Kinetics of 1,4-Dioxane Biodegradation by Monooxygenase-Expressing Bacteria

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1,4-Dioxane is a probable human carcinogen, and an important emerging water contaminant. In this study, the biodegradation of dioxane by 20 bacterial isolates was evaluated, and 13 were found to be capable of transforming dioxane. Dioxane served as a growth substrate for *Pseudonocardia dioxanivorans* CB1190 and *Pseudonocardia benzenivorans* B5, with yields of 0.09 g protein g dioxane^{-1} and 0.03 g protein g dioxane^{-1}, respectively. Cometabolic transformation of dioxane was observed for monooxygenase-expressing strains that were induced with methane, propane, tetrahydrofuran, or toluene including *Methylosinus trichosporium* OB3b, *Mycobacterium vaccae* JOB5, *Pseudonocardia* K1, *Pseudomonas mendocina* KR1, *Ralstonia pickettii* PKO1, *Burkholderia cepacia* G4, and *Rhodococcus* RR1. Product toxicity resulted in incomplete dioxane degradation for many of the cometabolic reactions. Brief exposure to acetylene, a known monooxygenase inhibitor, prevented oxidation of dioxane in all cases, supporting the hypothesis that monooxygenase enzymes participated in the transformation of dioxane by these strains. Further, *Escherichia coli* TG1/pBS(Kan) containing recombinant plasmids derived from the toluene-2- and toluene-4-monooxygenases of G4, KR1 and PKO1 were also capable of cometabolic dioxane transformation. Dioxane oxidation rates measured at 50 mg/L ranged from 0.01 to 0.19 mg hr^{-1} mg protein^{-1} for the metabolic processes, 0.1–0.38 mg hr^{-1} mg protein^{-1} for cometabolism by the monooxygenase-induced strains, and 0.17–0.60 mg hr^{-1} mg protein^{-1} for the recombinant strains. Dioxane was not degraded by *M. trichosporium* OB3b expressing particulate methane monooxygenase, *Pseudomonas putida* mt-2 expressing a toluene side-chain monooxygenase, and *Pseudomonas* JS150 and *Pseudomonas putida* F1 expressing toluene-2,3-dioxygenases. This is the first study to definitively show the role of monooxygenases in dioxane degradation using several independent lines of evidence and to describe the kinetics of metabolic and cometabolic dioxane degradation.

Introduction

1,4-Dioxane (hereafter referred to as dioxane) is widely used as a stabilizer for chlorinated solvents such as 1,1,1-trichloroethane (TCA). Dioxane is also used as a wetting agent in paper and textile processing and in the manufacture of several organic chemicals (*1*). Dioxane is a probable human carcinogen (*2*) that has been detected as a contaminant in surface waters and groundwaters (*3*–*5*). Volatilization and sorption are not significant attenuation mechanisms for dioxane due to its complete miscibility with water, low volatility (Henry's Law constant = 5×10^{-6} atm-m^{3}mol^{-1} at 20° C), and hydrophilic nature (log K_{ow} = −0.27) (*1*). Photocatalytic oxidation is effective in treating dioxane in water, but is often expensive, and for groundwater contamination, requires ex situ treatment with pump-and-treat operations (*6*, *7*). Although successful phytoremediation of dioxane by hybrid poplar trees has been reported (*8*), laboratory studies suggested that most of the dioxane transpired from leaf surfaces into the air instead of undergoing biodegradation.

The metabolism of dioxane in mammalian systems has been fairly well studied while evaluating the nature of its carcinogenicity. Evidence from animal studies indicates that dioxane biotransformation involves NADH-dependent oxidation by cytochrome P-450 monooxygenase enzymes (*9*, *10*). Several ethers, such as tetrahydrofuran and methyl *tert*-butyl ether, have also been shown to degrade via cytochrome P-450-catalyzed monooxygenation (*11*, *12*). Since bacterial monooxygenase enzymes are similar in structure, function, and reaction mechanisms to mammalian P-450 enzymes, it is logical that these enzymes may also have the ability to degrade dioxane in reactions analogous to their mammalian counterparts. Indeed, the broad specificity of monooxygenases induced by substrates such as methane (*13*), propane (*14*), toluene (*15*), butane (*16*), and ethene (*17*) has been widely reported.

Bioremediation may be an attractive option for the treatment of dioxane as it has been demonstrated that dioxane can be degraded by bacteria via both metabolic and cometabolic reactions. Aerobic biodegradation of high concentrations of dioxane by mixed cultures in industrial sludge has been previously reported (*18*–*21*). Bacterial strains *Mycobacterium vaccae* JOB5 (*22*) and *Pseudonocardia* K1 (*23*), as well as a fungus, *Aureobasidium pullmans* (*24*), have been shown to degrade dioxane when grown on alternate substrates, but dioxane oxidation did not support growth of these organisms. To date, only two bacterial strains, *Rhodococcus* strain 219 (*25*) and *Pseudonocardia dioxanivorans* CB1190 (*26*, *27*), and one fungus *Cordyceps sinensis* (*28*), have been reported to be capable of using dioxane as sole energy and carbon source. In addition, mineralization of dioxane to CO_2 has been reported for both growth-sustaining and cometabolic reactions (*21*, *25*, *27*). However, little research has been conducted to identify the responsible enzymes involved in the dioxane degradation or to quantify the kinetics of the degradation reactions.

