Active Site Engineering of the Epoxide Hydrolase from *Agrobacterium radiobacter* AD1 to Enhance Aerobic Mineralization of *cis*-1,2-Dichloroethylene in Cells Expressing an Evolved Toluene *ortho*-Monooxygenase

Lingyun Rui¹, Li Cao¹, Wilfred Chen², Kenneth F. Reardon³, and Thomas K. Wood¹*

¹Departments of Chemical Engineering and Molecular and Cell Biology, University of Connecticut, Storrs, CT 06269-3222

²Department of Chemical and Environmental Engineering, University of California, Riverside, CA 92521

³Department of Chemical Engineering, Colorado State University, Fort Collins, CO 80523-1370

*Corresponding author. Phone (860) 486-2483. Fax (860) 486-2959. Email twood@engr.uconn.edu

Running Title: Engineering an Epoxide Hydrolase for *cis*-DCE Degradation
SUMMARY

Chlorinated ethenes are the most prevalent groundwater pollutants, and the toxic epoxides generated during their aerobic biodegradation limit such processes. Hydrolysis of the toxic epoxide by epoxide hydrolases represents the major biological detoxification strategy; however, chlorinated epoxyethanes are not accepted by known bacterial epoxide hydrolases. Here, the epoxide hydrolase from *Agrobacterium radiobacter* AD1 (EchA), which enables growth on epichlorohydrin, was tuned to accept cis-1,2-dichloroepoxyethane as a substrate by accumulating beneficial mutations from three rounds of saturation mutagenesis at three selected active site residues: F108, I219, and C248 (no beneficial mutations were found at position I111). The EchA F108L/I219L/C248I variant co-expressed with a DNA-shuffled toluene *ortho*-monooxygenase, which initiates attack on the chlorinated ethene, enhanced the degradation of cis-dichloroethylene (*cis*-DCE) an infinite extent compared to wild-type EchA at low concentrations (6.8 µM) and up to 10 fold at high concentrations (540 µM). EchA variants with single mutations (F108L, I219F, or C248I) enhanced *cis*-DCE mineralization 2.5 fold (540 µM), and EchA variants with double mutations, I219L/C248I and F108L/C248I, increased *cis*-DCE mineralization 4 fold and 7 fold, respectively (540 µM). For complete degradation of *cis*-DCE to chloride ions, the apparent $V_{\text{max}}/K_m$ for the recombinant *Escherichia coli* strain expressing the EchA F108L/I219L/C248I variant was increased over five-fold as a result of the evolution of EchA. The EchA F108L/I219L/C248I variant also had enhanced activity for 1,2-epoxyhexane (two-fold) and the natural substrate epichlorohydrin (six-fold).
INTRODUCTION

Epoxide hydrolases (EHs; EC 3.3.2.3) hydrolyze an epoxide to its corresponding vicinal diol by the addition of a water molecule (1). In mammalian systems, epoxides are frequently found as intermediates in the catabolic pathways of various xenobiotics including unsaturated aliphatic and aromatic hydrocarbons (2-4). These intermediates are potentially harmful as the oxirane moiety of epoxides is electrophilically reactive and can form adducts with various cellular components including DNA, RNA, proteins, and other small molecules (5,6); hence, it is vital for the biological system to detoxify these reactive species. Together with the conjugation reaction catalyzed by glutathione S-transferases (GSTs), conversion of epoxides by EHs into chemically and toxicologically less-active diols constitutes the major mechanism for detoxification in mammalian systems (5).

While mammalian EHs have been extensively studied for detoxification, interest in microbial EHs has arisen primarily due to the potential of the enzymes as enantioselective biocatalysts (1,7,8). It is also of interest to investigate the detoxification role of microbial EHs and genetically adapt this universally-successful detoxification strategy to the process of aerobic, cometabolic biodegradation of chlorinated ethenes in which toxic epoxides form as the primary intermediates (6). Chlorinated ethenes, such as trichloroethylene (TCE) and cis-1,2-dichloroethylene (cis-DCE), constitute a large group of priority pollutants (126 chemicals of the federal Clean Water Act) (9,10). Since they are toxic, it is critical to remediate these compounds (11). While reductive dechlorination of chlorinated ethenes under anaerobic conditions has the risk of accumulation of the well-known carcinogen vinyl chloride (11-14), aerobic co-metabolic mineralization (conversion to chloride ion) of these compounds by microorganisms expressing various non-specific oxygenases suffers from the inability of the cell to detoxify the reactive chlorinated epoxyethanes that are the primary metabolites (6). The chlorinated epoxyethanes may cause covalent modification of cellular components, inactivation of enzymes, and even cell death, and thus greatly limit the transformation capacity (6,15-18).

Recently, a novel glutathione S-transferase from Rhodococcus sp. strain AD45 having activity toward cis-1,2-dichloroepoxyethane (cis-DCE epoxide) was coexpressed with an evolved toluene ortho-
monooxygenase (TOM), TOM-Green, in *E. coli* in our lab and showed significant detoxification of the reactive epoxide intermediates from *cis*-DCE, *trans*-1,2-dichloroethylene, and TCE (19) (Fig. 1). TOM is a three-component, diiron enzyme encoded by the *Burkholderia cepacia* G4 genes *tomA012345* (20), catalyzing hydroxylation of toluene to form 3-methylcatechol through the intermediate *o*-cresol (21). TOM also oxidizes TCE primarily to Cl⁻ and CO₂ *in vivo* (22,23) and aerobically degrades various other chlorinated ethenes (20,24,25). TOM-Green originated from the first DNA shuffling of a non-heme monooxygenase (TOM) and has enhanced activity for both TCE degradation and naphthalene oxidation due to a single amino acid substitution, V106A, in TomA3 (26).

In contrast to GSTs, which require glutathione as the cofactor for their enzymatic activity (27), EHs do not require a cofactor (1). Unfortunately, there are no EHs of microbial origin known to have activity toward chlorinated epoxyethanes. Nevertheless, a number of microorganisms contain EHs with various substrate ranges (28-32), and various directed evolution and rational protein engineering techniques may be used to alter enzymatic activity (33,34). Hence, it was investigated here whether an epoxide hydrolase could be tuned to accept chlorinated epoxyethanes as a substrate.

The EH from *Agrobacterium radiobacter* AD1 (EchA, GeneBank accession No. Y12804) (35) was chosen for protein engineering since its physiological substrate, epichlorohydrin (2-chloropropylene oxide), resembles chlorinated epoxyethanes. EchA (294 amino acids) contains a core domain with typical α/β hydrolase fold topology formed by an eight-stranded β sheet sandwiched by α helices, and an α-helical cap domain protruding from the core domain (36). The catalytic triad residues Asp107, Asp246, and His275 are located in a hydrophobic internal cavity between the two domains (36). The catalytic reaction follows a two-step mechanism involving an alkyl-enzyme ester intermediate, which is further hydrolyzed via the attack of a water molecule (36,37).

