Discovery of an Inducible Toluene Monooxygenase that Co-oxidizes 1,4-Dioxane and 1,1-Dichloroethylene in Propanotrophic *Azoarcus* sp. DD4

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Abstract

Cometabolic degradation plays a prominent role in bioremediation of commingled groundwater contamination (e.g., chlorinated solvents and the solvent stabilizer 1,4-dioxane [dioxane]). In this study, we untangled the diversity and catalytic functions of multi-component monooxygenases in *Azoarcus* sp. DD4, a gram-negative propanotroph that is effective in degrading dioxane and 1,1-dichloroethylene (1,1-DCE). Using a combination of knockout mutagenesis and heterologous expression, a toluene monooxygenase (MO) encoded by the *tmoABCDEF* gene cluster was unequivocally proved as the key enzyme responsible for the cometabolism of both dioxane and 1,1-DCE. Interestingly, in addition to utilizing toluene as a primary substrate, this toluene MO can also oxidize propane into 1-propanol. Expression of this toluene MO in DD4 appears inducible by both substrates (toluene and propane) and their primary hydroxylation products (m-cresol, p-cresol, and 1-propanol). These findings coherently explain why DD4 can grow on propane and express toluene MO for active co-oxidation of dioxane and 1,1-DCE. Furthermore, upregulation of *tmo* transcription by 1-propanol underlines the implication potential of using 1-propanol as an alternative auxiliary substrate for DD4 bioaugmentation. The discovery of this toluene MO in DD4 and its degradation and induction versatility renders broad applications spanning from environmental remediation and water treatment to biocatalysis in green chemistry.

Importance

Toluene MOs have been well recognized given their robust abilities to degrade a variety of environmental pollutants. Built upon previous research efforts, this study ascertained the untapped capability of a toluene MO in DD4 for effective co-oxidation of dioxane and 1,1-DCE, two of the most prevailing yet challenging groundwater contaminants. This report also aligns the induction

of a toluene MO with non-toxic and commercially accessible chemicals (e.g., propane and 1-propanol), extending its implication values in the field of environmental microbiology and beyond.

Keywords: 1,4-dioxane, aliphatic chlorinated hydrocarbons, toluene monooxygenase, propanotroph, cometabolic degradation

Introduction

As a possible human carcinogen, the common chlorinated solvent stabilizer, 1,4-dioxane (dioxane), has emerged with increasing concern nationwide and globally (1, 2). Its extreme hydrophilicity and stable cyclic structure preclude effective treatment by most physical and chemical approaches, such as adsorption, air sparging, and chemical oxidation (3). Recent microbiological efforts have promoted bioremediation as a promising alternative to mitigate dioxane contamination. Most of the characterized dioxane-degrading bacteria are Actinomycetes (e.g., *Pseudonocardia dioxanivorans* CB1190 (4) and *Mycobacterium dioxanotrophicus* PH-06 (5)) with relatively slow growth rates, low affinity to dioxane, and clumping behaviors, which limit their field applications (6-8). These unfavorable characteristics are exacerbated by the co-existence of 1,1-dichloroethylene (1,1-DCE), a major attenuation product of chlorinated solvents (e.g., 1,1,1-trichloroethane via abiotic process (9) and trichlorethylene via anaerobic biotransformation (10)), which is frequently detected at dioxane-impacted sites and displays potent inhibition to dioxane biodegradation (11-14). Thus, 1,1-DCE has been long recognized as a stumbling block hindering effective biological removal of dioxane and other coexisting pollutants.

Azoarcus sp. DD4 is a gram-negative bacterium that can cometabolize both dioxane and 1,1-DCE when it is fed with propane as the primary substrate (15). DD4 demonstrates many superior physiological properties well suited for field applications (e.g., fast and planktonic growth) (15). Dioxane and 1,1-DCE biotransformation pathways were predicted based on the degradation metabolites detected using modern mass spectrometry techniques (15). Detection of 2-hydroxyethoxyacetic acid (HEAA) suggests dioxane is oxidized via 2-hydroxylation, a catalytic process that inserts a hydroxyl group to the α-carbon adjacent to the oxygen enabling the subsequent ring cleavage. As for 1,1-DCE, epoxidation is postulated as the initial step to activate

63 the decomposition (15). Biotransformation of dioxane, 1,1-DCE, and propane are all terminated
64 once DD4 is exposed to acetylene, a suicide inhibitor to bacterial monooxygenases (MOs) (16).
65 Thus, converging lines of evidence demonstrate the pivotal role of MO(s) in initiating the
66 breakdown of dioxane and 1,1-DCE in DD4 (15).

67 Soluble di-iron monooxygenases (SDIMOs) represent a non-heme bacterial enzyme family
68 (17, 18) known for their substrate versatility and robust capability of degrading a plethora of
69 anthropogenic pollutants. SDIMOs can be divided into six groups based on their sequence
70 similarity and substrate preference (19). In dioxane metabolizers (e.g., CB1190 and PH-06) that
71 can grow with dioxane as the sole carbon and energy source, group-5 tetrahydrofuran (THF) MOs
72 (20) and group-6 propane MOs (21) have been shown to initiate the metabolism of dioxane via 2-
73 hydroxylation. Some other bacteria like DD4 can degrade dioxane via cometabolism, a fortuitous
74 oxidation process necessitating the supplement and induction of auxiliary substrates (e.g., propane
75 and toluene). A few propane- and toluene-inducible SDIMOs have been reported to be responsible
76 for dioxane cometabolism. Some propanotrophs (e.g., *Mycobacterium* sp. ENV421 (22),
77 *Mycobacterium vaccae* JOB5 (23), and *Rhodococcus ruber* ENV425 (24)) expressing group-5
78 and/or group-6 propane MOs have demonstrated their ability to co-oxidize dioxane when fed with
79 propane. Additionally, toluene-2-MO in *Burkholderia cepacia* G4, toluene-*p*-MO in *Ralstonia*
80 *picketii* PKO1, and toluene-4-MO in *Pseudomonas mendocina* KR1 exhibited dioxane degradation
81 abilities in both toluene-induced wild-type (wt) cultures and heterologous clones (6).
82 Biotransformation of 1,1-DCE has also been observed in SDIMO-expressing bacteria (10, 25),
83 such as the methanotrophic strain *Methylosinus trichosporium* OB3b (26) and the toluene/o-xylene
84 degrader *Pseudomonas stutzeri* OX1 (27). Hence, it is plausible to postulate that one or more

SDIMO(s) are responsible for the cometabolic degradation of dioxane and 1,1-DCE in the propanotrophic isolate, DD4.

The complete genome of DD4 has been recently sequenced using the long-read high-accuracy PacBio SMRT Sequencing platform (28). In this present study, with the combined assistance of genome annotation, gene knockout, and heterologous expression, the diversity and catalytic functions of SDIMOs in DD4 are examined with the focus on the cometabolic degradation of dioxane and 1,1-DCE. Their roles in oxidation of propane and other auxiliary substrates are elucidated through the characterization of degradation profiles in knockout mutants, as well as quantitative analysis of key metabolite production. Importantly, regulation of SDIMO expression in DD4 is assessed in association with these primary substrates and their metabolites to align propane assimilation with cometabolic degradation of dioxane and 1,1-DCE (15). This research advances our fundamental knowledge about the catalysis and induction versatility of SDIMOs, promoting the innovation of effective remedial operations that can be tailored to mitigate the co-contamination of dioxane and 1,1-DCE.