In this study, we evaluate the capabilities of monooxygenase-expressing bacteria for degrading dioxane, measure oxidation kinetics for metabolic and cometabolic degradation, and establish links between enzyme activity and dioxane degradation.

Materials and Methods

Chemicals. All chemicals used in medium preparation were of ACS reagent grade or better. Dioxane (99.8%) and toluene (99.5%) were obtained from Sigma-Aldrich, Milwaukee, WI. High purity methane, ethene, and propane gases (>99.9%) were purchased from Matheson Gas products. Deionized water from a Barnstead Nanopure II system was used for all experiments.

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Laboratory Strains. *Pseudonocardia dioxanivorans* CB1190 (*26*, *27*) was supplied by Dr. Rebecca Parales, University of California, Davis. *Pseudonocardia benzenivorans* (*29*), *Pseudonocardia sulfidoxydans* (*30*), *Pseudonocardia hydrocarbonoxydans* (*31*), and *Pseudonocardia* strain K1 (*23*) were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ). *Methylosinus trichosporium* OB3b (*13*) was purchased from the American Type Culture Collection (ATCC). *Mycobacterium vaccae* JOB5 (*22*) was supplied by Professor Daniel Arp, Oregon State University, and *Methylobium petroleophilum* PM1 (*32*) by Professor Kate Scow, University of California, Davis. The toluene-oxidizers *Pseudomonas mendocina* KR1 (*33*), *Ralstonia pickettii* PKO1 (*34*), *Burkholderia cepacia* G4 (*35*), *Pseudomonas putida* mt-2 (*36*), *Pseudomonas* sp. JS150 (*37*), and *Pseudomonas* putida F1 (*38*), were supplied by Professor Jerome Kukor, Rutgers University. *Rhodococcus* RR1 was previously isolated from gasoline contaminated soils (*39*). *Escherichia coli* pCR 2.1-TOPO was procured from Invitrogen (Carlsbad, CA). *Escherichia coli* strains TG1/pBS(Kan) containing constitutively expressed toluene monooxygenases (*40*−*42*) were provided by Professor Thomas Wood, Texas A&M University.

Culture Conditions. Strain CB1190 was grown in ammonium mineral salts (AMS) liquid medium (*27*) with 5 mM dioxane. All other monooxygenase-expressing strains were grown on nitrate mineral salts (NMS) medium (*43*) with inducing substrate amendments. Growth substrates were added to the medium from aqueous stock solutions to achieve final concentrations of 0.1 M for glucose and approximately 5 mM for dioxane, THF, toluene, and MTBE. Methane, ethene, and propane were supplied by injecting 25% (vol/vol) of the gas into the sealed liquid culture flasks. The copper salt was excluded from the medium when induction of soluble methane monooxygenase in OB3b was desired (*44*). Further, the activity of soluble methane monooxygenase was confirmed by the naphthalene oxidation assay (*45*). For strains PKO1 and JS150, NMS was amended with 0.1% (wt/vol) casamino acids to increase yield without affecting the expression of the oxygenase enzyme (*46*). The recombinant *E. coli* strains were grown in Luria Bertani broth (Becton Dickinson, Sparks, MD) containing 100 mg/L kanamycin. All cultures were incubated aerobically at 30 °C while shaking at 150 rpm and aseptic conditions were maintained to avoid contamination. The culture medium was always less than 20% of the total flask volume to prevent mass transfer limitations of oxygen and other gases. Cultures were also maintained on plates containing 1.5% Bacto agar and LB, R2A, AMS, or NMS medium.

Experimental Approach. The cells were harvested in mid to late exponential phase. Prior to adding dioxane, the gaseous growth substrates were removed to prevent inhibition by purging the cell cultures for 5 min with 300 mL/min nitrogen followed by transfer into new aerated vials. Nonvolatile growth substrates were removed by centrifuging at 15 000 xg for 5 min followed by resuspension in fresh NMS medium in new vials. Biodegradation experiments were conducted at 30 °C in 26 mL vials containing 5 mL cell suspensions. To investigate the effect of acetylene on dioxane degradation, the cells were exposed to 5% acetylene in the headspace for 10 min while shaking, followed by 5 min nitrogen stripping to remove residual acetylene gas. Nonexposed cells were treated identically, with no acetylene addition. Degradation experiments were conducted by adding various concentrations of dioxane to the experimental vials and measuring their disappearance over time. Degradation rates were calculated by averaging at least two slopes of dioxane disappearance within the first 30 min of the experiment. Transformation capacities (T_c; mg dioxane degraded (mg cell protein)$^{-1}$) were measured for cultures cometabolizing dioxane. Transformation capacities were calculated by subtracting the concentration of dioxane remaining in vials incubated for an additional 24 h after dioxane disappearance ceased from the initial total concentration of dioxane and normalizing to initial cell protein concentration. Product toxicity was examined by measuring the oxidation of primary growth substrate by cells after they had degraded dioxane. Duplicate or triplicate vials and cell-free controls were used in all experiments. Abiotic loss of dioxane in the controls was consistently less than 5%.

