Distinct Catalytic Behaviors between Two 1,4-Dioxane Degrading Monooxygenases: Kinetics, Inhibition, and Substrate Range

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Abstract

Monitored natural attenuation (MNA) and engineered bioremediation have been recognized as effective and cost-efficient *in situ* treatments to mitigate 1,4-dioxane (dioxane) contamination. Dioxane metabolism can be initiated by two catabolic enzymes, propane monooxygenase (PRM) and tetrahydrofuran monooxygenase (THM), belonging to the group 6 and 5 of soluble di-iron monooxygenase family, respectively. In this study, we comprehensively compared catalytic behaviors of PRM and THM when individually expressed in the heterologous host, *Mycobacterium smegmatis* mc^2-155. Kinetic results revealed a half-saturation coefficient (K_m) of 53.0 ± 13.1 mg/L for PRM, nearly four times lower than that of THM (235.8 ± 61.6 mg/L), suggesting PRM has a higher affinity to dioxane. Exposure with three common co-contaminants (1,1-dichloroethene, trichloroethene, and 1,1,1-trichloroethane) demonstrated PRM was also more resistant to their inhibition than THM. Thus, dioxane degraders expressing PRM may be more physiologically and ecologically advantageous than those with THM at impacted sites, where dioxane concentration is relatively low (e.g., 250 to 1,000 μg/L) with co-occurrence of chlorinated solvents (e.g., 0.5 to 8 mg/L), underscoring the need of surveying both PRM and THM encoding genes for MNA potential assessment. PRM is also highly versatile, which breaks down cyclic molecules (dioxane, tetrahydrofuran, and cyclohexane), as well as chlorinated and aromatic pollutants, including vinyl chloride, 1,2-dichloroethane, benzene, and toluene. This is the first report regarding the ability of PRM to degrade a variety of short-chain alkanes and ethene in addition to dioxane, unraveling its pivotal role in aerobic biostimulation that utilizes propane, isobutane, or other gaseous alkanes/alkenes (e.g., ethane, butane, and ethene) to select and fuel indigenous microorganisms to tackle the commingled contamination of dioxane and chlorinated compounds.

Keywords

1,4-Dioxane, Chlorinated Solvents, Soluble Di-iron Monooxygenase, Alkanes, Bioremediation, Monitored Natural Attenuation

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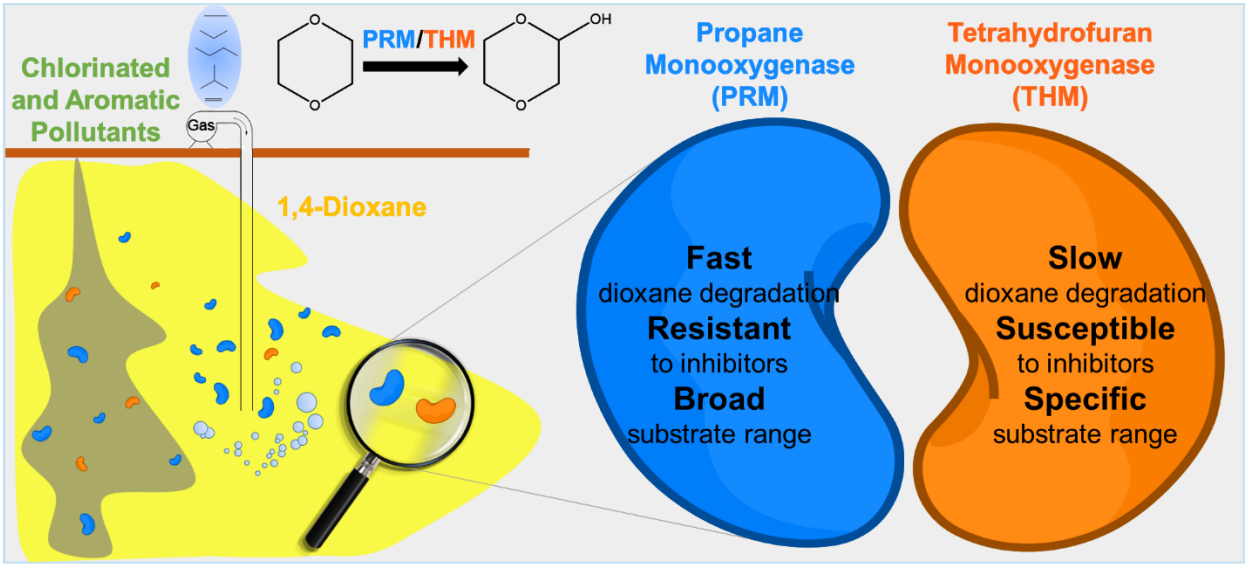

Introduction

1,4-Dioxane (dioxane) has been widely used as a stabilizer for chlorinated solvents particularly 1,1,1-trichloroethane (1,1,1-TCA)[1]. Dioxane has been classified as a possible human carcinogen by USEPA[2, 3] and listed as a "high priority" pollutant in the 2016 amendment of the Toxic Substance Control Act (TSCA)[4, 5]. As a cyclic ether, dioxane exhibits high mobility and persistency once released to the environment. It is recognized as one of the most frequently detected nonregulated pollutants in our water supplies and sources based on the national survey for the Third Unregulated Contaminant Monitoring Rule (UCMR3)[6]. Dioxane's extreme hydrophilicity and water miscibility may also lead to the formation of large dilute plumes with trace concentrations (e.g., < 1 mg/L[7]) in the subsurface[1, 8]. To date, pump-and-treat followed by carbon adsorption or advanced oxidation is a common practice to clean up dioxane in groundwater[9]. However, such *ex situ* remedial efforts are unremitting (e.g., for decades until the closure of the site cleanup) and costly considering the tremendous volume (e.g., over hundred million liters[10]) of polluted water and associated energy input for pumping and operation.

Monitored natural attenuation (MNA) and bioremediation, mainly relying on biodegradation by microorganisms, are cost-efficient and eco-friendly techniques for remediation of dioxane. A number of bacteria have been isolated and identified given their ability of growing with dioxane as their sole carbon and energy source via metabolism[11-13]. *Mycobacterium dioxanotrophicus* PH-06[11, 14] and *Pseudonocardia dioxanivorans* CB1190[12, 15] are two archetypic dioxane degrading strains. In both strains, dioxane degradation is initiated via a critical step named 2-hydroxylation, which inserts a hydroxyl group to the carbon adjacent to the oxygen and forms unstable intermediates that undergo a chain of biotic or abiotic reactions[11, 16]. Key dioxane

63 metabolites were found identical, PH-06 and CB1190 use two different enzymes to catalyze this
64 2-hydroxylation step for the initial ring cleavage of dioxane. In PH-06, we recently uncovered and
65 verified the dioxane catalytic function of a novel propane monooxygenase (PRM)[14, 17] encoded by
66 the gene cluster *prmABCD* located on a linear plasmid. In contrast, CB1190 expresses
67 tetrahydrofuran monooxygenase (THM)[16] encoded by *thmADBC* to oxidize dioxane and
68 tetrahydrofuran (THF). Though with relatively low sequence identity (< 40% for α subunits) and
69 different arrangement of core gene components, PRM and THM are phylogenetically related, both
70 belonging to the multi-component bacterial enzyme family, soluble di-iron monooxygenases
71 (SDIMOs)[14, 18, 19]. PRM and THM are categorized as subgroups 6 and 5 SDIMOs[14, 20, 21],
72 respectively, reflecting the potential divergence of their enzyme structures and catalytic behaviors.