Materials and Methods

Bacterial strains, plasmids, and growth conditions

The *Azoarcus* and *E. coli* strains and the plasmids used in this study are listed in Table S1. *E. coli* was grown in LB at 37°C following standard protocols (29). *Azoarcus* sp. DD4 was routinely grown aerobically at 30°C in VM-ethanol medium (30). For substrate degradation studies with either growing or resting cells or reverse transcription-quantitative PCR (RT-qPCR) analysis, nitrate mineral salts (NMS) medium supplemented with the indicated substrates was used as the growth medium.

Knockout mutagenesis of prm, bmo, and tmo genes in DD4

Knock-out mutants for *prm*, *bmo,* and *tmo* genes were constructed using in-frame deletion of respective gene clusters. Primers used to produce the homologous recombination arms were listed in Table S1. Upstream and downstream regions of the target genes were amplified by primers containing restriction sites and cloned in sequence into pBBRMCS-2 to generate the plasmid containing the deletion modification. The recombination insert was further subcloned into pK18mobsacB to generate the suicide plasmid that carries the recombinant sequence, which was then transferred into wt DD4 by triparental conjugation with the assistance of the helper strain *E. coli* (pRK2013). After incubation for 2 days at 30°C, cells were scraped from KON plates (31), suspended in KON liquid media, and eventually transferred on VM-ethanol plates containing 25 μg/mL kanamycin. Single colonies were obtained by re-streaking on VM-ethanol plates and then successful exconjugants were confirmed using colony PCR. Individual exconjugants were further selected for double recombination on 10% sucrose in VM-Ethanol agar without antibiotics. After being grown at 30°C for 48 h, strains with positive deletions of *prm*, *bmo* and *tmo* gene clusters were examined by gel electrophoresis. Details regarding the plasmid construction, triparental conjugation, and exconjugant selection are provided in the Supplemental Material.

Catalytic assays using growing cells

To identify the catalytic functions of three SDIMOs encoded by *prm*, *bmo*, and *tmo*, concentrations of corresponding substrates were monitored in the growing wt or deletion mutant cells. Unless otherwise stated, all the assays were operated in 160 mL serum vials in triplicate. The initial 0.1 mg protein biomass of wt or mutant DD4 was inoculated into 20 mL NMS medium supplemented with individual substrates, including propane, butane, toluene, and ethanol. The serum vials were sealed with butyl rubber stoppers and crimped with aluminum caps before being aerobically cultured at 30°C. For propane and butane degradation assays, an initial concentration

of 20 mg/L of propane (instrument grade, purity > 99.5%, Airgas) or butane (chemical pure, purity > 99.0%, Airgas) in the gas phase was injected. To avoid the inhibitory effects derived from high exposure of toluene, the initial aqueous phase concentration of toluene used was not allowed to exceed 80 mg/L. To cultivate DD4 mutants, ethanol was used as the primary substrate at an initial concentration of 200 mg/L. Abiotic controls were prepared without cell suspensions. At the selected incubation intervals, 700 μL of aqueous or 100 μL of headspace samples were removed and analyzed for the disappearance of the amended substrate by gas chromatography-flame ionization detection (GC-FID). For the MO inhibition observation, allylthiourea (ATU) was dosed to a concentration of 25 μM in the NMS media. Acetylene was injected to achieve 10% (v/v) of the headspace volume. Concentrations of acetylene were monitored by GC-FID to confirm no gas leakage during incubation.

To assess cometabolism of dioxane, wt DD4 or knockout mutants are grown in NMS initially supplemented with 200 mg/L ethanol (as substrate) and 10 mg/L dioxane. Dioxane concentrations were monitored over time by GC-FID. Analytical approaches regarding biomass and chemical measurements are detailed in the Supplemental Material.

Catalytic assays using resting cells

To test the transformation capabilities for 1,1-DCE, wt or mutant DD4 clones were pre-grown with ethanol (200 mg/L) in NMS media. The transformation assay was conducted in 35 mL serum vials containing 4.5 mL of 1×PBS and 0.5 mL of cell suspensions. The initial biomass was estimated as 1.5 mg total protein per vial. In the beginning, 1,1-DCE was dosed at 3.0 mg/L as the equilibrium aqueous phase concentration. Abiotic controls were prepared without cell suspensions. At selected incubation time, 100 μL headspace samples were removed and analyzed for the disappearance of 1,1-DCE by GC-FID.

Heterologously expression of toluene MO

A 4997 bp fragment of the *tmoABCDEF* gene cluster was amplified using TMO/pETF and TMO/pETR as primers and the genomic DNA of DD4 as the template. PCR mixtures (50 μL) consisted of 1 × PCR buffer, 100 nM dNTPs, 250 nM either primer, 1 unit of Pfu polymerase (Thermo, Carlsbad, CA) and 10 ng of the DD4 genomic DNA. Thermocycling conditions were: 98 ºC for 5 min, then 30 cycles of 98 ºC for 20 s and 62 ºC for 6 min, and 72 ºC for 10 min at the end. Restriction sites NdeI and NheI were introduced at their 5′ ends of the forward and reverse primers, respectively (Table S1). The PCR amplicon and vector pET-28a(+) were both digested with NheI and NdeI (New England Biolabs, Ipswich, MA). After gel purification, the linearized plasmid and PCR insert were ligated at a 1:3 (plasmid:insert) ratio at 16 ºC overnight with T4 DNA ligase (New England Biolabs, Ipswich, MA). The ligation mixture (1 μL) was then used to transform electrocompetent *E. coli* DH5α cells to generate pET-*tmo*. Recombinant plasmid pET-*tmo* was then harvested using the Zyppy™ Plasmid Mini-prep Kit (Zymo, Irvine, CA) and transformed to *E. coli* BL21(DE3) via electroporation with the MicroPulser™ Electroporator (Bio-Rad, Hercules, CA). For enzyme expression, BL21(DE3) transformants with the empty vector pET-28a(+) or recombinant plasmid pET-*tmo* were grown in LB medium containing 25 μg/mL kanamycin at 30 °C. When an OD at 600 nm raised to 0.6, IPTG (0.5 mM) was added to induce protein expression for an additional 4 h at 30 °C before cell harvesting. Production of toluene MO components in cell lysates was examined using SDS-PAGE analysis as detailed in the Supplemental Material.

To examine their degradation activities, IPTG-induced transformants BL21(DE3) (pET-*tmo*) and BL21(DE3) (pET-28a(+)) were washed after growth in LB media and suspended in 5 mL 1×PBS in 35-mL serum vials sealed with rubber caps. The initial biomass of transformants

was 1.5 mg total protein per vial. Initial amount of toluene, dioxane, 1,1-DCE, and propane was 1.43, 0.49, 0.84, and 2.28 μmol in each bottle, respectively, equivalent to 26.4, 2.73, and 8.6 mg/L toluene, 1,1-DCE, and dioxane in the aqueous phase and 3.5 mg/L propane in the gas phase based on the equilibrium following their Henry's Law constants (32). Abiotic controls were prepared without cell suspensions. Treatments were all incubated at 30 °C while being shaken at 175 rpm and performed in triplicate. Disappearance of the four added compounds was monitored by GC−FID. Instant degradation rates were calculated by averaging substrate disappearance in triplicate within the first 2 h, which was further normalized by the initial biomass as measured by total protein using the Bradford Assay (12, 14).

