combination of analytical techniques.

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-

TABLE 1. Products of 1,4-Dioxane Degradation by Monooxygenase-Expressing Bacteria

compound	accurate mass	C oxidation state	detection	growth of CB1190[a]
1,4-dioxane	88.0524	−1	GC-FID	yes
2-hydroxy-1,4-dioxane	104.0401	−0.5	FTICR-MS[a]	n.t.[b]
2-hydroxyethoxyacetaldehyde	104.0401	−0.5	FTICR-MS[a]	n.t.[b]
1,4-dioxane-2-one (dioxanone)	102.0246	0	FTICR-MS[b]	n.t.[c]
2-hydroxyethoxyacetic acid (HEAA)	120.0351	0	FTICR-MS[b]	n.t.[c]
1,2-dihydroxyethoxyacetic acid	136.0300	+0.5	FTICR-MS[a]	n.t.[b]
2-hydroxyethoxy-2-hydroxyacetic acid	136.0300	+0.5	FTICR-MS[a]	n.t.[b]
1,2-ethanediol (ethylene glycol)	62.04	−1	MS-MS	yes
2-hydroxyethanoic acid (glycolic acid)	76.02	+1	MS-MS	yes
2-hydroxyethanal (glycoaldehyde)	60.02	0	MS-MS	yes
ethanedial (glyoxal)	58.01	+2	MS-MS	yes
2-oxoethanoic acid (glyoxylic acid)	74.00	+2	MS-MS	yes
1,2-dihydroxydioic acid (oxalic acid)	89.99	+3	MS-MS	yes
formic acid	46.00	+2	MS-MS	yes
carbon dioxide	44	+4	^{14}C LSC	n.t.

[a] 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.

tainly formed, although 1,2-dihydroxyethoxyacetic acid might also be present. After the cleavage of the second ether bond, low molecular weight compounds such as ethylene glycol, glyoxal, glycoaldehyde, glycolic acid, glyoxylic acid, and oxalic acid are progressively formed in an oxidizing environment (Figure 5). These compounds are easily mineralized to CO_2 via common cellular metabolic pathways. To confirm this pathway, all intermediates were identified by using high-resolution mass spectrometry (Table 1).

Discussion

In this study, the intermediates and end products of dioxane degradation by several bacterial isolates were identified. In all cases, dioxane was mineralized without the accumulation of toxic byproducts. A biochemical pathway was derived from the products conclusively identified during dioxane degradation.

The degradation pathway of dioxane by mammalian P_{450}s was previously reported in studies investigating the carcinogenicity of dioxane (*21*, *22*). The major metabolite of dioxane was identified as HEAA. In a recent study, HEAA was detected as the terminal product of dioxane degradation by *Pseudonocardia* strain ENV478 (*14*). Neither mineralization of dioxane nor its assimilation into biomass was observed. Parales et al. (*12*) reported CO_2 production during 1,4-dioxane degradation by CB1190, but analyses of the culture medium as well as headspace did not reveal any accumulated intermediates. In contrast, HEAA was not detected during dioxane degradation by the fungus *Cordyceps sinensis*. The intermediates of dioxane degradation were identified as ethylene glycol, glycolic acid, and oxalic acid. The authors suggested that etherases or oxidases were involved in the degradation, but this was not confirmed during their study.

Although it has been previously reported that the first step of dioxane degradation is catalyzed by monooxygenases expressed by the strains tested in this study (*19*), the transient detection of pathway intermediates including HEAA leading to mineralization is a novel result. For strains cometabolizing dioxane, monooxygenases corresponding to the primary growth substrates were induced. These enzymes were responsible for carrying out the first oxidation of dioxane to produce HEAA. Acetylene is a known inhibitor of monooxygenases (*30*). Consequently, the non-appearance of HEAA in acetylene-treated cells further confirms that dioxane is degraded by monooxygenases. It is likely that the same monooxygenases also catalyze the attachment of a second oxygen atom to the carbon of the ether bond in HEAA. The strongest evidence for the second monooxygenation is the oxidation of HEAA in recombinant *E. coli* TG1(T4MO) cells, which express only a single monooxygenase, T4MO, derived from KR1. Attempts to confirm the role of T4MO in the second monooxygenation were hindered by the rapid polymerization of chemically synthesized HEAA/1,4-dioxane-2-one. Similarly, in the dioxane-grown strain CB1190, a putative 1,4-dioxane monooxygenase was responsible for the degradation of dioxane (*19*). Since THF-grown CB1190 and K1 degrade both THF and dioxane using a monooxygenase catalyzed oxidation (no growth or degradation after acetylene exposure), this enzyme is presumed to be homologous to a THF monooxygenase characterized in strain K1 (*20*). It is likely that the same enzyme catalyzed hydroxylation of another ether bond in HEAA leading to energy generation by downstream reactions conducive to the growth of CB1190 using dioxane as a sole carbon and energy source.

Unlike previous reports of HEAA as the terminal product of dioxane degradation, HEAA was further oxidized to carbon dioxide in all strains tested in this study. Vainberg et al. (14) concluded that ENV478 did not mineralize dioxane or use it as a growth substrate primarily due to its inability to degrade HEAA (*14*). In our study, CB1190 cells degrading dioxane for growth were able to overcome HEAA toxicity and also generate energy from downstream reactions to sustain growth. On the basis of 16SrRNA gene sequences, *Pseudonocardia* strain ENV478 is phylogenetically closer to strain K1 than CB1190 (*14*). Unlike CB1190, both ENV 478 and K1 cometabolize dioxane, but K1 does not accumulate HEAA like ENV478. While ENV478 can degrade MTBE, strains CB1190 and K1 do not. Further, CB1190 does not have a constitutive dioxane-degrading capability similar to that of strain ENV478. Minor differences in the monooxygenases of these strains could be causing differences in the affinity for HEAA and catalytic behavior. While strains cometabolizing dioxane exhibited limited transformation capacity for dioxane in the absence of primary substrates, they were able to degrade all HEAA formed to small organic molecules, which were metabolized by the tricarboxylic acid cycle, and subsequently mineralized to CO_2.

Compounds similar to dioxane, including ethers such as THF and methyl-*t*-butyl ether (MTBE), and heterocyclics such as morpholine are degraded by monooxygenase-expressing bacteria (*20*, *31*−*34*). Biodegradation pathways analogous to the dioxane degradation pathway have been reported for these compounds. THF is converted to butyrolactone following hydroxylation of the ether carbon in a *Rhodococcus* strain (*9*). In strain K1, butyrolactone is thought to be transformed into 4-hyroxybutanoic acid and succinate, which are metabolized by the tricarboxylic acid cycle (*20*). MTBE is converted to *t*-butyl alcohol (TBA) by a monooxygenase-

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 monooxygenase 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 ethoxyacetic acids produced during dioxane degradation.

This is the first report of the intermediates generated during growth-supporting degradation of dioxane by *P. dioxanivorans* CB1190, 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 mechanisms is proposed. Biodegradation of dioxane via this pathway is unlikely to cause accumulation of toxic byproducts in the environment.

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