73 Genes encoding THM (e.g., *thmA* and *thmB*) have been detected at sites historically
74 impacted by dioxane, indicating the existence of indigenous dioxane degrading microorganisms
75 by use of modern biotechnologies (e.g., quantitative PCR [qPCR][21-24] and microarray[25]).
76 Abundance of *thm* genes was positively correlated with the dioxane removal observed in bench-
77 scale microcosm and *in situ* Biotrap assays[21, 22, 24], supporting the significant contribution of
78 bacteria expressing THM to intrinsic dioxane attenuation in the field. The discovery of dioxane
79 degrading propanotrophs and the essential PRM enzyme in recent field demonstration studies
80 assayed and validated the dominance of *prm* genes after biostimulation with propane[26] and
81 bioaugmentation of some propanotrophs[27]. qPCR[20] and targeted gene sequencing[28] were used to
82 monitor the dioxane degradation by *prm*-harboring *Mycobacterium* spp. in non-contaminated
83 garden soil enrichments. These lines of evidence corroborate the prevalence of bacteria expressing
84 PRM in engineered or enriched environments with or without previous exposure of dioxane.

However, the contribution of naturally occurring bacteria expressing PRM to the overall dioxane attenuation at impacted sites remained unknown.

To discern dioxane degradation capabilities and influence of environmental factors, previous studies have been centered on characterizing type strains (e.g., CB1190[29-31] and PH-06[11, 20]). By fitting with Michaelis-Menten or Monod model, an array of dioxane degradation kinetic parameters (Table S1) have been generated, including the half-saturation coefficients (K_m) and maximum degradation velocities (V_{max}), as well as the inhibition constants (K_I) for common co-occurring chlorinated solvents. However, to interpret the dioxane attenuation naturally occurring in the field, these kinetic parameters may be of limited value for direct implication because (1) the data lack consistency due to variances in experimental operations among different research laboratories and (2) indigenous dioxane-degrading microbes living in the field may behave differently compared to these isolates grown in laboratory culture media. Though expressing the same enzymes (i.e., PRM and THM) to degrade dioxane, indigenous degraders may not only be phylogenetically and functionally disparate, but also display varied physiologies (e.g., biomass growth, nutrient assimilation, membrane transport, and stress resilience) that affect the overall catabolism effectiveness. An additional important impediment is the practice of normalizing the rate of compound removal to the amount of protein associated with the active cells (e.g., V_{max} values in Table S1). Wilson *et al.* suggested that the lab-derived kinetic parameters could be used along with data on the abundance of catabolic biomarkers to screen for intrinsic degradation activity[32]. Thus, normalization of degradation rates to the abundance of gene or transcript copies measured by quantitative polymerase chain reaction (qPCR) or reverse transcription- quantitative polymerase chain reaction (RT-qPCR) analyses can be extrapolated to the field system within some useful level of agreement.

To circumvent discrepancies derived from experimental operations and physiological differences among hosting bacteria, we heterologously expressed PRM and THM in competent cells *Mycobacterium smegmatis* mc^2-155 and compared their kinetic performance at the enzymatic level, which excludes other potentially interfering biological factors (e.g., molecular transport, gene regulation, global stress response). We further investigated the inhibitory effects of three chlorinated compounds (1,1-dichloroethene [1,1-DCE], trichloroethene [TCE], and 1,1,1-trichloroethane [1,1,1-TCA]) given their high co-occurrence frequency with dioxane at impacted sites[5, 33, 34]. In this study, substrate range of both dioxane degrading enzymes was surveyed to investigate their catalytic versatility, particularly toward prevailing chlorinated and aromatic pollutants, as well as short-chain alkane/alkene gases given their association with the success of biostimulation. We hypothesize distinct performances between PRM and THM in regard of dioxane degradation kinetics, susceptivity to environmental inhibitors, and catalytic versatility given their sequence dissimilarity and evolutionary divergence. The expression of both enzymes is unified in an identical heterologous system and monitored by RT-qPCR, thus allowing kinetic parameters to be normalized based on the transcript copy numbers of their encoding genes, providing useful quantitative data for field assessment. This research is of critical value to advance our fundamental understanding of dioxane degrading enzymes and enable the prediction of their environmental behaviors and contributions to dioxane biotransformation naturally occurring in the field or stimulated with auxiliary substrates.

Materials and Methods

Chemicals and Cultures

Propane, butane, isobutane, ethane, and ethene were purchased from Airgas (Radnor, PA) with the purity of 99.5% or higher. Dioxane, THF, trichloroethene (TCE), 1,1-dichloroethene (1,1-DCE), *cis*-1,2-dichloroethene (cDCE), *trans*-1,2-dichloroethene (tDCE), vinyl chloride (VC), 1,2-dichloroethane (1,2-DCA), toluene, benzene, methyl tert-butyl ether (MTBE), cyclohexane, chloramphenicol, and thiostrepton were purchased from Sigma-Aldrich (St. Louis, MO). Neat 1,1,1-trichloroethane (1,1,1-TCA) was bought from Ultra Scientific (North Kingstown, RI) and diluted with HPLC-grade (99.9%) methanol (Sigma-Aldrich). Bacterial strains PH-06 and mc^2-155 were originally obtained from Dr. Yoon-Seok Chang (POSTECH, Pohang, South Korea) and Dr. Nicolas Coleman (University of Sydney, Sydney, Australia); CB1190 was bought from DSMZ; *E. coli* DH5α was purchased from Thermo (Carlsbad, CA), and the plasmid pTip-QC2 was acquired from Dr. Tomohiro Tamura at AIST, Japan.