RNA Extraction and RT-qPCR analysis

To quantify the expression levels of the *tmo* gene cluster under different substrate conditions, RT-qPCR was conducted using *tmoA* as the target gene and 16S rRNA gene of DD4 as the housekeeping gene to normalize experimental variance. Primers specific for *tmoA* and 16S rRNA genes in DD4 were designed and listed in Table S1. A broad array of substrates and metabolites were first screened to assess if they can support the growth of DD4 and also stimulate dioxane and 1,1-DCE biodegradation activities. Growth of DD4 was monitored by OD at 600 nm when cultivated in NMS amended with individual substrates (50 mg/L) at 30°C for 24 h. After growth with different substrates, cells were washed and concentrated to an OD_{600} of 1.5 in 5 mL 1×PBS and tested for dioxane or 1,1-DCE removal after exposure for 1 h. Based upon these screening results, we selected toluene, m-cresol, p-cresol, 3-methylcatechol (3-MC), 4-methylcatechol (4-MC), propane, 1-propanol, propionaldehyde, and propionic acid for further RT-qPCR analysis considering their relevance to toluene and propane oxidation and distinct effects in inducing dioxane and 1,1-DCE biodegradation. DD4 cells were cultured using these selected

compounds at an initial dosage of 2.0 mM as the sole carbon source at 30 °C while being shaken at 150 rpm. As a control, pyruvate treatment was conducted as the control in parallel. When grown to the exponential phase, DD4 cells were harvested for total RNA extraction before substrate concentrations dropped below half of the initial dosage.

The total RNA was extracted using the PureLink RNA Mini Kit (Thermo, Carlsbad, CA) according to the manufacturer's protocol, in combination with an on-column PureLink DNase Treatment (Thermo, Carlsbad, CA) to eliminate the interference from DNA. cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Thermo, Carlsbad, CA) and then purified using the DNA Clean & Concentrator™-5 Kit (Zymo, Irvine, CA). RT-qPCR mixtures contained 1 μL of diluted cDNA (5 ng/μL), 10 μL of 2 × Power SYBR Green PCR Master Mix (Thermo, Carlsbad, CA), 0.3 μM of forward and reverse primers, and DNA-free water to a total volume of 20 μL. RT-qPCR was performed with a QuantStudio™ 3 Real-Time PCR System (Thermo, Carlsbad, CA) with the following temperature setup: 95 ºC for 10 min, and 40 cycles of 95 ºC for 15 s and 60 ºC for 1 min. Differential gene expression was quantified using the $2^{-\Delta\Delta Cq}$ method (8) and the expression fold change was calculated with the following formula.

$$\Delta\Delta C_{q,\text{ Target gene}} = (C_{q,\text{ Target gene}} - C_{q,\text{ Housekeeping gene}})_{\text{ Treatment}} - (C_{q,\text{ Target gene}} - C_{q,\text{ Housekeeping gene}})_{\text{ Control}}$$

Results and Discussion

DD4 harbors a diversity of SDIMO gene clusters

Five distinct SDIMO gene clusters (Figure 1 and Table S2) were identified scattered across the single chromosome of *Azoarcus* sp. DD4 (28). According to the sequence identity and arrangement order of their gene components, these SDIMO gene clusters (Figure 2) were categorized and designated as *prmABCD* encoding a group-5 propane MO, *bmoXYBZDC* encoding a group-3 butane MO, *tmoABCDEF* encoding a group-2 toluene MO, and *dmp1KLMNOP* and

223 *dmp2KLMNOP* encoding two group-1 phenol hydroxylases. On the basis of the 16S rRNA gene
224 sequence analysis, DD4 is most phylogenetically related to *Azoarcus* sp. BH72 and *Azoarcus*
225 *olearius* DQS-4 (15). Similarly, the *prm* and two *dmp* gene clusters are highly identical to those in
226 BH72 (33) and DQS-4 (34) (mostly > 92%) (Table S1), suggesting all three Betaproteobacteria
227 may be able to exploit propane and phenolic compounds as their substrates.

228 Compared to BH72 and DQS-4, DD4 harbors two unique SDIMO genes, *tmo* and *bmo*.
229 Closest homologues of *bmo*- and *tmo*-encoding proteins have been found in *Thauera butanivorans*
230 (35-37) and *Thauera* sp. 27 (38, 39), respectively. *BmoXYBZDC*-encoding butane MO has been
231 well characterized in *Thauera butanivorans* regarding its catalytic preference and regiospecificity,
232 which predominantly oxidizes terminal carbons of C4-C5 alkanes (35, 40). In DD4, all six key
233 *bmo* gene components have been identified, though *bmoZ*, *bmoD*, and *bmoC* were separated by
234 two genes of unknown functions (*hp1* and *hp2* in Figure 1 and Table S2). Homologues of these
235 two hypothetical proteins are found from Gammaproteobacteria (e.g., *Solimonas* and
236 *Methylohalobius*), rather than Betaproteobacteria as *Azoarcus* or *Thauera*. The existence of
237 internal spacer genes in a *bmo* or SDIMO gene cluster has been sparsely reported. However, the
238 encoded butane MO in DD4 retains its catalytic activity towards butane with the presence of these
239 two inserted genes, as revealed by the following mutagenesis assays.

240 The *tmo* gene cluster in DD4 consists of all six components essential for group-2 toluene
241 MOs (Figure 1 and Table S2). Immediately downstream of the *tmoABCDEF* gene cluster, three
242 genes were identified on the same strand of the sequence, including *tmoX*, *tmoS*, and *tmoT*. *TmoX*
243 encodes a putative membrane channel protein promoting the transport of toluene from the outside
244 of the cell (41). TmoST likely represents a dual regulation system (42). Presence of toluene or
245 other inducers may trigger the phosphorylation of TmoS. Then, the phosphorylated TmoS reacts

with TmoT and transfers the phosphate to TmoT. When phosphorylated, TmoT binds to the promoter of the *tmo* gene cluster and facilitates its transcription. These three genes have been identified scattering in the genomes of archetypical toluene degraders, such as toluene dioxygenase (TOD) expressing *Pseudomonas putida* F1 and toluene-4-MO expressing *P. mendocina* KR1 (42). To our knowledge, a consecutive *tmoABCDEFXST* gene cluster encoding comprehensive functions covering toluene biocatalysis, transport, and signal transduction has not been previously reported. The integrity of this gene cluster may enable efficient dissemination of toluene catabolism abilities via horizontal gene transfer at toluene-impacted environments.

Deletion of the tmo gene inactivates biotransformation of both dioxane and 1,1-DCE in DD4

To discern the contribution of SDIMOs to dioxane and 1,1-DCE degradation in DD4, knockout mutants were created using homologous recombination with the sucrose counter-selectable suicide vector, pK18*mobsacB*. We primarily deleted *prm*, *bmo*, and *tmo* genes, given their potential roles in cometabolism of dioxane and chlorinated aliphatic hydrocarbons (CAHs). Successful deletion of target SDIMO genes was verified by PCR and gel electrophoresis in comparison with amplicons using genomic DNA from wild-type DD4 (wt DD4) as the template (Figures 1 and S1). Ethanol was used as a carbon source for knockout mutants, since it can not only support the growth of DD4 regardless of the presence or absence of the various SDIMO genes (Figure S2), but also stimulate active dioxane/1,1-DCE degradation in wt DD4 (Table S3).