We reasoned the substrate range of the enzyme may be tailored to accept a chlorinated epoxyethane based on the three-dimensional structure of EchA (PDB accession code 1EHY) (36), an understanding of the molecular-level properties of this enzyme (36), and its relatedness to similar EH enzymes (2,38) and haloalkane dehalogenase (DhlA) (39,40). Saturation mutagenesis was used rather than site-directed
mutagenesis to introduce all possible mutations at one site to explore a larger fraction of the protein sequence space (41). This is the first report of targeted mutagenesis of epoxide hydrolases at these positions, of an epoxide hydrolase with activity toward chlorinated epoxyethanes, and of enhancing cis-DCE degradation by combining an evolved monooxygenase and evolved epoxide hydrolase to detoxify the reactive intermediates.

EXPERIMENTAL PROCEDURES

Chemicals, organisms, and growth conditions. All materials were of highest purity available and purchased from Fisher Scientific Company (Pittsburgh, PA) except for epichlorohydrin (Acros Organics, Morris Plains, NJ), betaine (Sigma Chemical Co., St. Louis, MO), and cis-DCE (TCI America, Inc., Portland, OR). E. coli TG1 (42) was used for cloning and gene expression. Recombinant strains were routinely grown at 37°C in Luria-Bertani (LB) broth (43) supplemented with kanamycin (Kan, 100 µg/mL) and chloramphenicol (Cam, 50 µg/mL) to maintain plasmids unless otherwise stated. All whole-cell experiments used LB + Kan + Cam cultures inoculated from single, fresh colonies; exponential-phase cells were harvested at an optical density at 600 nm (OD) of ∼1.5. Isopropyl β-D-thiogalactopyranoside (IPTG, 0.5 mM) was used to induce TOM-Green which was under control of the tac-lacUV5 tandem promoter in plasmid pMMB206 (44) and also to induce EchA under control of the lac promoter in pBS(Kan) (26); IPTG was added at an OD of 0.2-0.3 for 2 hrs. The exponentially-grown cells were washed three times with one volume of Tris-HNO₃ buffer (50 mM, pH 7.0) to remove interfering byproducts and trace chloride (26).

Protein analysis and molecular techniques. Total cellular protein for the exponentially-growing culture was determined with the Total Protein Kit (Sigma Chemical Co.), and expression of recombinant proteins was analyzed with standard Laemmli discontinuous sodium dodecyl sulfate-12% polyacrylamide gels (SDS-PAGE) (43). Plasmid DNA was isolated using a Midi or Mini Kit (Qiagen, Inc., Chatsworth, CA), and polymerase chain reaction (PCR) products were purified with a Wizard® PCR Preps DNA Purification System (Promega Corporation, Madison, WI). DNA fragments were isolated from agarose
gels using a QIAquick Gel Extraction Kit (Qiagen, Inc.). *E. coli* was transformed using electroporation with Gene Pulser/Pulse Controller (Bio-Rad Laboratories, Hercules, CA) at 15 kV/cm, 25 µF, and 200 Ω.

**PCR amplification and plasmid construction.** To stably and constitutively express the EH from *A. radiobacter* AD1, the echA gene was amplified by PCR using plasmid pEH20 (35) as the template with the forward primer 5’-ATAGCGGTACCACAACGGTTTCCCT-3’ and reverse primer 5’-ATTGCTGTCGACCAGTCATGCTAGCC-3’ where underlining indicates the *KpnI* and *SalI* restriction enzyme sites, respectively. The PCR amplification was performed with *Pfu* DNA polymerase (Stratagene Corporate, La Jolla, CA) using a PCR program of 30 cycles of 94°C for 45 sec, 55°C for 45 sec, 72°C for 2 min, and a final extension of 72°C for 10 min. The PCR fragment was double-digested with *KpnI* and *SalI* and ligated into pBS(Kan) at the same restriction sites, yielding pBS(Kan)EH (Fig. 2).

The six genes tomA0-tomA5 of TOM-Green were obtained from plasmid pBS(Kan)TOM-Green (26) after *EcoRI* and *PvuI* restriction digestion and purification from an agarose gel. The resulting 5345-bp fragment was ligated into pMMB206 after digestion with the same restriction enzymes, resulting in pMMB206-TOM-Green (Fig. 2).

**Saturation mutagenesis of EchA.** A gene library encoding all possible amino acids at positions F108, I111, I219, and C248 of EchA in pBS(Kan)EH was constructed by replacing the target codon with NNN via overlap-extension PCR (41). Four pairs of degenerate primers, F108 Front/F108 Rear, I111 Front/I111 Rear, I219 Front/I219 Rear, and C248 Front/C248 Rear (Table 1) were designed to randomize codons F108, I111, I219, and C248 in the nucleotide sequence respectively, and two additional primers for cloning were EH Front and EH Rear (Table 1), which were upstream and downstream of the natural *KpnI* and *SacI* restriction sites flanking the echA gene (Fig. 2). To minimize random point mutations, *Pfu* DNA polymerase was used in the PCR. Addition of betaine (1 M) in the PCR mixture was used to improve the amplification of DNA by reducing the formation of secondary structure in the GC-rich region when necessary (45). In the first round of saturation mutagenesis, pBS(Kan)EH was used as the template and mutagenesis was performed individually at sites F108, I111, I219, and C248. In the second round of mutagenesis, pBS(Kan)EH C248I (containing amino acid substitution C248I in EchA) was used as the
template and sites F108 and I219 were randomized individually. In the third round, pBS(Kan)EH F108L/C248I (containing amino acid substitutions F108L and C248I in EchA) was used as the template and site I219 was subjected to saturation mutagenesis. Two degenerate PCR fragments were produced for each site with 463 bp and 749 bp for site F108, 457 bp and 754 bp for site I111, 800 bp and 414 bp for site I219, and 853 bp and 327 for site C248. After purifying from agarose gels, the two fragments for each site were combined at a 1:1 ratio as templates to obtain the full-length PCR product with the EH Front and EH Rear primers. The resulting randomized PCR product (1167 bp) was cloned into pBS(Kan)EH after double digestion with \( \text{KpnI} \) and \( \text{SacI} \), replacing the corresponding fragment in the original plasmid.