Oxygenase Activity Assays. To monitor for monooxygenase enzyme expression by bacteria on agar plates, a colorimetric naphthalene assay was performed (*47*). A few naphthalene crystals were sprinkled in the lid of Petri dishes. The inverted dishes were stored in air at room temperature. After 15 min incubation, the dishes were opened and sprayed with freshly hydrated 5-mg/mL *o*-dianisidine (tetrazotized; zinc chloride complex, Sigma Chemical Company, St. Louis, MO). The appearance of a purple-red color indicated monooxygenase activity because naphthalene is oxidized to a mixture of 1-naphthol and 2-naphthol by monooxygenases. The naphthols were colorimetrically detected by reaction with tetrazotized *o*-dianisidine to form purple diazo dyes. A similar assay was performed in samples taken from liquid cultures (*45*). Toluene dioxygenase activity was confirmed by indole oxidation assay (*48*). Duplicate uninoculated media controls were also tested.

Analytical Methods

Biomass Quantification. Cell biomass was quantified as total protein concentration (*49*). Bovine serum albumin was used as a standard for the Coomassie Plus protein assay kit (Pierce Chemical Company, Rockford, IL). Serial dilutions were prepared to achieve final protein concentrations within the linear range of the assay.

Gas Chromatography. Liquid culture samples were used to analyze for dioxane by filtering with 0.45 μm syringe filters to eliminate the cells and injecting 5 μL samples 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 °C, 120 °C, and 250 °C, respectively. The dioxane peak was observed at a retention time of 6 min. Calibration curves were prepared from standards generated over concentration ranges that spanned those of the experiments, and the detection limit was 1 mg/L.

Respirometry. The consumption of dissolved oxygen during dioxane degradation was monitored using a YSI 5300A biological oxygen monitor (YSI Life Sciences, Yellow Springs, OH). Dioxane-free controls were used to correct for cellular respiration.

Results

Degradation of Dioxane by Bacterial Strains. *Strains Capable of Growth on Dioxane.* Two of the bacterial strains used in this study were capable of sustained growth on dioxane as a sole carbon and energy source (Table 1). *P. dioxanivorans* CB1190 (*26*, *27*) has been previously reported to grow on dioxane while the ability of *P. benzenivorans* B5 to do so has not been shown previously. Brief exposure to acetylene gas inhibited dioxane degradation by both cultures and neither culture was capable of growth on dioxane in the presence of acetylene. Although dioxane-degrading activity was not recovered when acetylene was removed from the cell cultures, suggesting irreversible enzyme inactivation, the cells were able to grow on substrates not requiring monooxygenase activity (e.g., glycolate, oxalate) and were able to regenerate dioxane-degrading activity presumably by de novo synthesis of undamaged monooxygenases. Since acetylene is a suicide

TABLE 1. Bacterial Strains Tested for the Ability to Degrade 1,4-Dioxane

bacterial strain	growth substrate	oxygenase expressed[a]	dioxane degradation
Pseudonocardia dioxanivorans CB1190	dioxane	unknown	yes
Pseudonocardia benzenivorans B5	dioxane	unknown	yes
Pseudonocardia K1	THF	tetrahydrofuran MO (*11*)	yes
Pseudonocardia K1	toluene	tetrahydrofuran MO (*11*)	yes
Pseudonocardia sulfidoxydans	THF	unknown	no
Pseudonocardia hydrocarbonoxydans	glucose	unknown	no
Methylosinus trichosporium OB3b	methane	soluble methane MO (*13*)	yes
Methylosinus trichosporium OB3b	methane	particulate methane MO (*49*)	no
Mycobacterium vaccae JOB5	propane	propane MO (*12*)	yes
Rhodococcus RR1	toluene	unknown	yes
Methylibium petroleiphilum PM1	MTBE	unidentified MO	no
Methylibium petroleiphilum PM1	toluene	unidentified MO	no
Burkholderia cepacia G4	toluene	toluene-2-MO (*35*)	yes
Ralstonia pickettii PKO1	toluene	toluene-*p*-MO (*34*)	yes
Pseudomonas mendocina KR1	toluene	toluene-4-MO (*33*)	yes
Pseudomonas putida mt-2	toluene	toluene-side chain-MO (*36*)	no
Escherichia coli TG1(T2MO)	LB broth	toluene-2-MO (*42*)	yes
Escherichia coli TG1(T*p*MO)	LB broth	toluene-*p*-MO (*40*)	yes
Escherichia coli TG1(T4MO)	LB broth	toluene-4-MO (*40*)	yes
Escherichia coli TG1(TMO)	LB broth	toluene-*o*-xylene-MO (*41*)	no
Pseudomonas JS150	toluene	toluene-2,3-DO (*37*)	no
Pseudomonas putida F1	toluene	toluene-2,3-DO (*38*)	no
Escherichia coli pCR 2.1-TOPO	LB broth	none	no

[a] The dominant enzyme expected to be induced/expressed under the growth conditions is indicated along with its citation. In some cases, the identity of the dominant enzyme was undetermined. Strain OB3b was grown in the absence of copper to induce for the soluble methane monooxygenase (sMMO) and in the presence of copper to induce for the particulate form (pMMO); the naphthol assay was used for confirmation. MO, monooxygenase; DO, dioxygenase.