Heterologous Expression of PRM and THM

A 4.0 kb fragment of the *prmABCD* cluster and a 4.3 kb fragment of the *thmADBC* cluster were amplified and cloned into the plasmid (pTip-QC2)[14, 16, 35] via digestion and ligation to generate plasmids pTip-*prmABCD* and pTip-*thmADBC*, respectively. Successfully ligated plasmids were first transformed in *E. coli* DH5α cells and screened on Luria-Bertani (LB) agar plates with ampicillin (50 μg/mL). After culturing in LB media for 24 h, *E. coli* transformants were harvested for plasmid extraction using the Zyppy™ Plasmid Miniprep Kit (Zymo Research Corp, Irvine, CA). Then, 50 ng of plasmid pTip-*prmABCD,* pTip-*thmADBC,* or empty vector pTip-QC2 was used to transform electrocompetent *Mycobacterium smegmatis* mc^2-155 cells via electroporation at 1.8 kV/cm for 4.5 ms using the MicroPulser™ Electroporator (Bio-Rad, Hercules, CA). After screening on LB plate with chloramphenicol (34 mg/L), the successful

transformants were designated as mc^2-155(pTip-*prmABCD*), mc^2-155(pTip-*thmADBC*), and mc^2-155(pTip-QC2), respectively. Transformants were then cultured with LB broth dosed with thiostrepton (2 mg/L) to induce the expression for 48 h. Comparable transcription levels (Figure S1) of inserted *prm* and *thm* gene clusters in transformants were checked by reverse transcription quantitative PCR (RT-qPCR) before processing enzyme comparison assays. Detailed transformation procedures can be found in the supporting information (SI) based on the methods developed by Sales *et al.*[16] and Deng *et al.*[14]. The heterologous expression procedures were designed and verified to ensure an identical transcription of both PRM and THM expressed with active catalytic functions. First, the sequence accuracy was ensured since high fidelity polymerase was used to amplify the complete *prm* and *thm* gene clusters from the genomic DNA. This greatly reduced the chance of function discrepancies caused by PCR-derived mutations. Second, transcription of the inserted genes was solely regulated by thiostrepton to induce the promoter system embedded on pTip-QC2. Gene clusters were inserted from their start codons (ATG) of *prm* or *thm*'s α-subunits without their original promoters or regulators in wild-type strains PH-06 or CB1190. Third, complete *prm* and *thm* gene clusters were cloned with the same initial restriction site, *Nde*I, at their 5' ends into the expression shutter vector, pTip-QC2. Thus, the start of the *prm* and *thm* transcripts were identical, allowing the consensus of ribosome binding to initiate the translation. Last but not the least, the gene expression shutter vector, pTip-QC2, plasmid proliferation host (*E. coli* DH5α), and expression host (*Mycobacterium smegmatis* mc^2-155), have all been successfully employed to express THM, PRM, and other SDIMOs in our lab and others[14, 16, 36-38]. This set of expression system enabled effective production of SDIMOs with catalytic functions comparable with wild-type strains.

Enzyme Kinetics and Inhibition Tests

After cultivation and induction as described above, transformant cells were washed twice and re-suspended in phosphate-buffered saline (PBS) to achieve an optical density (OD) of approximately 2.0 at 600 nm. Dioxane was then spiked to achieve the initial concentrations of 10, 40, 80, 160, 320, and 640 mg/L to perform the kinetic assays. Such high initial concentrations were used because PRM and THM both exhibited high K_m and V_{max} values, which were in good agreement with previous studies[20, 29, 31, 39] using wild-type strains (Table S1). Two liquid samples (600 µL) were collected, including one at the beginning and the other after 3 h of the enzymatic reaction in each batch test. Samples were then filtered using 0.22 µm Nylon syringe filters and kept in glass vials at 4 °C prior to the gas chromatograph (GC) analysis. Instant degradation rates were calculated by averaging dioxane disappearance in triplicate within the first 3 h, which were further normalized by the initial protein concentration[30] measured by the Bradford Assay[40]. In addition, to evaluate dioxane degradation kinetics under environment-relevant dioxane contaminations, resting cells were exposed to 1.0 and 0.2 mg/L of dioxane, respectively. All treatments were conducted in triplicate and negative controls were prepared with autoclaved biomass. The significance level among different treatments was statistically determined using the Student's *t*-test.

To assess the inhibition effects from the presence of chlorinated solvent compounds (i.e., 1,1-DCE, TCE, and 1,1,1-TCA), harvested transformant cells were first exposed to the desired concentrations (0-8 mg/L in the aqueous phase) of inhibitors for 20 min, allowing complete portioning of volatile inhibitors in the batch setup and sufficient contact between enzymes and inhibitors. Based on our preliminary tests with varying pre-exposure durations (data not shown), pre-exposure of 20 min is optimal to prevent rapid dioxane degradation by inhibitor-free enzymes without significant impact to enzyme activities, which could greatly affect the estimation of

degradation rates. After the pre-exposure, dioxane was spiked at varying initial concentrations and its disappearance was measured at 3 h. Calculation of the concentrations of chlorinated solvents in aqueous phase were based on the mass balance and Henry's law equilibrium using the following equation.

$$V_{stock} \times C_{stock} = V_{aq} \times C_{aq} + V_{gas} \times \frac{C_{aq}}{H_c}$$

Where, C_{stock} and C_{aq} are the concentrations of chlorinated compounds in stock solution and aqueous phase; V_{stock}, V_{aq}, and V_{gas} are the volumes of stock solution, aqueous phase, and headspace, respectively. H_c is the dimensionless Henry's constant of a specific chlorinated compound[41]. All dioxane degradation rates were first fitted to the non-linear Michaelis-Menten model (Equation S1) to compute apparent kinetic values, which were then fitted with three inhibition equations (Equation S2-S7) (i.e., competitive, noncompetitive, and uncompetitive) to estimate their inhibition factors and distinguish the dominant inhibition mechanism.

Substrate Range Characterization

Three transformants, mc^2-155(pTip-*prmABCD*), mc^2-155(pTip-*thmADBC*), and mc^2-155(pTip-QC2), were harvested using the procedures as mentioned above. Five milliliters of resuspended cells were transferred to 35-mL sealed serum bottles and then exposed to 19 selected compounds individually to assess if significant degradation occurs in comparison with abiotic controls prepared with PBS with 0.1% Tween 80 as the medium. These tested compounds are categorized into four groups, embracing (1) cyclic and branched ethers (dioxane, THF, MTBE) and a structural analogue (cyclohexane), (2) short-chain alkane/alkene gases (ethane, propane, butane, isobutane, and ethene), (3) aromatic compounds (e.g., toluene, benzene), and (4)

chlorinated aliphatic hydrocarbons (1,1-DCE, tDCE, cDCE, 1,1-DCA, 1,2-DCA, VC, TCE, and 1,1,1-TCA). The exposure dosage of each compound is listed in Table S2. MTBE, cyclohexane, alkanes, aromatic compounds, and chlorinated solvents were detected in the headspace; dioxane and THF were measured in the filtered aqueous solutions. Concentrations of these compounds were monitored by GC coupled with a flame ionization detector (FID) detector or mass spectrometry (MS) with key analytical details (e.g., retention time and target ions) indicated in Table S2. As concentrated non-growing transformant cells were used in these assays, degradation rates were estimated based on the disappearance of each tested compound with the first 4 h of incubation. Samples were also collected at 24 h after the exposure, which were analyzed to verify the occurrence and extent of degradation. All experiments were conducted in triplicate to avoid discrepancy among individual tests and minimize system errors. Significant degradation was only recognized by the Student's *t*-test when the substrate disappearance in clones expressing PRM or THM within first 4 h is statistically greater ($p < 0.05$) than (1) the abiotic loss observed in negative controls and (2) the biotic loss in mc^2-155(pTip-QC2) transformant cells which contain the empty vector. The degradation ability was verified based on the observation of (1) continuous substrate depletion at 24 h and (2) degradation exhibited by the wild type strains, PH-06 and CB1190. PH-06 and CB1190, which were grown with 50 mL of ammonium mineral salts (AMS) and 500 mg/L of dioxane as a growing substrate in 160 mL serum bottles. Cells were harvested at their exponential phase and diluted to OD_{600} around 1.0 by PBS with 0.1% Tween-80.