**Screening for enhanced cis-DCE degradation.** Evolved EchA activity toward cis-DCE epoxide was found indirectly by monitoring the concentration of chloride ion released from cis-DCE epoxide (generated by TOM-Green oxidation of cis-DCE) degradation by the evolved EchA (Fig. 1). TG1 cells harboring plasmids pMMB206-TOM-Green and pBS(Kan)EH variants were grown in 96-well plates, washed three times with Tris-HNO\(_3\) buffer (50 mM, pH 7.0), and contacted with shaking at 37°C in an airtight chamber, 23 cm by 20 cm by 23 cm, with cis-DCE vapor (2 mL) for 18 hrs. The inorganic chloride ion generated from the mineralization of cis-DCE by whole cells were detected by adding 40 \( \mu \text{L} \) 0.25 M Fe(NH\(_4\))(SO\(_4\))\(_2\) in 9 M HNO\(_3\) and 40 \( \mu \text{L} \) of saturated Hg(SCN)\(_2\) in 95% ethanol to the 200 \( \mu \text{L} \) of supernatant in each well of the 96-well plate and measured at 450 nm (26).

**Extent and kinetics of cis-DCE mineralization.** For determining the extent of mineralization of cis-DCE as indicated by Cl\(^-\) production from the best colonies identified by the 96-well screening, the exponentially-growing cells were washed three times with Tris-HNO\(_3\) buffer (50 mM, pH 7.0), and then the cell suspension (2.5 mL) was adjusted to an OD of 3, sealed in 15-mL glass serum vials, and contacted with cis-DCE at an initial liquid concentration of 540 \( \mu \text{M} \) [based on a Henry’s law constant of 0.17 (46), 2.5 \( \mu \text{mol} \) of cis-DCE was injected to the cells in 5 \( \mu \text{L} \) dimethyl formamide (DMF) at 0.2 vol\%]. IPTG (0.5 mM) was added along with 5 mM sodium succinate (as a substrate to produce NADH). After 2 hrs of incubation at 37°C and 250 rpm, the whole-cell reaction was quenched by heating the vials in boiling water for 90 sec and centrifuging \((16,000 \times \text{g}, 4 \text{ min})\) to collect the supernatant. Chloride ions
concentrations in 500 µL of supernatant were measured spectrophotometrically at 460 nm as indicated above. Cells contacted with the same amount of DMF were used as the negative control, and at least three independent experiments were analyzed.

To determine the kinetics of \textit{cis}-DCE mineralization, the OD of the cell culture was 1.2, and the initial \textit{cis}-DCE concentrations were 6.8 to 540 µM (using different stock solutions of 6.25 mM, 25 mM, 125 mM, and 500 mM in DMF at 0.2-0.4 vol%). The supernatant chloride ion concentrations generated from mineralizing \textit{cis}-DCE for each concentration were measured at 9 min for 6.8 µM and 13.5 µM, 15 min for 27 µM, 21 min for 54 µM, 38 min for 135 µM, 56 min for 270 µM, and 67 min for 540 µM; the contacting times were varied to detect significant Cl\(^{-}\) while maintaining the mineralization rate in the linear range for each \textit{cis}-DCE concentration. Parallel experiments determining the \textit{cis}-DCE degradation rate were conducted using gas chromatography (GC) to monitor \textit{cis}-DCE depletion as described previously (25). Headspace samples from the same cell suspensions contacted with \textit{cis}-DCE at various concentrations in the \textit{cis}-DCE kinetics experiments were analyzed before the cells were quenched to determine Cl\(^{-}\) production.

**Purification of EchA.** Both wild-type EchA and variant F108L/I219L/C248I were expressed in TG1/pBS(Kan) for enzyme purification, and the method of Rink et al.(35) was adopted with modification. Exponentially-growing pre-cultures were diluted 1:100 in 3 L of fresh LB containing 100 µg/mL Kan and incubated with shaking at 37°C. IPTG (1 mM) was added when the cell OD reached 0.3, and the culture was incubated at 25°C overnight with aeration. Cells were harvested, washed by centrifugation at 10,000 \( \times \) g, 4°C for 10 min with TEMAG buffer (10 mM Tris-SO\(_4\), 1 mM EDTA, 1 mM \( \beta \)-mercaptoethanol, 0.02% sodium azide and 10 % glycerol, pH 7.5), and resuspended in 30 mL of the same buffer. Cells were disrupted by a French® Pressure Cell Press (Spectronic Instruments, Rochester, NY) and centrifuged at 20,000 \( \times \) g, 4°C for 30 min. Anion exchange was performed by applying 30 mL of the supernatant to 30-mL of DEAE Sepharose (Sigma-Aldrich) (47) and proteins were eluted with a continuous gradient ammonium sulfate in TEMAG (0 M – 1 M). Fractions with the highest enzymatic activity were pooled and dialyzed against TEMAG buffer overnight at 4°C and were further purified via
size exclusion chromatography by adding 1.5 mL to 80-mL Sephacryl S-100 HR (Sigma-Aldrich). Fractions with purified EchA (with the highest EH activity and the highest purity as visualized on SDS-PAGE) were pooled and dialyzed against TEMAG buffer overnight. The final product was stored at –20°C with glycerol (10% v/v) for future use; variant F108L/I219L/C248I and wild-type enzyme were purified from 10% to 80% and 90%, respectively. Activity of column fractions was determined using a polypropylene 96-well plate format with styrene oxide (5 mM) as the substrate rather than the reported epichlorohydrin (35). Column fractions (10 µL) added to 136 µL TE buffer were incubated with 5 mM styrene oxide at 37°C for 15 minutes followed by the sequential addition of 100 mM 4-nitrobenzylpyridine (75 µL) and triethylamine (75 µL). The chromogenic reaction of styrene oxide with 4-nitrobenzylpyridine was measured at 620 nm with a Multiskan reader (Fisher Scientific), and disappearance of the blue color indicated the disappearance of styrene oxide.

**EH assays.** As a preliminary assay, whole cells of TG1/pBS(Kan)EH (grown in LB + 100 µg/mL Kan) were tested for EchA activity in *E. coli* using a chromogenic reaction of the epoxide epichlorohydrin with 4-nitrobenzylpyridin (35). The assay was performed in 1.5-mL microcentrifuge tubes with 100 µL of exponentially-grown cells contacted with 10 mM epichlorohydrin in 400 µL TE buffer (50 mM Tris-SO₄, 1 mM EDTA, pH 9.0) for around 1 hr at room temperature, then 250 µL of 4-nitrobenzylpyridine (100 mM in 80 vol% ethylene glycol and 20 vol% acetone) was added, the cells were heated at 80°C for 10 min, and 250 µL triethylamine (1:1 in acetone) was added. The blue color was proportional to the remaining epichlorohydrin. Purified enzyme (2.5 µg) was also tested at 5 mM epichlorohydrin at 37°C for 30 min and with 4-nitrobenzylpyridine heated at 50°C for 30 min.