TABLE 2. Estimated Kinetic Parameters[a] of 1,4-Dioxane Biodegradation by a Variety of Cultures[b]

culture (reference)	k_{max} (mg dioxane hr^{-1} mg protein^{-1})	K_s (mg/L)	Y (mg protein mg dioxane^{-1})	Y (e^- eq protein e^- eq dioxane^{-1})	μ_{max} (day^{-1})
CB1190 (this study)	1.1 ± 0.008	160 ± 44	0.09	0.07	2.4
CB1190 (*27*)	1.98		0.02	0.01	0.8
CB1190 (*63*)	0.92 ± 0.29[c]		0.01		-
B5 (this study)	0.1 ± 0.006	330 ± 82	0.03	0.008	0.07
enrichment culture (*21*)	0.05 ± 0.003	13 ± 7.6	-		-
industrial activated sludge (*18*)	0.12	9.9	0.12	0.09	0.34
industrial activated sludge (*20*)		182			0.127

[a] k_{max}, maximum substrate degradation rate; K_s, substrate half-saturation constant; μ_{max}, maximum specific growth rate; Y, cell yield. [b] The following conversions were used: 1 g protein = 0.5 g VSS = 0.4 g TSS = 0.43 g COD (*64, 65*). Molecular weights: dioxane, 88; generic protein, 113. [c] This value is the specific substrate degradation rate at initial dioxane concentration of 100 mg/L.

substrate of both soluble and particulate MMO of methanotrophs (*44*, *50*−*52*), and an inhibitor of monooxygenase activity of several other cultures (*15*, *53*, *54*), these results suggest that the enzymes catalyzing the initial oxidation of dioxane in strains CB1190 and B5 are monooxygenases. Further, dioxane degradation did not occur in the absence of molecular oxygen; and stoichiometric uptake of dissolved oxygen accompanied dioxane degradation by CB1190 within the first minute. The cell yields for strains CB1190 and B5 were 0.09 ± 0.002 mg protein (mg dioxane)$^{-1}$ and 0.03 ± 0.002 mg protein (mg dioxane)$^{-1}$, respectively. When expressed as electron equivalents these yields were 0.07 e^- eq protein (e^- eq dioxane)$^{-1}$ and 0.008 e^- eq protein (e^- eq dioxane)$^{-1}$, respectively (Table 2). The growth kinetics for CB1190 were well described by the Monod equation, with a maximum dioxane degradation rate (k) and half-saturation concentration (K_s), computed as 1.1 ± 0.008 mg dioxane hr^{-1} (mg protein)$^{-1}$ and 160 ± 44 mg/L, respectively, as calculated by a nonlinear regression fit of the model to the data (Figure 1a). Similarly, k and K_s values for strain B5 were calculated as 0.1 ± 0.006 mg dioxane hr^{-1} (mg protein)$^{-1}$ and 330 ± 82 mg/L, respectively (Figure 1b).

Cometabolic Transformation of Dioxane. A variety of bacterial strains capable of expressing mono- or dioxygenase enzymes were investigated for cometabolic biodegradation of dioxane. The cells were initially grown on primary substrates, such as hydrocarbons or ethers, which induce for their respective oxygenase enzymes (Table 1). Several of the monooxygenase-expressing bacteria were able to degrade dioxane, including those induced with methane, propane, THF, and toluene.

Interestingly, although *Pseudonocardia* K1 (*23*) degraded dioxane when induced with tetrahydrofuran, *P. sulfidoxydans* (*30*) and *P. hydrocarbonoxydans* (*31*), did not, even though both strains are taxonomically very close to the two *Pseudonocardia* strains capable of growth on dioxane. When induced by methane, *M. trichosporium* OB3b expresses the particulate form of methane monooxygenase (pMMO) in the presence of copper, and soluble methane monooxygenase (sMMO) under copper-deficient conditions (*44*). This strain was capable of dioxane transformation when expressing sMMO but not when expressing pMMO (Figure 2). *My. vaccae* JOB5 was also capable of dioxane transformation when expressing a propane monooxygenase, as previously reported (*22*). Surprisingly, MTBE-metabolizing *Methylibium petroleiphilum* PM1 (*32*) did not degrade dioxane even though both MTBE and dioxane contain ether bonds.