Genomic Comparison

Genomes of 10 Actinomycetes in the genera of *Mycobacterium*, *Pseudonocardia*, and *Rhodococcus* that carry complete genes clusters of *prmABCD* or *thmADBC* were retrieved from

National Center for Biotechnology Information (NCBI). The sequence alignment was conducted using Mauve 2.4.0[42] with the default parameters.

Results and Discussion

PRM Exhibits Higher Affinity to Dioxane than THM

In comparision with THM, PRM exhibited a higher affinity to dioxane since the K_m of PRM (53.0 ± 13.1 mg/L) was significantly lower ($p < 0.05$) than that of THM (235.8 ± 61.6 mg/L) (Figure 1, Table S1). The V_{max} values for PRM and THM were estimated as 0.040 ± 0.003 and 0.055 ± 0.007 mg-dioxane/h/mg-protein, respectively. On the basis of our RT-qPCR analysis (Figure S1), V_{max} of PRM and THM can be converted to (9.52 ± 0.71) × 10^{-12} and (1.13 ± 0.14) × 10^{-11} mg dioxane/h/transcript copy, respectively. These values may be of significant value to evaluate real-time dioxane degradation activities in the field when total RNA is recovered from environmental samples[32]. V_{max} of PRM is significantly smaller than THM ($p < 0.05$), indicating PRM has a relatively lower maximum catalytic capacity for dioxane transformation. However, when dioxane concentration is lower than 430 mg/L, PRM surpasses THM in dioxane degradation rate, primarily due to its greater affinity to dioxane. This was evident by the faster dioxane biotransformation observed under two environment-relevant dioxane concentrations commonly found in the field (Figure 1B). When the transformant cells exposed to an initial dioxane concentration of 1082.5 ± 29.3 μg/L, the dioxane biotransformation rate by PRM was 0.42 ± 0.01 μg dioxane/h/mg protein, equivalent to (1.00 ± 0.02) × 10^{-13} mg dioxane/h/transcript copy. This was two times as high as that of THM (0.20 ± 0.01 μg dioxane/h/mg protein, equivalent to (4.12 ± 0.21) × 10^{-14} mg dioxane/h/transcript copy). When we lowered the initial dioxane concentration to around 250 μg/L, PRM (0.11 ± 0.01 μg dioxane/h/mg protein, equivalent to (2.62 ± 0.23) × 10^{-}

^{14} mg dioxane/h/transcript copy) retained higher efficiency in dioxane degradation than THM (0.04 ± 0.01 μg dioxane/h/mg protein, equivalent to (0.82 ± 0.21) × 10^{-14} mg dioxane/h/transcript copy). Since dioxane concentration is generally lower than 1 mg/L in groundwater[33] and rarely exceeds 100 mg/L at impacted sites, it can be speculated that bacteria that express PRM are more advantageous compared to those with THM given their higher efficiency in exploiting low or trace levels of dioxane for metabolism (Figure 1).

Our enzymatic kinetic results are in good agreement with some previous dioxane degradation kinetic studies using wild type model dioxane degraders that actively express these two enzymes essential for dioxane metabolism (Table S1). For instance, He *et al.*[20] observed a stronger affinity for dioxane in PH-06 that expresses PRM than CB1190 that expresses THM. Relatively high K_m and V_{max} values were also reported in an early study that characterize dioxane degradation kinetics in CB1190[31]. However, results from some other investigations[29, 30, 39] in CB1190 dioxane degradation kinetics were at variance (Table S1). The variation in kinetic coefficients among studies is attributed, at least in part, to the differences in (1) culturing conditions and (2) dioxane exposure duration in the degradation tests[39, 43]. Different culturing media, temperatures, and initial biomass concentrations may affect overall microbial activities and induction of the specific degradation enzyme(s). Dioxane exposure duration is also a critical parameter for the estimation of the kinetic coefficients. These reported studies exposed cells to dioxane for a period ranging from 0.5 to 8 h. Short exposure time may result in an underestimation of degradation rates as cells may take time to acclimate to a new environment. However, long exposure time may cause unwanted biomass growth, as CB1190 cells can grow with dioxane, particularly in the high concentrations dosed in the testing system. In this case, dioxane degradation rates could be overestimated, introducing the extrapolation inaccuracy of V_{max} and K_m

using the Michaelis-Menten model that assumes non-growth condition. In our study, we employed expressing cells that do not grow with dioxane and a median exposure duration of 3 h to improve the measurement consistency for dioxane degradation rates.

1,1-DCE Is the Most Potent Inhibitor to Both PRM and THM

For both PRM and THM, the inhibitory effects of three tested chlorinated compounds were ranked as: 1,1-DCE > TCE > 1,1,1-TCA (Figure 2). The dioxane removal efficiency of PRM dropped from 85.3 ± 12.9% in inhibitor-free PBS solution to 45.8 ± 15.4% with the presence of 2 mg/L of 1,1-DCE. TCE also significantly reduced the dioxane removal efficiency to 52.0 ± 4.1% ($p < 0.05$). However, the influence of 1,1,1-TCA to PRM-catalyzed dioxane degradation was negligible when dosed with the same concentration (i.e., 2 mg/L). A similar inhibitory order of these three chlorinated compounds was also observed in transformant cells expressing THM (Figure 2). In PBS solution without any chlorinated inhibitors, cells expressing THM can eliminate 81.2 ± 6.0% of the initial dioxane after 3 h. The addition of 2 mg/L of 1,1-DCE, TCE, and 1,1,1-TCA greatly inhibited the dioxane degradation by THM and reduced the removal efficiencies to 20.0 ± 9.7, 24.0 ± 2.8, and 49.5 ± 8.2%, respectively. This inhibitory order is in concert with previous inhibition tests using growing cells of CB1190 by Zhang[29]. The consensus between our enzyme study and their pure culture assay suggest the observed inhibition of chlorinated compounds to dioxane degradation is dominantly governed by the direct interaction between inhibitory molecules and catalytic enzymes, though these inhibitors may also negatively affect the degrading bacteria by inducing universal stress, repressing gene expression, impeding substrate transport, and/or interrupting membrane integrity[29].

1,1-DCE has been well recognized as a potent inhibitor to SDIMOs, such as group-3 methane monooxygenase[44, 45], group-3 butane monooxygenase[46], and group-2 toluene-4-monooxygenase[30], as well as many other bacterial catabolic enzymes (e.g., ammonium monooxygenase[47]). 1,1-DCE can incur an irreversible loss of butane monooxygenase activity in alkane degrading *Pseudomonas butanovora*[46]. Our study using heterologous expression cells provides the first evidence unequivocally revealing the inhibition of 1,1-DCE to group-6 and group-5 SDIMOs that are responsible for dioxane metabolism. The inhibition of 1,1-DCE may be attributed to its steric and chemical properties (e.g. polarity and degree of unsaturation and chlorination). The double bond in 1,1-DCE confers to a greater reactivity compared to 1,1,1-TCA. Furthermore, 1,1-DCE has a carbon with two chlorine atoms paired with a carbon with no chlorine. In contrast, TCE has a carbon with two chlorine atoms paired with a carbon with one chlorine atom. Such asymmetry of the double bound in 1,1-DCE may result in a higher reactivity than TCE[29].