EchA specific activity was also determined with whole cells using the substrate 1,2-expoxyhexane; cells prepared the same way as for the *cis*-DCE mineralization experiments (2.5 mL with contact OD ~ 2.0) were sealed in 15 mL glass vials and used for the assay, and headspace concentrations of 1,2-expoxyhexane (5 mM) were determined by monitoring headspace samples with GC. The GC (Agilent 6890 series) was equipped with a 0.10% AT-1000 packed column (Alltech, length 1.829 m, inner diameter 3.175 mm, film thickness 2.159 mm) and a flame-ionization detector (FID). The FID was
supplied with hydrogen (30 mL/min) and air (300 mL/min), and nitrogen was used as the carrier gas (20 mL/min). Headspace samples injected into the GC every 15 min were analyzed isothermally at 200°C and the retention time of 1,2-expoxyhexane was 1.7 min under such conditions. TG1 pMMB206-TOM-Green pBS(Kan) was used as the negative control.

To determine the specific activity of purified EchA towards 1,2-expoxyhexane, 1 or 20 µg EchA was added to 2.5 mL Tris-HCl buffer (50 mM, pH 7.4) in a sealed 15-mL glass vial and reacted with 0.025 to 5 mM 1,2-expoxyhexane. The activity was determined using GC by monitoring substrate depletion with headspace samples injected every 15 min (same GC conditions as with the whole cell experiments). The Henry’s law constant for 1,2-epoxyhexane was estimated as 0.089 using extraction with ethyl acetate.

**TOM-Green activity and DNA Sequencing.** To ensure relatively constant TOM-Green activity during cis-DCE degradation with the various EchA variants, parallel, whole-cell naphthol synthesis assays were conducted by incubating the same cells that were used for cis-DCE degradation with naphthalene in the absence of cis-DCE to monitor TOM-Green activity as described previously using tetrazotized o-dianisidine and a spectrophotometric assay (26). A dye terminator cycle sequencing protocol based on the dideoxy method of sequencing DNA developed by Sanger et al. (48) was used to sequence both strands of wild-type and mutant EchA(49).

**Homology structural modeling.** The three dimensional coordinates of the EchA variants were generated with SWISS-MODEL Server (50-52) using a structure model of wild-type EchA as the template (36), and visualized with Swiss-PdbViewer (50-52). The use of the structure model instead of the original X-ray structure of EchA as the structural template for homology modeling was due to the fact that the X-ray structure was obtained from an inactive enzyme possibly with false crystal packing forces, which resulted in one of the catalytic triad residues, Asp246, to be positioned outside of the active site (36); here we used the EchA structure model with the loop containing Asp246 rebuilt in the more likely active conformation of EchA (36) both because it represents a common picture of active site of α/β hydrolase-fold enzymes (36) and was further confirmed by mutagenesis studies (37).
RESULTS

Plasmid construction. To create clone libraries via electroporation and reliably screen them for enhanced EH activity, a stable plasmid that expresses EchA constitutively, pBS(Kan)EH (Fig. 2), was constructed that utilizes a constitutive lac promoter and kanamycin resistance gene. Use of kanamycin circumvents segregational instability and avoids feeder colonies that are associated with ampicillin resistance vectors. The resulting epoxide hydrolase expressed in E. coli TG1 had activity towards its natural substrate epichlorohydrin (10 mM) based on the preliminary EH assay using 4-nitrobenzylpyridine (data not shown). TOM-Green was expressed from pMMB206-TOM-Green (Fig. 2), a wide-host-range, low-copy-number vector that is compatible with pBS(Kan)EH.

Saturation mutagenesis, screening, and sequencing analysis. Saturation mutagenesis was performed individually on the four EchA sites F108, I111, I219, and C248, which we chose based on their close vicinity to the catalytic triad residues (D107, D246, and H275; Fig. 3) (36). Two of these residues, F108 and C248, were hypothesized previously to influence substrate binding in this or a related enzyme, although mutagenesis was not performed at these sites (2,36). By cloning DNA fragments from saturation mutagenesis back into the corresponding position of pBS(Kan)EH, all possible amino acids were introduced at the three sites respectively. A library containing ~2,000 colonies for each site was obtained and about 300 of those colonies were screened in 96-well plates for cis-DCE degradation since 292 independent clones from saturation mutagenesis at one site need to be screened for a 99% probability that each possible codon has been tested (49).

Whole cells expressing the EchA variants and TOM-Green with enhanced cis-DCE mineralization, as indicated by increased Cl− released, were found from three of the mutagenesis libraries, and the beneficial amino acid substitutions were F108L, I219F, and C248I, indicating each of these positions is important for adapting EchA to the substrate cis-DCE epoxide (Table 2). No beneficial amino acid substitution was found at position I111. Although the three variants enhanced cis-DCE mineralization to a similar extent when co-expressed with TOM-Green (Table 2, 2.4 to 2.7-fold), the C248I mutation was slightly superior so it was used as a new template for a second round of saturation mutagenesis to combine the beneficial
mutations at positions F108 and I219; saturation mutagenesis was used to introduce the new residues at these positions rather than site-directed mutagenesis since it was not clear that how the three positions would interact.

Around 300 colonies from each of the two resulting libraries were again screened for improved cis-DCE mineralization activity using 96-well microtiter plates. The beneficial mutations that resulted in further improvements in cis-DCE mineralization from the two libraries were F108L/C248I (7.1-fold) and I219L/C248I (4.2-fold). As EchA F108L/C248I enhanced cis-DCE mineralization more than EchA I219L/C248I, it was used as the new template for a third round of saturation mutagenesis at position I219. The same-sized library was screened, and four positive variants were found, all containing I219L. Thus, the best EchA variant for enhancing cis-DCE mineralization was created by three rounds of saturation mutagenesis with amino acid substitutions F108L, I219L, and C248I. The mutation I219F that was discovered in the first round of saturation mutagenesis as beneficial was lost in the further mutagenesis experiments, which indicates I219F might not be compatible with other mutations at C248 and/or F108. The whole process shows that beneficial mutations can be quickly accumulated by multiple rounds of saturation mutagenesis and screening relatively small libraries.

Enhanced cis-DCE mineralization by the evolved EchA. As cis-DCE epoxide is commercially unavailable and short-lived with a half life of 72 hrs (16), cis-DCE mineralization was used as the indirect assay to characterize evolved EchA. In evaluating cis-DCE mineralization, TOM-Green in pMMB206-TOM-Green was always expressed to initiate the degradation reaction by forming cis-DCE epoxide. Because the mineralization of cis-DCE is the concerted reaction by both TOM-Green and EchA, whole cells were used. Naphthol synthesis assays were used to monitor TOM-Green activity in the cis-DCE degradation experiments of EchA mutants F108L/C248I and F108L/I219L/C248I to ensure the difference in cis-DCE mineralization rate was not caused by differences in TOM-Green activity. It was assumed that EchA should have no effect on naphthol formation either because no naphthalene epoxide was formed during the TOM-Green transformation or because naphthalene epoxide (if formed) was not within the

Rui et al., UConn
substrate range of EchA. TOM-Green activity was relatively constant with each EchA isoform (Table 3) at approximately 1 nmol/min•mg protein at 0.24 mM naphthalene.