A variety of organisms that predominantly express toluene mono- and dioxygenases were also tested for their dioxane degrading potential. *Pseudomonas mendocina* KR1 expresses

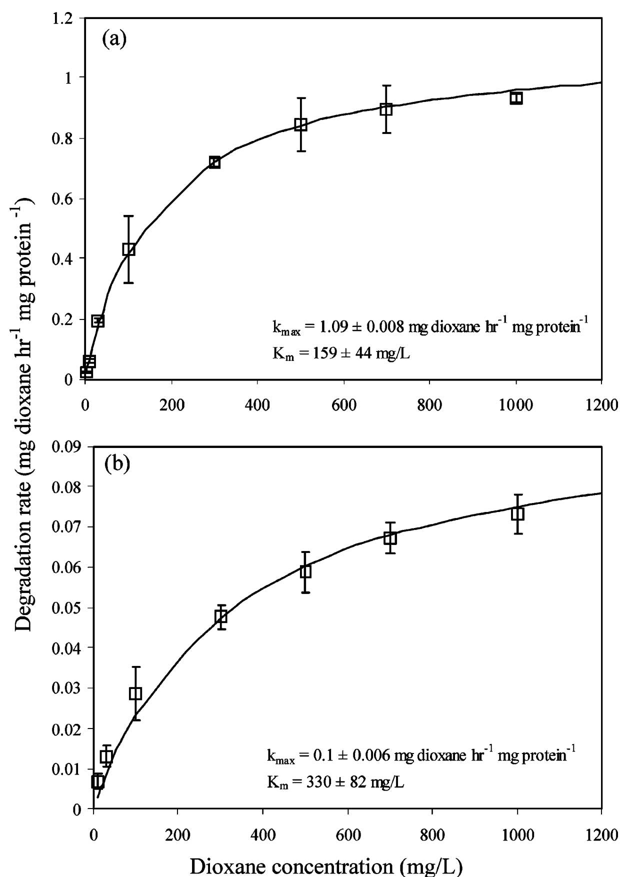

FIGURE 1. Monod plot of dioxane degradation by (a) *Pseudonocardia dioxanivorans* CB1190 and (b) *Pseudonocardia benzenivorans* B5 over a range of concentrations. The rates were calculated as average of slopes during the first 30 min of adding various concentrations of dioxane to the cultures. The initial protein densities were 78 mg/L and 49 mg/L for CB1190 and B5, respectively, and the increase in protein was negligible within 30 min. The parameters k_{max} and K_m were calculated by nonlinear regression minimizing the difference between measured and modeled rates at each concentration. The error bars represent the range of calculated values from each replicate.

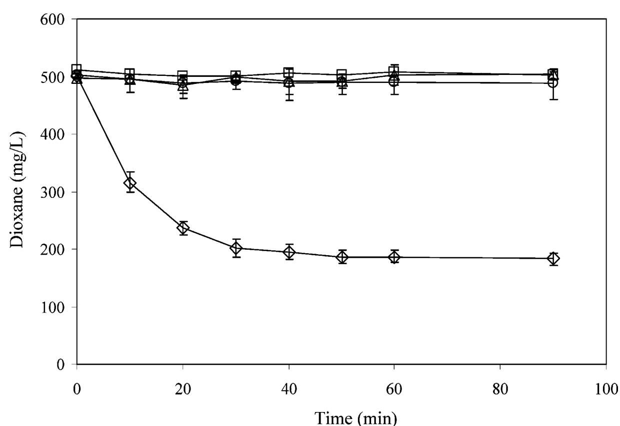

FIGURE 2. Dioxane transformation by *Methylosinus trichosporium* OB3b (~160 mg protein/L). ◇, cells expressing soluble methane monooxygenase (sMMO); △, cells expressing sMMO exposed to acetylene; ○, cells expressing particulate MMO; □, abiotic control. The error bars represent the range of triplicates.

toluene-4-monooxygenase (T4MO) (*33*), *Ralstonia pickettii* PKO1 expresses toluene-*para*-monooxygenase (T*p*MO) similar to T4MO of KR1 (*34*), *Burkholderia* cepacia G4 expresses a toluene-2-monooxygenase (T2MO) (*35*), and *Pseudomonas putida* mt-2 expresses toluene-side chain-monooxygenase (TMO) (*36*). *Pseudomonas putida* F1 (*38*) and *Pseudomonas* JS150 (*37*) express toluene-2,3-dioxygenases. In addition, *Rhodococcus* sp. RR1 expresses an uncharacterized oxygenase when grown on toluene (*39*). The strains expressing T2MO, T4MO, and the unidentified oxygenase degraded dioxane while those expressing TMO or toluene dioxygenase did not have this capability. Further, *Escherichia coli* strain pCR 2.1-TOPO, which does not express an oxygenase, was unable to biodegrade dioxane.

Brief exposure to acetylene gas inhibited dioxane degradation by all cometabolic cultures (e.g., Figures 1 and 2) and short-term (<1 min) dioxane transformation was accompanied by stoichiometric oxygen uptake, supporting the hypothesis that the monooxygenase is responsible for dioxane degradation.

Application of a colorimetric assay based upon naphthol production that was previously developed as a rapid method for detecting monooxygenase activity (*45*, *47*) demonstrated that dioxane degradation was positively correlated with monooxygenase activity. Further, brief exposure to acetylene inhibited naphthol production by all strains analogous to the results with dioxane degradation. In contrast, the colorimetric assay generated negative results for all strains incapable of dioxane degradation.