PRM Is Less Susceptible to Chlorinated Solvent Inhibition than THM

Based on the best fitness (i.e., highest coefficient of determination [R^2]) with the nonlinear Michaelis-Menten model and its derived equations, negative effects of 1,1-DCE and 1,1,1-TCA on dioxane degradation by PRM and THM might be dominated by noncompetitive inhibition (Figure 3, Table S3, and Figure S2). Previous investigation by Mahendra[30] also revealed noncompetitive inhibition for 1,1-DCE and 1,1,1-TCA on dioxane degradation kinetics using live cells of CB1190 (Table S4). Thus, 1,1-DCE and 1,1,1-TCA may bind to an allosteric site (non-active site) on PRM and THM and trigger desensitization of the active site, conducive to the decrease in overall catalytic performance[48]. Unlike 1,1-DCE and 1,1,1-TCA, TCE was inclined to

inhibit both enzymes via competitive inhibition (Figure 3, Table S3, and Figure S2). The presence of TCE may compete with dioxane for the active sites on PRM and THM, resulting in a decreased affinity. Such inhibition may be alleviated when dioxane concentrations are sufficiently high to outcompete TCE. Over the course of dioxane kinetic assays, no significant change was observed in concentrations of three chlorinated compounds (data not shown), precluding negative effects caused by toxic products derived from intracellular reactions of these chlorinated compounds.

It is noted that the R^2 values representing the fitness of empirical data to varying inhibition models were close for some cases in this study and in many previous studies[29, 30, 49-52]. This insufficient resolution inherently presented in kinetic studies may result from the mixed inhibitory mechanisms, systematic errors, and unweighted regression approaches. Our experiments were carried out with whole cells that actively express enzymes of interest, rather than purified enzymes considering the technical challenges in *in vitro* purification. Substrate transport to enzymes and other cellular dynamic processes may thus influence our inhibition observations[53]. On the other hand, nonlinear regression with the classic Michaelis-Menten model is quite robust in estimating apparent K_m and V_{max} values and can work fairly well even when the errors are not Gaussian-distributed[54]. Comprehensively weighing the shifting of these kinetic parameters in response to a series of inhibitor concentrations, the fitness with different inhibition models is the most frequently used and well-received approach to interpret enzyme-substrate inhibition mechanisms and estimate inhibition constants providing implications for scaling the inhibition potencies.

Remarkably, PRM is less susceptible than THM to the inhibition of all three chlorinated solvents tested in this study. As depicted in Figure 2, under a same concentration of any chlorinated solvent (i.e., 2 mg/L), the initial 10 mg/L of dioxane was removed in a significantly greater extent

in transformant cells expressing PRM than those that express THM. This was also echoed by the computed inhibition constants K_I based on our experimental results (Table S3). For each chlorinated solvent, the best described inhibition mechanism was identical for PRM and THM (Table S4); further, K_I values were always greater for cells expressing PRM. These results suggested that PRM is more resistant to the inhibition of chlorinated solvents than THM. Considering that chlorinated solvents are common co-contaminants of dioxane[33, 55], microorganisms expressing PRM may be catalytically more active and enduring in the proximity of the source zone where dioxane and chlorinated solvents co-occur.

PRM Has a Broader Substrate Range than THM

As expected, PRM and THM are both efficient in transforming cyclic ethers, including dioxane (0.287 ± 0.010 and 0.171 ± 0.042 μmol/h/mg, respectively) and THF (0.368 ± 0.055 and 0.497 ± 0.036 μmol/h/mg) (Table 1). Additionally, both PRM and THM can degrade cyclohexane, a structural analog of dioxane. This is the first report that aligns PRM and THM with cyclohexane degradation, which was previously observed in wild type dioxane degrader PH-06[11]. However, degradation of this 6-membered carbocyclic alkane was much slower (0.098 ± 0.001 and 0.066 ± 0.011 μmol/h/mg for PRM and THM, respectively) in comparison to the 6-membered heterocyclic dioxane. It is also interesting to notice that PRM exhibited significantly higher degradation rates ($p < 0.05$) for six-membered ring compounds (dioxane and cyclohexane) than THM. Reversibly, THM is faster in degrading the five-membered ring THF. The varied degradation efficiencies on different substrates could partially result from the fitness of substrate molecules with the active site or the transport channel of the catalytic enzyme. MTBE is a highly branched ether pollutant

of emerging water concern, since it has been widely used as oxygenate for gasoline[56]. However, neither PRM nor THM can degrade MTBE.

Short-chain (C1-C4) alkanes and alkenes are primary substrates of many subgroups of SDIMOs[57]. They also play an important role in the regulation of SDIMO expression in bacteria. In our transformation surveys (Table 1), PRM exhibited exceptional ability to degrade all alkanes (C2-C4) and the C2 alkene (i.e., ethene) tested in this study. Ethene showed the highest degradation rate (0.487 ± 0.047 μmol/h/mg), followed by propane (0.307 ± 0.045 μmol/h/mg), butane (0.246 ± 0.050 μmol/h/mg), isobutane (0.208 ± 0.084 μmol/h/mg), and ethane (0.127 ± 0.053 μmol/h/mg). Homologues to the PH-06 group-6 PRM have been previously identified in dioxane cometabolizers that grow on propane or isobutane, such as *Mycobacterium* sp. ENV421[58] and *Rhodococcus rhodochrous* 21198[59, 60] (Table 2). Further, the presence of propane can also upregulate the polycistronic transcription of the *prmABCD* clusters in PH-06[14] and ENV421[61, 62], which subsequently promoted the activity of dioxane biotransformation. Our study revealed this single PRM enzyme can degrade both dioxane and gaseous alkanes. This novel finding unveiled the plausible linkage between propane/isobutane assimilation and dioxane degradation as evident in the mentioned wild-type strains.

Besides propane and isobutane, PRM can oxidize a greater range of short-chain alkanes and alkenes, including ethene, ethane, and butane. This is in concert with the previous observations that some *prmABCD*-harboring microorganisms can grow on a wide variety of alkane/alkene gases though their ability to degrade dioxane has yet been characterized (Table 2). For instance, *Rhodococcus* sp. BCP1[63] can grow on all C2-C7 linear alkanes, which also induced the expression of its group-6 SDIMO. Similarly, *Mycobacterium chubuense* NBB4 can grow on C2-C4 alkanes

393 and ethene[57]. It is noted that these Actinomycetes express a diversity of SDIMOs and other
394 enzymes (e.g. cytochrome P450 and dehydrogenase) that may also contribute to the observed
395 alkane and alkene oxidation[57, 64]. However, this is the first study to ascertain the ability of group-
396 6 SDIMO for the oxidation of C2-C4 alkanes (linear or branched) and ethene.