In addition, the EchA expression levels of all the mutants listed in Table 2 was characterized using SDS-PAGE (41). The TOM-Green α (size 54.4 kDa) and β (size 37.7 kDa) subunits were clearly seen as well as EchA (size 34 kDa), and the expression level was the same for all the EchA mutants as well as for TOM-Green (data not shown). Hence, the enhancements in cis-DCE activity were not due to changes in protein expression.

The enhancements in cis-DCE mineralization at 540 µM initial substrate concentration by whole cells expressing TOM-Green and the EchA variants created in the first, second, and third rounds of saturation mutagenesis are listed in Table 2. In comparing the enhancement of cis-DCE mineralization by EchA variant to the wild-type, the part of cis-DCE mineralized of by TOM-Green alone (in TG1/pMMB206-TOM-Green/pBS(Kan)) was subtracted as background signal as no EchA was involved. It is shown in Table 2 that there was only slight increase in cis-DCE mineralization rate by wild-type EchA compared to the EchA− strain, indicating that cis-DCE epoxide is a poor substrate of wild-type EchA. Although the single mutation variants at the three separate sites (F108, I219, and C248) did not result in a large change in cis-DCE mineralization enhancement, combination of beneficial mutations did lead to a step-by-step improvement and finally brought about 10-fold enhancement in cis-DCE mineralization rate with the variant containing the triple mutations F108L/I219L/C248I (Table 2). As the cell systems are isogenic, there was equivalent EchA protein expression level, and there was similar TOM-Green activity, these results indicate that the EchA mutants, especially F108L/I219L/C248I, were tailored to accept cis-DCE epoxide within their substrate range and to participate in the biological degradation of cis-DCE epoxide generated as the primary intermediate by TOM-Green.

**Kinetics of cis-DCE mineralization by the best EchA variant.** EchA F108L/I219L/C248I co-expressed with TOM-Green was further characterized for enhancement in cis-DCE mineralization rate at different substrate concentrations, and the saturation constants, apparent $V_{\text{max}}$ and apparent $K_m$ for the co-expression system, were obtained (Table 3). Whole cells expressing EchA variant F108L/I219L/C248I
had enhanced cis-DCE mineralization at all the substrate concentrations with the largest difference at lower cis-DCE concentrations (6.8-27 µM) as there was no detectable activity with wild-type EchA below 25 µM, and this was reflected by 40% reduction in the apparent K_m with EchA F108L/I219L/C248I. Thus, EchA F108L/I219L/C248I not only elevated the apparent V_max for cis-DCE mineralization, but also increased the affinity towards cis-DCE.

Although we expected an enhancement in the cis-DCE degradation rate as well (initial disappearance rate), the parallel experiments monitoring cis-DCE degradation via GC did not show a significant difference in the initial degradation rates between the strains with wild-type EchA and the F108L/I219L/C248I variant (data not shown). For example, at an initial liquid cis-DCE concentration of 135 µM, about 55% cis-DCE was consistently depleted within 38 min for both strains. However, for the F108L/I219L/C248I variant, the degraded cis-DCE was almost completely mineralized as indicated by the Cl^- production, while only 36% of the degraded cis-DCE was mineralized with wild-type EchA. As the two strains are isogenic with only three amino substitutions, the enhanced Cl^- formation arises from the additional conversion route of cis-DCE epoxide by the evolved EchA (Fig. 1).

Enhanced 1,2-epoxyhexane and epichlorohydrin hydrolysis. To obtain direct evidence that the EchA isoforms were functionally expressed in the system, EH activity towards an epoxide was examined. Though cis-DCE epoxide would be the best substrate for this study, it is commercially unavailable and difficult to synthesize and utilize (16), so 1,2-epoxyhexane, a good substrate of wild-type EchA (35), was chosen as the alternative substrate to determine EH activity of wild-type EchA, EchAF108L/C248I, and EchA F108L/I219L/C248I. The same whole-cell system used for the cis-DCE mineralization experiments, TG1/pMMB206-TOM-Green/pBS(Kan)EH, was used for determining EH activity. For whole cells, there was a 2.1-fold increase in the 1,2-epoxyhexane activity by EchA F108L/I219L/C248I compared to the wild-type enzyme (Table 3). To corroborate these results, purified EchA was tested, and the k_cat for 1,2-epoxyhexane hydrolysis with the F108L/I219L/C248I variant and wild-type enzymes were 8.4/sec and 3.6/sec, respectively (K_m values of 43 µM and 20 µM, respectively); hence, there was a similar activity enhancement as that obtained with whole cells. Further, the increase in the 1,2-epoxyhexane hydrolysis
rate seemed to follow the same trends as the enhancement in *cis*-DCE mineralization: a gradual increase was seen as the beneficial mutations were combined as indicated by the intermediate activity of the dual mutant EchA F108L/C248I towards 1,2-epoxyhexane (156 ± 20 nmol/min•mg protein with whole cells). A six-fold improvement in epichlorohydrin hydrolysis was also obtained using purified enzymes (94 ± 8 µmol/min•mg for the F108L/I219L/C248I variant vs. 16 ± 2 µmol/min•mg for the wild-type enzyme). Hence, EchA was optimized for more than just *cis*-DCE epoxide by the three mutations.

**DISCUSSION**

It is clearly shown in this paper that by active site engineering at carefully selected residues (EchA F108, I219, and C248) and by accumulating beneficial mutations via saturation mutagenesis, EchA was engineered to accept *cis*-DCE epoxide as a substrate. This is significant since the aerobic biodegradation of chlorinated ethenes requires the detoxification of the reactive epoxides formed as the primary intermediates after oxygenase attack. To our knowledge, this is the first report of protein engineering of epoxide hydrolases at these or analogous sites for any application.