The rates of dioxane cometabolism varied from 0.1 to 0.4 mg hour^{-1} (mg protein)$^{-1}$ (Table 3). Among the toluene monooxygenases, cells expressing T4MO and T*p*MO degraded dioxane approximately four times faster rates than cells expressing T2MO when incubated at similar cell densities (100 mg protein L^{-1}).

Cometabolism of Dioxane by Recombinant E. coli Expressing Toluene Monooxygenases. To confirm that dioxane degradation was catalyzed by monooxygenase enzymes, four strains of *E. coli* TG1/pBS(Kan) containing plasmids coding for distinct toluene monooxygenases were tested. These bacteria were designed to constitutively express the T4MO derived from KR1, T*p*MO derived from PKO1, T2MO derived from G4, and T*o*MO (toluene-*o*-xylene monooxygenase) derived from *Pm. stutzeri* OX1 (*40*−*42*). The advantage of expressing target enzymes in the same host is that the effects of individual strain differences are neutralized. Dioxane transformation behavior of recombinant strains mirrored that of their respective source strains (Figure 3a-3c). Surprisingly, T*o*MO was unable to degrade dioxane even though its toluene oxidation behavior is similar to the aromatic monooxygenases, T4MO and T2MO. Recombinant *E. coli* strains TG1(T4MO), TG1(T*p*MO), and TG1(T2MO) cometabolically degraded dioxane with rates 30−50% lower than those of their source strains (Table 3). Acetylene exposure resulted in the loss of both dioxane- and toluene-degrading activities of all recombinant strains.

Product Toxicity from Dioxane Biodegradation. For most of the cultures that degraded dioxane cometabolically, degradation ceased before the dioxane was completely consumed, indicating that the resting cells have a limited transformation capacity for dioxane (Figures 2 and 3). The measured transformation capacities for each culture are listed in Table 3. Since the primary substrate was removed to prevent competition with dioxane during the cometabolic degradation reactions, this phenomenon could be caused by limited reductant (NADH) supply to fuel the monooxygenase reaction, toxicity due to dioxane, and/or toxicity due to dioxane metabolites. To evaluate whether reductant limitation was responsible, strain OB3b was augmented with 20 mM sodium formate during incubation with dioxane (*55*). Formate provides reducing equivalents to OB3b without affecting monooxygenase activity. The addition of formate did not have any effect on the rate or extent of dioxane transformation (data not shown), indicating that reductant

TABLE 3. Rates of 1,4-Dioxane Degradation by Monooxygenase-Expressing Cultures

bacterial strain	initial dioxane concentration (mg/L)	dioxane degradation rate (mg/hr/ mg protein)[a]	transformation capacity (mg dioxane/ mg protein) [b]
P. dioxanivorans CB1190	50	0.19 ± 0.007	
P. benzenivorans B5	50	0.01 ± 0.003	
Pseudonocardia K1 (THF)	25	0.26 ± 0.013	0.22 ± 0.014
Pseudonocardia K1 (toluene)	25	0.16 ± 0.006	0.23 ± 0.015
M. trichosporium OB3b (sMMO)	50	0.38 ± 0.02	2.3 ± 0.015
My. vaccae JOB5	88	0.40 ± 0.06	1.4 ± 0.007
Rhodococcus RR1	50	0.38 ± 0.03	0.32 ± 0.003
B. cepacia G4	50	0.10 ± 0.006	0.26 ± 0.008
R. pickettii PKO1	50	0.31 ± 0.007	0.29 ± 0.01
Pm. mendocina KR1	50	0.37 ± 0.04	0.38 ± 0.006
E. coli TG1(T2MO)	50	0.06 ± 0.008	0.29 ± 0.005
E. coli TG1(T*p*MO)	50	0.17 ± 0.01	0.27 ± 0.016
E. coli TG1(T4MO)	50	0.26 ± 0.03	0.30 ± 0.02

[a] The degradation rates were calculated as the average of at least two slopes within the first 30 min of adding dioxane to the cultures, and were normalized to total protein concentration. Abiotic losses of dioxane were less than 5%. [b] Transformation capacities were calculated for cultures cometabolizing dioxane by dividing the total concentration of dioxane degraded by initial cell protein concentration. The errors represent the range of replicates.