Notably, deletion of the *tmo* gene was conducive to the complete disability of dioxane transformation in the Δ*tmo* DD4 mutant (Figure 3). When grown with ethanol, wt DD4 was capable of proliferation (Figure S2) and concurrently degraded the initial 11.0 mg/L of dioxane to below our method detection limit (MDL) (i.e., 0.1 mg/L) till Day 4 (Figure 3). Though experiencing a lag phase that lasted for two days (Figure S2), Δ*prm* and Δ*bmo* DD4 mutants were

able to resume their growth and transform approximately 79.4% and 77.4% of the initially dosed dioxane, respectively. However, no significant removal of dioxane was observed in the Δ*tmo* DD4 mutant, though continuous cell growth was evident by the increase in turbidity of the culture media over 4 days of incubation (Figure S2). This indicated that an intact *tmo* gene cluster is essential for the dioxane degradation activity in DD4. This finding was out of our expectation, in part because (i) propane was initially identified as the primary substrate to sustain the dioxane cometabolism of DD4 (15) and (ii) no previous report has revealed group-2 toluene MOs are inducible by propane. To validate our knockout assays, dioxane degradation capability of wt DD4 was tested when it was fed with toluene as the sole carbon and energy source. As shown in Figure 4, DD4 can grow on toluene and concurrently degrade dioxane. With two consecutive toluene amendments (~58 mg/L per time), DD4 steadily degraded 16.4 mg/L of dioxane within 9 days of incubation. This demonstrated that toluene can sustain the growth of DD4 and stimulate the cometabolic oxidation of dioxane, supporting the contribution of *tmo* in dioxane cometabolism.

Further assays using resting cells pregrown with ethanol indicated this *tmo* gene is also tied to 1,1-DCE biotransformation in DD4 (Figure 5). Compared with the rapid 1,1-DCE depletion by wt DD4, no 1,1-DCE removal was observed by the Δ*tmo* DD4 mutant. In contrast, deletion of the *prm* gene didn't significantly affect the biotransformation of 1,1-DCE. Knockout mutant Δ*bmo* exhibited 27.6% less 1,1-DCE removal than wt DD4 over the incubation course of 20 h. This decrease in 1,1-DCE removal is probably attributed to the encoded butane MO taking part in certain metabolic process(es). Deletion of this *bmo* gene may negatively affect overall degradation performance of resting cells by diminishing cellular energy efficiency or impeding detoxification response to stressors (e.g., DCE epoxide formed from 1,1-DCE oxidation (15)). This hypothesis is also supported by the observation of the delayed growth incurred to this Δ*bmo* mutant when fed

with ethanol (Figure S2). Furthermore, the Δ*tmo* DD4 mutant carries the complete *bmo* gene cluster and presumably expresses active butane MO. However, it has completely lost the ability to oxidize 1,1-DCE. Thus, the integrity of the *bmo* gene cluster appears independent from the catalytic capability of degrading 1,1-DCE in the Δ*tmo* DD4 mutant. Collectively, all these lines of evidence corroborate that the *tmo*-decoding toluene MO is responsible for co-oxidation of dioxane and 1,1-DCE in DD4, precluding the involvement of other SDIMOs. Slowed dioxane or 1,1-DCE removal in *prm*- and *bmo*-knockout mutants was probably due to inadvertent molecular detriments to cellular metabolism.

Toluene MO in DD4 can initiate the oxidation of toluene, dioxane, 1,1-DCE, and propane

To further characterize its catalytic function in DD4, this toluene MO was heterologously expressed in *E. coli* BL21(DE3). After induction with IPTG, BL21(DE3) transformed with pET-*tmo*, the high expression vector that carries the whole *tmoABCDEF* gene cluster, can produce a catalytically active toluene MO complex. This toluene MO-expressing transformant can catalyze the oxidation of its primary substrate toluene (Figure 6A). This was also supported by the formation of blue colonies cultured in LB media owing to the ability of toluene MOs to convert indole to indigo (43, 44) (Figure S3). Furthermore, transformation of dioxane, 1,1-DCE, and propane was positively detected in the toluene MO-expressing transformant (Figure 6). In contrast, the control BL21(DE3) transformant carrying the empty plasmid pET-28a(+) could neither turn blue (as an indication of no indigo generation) (Figure S3) nor degrade any of the tested substrate compounds (Figure 6). This heterologous expression assay unequivocally demonstrates the catalysis of toluene, dioxane, 1,1-DCE, and even propane by this group-2 toluene MO in DD4.

The expressed toluene MO was most efficient in transforming toluene at a rate of 14.7 μg toluene/mg protein/h. The initial biotransformation rate for dioxane in the first 2 h was estimated

as 1.3 μg dioxane/mg protein/h (Figure 6B). We noticed that the dioxane oxidation rate of this transformant was at least one order of magnitude lower than that exhibited by resting wt DD4 cells (23 μg dioxane/mg protein/h) (15), possibly due to the production of insoluble protein aggregates that lack of biocatalyst activity as observed in Figure S4.

For 1,1-DCE, the instant biotransformation rate was estimated as 12.0 μg 1,1-DCE/mg protein/h in the toluene MO-expressing transformant (Figure 5C). 1,1-DCE cometabolism has been reported in bacteria expressing SDIMOs. Iwashita et al. found that the toluene-2-MO, classified as a group-1 SDIMO, and its shuffle mutants from *B. cepacian* G4 toluene MO can degrade 1,1-DCE (45). In subgroup 3, methane MO can oxidize 1,1-DCE into 1,1-DCE epoxide in *M. trichosporium* OB3b (26). Further, belonging to the same SDIMO subgroup 2 as the toluene MO of DD4, the toluene/o-xylene MO of *P. stutzeri* OX1 can co-oxidize 1,1-DCE and many other recalcitrant CAHs (e.g., PCE, TCE, and chloroform) while releasing free chloride as an indication of mineralization (27, 46). However, no significant dioxane removal was observed in *E coli.* transformant expressing this specific toluene/o-xylene MO (6).

It is surprising that the toluene MO in DD4 can also degrade propane with a transformation rate of 2.1 μg propane/mg protein/h (Figure 5D). No prior studies have reported propane or other short-chain alkanes as a substrate for group-2 toluene MOs. The ability to oxidize propane by the toluene MO in DD4 partially explains why propane is effective in sustaining the growth of DD4 and the transformation of dioxane and 1,1-DCE at the same time.

Toluene MO in DD4 catalyzes two successive toluene oxidation steps

The catalytic function of this new toluene MO in DD4 was further characterized using the constructed expression clone. After the toluene MO-expressing transformant was exposed to toluene for 20 h, three metabolites (p-cresol, m-cresol, and 4-methylcatechol [4-MC]) were

detected by high-performance liquid chromatography (HPLC) (Figure S5A). No 3-methylcatechol (3-MC) or other metabolites were noticeably detected. Negative controls with the BL21(DE3) transformant carrying the empty vector exhibited no significant abiotic loss of toluene over the course of the experiment (Figure S5B). Detection of both p-cresol and m-cresol revealed the ability of toluene MO in DD4 to attack both the para- and meta-carbons of toluene via hydroxylation. 4-MC was only detected at 20 h, indicating the toluene MO may be able to insert a second hydroxyl group to p-cresol and/or m-cresol. To further verify this secondary hydroxylation by the toluene MO, toluene MO-expressing transformant cells were exposed to p-cresol and m-cresol, respectively. Interestingly, 4-MC was observed as the only metabolite of either p-cresol or m-cresol via the oxidation of toluene MO (Figure S5C and S5E). Though there was no significant abiotic loss of p- and m-cresol in the BL21(DE3) transformant carrying the empty vector (Figure S5D and S5F), disappearance of 4-MC was observed in the transformant clones with or without the *tmo* gene cluster (Figure S6). This decay of 4-MC was probably due to the biodegradation by the BL21(DE3) host cells (47) and/or abiotic transformation, explaining the major molar discrepancy between the production of 4-MC and the reduction of p- or m-cresol as observed in Figure S5C and S5E.