For the rational redesign of EchA, the important residues must first be identified. The choice of sites here was based on the investigation of the active site of EchA and structural comparison with other related enzymes, including the haloalkane dehalogenase from *Xanthobacter autotrophicus* (DhlA) (39,40) (PDB accession code 2HAD), marine soluble epoxide hydrolase (PDB accession code 1CQZ) (38), and *Aspergillus niger* epoxide hydrolase (AnEH, PDB accession code 1QO7) (2). The structural model of a human microsomal epoxide hydrolase based on AnEH was also considered (2). These enzymes contain the canonical α/β hydrolase fold with conserved catalytic triad (2), indicating their common phylogenetic origin; however, there are many structural differences due to their low sequence homology (20-30% amino acid identity in the core region) which yields an extremely-versatile substrate range (2). Based on the hypervariability at key structural residues that may contribute to the shape and substrate binding properties of the active site cavity (2,36,38,39), and in turn might affect the substrate specificity, we altered the active site residues Phe108 and Cys248 of EchA for acceptance of a new chlorinated substrate.
Phe108 is in close vicinity to the substrate (Fig. 3) as it is located next to the nucleophile Asp107, which initiates the hydrolysis reaction by attacking the substrate (36), and contributes to the formation of the structurally-conserved oxyanion hole, which is needed to stabilize the negatively-charged transition state occurring in hydrolysis (36). In addition, Phe108 has been suggested to be involved in substrate binding (36). Despite its structural and functional importance, the equivalent residues of Phe108 in the related enzymes vary considerably, with Trp125 in DhIA (39), Trp227 in human microsomal EH (mEH) (2), Trp334 in marine liver cytosolic EH (sEH) (38), Ile193 in AnEH (2), and Phe108 in EchA (36).

Cys248 is one residue away from the catalytic acidic residue Asp246 (36). Its equivalent residue in AnEH, Cys350, is a constituent of the active site wall and was proposed to contribute to the geometry and character of the active site cavity (2). In addition, the side chain of Leu262, the equivalent residue in DhIA, appears to block the tunnel that connects the active site cavity with the outside solvent region (39). Cys248 is also a hypervariant codon with the equivalent residues in other related enzymes as Cys350 in AnEH (2), Phe406 in human mEH (2), Val497 in sEH (38), and Leu262 in DhIA (39). We reasoned that mutating Cys248 may bring subtle effects on the specificity and reactivity of the enzyme.

Although there is no evidence showing that Ile219 interacts directly with substrate during the reaction nor has it been previously identified as influencing catalytic activity, we determined that it has van der Waals contact with both Phe108 and Tyr215 (within 4 Å; Fig. 3); Tyr215 was suggested to function as the proton donor in the catalytic mechanism of EchA (53) and was thought to direct initial substrate binding and positioning in the active center (2). As this Tyr residue role is conserved in other EHs (2), direct mutation at this residue could cause drastic changes in the active site properties, whereas we reasoned that mutation at Ile219, which interacts with Tyr215, could bring some subtle, beneficial effects. Change in the side chain of Ile219 was thought to bring slight changes in the position or orientation of Tyr215 as well as Phe108 and in turn could influence substrate binding.

We also tried saturation mutagenesis at position Ile111 as it is also in the vicinity of one of the catalytic residues, Asp107 (Fig. 3) and seems to be a hypervariant residue with Phe128 in DhIA (39), Phe196 in AnEH, and Leu230 in mEH (2). However we did not obtain any variant with enhanced cis-
DCE mineralization when coexpressed with TOM-Green; hence, its role may be more structural than catalytic.

Concerted effects from the changes of the three residues (F108L/I219L/C248I) may optimize the size, shape, and hydrophobic character of the active site to facilitate binding and stabilization for cis-DCE epoxide and its transitional state intermediates. Interestingly, engineering EchA for the poor substrate cis-DCE epoxide also improved activity for both 1,2-epoxyhexane (Table 3) and epichlorohydrin. Hence, the substrate specificity of EchA may be extended further to epoxides of other chlorinated ethenes, such as TCE and tetrachloroethylene (PCE), by protein rational design or directed evolution. Further, in combination with metabolic pathway engineering, the chlorinated epoxyethanes may be channeled into productive metabolic pathways, potentially allowing chlorinated ethenes to be utilized as a sole carbon and energy source, since the inability of various chlorinated ethenes to support growth is not due to lack of energy during conversion (6), but because no suitable enzyme system is able to harvest the energy.

REFERENCES

1. Weijers, C. A. G. M., and Bont, J. A. M. d. (1999) *J. Mol. Catal., B Enzym.* 6, 199-214
2. Zou, J., Hallberg, B. M., Bergfors, T., Oesch, F., Arand, M., Mowbray, S. L., and Jones, T. A. (2000) *Structure* 8, 111-122
3. Fretland, A. J., and Omiecinski, C. J. (2000) *Chem. Biol. Interact.* 129, 41-59
4. Hernandez, O., and Bend, J. R. (1982) in *Metabolic Basis of Detoxification* (Jakoby, W. B., Bend, J. R., and Caldwell, J., eds), Academic Press, New York, NY
5. Archelas, A., and Furstoss, R. (1998) *Trends Biotechnol.* 16, 108-116
6. van Hylckama Vlieg, J. E. T., and Janssen, D. B. (2001) *J. Biotechnol.* 85, 81-102
7. Faber, K., Mischitz, M., and Kroutil, W. (1996) *Acta Chem. Scand.* 50, 249-258
8. Steinreiber, A., and Faber, K. (2001) *Curr. Opin. Biotechnol.* 12, 552-558
9. Bradley, P. M., and Chapelle, F. H. (1998) *Environ. Sci. Technol.* 32, 553-557
10. McCarty, P. L. (1997) *Science* 276, 1521-1522
11. Henschler, D. (1994) Angew. Chem. Int. Ed. Engl. 33, 1920-1935
12. Bolt, H. M., Lair, R. J., and Filser, J. G. (1982) Biochem. Pharmacol. 31, 1-4
13. Carter, S. R., and Jewell, W. J. (1993) Water Res. 27, 607-615
14. Magnuson, J. K., Stern, R. V., Gossett, J. M., Zinder, S. H., and Burris, D. R. (1998) Appl. Environ. Microbiol. 64, 1270-1275
15. Newman, L. M., and Wackett, L. P. (1997) J. Bacteriol. 179, 90-96
16. van Hylckama Vlieg, J. E. T., de Koning, W., and Janssen, D. B. (1996) Appl. Environ. Microbiol. 62, 3304-3312
17. van Hylckama Vlieg, J. E. T., de Koning, W., and Janssen, D. B. (1997) Appl. Environ. Microbiol. 63, 4961-4964
18. Yeager, C. M., Bottomley, P. J., and Arp, D. J. (2001) Appl. Environ. Microbiol. 67, 2107-2115
19. Rui, L., Kwon, Y. M., Reardon, K. F., and Wood, T. K. (2004) Environ. Microbiol. 6, 491-500
20. Shields, M. S., and Francesconi, S. C. (1996) in U. S. Patent 5,543,317, U.S. A
21. Shields, M. S., Montgomery, S. O., Chapman, P. J., Cuskey, S. M., and Pritchard, P. H. (1989) Appl. Environ. Microbiol. 55, 1624-1629
22. Luu, P. P., Yung, C. W., Sun, A. K., and Wood, T. K. (1995) Appl. Microbiol. Biotechnol. 44, 259-264
23. Nelson, M. J. K., Montgomery, S. O., O'Neill, E. J., and Pritchard, P. H. (1986) Appl. Environ. Microbiol. 52, 383-384
24. Shields, M. S., Reagin, M. J., Gerger, R. R., Somerville, C., Schaubhut, R., Campbell, R., and Hu-Primmer, J. (1994) in Bioremediation of Chlorinated and Polycyclic Aromatic Hydrocarbon Compounds (Hinchee, R. E., Leeson, A., Semprini, L., and Ong, S. K., eds), pp. 50-65, Lewis Publishers, Boca Raton, FL
25. Shim, H., and Wood, T. K. (2000) Biotechnol. Bioeng. 70, 693-698
26. Canada, K. A., Iwashita, S., Shim, H., and Wood, T. K. (2002) J. Bacteriol. 184, 344-349
27. Vuilleumier, S. (1997) J. Bacteriol. 179, 1431-1441
28. Jacobs, M. H., Van den Wijngaard, A. J., Pentenga, M., and Janssen, D. B. (1991) *Eur. J. Biochem.* **202**, 1217-1222