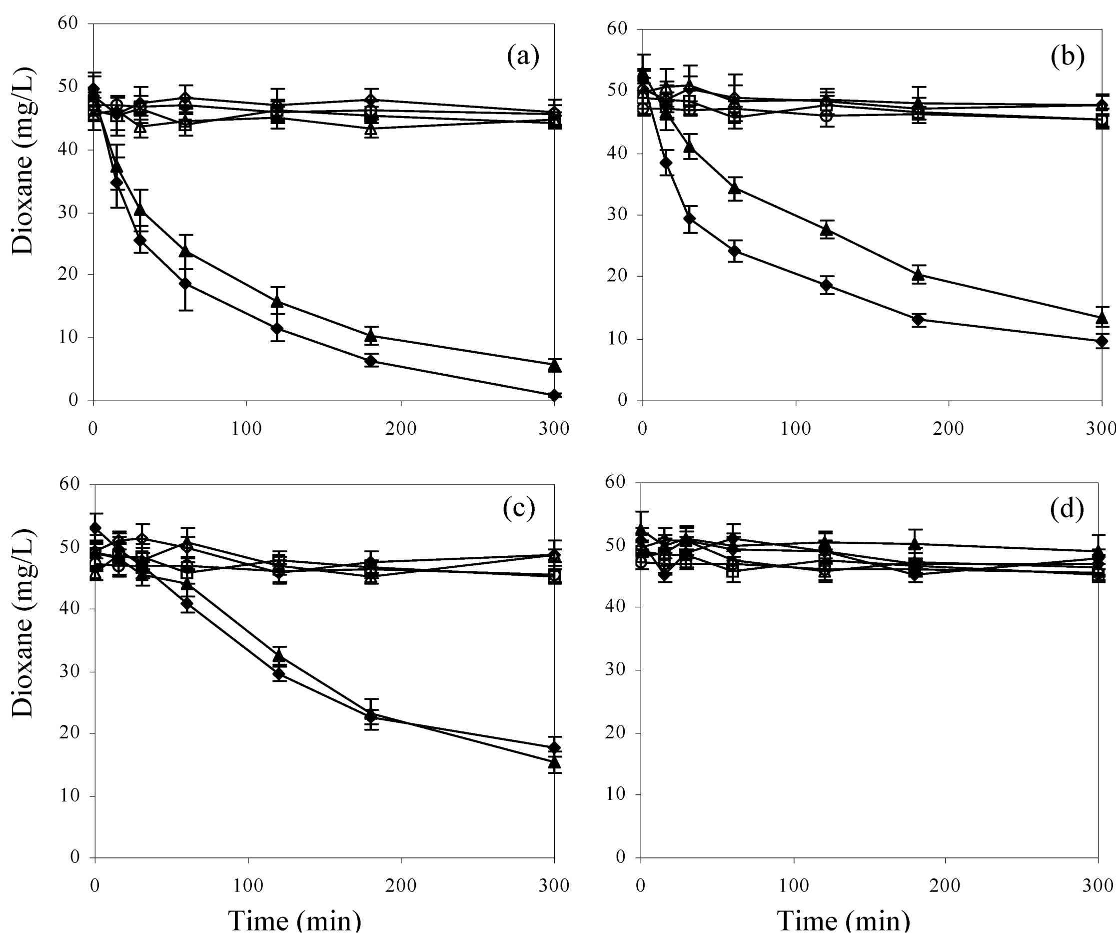

FIGURE 3. Dioxane transformation by source strains and recombinant strains expressing toluene monooxygenases. (a) *Pm. mendocina* KR1 and *E. coli* TG1(T4MO) expressing toluene-4-MO, (b) *R. pickettii* PKO1 and *E. coli* TG1(T*p*MO) expressing toluene-4-MO; (c) *B. cepacia* G4 and *E. coli* TG1(T2MO) expressing toluene-2-MO; (d) *Pm. putida* mt-2 expressing toluene-side chain-MO and *E. coli* TG1(T*o*MO) expressing toluene-*o*-xylene-MO. ◆, source strain; ▲, recombinant strain; ◇, acetylene-exposed source strain; △, acetylene-exposed recombinant strain; ○, *E. coli* without monooxygenase insert; □, abiotic control. The error bars represent the range of duplicates.

limitation was not responsible for the observed cessation of cometabolic dioxane oxidation. Similarly, it is unlikely that dioxane exerts direct toxicity to the cells at the concentrations tested given that CB1190 is able to degrade dioxane at concentrations above 1000 mg/L (Figure 1), while strains JOB5, OB3b, and RR1 tolerate dioxane concentrations as high as 500 mg/L (for example, Figure 2 for OB3b). In contrast, propane oxidation was negligible in propane-grown JOB5 cells after they had degraded 140 mg/L dioxane over a 2-h time period, even after 24 h incubation. Similarly, the oxidation of methane by OB3b cells and the degradation of toluene by strains KR1, PKO1, and G4 were completely inhibited after they had degraded dioxane, suggesting that dioxane transformation products exert irreversible toxicity to the monooxygenase enzyme and/or the cells in general.

Discussion

With recent advances in analytical methods and growing public awareness of the occurrence of dioxane in drinking water supplies, this contaminant is emerging as an important threat to water resources. Consequently, a better understanding of biodegradation as an attenuation mechanism for dioxane in the environment is needed.