Accompanying the disappearance of initial 0.86 mM toluene, production of p-cresol (0.72 mM), m-cresol (0.06 mM), and 4-methylcatechol (0.09 mM) was observed at 20 h (Figure S5A). As shown in Figures S5C and S5E, degradation rates of p-cresol and m-cresol to 4-MC were indistinguishable ($p > 0.05$). Assuming conversion rates of m-cresol and p-cresol to 4-MC were identical, stoichiometric generation of p- and m-cresol at a ratio of 12:1 was estimated from the oxidation of toluene. Therefore, toluene MO in DD4 is a multi-hydroxylating enzyme that oxidizes toluene to p-cresol (mainly) and m-cresol followed by the insertion of a second hydroxyl group to

form 4-MC. The ring structure of 4-MC can be further broken down by catechol 2,3-dioxygenase (C23O) via meta cleavage, entering the TCA cycle as depicted in Figure S7. DD4 genome contains three copies of C23O genes along with other genes that participate in the downstream metabolism (Table S4).

Phylogenetic analysis of α subunits revealed the toluene MO in DD4 is more closely related to the archetypic toluene-*p*-MO in PKO1 (71%) and toluene/o-xylene MO in OX1 (72%) than toluene-4-MO in KR1 (69%) and toluene-2-MO in G4 (26%) (Figure 2 and Table S5). This was also echoed by the sequence identity analysis for other MO subunits (Table S5). In good agreement with their evolutionary relationship, catalytic versatility exhibited by the DD4 toluene MO is somewhat more similar to toluene-*p*-MO in PKO1 and toluene/o-xylene MO in OX1. The recombinant toluene-*p*-MO from PKO1 was shown to oxidize toluene at both para-and meta-positions. The formation ratio of p- and m-cresol was determined as around 9:1 stoichiometry with the assistance of gas chromatography and NMR analyses (48). However, this toluene-*p*-MO was not able to further oxidize p- or m-cresol in PKO1 (49). In OX1, the toluene/o-xylene MO can not only attack toluene at all three positions (para and ortho primarily) but also proceed with the secondary hydroxylation to generate 4-MC (from p- and m-cresols) and 3-MC (from o-cresol) (50, 51). Contrastingly, toluene-4-MO in KR1 (52) and toluene-2-MO in G4 (53) can only attack toluene at the para- and ortho-position, respectively, exhibiting relatively high regiospecificity.

With recent discovery of more group-2 toluene MOs, their regiospecificity and substrate range are of great variance. For instance, benzene MO in *Pseudomonas aeruginosa* JI104 and toluene MO in *Burkholderia cepacia* AA1 (Figure 2) are closely related to the toluene MO in DD4 with high sequence identities (79% and 76%, respectively). The benzene MO in JI104 exhibited relaxed regiospecificity toward toluene, producing three metabolites, o-, m-, and p-cresol, at a ratio

of 1:2:1.7 (54). However, the toluene MO in AA1 is highly specific, which may only catalyze at the meta-position (55). Thus, it is promiscuous to categorize the catalytic functions of group-2 SDIMOs on the basis of their refined phylogenies (Figure 2). Further molecular characterization and computational efforts are underway to unravel the catalytic diversity of group-2 SDIMOs in association with their enzyme configurations and key residues that govern substrate interactions.

Expression of toluene MO is inducible by both substrates and their hydroxylated products

DD4 resting cells harvested from toluene, m-cresol, p-cresol, propane, or 1-propanol can degrade both dioxane and 1,1-DCE (Table S3), whereas no significant dioxane or 1,1-DCE degradation was observed in DD4 cells cultured with 4-MC, 3-MC, propionaldehyde, propionic acid, succinate, or pyruvate. To gain further insight into *tmo* regulation in DD4, differential expression of *tmoA,* encoding the large hydroxylase subunit of the toluene MO, was examined using RT-qPCR. Notably, *tmoA* expression was upregulated not only by growth substrates (i.e., toluene and propane), but also their primary hydroxylated metabolites (i.e., p-cresol, m-cresol, and 1-propanol) (Figure 7). When DD4 was fed with toluene, the highest level of *tmoA* expression (4.81±0.90 fold) was observed in comparison with the control cells cultivated on pyruvate. Significant upregulation of *tmoA* was also detected in DD4 cells pregrown on propane (2.93±0.47 fold), p-cresol (2.79±1.03 fold), m-cresol (2.60±0.19 fold), or 1-propanol (2.16±0.30 fold). When grown with downstream metabolites of 1-propanol or cresol oxidation (e.g., propionaldehyde, propionic acid, or 4-MC), no positive expression of *tmoA* was observed compared to pyruvate-fed cells. Induction of toluene MO expression in DD4 revealed by our RT-qPCR results is in good agreement with the observation of active cometabolism of dioxane and 1,1-DCE after DD4 was fed with toluene, propane, and their primary hydroxylated metabolites, but not the secondary metabolite, 4-MC, or other lower pathway products (e.g., propionaldehyde) (Table S3).

Note that the RT-qPCR analysis used in our study is inadequate to rule out two possibilities. First, induction by toluene and propane may be partially attributed to their primary metabolites (e.g., cresols and 1-propanol). When DD4 cells are grown with toluene or propane, their primary metabolites are formed. Though these intermediate metabolites are transient, subject to rapid degradation, they may induce the expression of the *tmo* gene cluster to some extent. Second, along with this toluene MO, DD4 expresses other MOs (e.g., group-1 phenol hydroxylases, group-5 propane MO, group-3 butane MO, and particulate MO) that may also oxidize toluene or propane (Figure S8 and detailed discussion in the Supplemental Material), generating cresols or 1-propanol, which in return can enhance the transcription of *tmo*. In these two scenarios, production of inducible metabolites by the toluene MO and other MOs in DD4 complicates the analysis of the extract regulation triggered by toluene or propane. Thus, further research utilizing quantitative induction assays such as a Δ*tmo* mutant fused with a green fluorescent protein (GFP) reporter system or *in vitro* assays with purified regulatory proteins and promoter DNA is needed to differentiate between these alternatives. However, our RT-qPCR results qualitatively prove that toluene and propane are both inducers of the toluene MO, since their exposures resulted in upregulation of the *tmoA* transcription to significantly higher levels ($p < 0.05$) than their corresponding metabolites (e.g., p- and m-cresols and 1-propanol, respectively) (Figure 7). Further, when RNA was extracted for RT-qPCR analysis, cells were harvested before half of the dosed substrate was degraded, ensuring induction resulted predominantly from the much greater exposure of the substrate (toluene or propane) compared to its metabolites. Our results also preclude the direct induction from dioxane and 1,1-DCE, because (i) DD4 won't grow with these two compounds without the supplement of carbon substrates and (ii) DD4 grown with pyruvate or succinate (non-inducers) won't degrade dioxane or 1,1-DCE (Table S3).