29. Visser, H., Bont, J. A. M. d., and Verdoes, J. C. (1999) *Appl. Environ. Microbiol.* **65**, 5459-5463

30. Visser, H., Vreugdenhil, S., Bont, J. A. M. d., and Verdoes, J. C. (2000) *Appl. Microbiol. Biotechnol.* **53**, 415-419

31. Misawa, E., Chion, C. K. C. K., Archer, I. V., Woodland, M. P., Zhou, N. Y., Carter, S. F., Widdowson, D. A., and Leak, D. J. (1998) *Eur. J. Biochem.* **253**, 173-183

32. Nakamura, T., Nagasawa, T., Yu, F., Watanabe, I., and Yamada, H. (1994) *Appl. Environ. Microbiol.* **60**, 4630-4633

33. Brannigan, J. A., and Wilkinson, A. J. (2002) *Nat. Rev. Mol. Cell Biol.* **3**, 964 - 970

34. Stemmer, W. P. C. (1994) *Nature* **370**, 389-391

35. Rink, R., Fennema, M., Smids, M., Dehmel, U., and Janssen, D. B. (1997) *J. Biol. Chem.* **272**, 14650-14657

36. Nardini, M., Ridder, I. S., Rozeboom, H. J., Kalk, K. H., Rink, R., Janssen, D. B., and Dijkstra, B. W. (1999) *J. Biol. Chem.* **274**, 14579-14586

37. Nardini, M., Rink, R., Janssen, D. B., and Dijkstra, B. W. (2001) *J. Mol. Catal., B Enzym.* **11**, 1035-1042

38. Argiriadi, M. A., Morisseau, C., Hammock, B. D., and Christianson, D. W. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 10637-10642

39. Franken, S. M., Rozeboom, H. J., Kalk, K. H., and Dijkstra, B. W. (1991) *EMBO J.* **10**, 1297-1302

40. Verschueren, K. H. G., Seljee, F., Rozeboom, H. J., Kalk, K. H., and Dijkstra, B. W. (1993) *Nature* **363**, 693-698

41. Sakamoto, T., Joern, J. M., Arisawa, A., and Arnold, F. H. (2001) *Appl. Environ. Microbiol.* **67**, 3882-3887

42. Gibson, T. J. (1984), Cambridge University, Cambridge, England
43. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Second Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

44. Morales, V. M., Backman, A., and Bagdasarian, M. (1991) *Gene* 97, 39-47

45. Baskaran, N., Kandpal, R. P., Bhargava, A. K., Glynn, M. W., Bale, A., and Weissman, S. M. (1996) *Genome Res.* 6, 633-638

46. Dolfing, J., van den Wijngaard, A. J., and Janssen, D. B. (1993) *Biodegradation* 4, 261-282

47. Spelberg, J. H. L., Rink, R., Kellogg, R. M., and Janssen, D. B. (1998) *Tetrahedron: Asymmetry* 9, 459-466

48. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* 74, 5463-5467

49. Rui, L., Kwon, Y. M., Fishman, A., Reardon, K. F., and Wood, T. K. (2004) *Appl. Environ. Microbiol.* 70, 3246-3252

50. Schwede, T., Kopp, J., Guex, N., and Peitsch, M. C. (2003) *Nucleic Acids Res.* 31, 3381-3385

51. Peitsch, M. C. (1993) *Bio/Technology* 13, 658-660

52. Guex, N., and Peitsch, M. C. (1997) *Electrophoresis* 18, 2714-2723

53. Rink, R., Kingma, J., Lutje Spelberg, J. H., and Janssen, D. B. (2000) *Biochemistry* 39, 5600-5613

54. Perry, R. H., and Chilton, C. H. (1973) *Chemical Engineers' Handbook*, Fifth Ed., McGraw-Hill Book Company, New York, NY

**ACKNOWLEDGEMENTS**

This research was supported by the National Science Foundation (BES-9911469 and BES-0331416). We appreciate the gifts of plasmid pEH20 by Prof. Dick Janssen and of the EchA structure model by Prof. Bauke Dijkstra (both of the University of Groningen). We thank Ying Tao of the Wood laboratory for measuring the Henry’s law constant.
FIGURE LEGENDS

**Figure 1.** Metabolic engineering to enhance *cis*-DCE mineralization by cloning an evolved epoxide hydrolase (EchA) along with an evolved toluene *ortho*-monooxygenase (TOM-Green) (adapted from van Hylckama Vlieg and Janssen (6)). Steps 1 and 2 are the two possible spontaneous transformation pathways for *cis*-DCE epoxide, while step 3 (this work) and 4 (19) represent two major detoxification strategies in with *cis*-DCE epoxide may be biologically converted by either an epoxide hydrolase or glutathione S-transferase (IsoILR1).

**Figure 2.** Plasmid maps of pBS(Kan)EH and pMMB206-TOM-Green. Cloning restriction enzyme sites are listed. TOM-Green is a variant of toluene *ortho*-monooxygenase from DNA shuffling with the substitution V106A in *tomA3* (26).