Monooxygenase-expressing bacteria belonging to at least six different genera were demonstrated to degrade dioxane with a wide range of rates. While dioxane cometabolism by strains K1 and JOB5 has been previously reported, this is the first study to describe the cometabolic transformation of dioxane by OB3b and the toluene-oxidizing strains. This is also the first report of the ability of strain B5 to utilize dioxane as sole carbon and energy source, expanding the number of isolates reported to have this capability from three to four (bacteria *Rhodococcus* 219 and *Pseudonocardia* strains CB1190 and B5, and fungus *Cordyceps sinensis* (*25*−*29*)). The role of monooxygenases in catalyzing dioxane degradation was demonstrated in a variety of ways. First, dioxane degradation was observed when the cells were grown on substrates that induce for specific monooxygenases. The expression of monooxygenases in each strain was independently confirmed by the strains' abilities to oxidize naphthalene to naphthol. Further, brief exposure to acetylene gas, which serves as an irreversible inhibitor of specific monooxygenases (*15*, *51*−*53*, *56*), inhibited the degradation of dioxane by all the monooxygenase-expressing bacterial strains. Lastly, *E. coli* mutants containing cloned toluene monooxygenases derived from strains KR1, PKO1, and G4 degraded dioxane, and were also inactivated by exposure to acetylene, mimicking the behavior of their source strains. In toto, these data provide strong evidence for the participation of monooxygenases in catalyzing dioxane degradation.

The rates of dioxane degradation in this study varied from 0.01 to 0.94 mg hr^{-1} (mg protein)$^{-1}$ for bacterial isolates. The variability in maximum dioxane degradation rates (k_{max}) in this study and the literature was about 40-fold (0.05−1.98 mg dioxane hr^{-1} (mg protein)$^{-1}$) for pure and mixed cultures, with no clear trend associated with metabolic or cometabolic degradation (Table 2). The maximum growth rates varied over 2 orders of magnitude (0.07−2.4 day^{-1}), as did the half-saturation concentrations (9.9−330 mg/L). While CB1190 exhibited the fastest reported dioxane degradation rates, growth rates on dioxane reported for industrial activated sludge were of a similar order (*18*, *20*). In general, the measured growth rates (μ_{max}) are quite low, mainly because the yields of all cells growing on dioxane are low. The yields are consistently low for all cultures reported in the literature, even on e^- eq/ e^- eq basis.

Cometabolic oxidation of dioxane did not proceed to completion in cases where initial dioxane concentration exceeded the transformation capacity. This phenomenon has also been previously described for dioxane (*20*, *22*, *57*). The theoretical dioxane transformation capacity of a microbial consortium was reported to be 1.0 ± 0.36 mg dioxane mg protein^{-1} by Zenker et al. (*57*), in good agreement with the observed T_c values of the isolates tested in this study that ranged between 0.22 and 2.3 mg dioxane (mg protein)$^{-1}$. Zenker et al. (*57*) concluded that for their enrichment culture, the observed loss of dioxane cometabolizing activity was caused by cofactor depletion. In this study, the lack of primary substrate degradation activity following dioxane transformation suggests that irreversible toxicity by dioxane transformation products was responsible for the activity loss with the monooxygenase-expressing isolates. Roy et al. (*20*) also reported accumulation of toxic products during biodegradation of dioxane by industrial waste sludge. In their study, toxicity to cells was measured in terms of a decrease in ATP luminescence as well as a decrease in oxygen demand. Samples containing accumulated metabolites demonstrated a higher level of toxicity than equivalent samples with pure dioxane. In mammalian cells, the major dioxane metabolite, 1,4-dioxane-2-one, is considerably more toxic than dioxane (*9*), with a lethal dose to rats that is about one-tenth that of dioxane.

While most monooxygenase-expressing strains tested in this study degraded dioxane, bacteria containing particulate MMO, side-chain TMO, and T*o*MO did not. Additionally, these monooxygenases did not test positive in the naphthalene oxidation assay, as has been previously observed (*45*, *56*, *58*). One likely reason for the lack of naphthalene- and dioxane-oxidizing activity observed for the pMMO and the side-chain TMO is the different catabolic properties and substrate affinities of those oxygenases (*35*, *59*). For example, the main products of trichloroethene (TCE) oxidation by pMMO are TCE epoxide and glyoxal (*60*), while sMMO produces chloral hydrate or TCE epoxide depending upon the methanotrophic strain (*61*). While all the tested monooxygenases that have been characterized are known to be multicomponent enzymes (*11*, *13*, *33*−*35*, *49*), and all but pMMO contain diiron centers, there were no observable trends with respect to enzyme structure and dioxane degradation.

The naphthalene oxidation assay has previously been applied to detect the monooxygenase activity of OB3b (*45*) and KR1 (*62*). In this study, the colorimetric assay demonstrated correlation between naphthalene oxidation and dioxane oxidation in all tested species. Since this assay is simple, rapid, and inexpensive to perform, it could be an attractive screening tool for predicting dioxane degradation activity of unknown cultures.

This study identifies phylogenetically distributed bacteria and reports kinetics of metabolic and cometabolic biodegradation of dioxane by monooxygenase-expressing isolates, confirms the role of monooxygenases in the degradation using several independent lines of evidence, and provides evidence for product toxicity associated with the dioxane transformation. These results provide a basis for modeling the kinetics of dioxane degradation. Further, the identification of bacteria capable of dioxane degradation, and the responsible enzymes, will facilitate the optimization of systems to enrich for these organisms and the expression of applicable enzymes in the field.

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