Nonetheless, this study provides multiple lines of evidence to establish a short-chain gaseous alkane (i.e., propane) as both the substrate and inducer of a group-2 toluene MO, though it is not unusual for SDIMOs to be flexibly regulated by a wide range of substrates and their hydroxylated products. Toluene-2-MO from G4 can be induced by toluene, phenol, and TCE (56). In PKO1, toluene-*p*-MO expression was upregulated by toluene, benzene, and ethylbenzene (57), as well as m-cresol (58). Similarly, toluene-4-MO from KR1 can be positively regulated by toluene, CAHs (e.g., TCE, perchloroethylene, cis-1.2-dichloroethylene, and chlorothene) (59), and p-cresol (42). Elevated toluene degradation activities were also observed when KR1 was fed with medium-chain alkanes, such as hexane, pentane, and octane (59). For the toluene/o-xylene MO in OX1, toluene was a less effective inducer than one of the hydroxylated products, o-cresol (60). However, none of these previous studies have revealed the induction of group-2 toluene MOs by propane, which is applicable for bioremediation (e.g., biostimulation and bioaugmentation) in the field.

Importantly, upregulation of *tmo* by 1-propanol should be also noted, considering the implication potential of using 1-propanol as an alternative auxiliary substrate for DD4 bioaugmentation. Compared to propane, 1-propanol is a water-miscible liquid, which can be advantageous for streamlining the engineering efforts for field injection. As 1-propanol is not a substrate for this toluene MO, growth with 1-propanol is unlikely to pose competitive inhibition to fortuitous degradation of dioxane or 1,1-DCE, which is a common practice concern when primary substrates (e.g., propane) are used to stimulate cometabolism (61). In addition, 1-propanol is well suited for scalable applications, since it is non-toxic, commercially available, and relatively inexpensive. Collectively, this study presents adequate evidence establishing a link between a newly identified group-2 toluene MO with propane (a short-chain alkane gas) and 1-propanol (a primary alcohol) degradation and/or induction. This extends our horizon in understanding the

453 substrate range and induction of SDIMOs for potential exploitation of their catalytic functions in
454 natural and engineered systems.

455 Toluene MOs are known to be valuable for bioremediation given their versatile abilities to
456 degrade common pollutants, such as aromatic compounds, CAHs, and polycyclic aromatic
457 hydrocarbons (PAHs) (59, 62-64). However, application of toluene MO-expressing bacteria for
458 cometabolic bioremediation has been greatly hindered by the necessity of using toluene as the
459 substrate, incurring risks of discharging this hazardous chemical into the environment. Though the
460 genetic and biochemical induction mechanisms need to be further refined, this study uncovered a
461 spectrum of non-hazardous gaseous and liquid substrates (e.g., propane and 1-propanol) that can
462 induce the expression of the toluene MO in DD4. Concurrently, the promiscuity of the toluene MO
463 allows the cometabolic degradation of dioxane, 1,1-DCE, toluene, and some other persistent
464 pollutants that may co-occur in the field. Overall, our findings provide a foundation for the
465 development of intrinsic bioremediation strategies with diverse auxiliary substrate options to
466 enhance the cleanup effectiveness and field engineering practicality.

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473 We declare no competing financial interest.

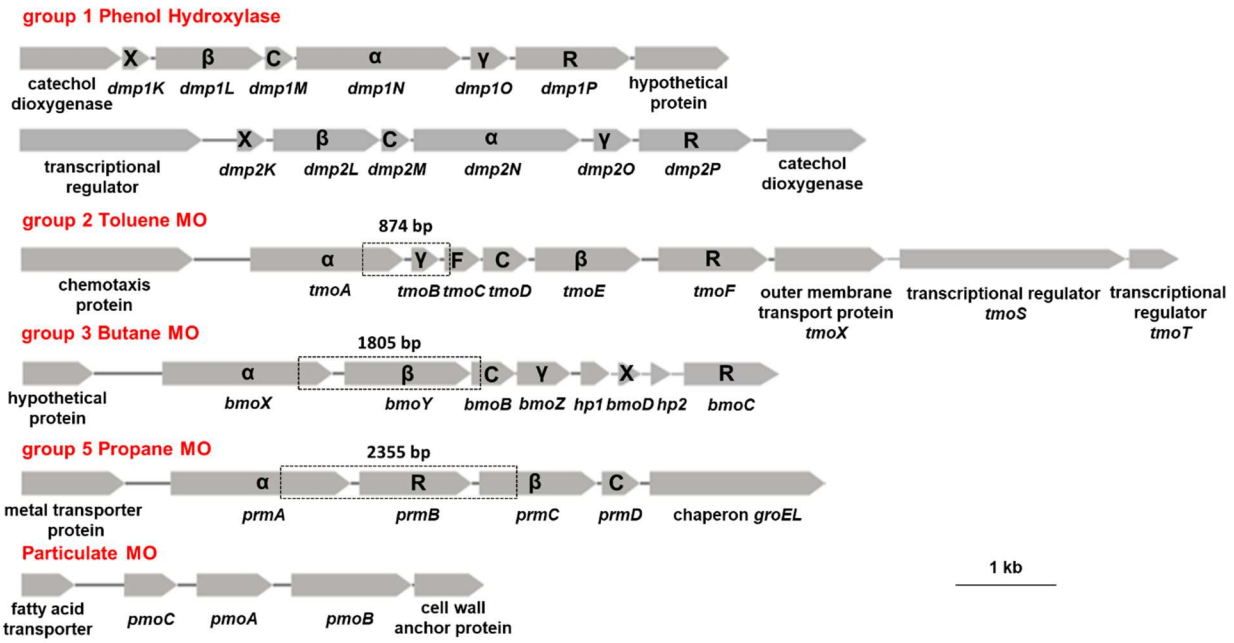


Figure 1. Organization of five SDIMO and one copper-based particulate MO gene clusters in *Azoarcus* sp. DD4. ORFs are depicted as arrows in grey shade. Gene names for the SDIMO gene clusters and their flanking genes are described below. SDIMO components are labeled with abbreviated symbols, including alpha (α), beta (β), and gamma (γ) hydroxylase, coupling protein (C), ferredoxin component (F), reductase (R), and protein with unknown function (X). For knockout mutagenesis, deletion fragments are framed in the rectangles with dotted lines.

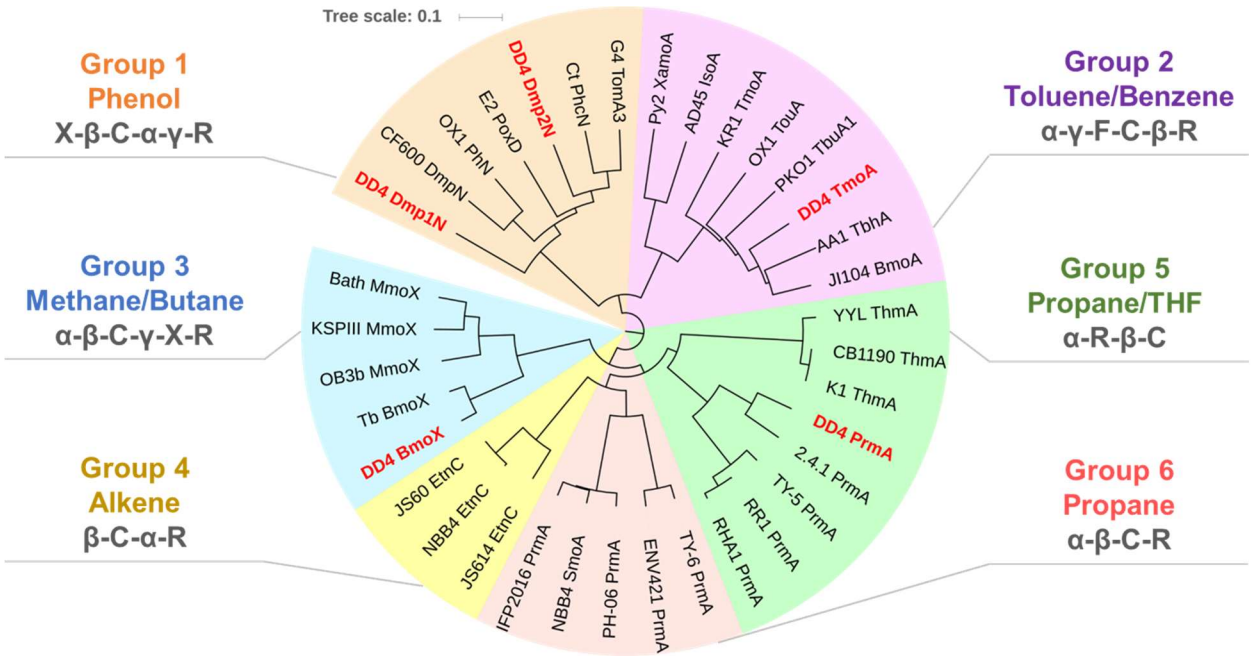


Figure 2. Neighbor-joining tree showing the phylogenetic relationship of five SDIMOs (highlighted in red) in *Azoarcus* sp. DD4 with representatives from all the six subgroups of SDIMOs. This phylogenetic tree is constructed based on the alignment of amino acid sequences of their alpha subunits. Key SDIMO components are abbreviated as alpha (α), beta (β), and gamma (γ) hydroxylase, coupling protein (C), reductase (R), ferredoxin component (F), and protein with unknown function (X).

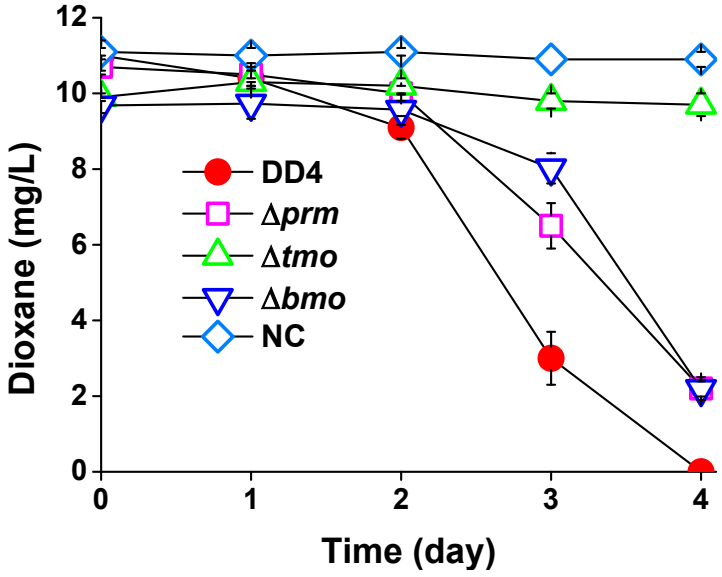
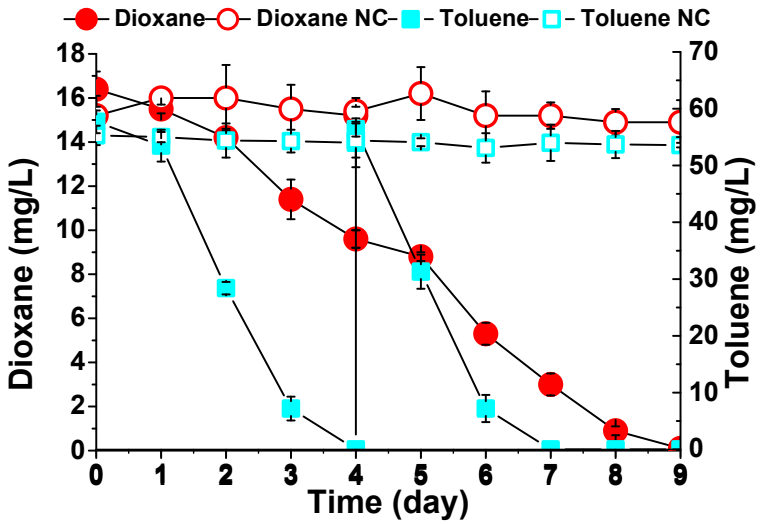


Figure 3. Dioxane degradation by wild-type and SDIMO-deleted growing DD4 cells that were fed with ethanol (200 mg/L) at time 0. Negative controls (NC) were prepared without cells. Data are the average of triplicates and error bars indicate their standard deviations.



492

493 **Figure 4**. Dioxane degradation by DD4 using toluene as the sole carbon and energy source. To
494 avoid the inhibition from the high concentration of toluene, toluene was initially amended at the
495 aqueous phase concentration of 57.7 mg/L. Same amount of toluene was re-amended when toluene
496 was fully consumed on day 4. Error bars indicate standard deviations among triplicates.

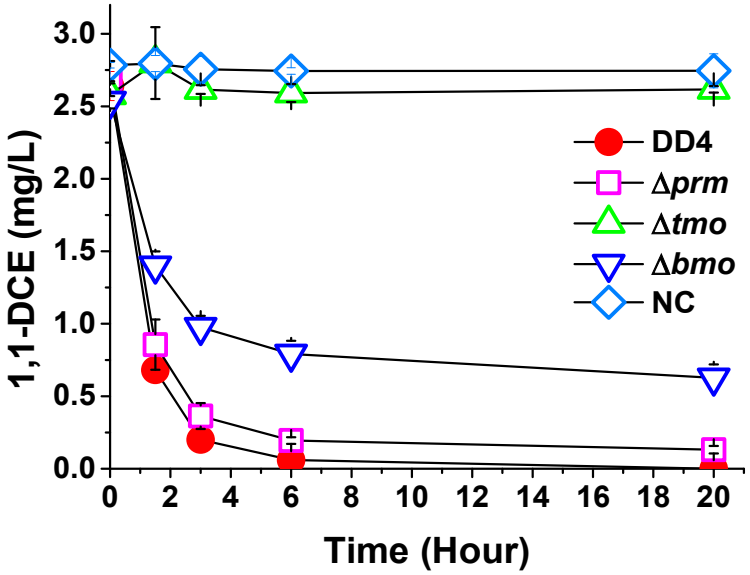


Figure 5. Degradation of 1,1-DCE by resting cells of wild-type and SDIMO gene-deleted DD4 that were precultured with ethanol. Negative controls (NC) were prepared without cells. Data are the average of triplicates and error bars indicate their standard deviations.

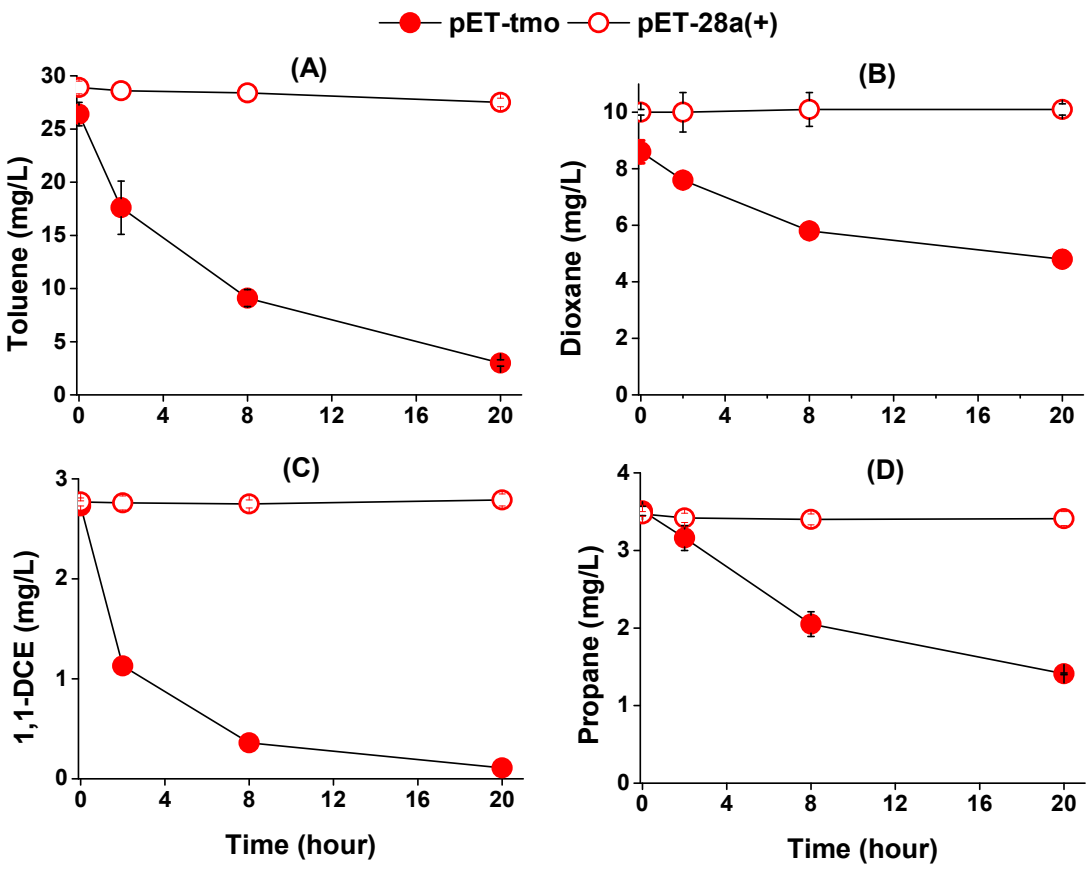


Figure 6. Degradation of toluene (A), dioxane (B), 1,1-DCE (C), and propane (D) by *E. Coli* BL21(DE3) heterologously expressing DD4 toluene MO. Control experiments were prepared with BL21(DE3) carrying the empty vector pET-28a(+). All transformants were induced with 0.5 mM IPTG prior to the substrate exposure. For toluene and 1,1-DCE, aqueous phase concentrations are calculated at equilibrium based on their Henry's Law constants. Concentrations of dioxane and propane are considered in the aqueous and gas phases, respectively. Error bars indicate standard deviations among triplicates.

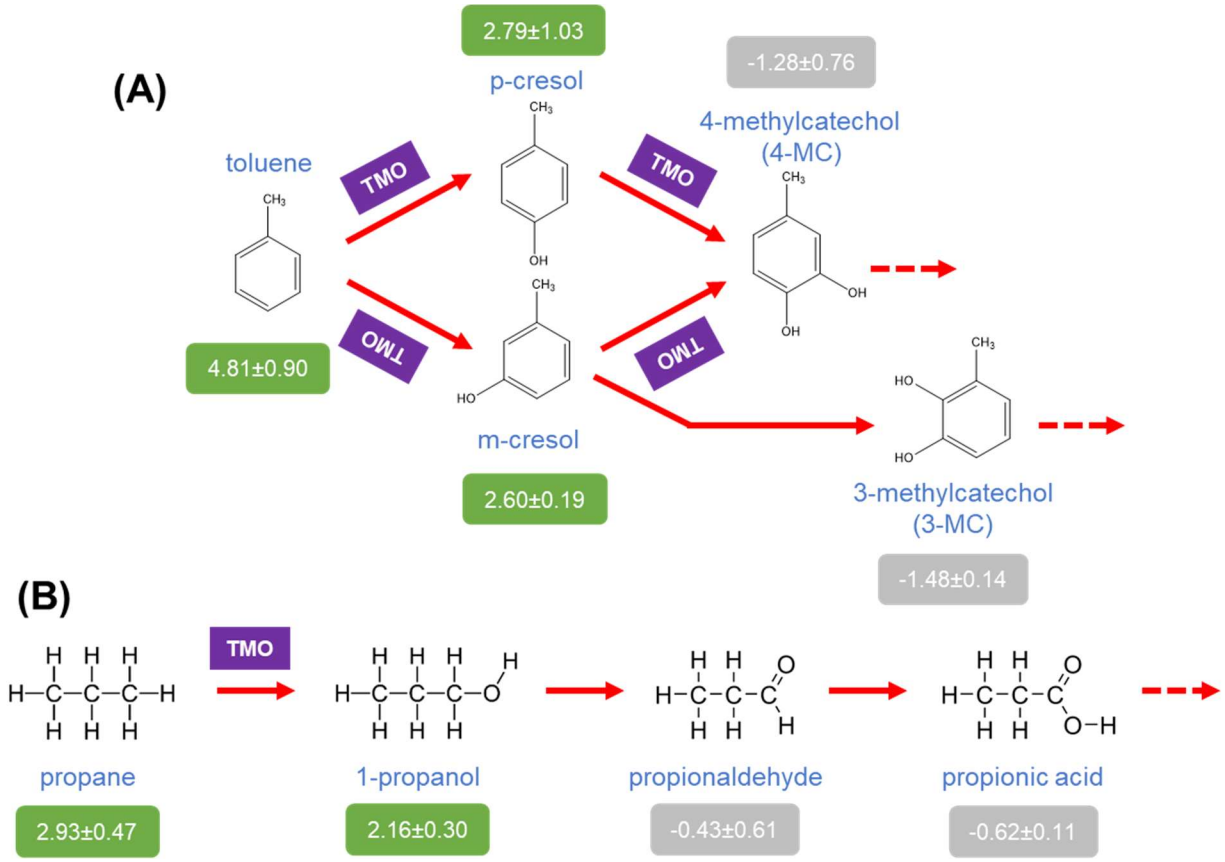


Figure 7. Degradation pathways of toluene and propane initiated by the toluene MO of DD4. Expression fold changes of the *tmoA* gene are indicated below each compound that DD4 was fed with as the sole carbon and energy source. Data were normalized to a treatment in which DD4 was fed with pyruvate. Green boxes highlight significant upregulation of the *tmoA* gene compared to the pyruvate control. 16S rRNA gene of DD4 was used as the housekeeping gene for error control.

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