397 Chlorinated solvents and aromatic compounds represent two groups of groundwater
398 pollutants commonly found in contaminated aquifers[33, 65, 66]. We assessed the capability of PRM
399 and THM of degrading these common co-contaminants. Notably, PRM degrades both VC and 1,2-
400 DCA, though the degradation rates were relatively low (0.060 ± 0.007 and 0.038 ± 0.005
401 μmol/h/mg for VC and 1,2-DCA, respectively) (Table 1). This suggests the active site of PRM can
402 weakly react with VC and 1,2-DCA, despite of low affinity. Particularly, VC is a carcinogenic
403 pollutant commonly accumulated as an undesirable metabolite via anaerobic dehalogenation in
404 TCE-contaminated aquifers[67, 68]. Thus, presence of bacteria expressing PRM can in addition
405 synchronize the removal of dioxane and VC co-occurring at the chlorinated solvent sites. PRM
406 can also degrade benzene and toluene at the degradation rates of 0.106 ± 0.011 μmol/h/mg and
407 0.345 ± 0.039 μmol/h/mg, respectively. Ability to degrade these two aromatic compounds was
408 validated using PH-06 cells actively expressing PRM as they were grown with propane. As major
409 gasoline constituents, benzene and toluene are contaminants prevalently detected in groundwater.
410 Compared with toluene, benzene is more toxic and recalcitrant with strict regulation by EPA[69]. To
411 break the aromatic ring, dihydroxylation is imperative to insert two hydroxyl groups at adjacent
412 aromatic carbon positions. This can be achieved by two sequential oxidations catalyzed by
413 monooxygenases or a simultaneous oxidation by dioxygenases[70]. This is the first study report that
414 PRM has the capability of degrading aromatic compounds, such as benzene and toluene. Overall,
415 PRM's versatile degradation capability of degrading a broad spectrum of common groundwater

pollutants (e.g., benzene, toluene, VC, and 1,2-DCA) underscores its value for environmental remediation.

Transformant cells expressing THM did not show degradation capability toward any of the alkanes, alkenes, chlorinated and aromatic compounds in our tests (Table 1). This demonstrates that THM is highly specific to cyclic compounds. In contrast, PRM has a much broader substrate range, unveiling greater potential for *in situ* and *ex situ* treatments of commingled contaminations. Even better, expression of PRM may also enable microorganisms to assimilate other carbon sources, such as propane and isobutane, for cell growth, and support decomposition of a variety of pollutants. Collectively, this group-6 PRM displays unparalleled catalytic versatility towards various types of small molecules including alkane, alkene, cyclic, chlorinated, or aromatic[71]. In our previous paper[14], we named this type of group-6 SDIMOs as PRM after its first discovery in the propanotroph, *Mycobacterium* sp. TY-6[72]. They were also designated as "short chain alkane-oxidizing monooxygenase (SCAM)" in other reports[59]. We propose the nomenclature of this group-6 SDIMOs can be unified in the future.

Environmental Implications for Monitored Natural Attenuation of Dioxane

Besides PH-06 and CB1190, many other Actinomycetes also harbor *prm* and *thm* genes (Table 2). Though not all were verified at the molecular level, it is prudent to assume that these strains can utilize PRM or THM for the initial breakdown of dioxane. It is interesting to note that these *prm* and *thm* harboring bacteria were isolated from geographically disparate locations (e.g., Asian, Europe, and America). However, sequences of their multicomponent gene clusters *prmABCD* and *thmADBC* are highly conservative with minimum identities of 86% and 94%, respectively, even with the consideration of the spacers and overlaps between gene components.

438 It is also notable that most of these gene clusters are localized on plasmids (Table 2) and/or
439 adjacent to mobile elements. For instance, the *prmABCD* gene cluster in PH-06 is carried by a
440 transposon cassette flanked by insertion sequences[14]. The meticulous examination (Figure S3)
441 revealed all gene clusters are intact without noticeable internal rearrangements. In addition,
442 upstream and downstream sequences (the colored blocks shown in Figure S3) of the *prm* or *thm*
443 gene cluster also demonstrated high homology suggesting a consensus origin. These converging
444 lines of evidence corroborate that dioxane degradation genes *prm* and *thm* are disseminated via
445 horizontal gene transfer (HGT), enabling the intercellular spreading of dioxane catabolism across
446 species.

447 In contaminated aquifers, HGT of *prm* and *thm* may occur among indigenous
448 microorganisms at varying frequencies in response to the concentration of dioxane as the selective
449 pressure[24, 73, 74]. Our enzymatic study suggests that transfer of *prm* may be both physiologically
450 and ecologically more profitable than *thm*. This is because (1) PRM displays a faster dioxane
451 catabolism at field-relevant dioxane concentrations (e.g., < 1 mg/L); (2) such dioxane degradation
452 activity of PRM is also less affected by the inhibition of chlorinated solvents; (3) PRM enables the
453 assimilation of short-chain alkanes and biotransformation of cyclic, chlorinated, and aromatic
454 pollutants which commonly co-occur in the contaminated aquifers. Therefore, it is plausible to
455 postulate that dioxane metabolizing microbes, like PH-06, which express PRM may be more
456 abundant and/or active at sites impacted by commingled contamination of dioxane and chlorinated
457 solvents than those employing THM-mediated catabolism. Note that field environment is
458 staggeringly complexed in comparison with the laboratory condition we conducted in our kinetic
459 assays. For instance, growth substrates other than dioxane may compete with the dioxane
460 degrading enzymes or suppress their expression due to metabolic flux dilution and catabolite

repression[24]. On the other hand, availability of other substrates may promote cellular growth in general. Further, intrinsic activities of these dioxane degrading enzymes may also be regulated by a wide spectrum of environmental factors (e.g., inhibiting compounds, temperatures, pH, nutrient, oxygen availability, presence of competitors). However, considering chronic acclimation, all these factors together will, in return, affect the native abundance of dioxane degrading microbes, as well as the frequency of these key catabolic genes (e.g., *prmA* and *thmA*) carried by them, permitting the use of these genes as effective biomarkers to assess dioxane attenuation potentials.

Unfortunately, dioxane attenuation potentials may have been long underestimated as previous efforts have merely focused on the quantification of *thm* genes which code for THM. This underscores the need for the complete molecular survey of both *prm* and *thm* genes to assess the abundance and activity of native dioxane degraders in the field. Together with other lines of evidence (e.g., field monitoring, laboratory microcosm assays, isotopic fractionation, and geochemical indication), comprehensive biomarker analysis will facilitate the justification to select or reject MNA for the mitigation of dioxane. This may elicit significant reduction of field remediation efforts and associated costs at sites where pump-and-treat is actively employed.

Environmental Implications for Biostimulation with Short-Chain Alkane/Alkene Gases

In addition to MNA, biostimulation is an alternative that can effectively accelerate the cleanup of dioxane in the field. A pilot trial lasting over 9 months demonstrated amendment of propane and oxygen into recirculating groundwater sustained an effective removal of dioxane, 1,2-DCA, and other chlorinated compounds at the former air force base site[26]. Ethane and isobutane were also reported for spurring monooxygenase-driven cometabolism of dioxane in aquifers[60, 75]. In this study, we unequivocally proved that PRM can degrade both dioxane and short-chain

483 alkane/alkene gases, explaining that PRM may contribute to the dioxane cometabolism observed
484 in previous field and microcosm tests for alkane biostimulation[27, 60, 75]. However, the presence of
485 *prm* genes doesn't guarantee their ability to carry out catabolic dioxane degradation. Dioxane
486 cometabolism can be hindered by field factors, such as the lack of inorganic nutrients or inhibition
487 of the auxiliary substrate[60]. Thus, further investigation regarding the PRM-associated dioxane
488 metabolism or cometabolism are needed to guide for field applications.

489 We also note that, contribution of bacteria expressing THF to short-chain alkane
490 biostimulation should not be precluded. Though THM is highly specific to heterocyclic ethers,
491 many of *thm* harboring bacteria also carry other SDIMOs genes enabling the assimilation of short-
492 chain alkanes/alkenes. Taking the archetypic THM-mediated dioxane degrader CB1190 as an
493 example, it also carries a group-5 propane monooxygenase gene cluster in the chromosome[76] and
494 its propane degradation capacity was verified in our lab (data not shown). Further investigation is
495 needed to assess the effectiveness of propane and other short-chain alkanes or alkenes for bacteria
496 that carry both *thm* and some other SDIMO genes. However, curing of *thm* carrying plasmids may
497 be of concern. In our previous study, CB1190 tends to lose redundant plasmids (e.g., the plasmid
498 that carries *thm*) when it is fed with substrates that are readily biodegradable (e.g., 1-butanol and
499 acetate)[24]. Further, in aquifers, the case becomes more intricate, particularly when *prm* harboring
500 bacteria co-exist. Again, this calls for a comprehensive survey of PRM, THM, and other SDIMO
501 genes that are associated with dioxane cometabolism and the assimilation of the selected auxiliary
502 substrate, which facilitate the design and monitoring of the intrinsic biostimulation. Nonetheless,
503 primary attention is recommended to be made to PRM given their unique and synchronic ability
504 of transforming dioxane and other pollutants and assimilating gaseous alkane/alkene substrates.

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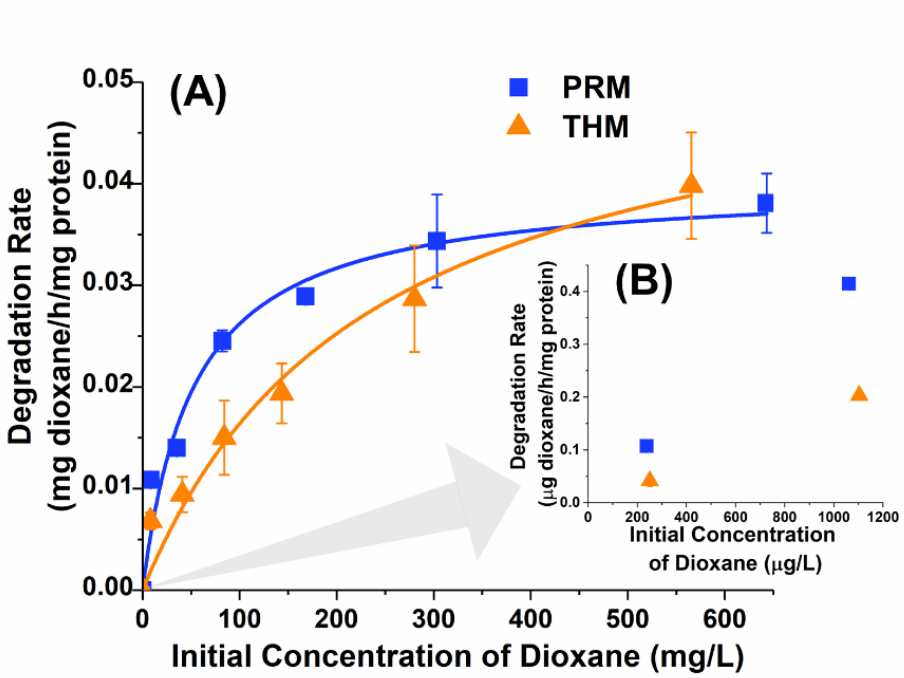
We declare no competing financial interest.

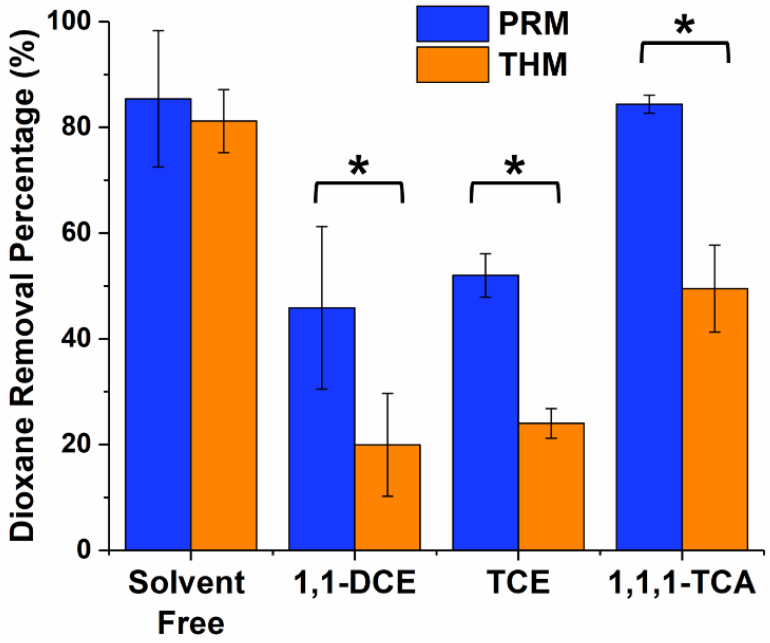
Supporting Information Available

Experimental details for cloning and induction of PRM and THM, quantitative assessment of gene expression, enzyme kinetic modeling, and analytical methods; tables showing the comparison of dioxane biodegradation kinetic parameters in this study and other previous publications (Table S1), GC-FID and GC/MS analytical details (Table S2), inhibition kinetic parameters for dioxane degradation by PRM and THM expressing transformants with the presence of three chlorinated compounds (Table S3), comparison of inhibition constants and mechanisms between this study and other previous publications (Table S4), and abiotic and biotic loss of selected substrates observed in abiotic control and transformant cells carrying the empty vector, respectively (Table S5); figures depicting uniform gene expression in the heterologous host (Figure S1), best-fitted inhibition regression (Figure S2), and molecular evidence implying shared origins among PRM- or THM-encoding genes (Figure S3).



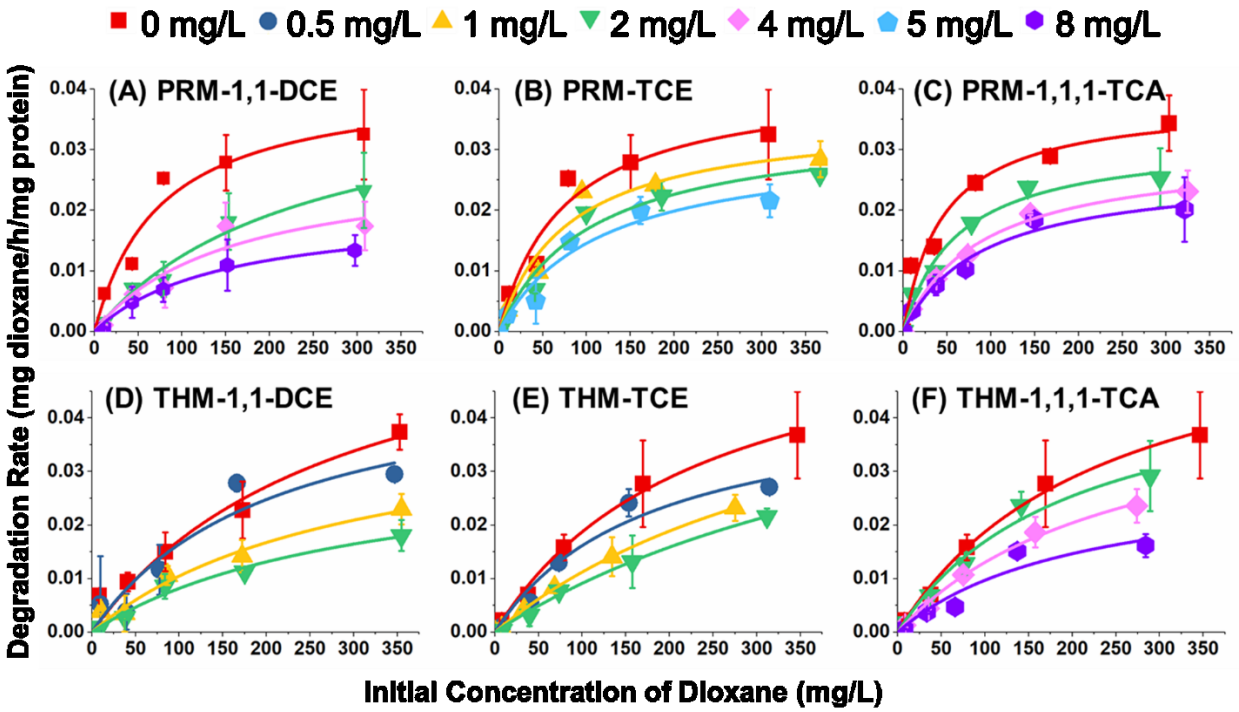
526

527 **Figure 1.** (A) Michaelis-Menten curves exhibiting dioxane degradation kinetics by transformant
528 cells expressing PRM (blue square) and THM (orange triangle). Dioxane degradation at
529 environment-relevant concentrations were shown in the inserted figure (B).



530

531 **Figure 2.** Inhibition of dioxane biodegradation by three chlorinated solvents in transformant cells
532 expressing PRM and THM. Cells were pre-exposed to 2 mg/L of each chlorinated solvent and then
533 assessed their dioxane removal efficiencies in the contact time of 3 h with an initial dioxane
534 concentration of 10.0 mg/L. Error bars represent the standard deviation of triplicates. Asterisk
535 marks represent significant ($p < 0.05$) dioxane removal differences between PRM and THM.



536

537 **Figure 3.** Enzyme inhibition kinetics by the Michaelis-Menten model for PRM (A, B, C) and THM
538 (D, E, F) with the presence of 1,1-DCE (A, D), TCE (B, E), and 1,1,1-TCA (C, F). Degradation
539 rates were estimated as the average of the dioxane disappearance among triplicates within the
540 contact duration of 3 h and normalized towards the initial protein concentrations. No significant
541 change in three inhibitor concentrations was observed during these assays.

Table 1. Substrate range of PRM and THM and accordant degradation rates.

Substrate	Degradation Rate (µmol/h/mg protein)	
	PRM	THM
Ethers/Analogs		
Dioxane	0.287 ± 0.010	0.171 ± 0.042
THF	0.368 ± 0.055	0.497 ± 0.036
Cyclohexane[a]	0.098 ± 0.001	0.066 ± 0.011
MtBE	-	-
Short-chain Alkanes/Alkene		
Ethene	0.487 ± 0.047	-
Ethane	0.127 ± 0.053	-
Propane	0.307 ± 0.045	-
Butane	0.246 ± 0.050	-
Isobutane	0.208 ± 0.084	-
Aromatics		
Benzene	0.106 ± 0.011	-
Toluene	0.345 ± 0.039	-
Chlorinated Aliphatic Hydrocarbons		
VC	0.060 ± 0.007	-
1,2-DCA	0.038 ± 0.005	-
1,1-DCE	-	-
cDCE	-	-
tDCE	-	-
TCE	-	-
1,1,1-TCA	-	-

Green cells represent significant degradation (p<0.05) exhibited by the transformant cells expressing PRM or THM in comparison with both (1) the abiotic control and (2) biotic control with transformant cells carrying the empty vector;

Red cells represent substrate depletion was not observed or not significantly different from either abiotic or biotic control treatment.

[a] Degradation rates for cyclohexane were calculated based on the concentration difference between 4 and 24 h due to a prolonged equilibrium of this chemical in the sealed bottles.

551 **Table 2.** Bacteria harboring the complete gene clusters of *prmABCD* and *thmADBC*.

Strain Name	Dioxane Degradation	Other Inducible Substrate	Gene Localization	Geographic Location	Gene Cluster Identity (%)	Reference
***Prm* Harboring Bacteria**						
Mycobacterium dioxanotrophicus PH-06	m	propane	plasmid	Pohang, South Korea	100	11
Mycobacterium sp. ENV421	ca	propane	ic	New Jersey, US	88.84	27, 58, 61
Rhodococcus rhodochrous strain 21198	ca	propane/isobutane	ic	Japan	86.24	59, 60, 77
Rhodococcus aetherovorans BCP1	u	C2-C7 alkanes	plasmid	Bologna, Italy	86.24	63, 78
Mycobacterium chubuense NBB4	u	ethene/C2-C4 alkanes	plasmid	New South Wales, Australia	86.51	57, 79
***Thm* Harboring Bacteria**						
Pseudonocardia dioxanivorans CB1190	m	THF	plasmid	South Carolina, US	100	12, 76
Pseudonocardia sp. N23	m	THF	ic	Japan	97.1	80
Pseudonocardia sp. K1	ct	THF	ic	Göttingen, Germany	94.86	81, 82
Pseudonocardia sp. ENV478	ct	THF	ic	New Jersey, US	96.84	83, 84
Rhodococcus ruber YYL	u	THF	plasmid	Zhejiang, China	99.74	85

552 m = metabolism

553 ca = cometabolism with alkane gases

554 ct = cometabolism with THF

555 u = unknown

556 ic = incomplete genome with major gaps (precluding the determination of localization of *prm* or
557 *thm* genes)

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