**Figure 3.** Active site of EchA F108L/I219L/C248I based on homology structural modeling. The catalytic triad residues Asp107, Asp246, and His275 (green) are located in a predominantly hydrophobic internal cavity between the core domain (gray) and the cap domain (black). The three amino acid substitutions Leu108, Leu219, and Ile248 (red) are superimposed on the wild-type residues Phe108, Ile219, and Cys248 (blue). Tyr215 (purple), a proton-donor residue, has van der Waals contact with Ile219.
Table 1. Oligonucleotide primers used for saturation mutagenesis at positions F108, I111, I219, and C248 of EchA.

| Primer   | Sequences                                                                 |
|----------|---------------------------------------------------------------------------|
| F108 Front | AGGCGTACGTCGTTGGCCATGACNNNGCGGCCATC                                      |
| F108 Rear  | TTATGGAGGACGATGCGCCGCGNNNTCATGGCCAAC                                      |
| I111 Front | ACGTCGTTGGCCATGACTTCGCGCCGCGNNNGTCCTCC                                    |
| I111 Rear  | ATGAATTATGGAGGACNNNGGGCCGGAAGTCATG                                      |
| I219 Front | CTTCAACTACTATCGTCGCAACNNNAGGCCCCGATG                                    |
| I219 Rear  | ACAGAGCGGCATCGGGCCTNNNGTTGGCAGGATAG                                    |
| C248 Front | ATATGGGTTTGGGAGATACTNNNGTGCCCTATGC                                     |
| C248 Rear  | TCAATGAGTGGAGCATAGGGCACNNNAGTATCTCC                                     |
| EH Front\(^1\) | AGCTATGACCATGATTACGCCAAGC                                               |
| EH Rear\(^2\) | CGTTGAAAACGACGGCCAGTGA                                                   |

\(^1\) Utilizing the natural KpnI restriction site upstream of echA.

\(^2\) Utilizing the natural SacI restriction site downstream of echA.
Table 2. Enhanced cis-DCE mineralization by whole cells expressing EchA variants after saturation mutagenesis at sites F108, I219, and C248. Strain TG1/pMMB206-TOM-Green/pBS(Kan)EH was used to simultaneously express EchA (wild-type and mutants) and TOM-Green.

| Enzyme               | Mineralization rate of cis-DCE<sup>2,3,4</sup> | Chloride ion release in 2 hr<sup>4</sup> |
|----------------------|-----------------------------------------------|-----------------------------------------|
|                      | Rate, nmol/min•mg protein | Fold increase<sup>5</sup> | Cl<sup>−</sup>, µM | Mineralization, % | Fold increase<sup>5</sup> |
| 1EchA<sup>−</sup>     | 2.35 ± 0.23                      | 1.0                                | 357 ± 24          | 17.8%            | 1.0                  |
| wild-type EchA       | 2.88 ± 0.25                      | 1.0                                | 420 ± 34          | 21.0%            | 1.0                  |
| F108L                | 3.60 ± 0.56                      | 2.4                                | 519 ± 45          | 26.0%            | 2.6                  |
| I219F                | 3.72 ± 0.61                      | 2.6                                | 544 ± 54          | 27.2%            | 3.0                  |
| C248I                | 3.78 ± 0.22                      | 2.7                                | 548 ± 7           | 27.4%            | 3.0                  |
| I219L/C248I          | 4.55 ± 0.67                      | 4.2                                | 606 ± 24          | 30.3%            | 4.0                  |
| F108L/C248I          | 6.13 ± 0.48                      | 7.1                                | 898 ± 137         | 44.9%            | 8.7                  |
| F108L/I219L/C248I    | 7.57 ± 0.21                      | 9.9                                | 1036 ± 37         | 51.8%            | 10.9                 |

<sup>1</sup>EchA<sup>−</sup>: TG1/pMMB206-TOM-Green/pBS(Kan)

<sup>2</sup>Determined via chloride ion release after 2 hr contact (cf. Table 3 with 67 min contact)

<sup>3</sup>Total protein: 0.18 mg protein/mL·OD

<sup>4</sup>Initial cis-DCE concentrations was 540 µM calculated based on Henry’s Law with Henry’s constant 0.17 (46) (1 mM were added as if all the volatile organic was in the liquid phase)

<sup>5</sup>Strains expressing EchA variants compared to that of wild-type EchA after subtracting the chloride generated by TG1/pMMB206-TOM-Green/pBS(Kan) (no epoxide hydrolase control)
Table 3. Kinetics of cis-DCE mineralization by whole cells expressing the best EchA variant, F108L/I219L/C248I, their activity toward 1,2-epoxyhexane, and TOM-Green activity via naphthalene oxidation.

| Enzyme¹ | 6.8 μM | 13.5 μM | 27 μM | 54 μM | 135 μM | 270 μM | 540 μM | Apparent Vₘₐₓ, nmol/min•mg protein | Apparent Kₘ, μM | 1,2-Epoxyhexane hydrolysis rate, nmol/min•mg protein³,⁵ | Naphthalene formation rate, nmol/min•mg protein³,⁶ |
|---------|--------|--------|-------|-------|--------|--------|--------|-------------------------------|----------------|---------------------------------|---------------------------------|
| EchA⁻    | 1.1 ± 0.2 | 2.5 ± 0.1 | 2.7 ± 0.1 | 3.4 ± 0.4 | 4.4 ± 0.8 | 4.5 ± 0.5 | 4.5 | 13 | 0 | 0.97 ± 0.09 |
| wild-type EchA | 0.2 ± 0.2 | 2.6 ± 0.3 | 3.1 ± 0.1 | 4.5 ± 0.2 | 5.2 ± 0.7 | 4.8 ± 0.0 | 5.4 | 14 | 118 ± 12 | 1.03 ± 0.02 |
| F108L/I219L/C248I | 4.4 ± 1.1 | 8.7 ± 0.3 | 11.1 ± 1.4 | 11.3 ± 0.5 | 13.3 ± 1.8 | 11.4 ± 0.5 | 11.0 ± 0.2 | 13.3 | 8.1 | 253 ± 18 | 0.91 ± 0.04 |

¹EchA⁻: TG1/pMMB206-TOM-Green/pBS(Kan); wild-type EchA: TG1/pMMB206-TOM-Green/pBS(Kan)EH; F108L/I219L/C248I: TG1/pMMB206-TOM-Green/pBS(Kan)EH F108L/I219L/C248I
²Determined via chloride ion release after 67 min contact time (cf. Table 2 with 2 hr contact time)
³Total protein was 0.18 mg protein/(mL·OD)
⁴Initial cis-DCE concentrations were calculated based on Henry’s Law with a Henry’s constant of 0.17 (46)
⁵Determined via gas chromatography by monitoring 1,2-epoxyhexane degradation using whole cells (5 mM initial concentration)
⁶Naphthalene was added at 5 mM although its solubility is 0.24 mM in water (54)
Figure 1

Cell death due to non-specific covalent modification of cell constituents directly or after intramolecular rearrangement.

Other small molecules

Epoxide hydrolase

Glyoxal

Glyoxal + GSH

Glutathione S-transferase

Glyoxal
Figure 2
Active site engineering of the epoxide hydrolase from agrobacterium radiobacter AD1 to enhance aerobic mineralization of cis-1,2-dichloroethylene in cells expressing an evolved toluene ortho-monoxygenase

Lingyun Rui, Li Cao, Wifred Chen, Kenneth F. Reardon and Thomas K. Wood

*J. Biol. Chem.* published online August 30, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M407466200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts