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Metabolic pathway engineering to enhance aerobic degradation of chlorinated ethenes and to reduce their toxicity by cloning a novel glutathione *S*-transferase, an evolved toluene *o*-monooxygenase, and γ-glutamylcysteine synthetase

Lingyun Rui,[1] Young Man Kwon,[1] Kenneth F. Reardon[2] and Thomas K. Wood[1]*

[1]*Departments of Chemical Engineering and Molecular and Cell Biology, University of Connecticut, Storrs, CT 06269-3222, USA.*

[2]*Department of Chemical Engineering, Colorado State University, Fort Collins, CO 80523-1370, USA.*

Summary

Aerobic, co-metabolic bioremediation of trichloroethylene (TCE), *cis*-1,2-dichloroethylene (*cis*-DCE) and other chlorinated ethenes with monooxygenase-expressing microorganisms is limited by the toxic epoxides produced as intermediates. A recombinant *Escherichia coli* strain less sensitive to the toxic effects of *cis*-DCE, TCE and *trans*-1,2-dichloroethylene (*trans*-DCE) degradation has been created by engineering a novel pathway consisting of eight genes including a DNA-shuffled toluene *ortho*-monooxygenase from *Burkholderia cepacia* G4 (TOM-Green), a newly discovered glutathione *S*-transferase (GST) from *Rhodococcus* AD45 (IsoILR1), found to have activity towards epoxypropane and *cis*-DCE epoxide, and an overexpressed *E. coli* mutant γ-glutamylcysteine synthetase (GSHI*). Along with IsoILR1, another new *Rhodococcus* AD45 GST, IsoILR2, was cloned that lacks activity towards *cis*-DCE epoxide and differs from IsoILR1 by nine amino acids. The recombinant strain in which TOM-Green and IsoILR1 were co-expressed on separate plasmids degraded 1.9-fold more *cis*-DCE compared with a strain that lacked IsoILR1. In the presence of IsoILR1 and TOM-Green, the addition of GSH1* resulted in a sevenfold increase in the intracellular GSH concentration and a 3.5-fold improvement in the *cis*-DCE degradation rate based on chloride released (2.1 ± 0.1 versus 0.6 ± 0.1 nmol min^{-1} mg^{-1} protein at 540 μM), a 1.8-fold improvement in the *trans*-DCE degradation rate (1.29 ± 0.03 versus 0.71 ± 0.04 nmol min^{-1} mg^{-1} protein at 345 μM) and a 1.7-fold improvement in the TCE degradation rate (6.8 ± 0.24 versus 4.1 ± 0.16 nmol min^{-1} mg^{-1} protein at 339 μM). For *cis*-DCE degradation with TOM-Green (based on substrate depletion), V_{max} was 27 nmol min^{-1} mg^{-1} protein with both IsoILR1 and GSHI* expressed compared with V_{max} = 10 nmol min^{-1} mg^{-1} protein for the GST$^-$ GSHI*$^-$ strain. In addition, cells expressing IsoILR1 and GSHI* grew 78% faster in rich medium than a strain lacking these two heterologous genes.

Received 30 July, 2003; revised 26 November, 2003; accepted 19 December, 2003. *For correspondence. E-mail twood@engr.uconn.edu; Tel. (+1) 860 486 2483; Fax (+1) 860 486 2959.

Introduction

Trichloroethylene (TCE), *cis*-1,2-dichloroethylene (*cis*-DCE) and other chlorinated ethenes constitute a large group of priority environmental pollutants (Krumme *et al.*, 1993; Bradley and Chapelle, 1998). Remediation of sites contaminated with these compounds is critical because chlorinated ethenes are toxic (Coleman *et al.*, 2002a), and natural anaerobic degradation often leads to even more toxic compounds such as vinyl chloride, a well-known carcinogen (Coleman *et al.*, 2002b). Bioremediation can provide a safe and economical alternative to commonly used physical–chemical methods (Pieper and Reineke, 2000). However, with the exception of vinyl chloride (Hartmans and Bont, 1992) and *cis*-DCE (Coleman *et al.*, 2002a), co-metabolism is currently the only option for aerobic biodegradation of most of the chlorinated ethenes including tetrachloroethene (PCE), TCE, 1,1-dichloroehene (1,1-DCE) and *trans*-1,2-dichloroethene (*trans*-DCE) (Ryoo *et al.*, 2000; van Hylckama Vlieg and Janssen, 2001). In co-metabolism, a non-specific oxygenase with a broad substrate range catalyses the biotransformation of the chlorinated ethenes without providing energy or carbon to the organism (van Hylckama Vlieg and Janssen, 2001).

Toluene *ortho*-monooxygenase (TOM) of *Burkholderia cepacia* G4 was originally studied for its ability to oxidize TCE (Nelson *et al*., 1986). In addition to TCE, TOM can also degrade other chlorinated ethenes such as *cis*-DCE and *trans*-DCE (Shim and Wood, 2000; Canada *et al*., 2002); hence, there is considerable interest in using TOM for bioremediation (Krumme *et al*., 1993; Winkler *et al*., 1995). TOM-Green, a DNA shuffling variant from our laboratory, has an enhanced degradation rate for TCE (2.5 ± 0.3 versus 1.39 ± 0.05 nmol min^{-1} mg^{-1} protein at 67 μM initial liquid concentration), 1,1-dichloroethylene and *trans*-DCE (Canada *et al*., 2002). A disadvantage of co-metabolic conversions of chlorinated ethenes by TOM and other monooxygenases is the production of reactive epoxide intermediates that make such bioremediation processes intrinsically unstable (van Hylckama Vlieg and Janssen, 2001).

Under aerobic conditions, epoxides are generally formed by monooxygenases when they initiate attack on chlorinated ethenes (Fox *et al*., 1990; van Hylckama Vlieg *et al*., 1996; van Hylckama Vlieg and Janssen, 2001). These chlorinated epoxyethanes are electrophilic and may covalently modify various cellular constituents including DNA, RNA, lipids, proteins and other small molecules and, in turn, cause turnover-dependent inactivation of the biocatalyst and death of the cell (Oldenhuis *et al*., 1991; van Hylckama Vlieg *et al*., 1996; Newman and Wackett, 1997; van Hylckama Vlieg and Janssen, 2001). Conversion of *cis*-DCE by *Methylosinus trichosporium* OB3b expressing soluble monooxygenase was accompanied by rapid killing during the transformation, which indicated that more toxic products were generated (van Hylckama Vlieg *et al*., 1997). Both the viability of *B. cepacia* G4 expressing TOM and TOM activity decrease upon TCE oxidation (Sun and Wood, 1997; Yeager *et al*., 2001), and TCE oxidation renders the cells ultrasensitive to oxidative stress (Yeager *et al*., 2001). Even aerobic growth on vinyl chloride is unstable because of the accumulation of the chlorinated epoxide (Hartmans and Bont, 1992). Therefore, it is desirable to find a mechanism for biological detoxification of these reactive intermediates.

In higher organisms, glutathione *S*-transferases (GSTs) are involved in the biotransformation of chlorinated epoxyethanes derived from chlorinated ethenes, such as TCE and tetrachloroethylene (Dekant and Henschler, 1999). In bacteria, only a GST from *Rhodococcus* sp. strain AD45 (IsoI) has been reported with activity towards *cis*-1,2-dichloroepoxyethane (van Hylckama Vlieg *et al*., 1998; 1999) (although no activity for this substrate was found when the gene was expressed in *Escherichia coli*). GSTs catalyse the conjugation reaction between electrophilic compounds and glutathione (GSH) (Field and Thurman, 1996). GSH conjugation to the epoxide results in the opening of the epoxide ring (e.g. fosfomycin) to protect against oxidative damage (Vuilleumier, 1997). In *E. coli*, biosynthesis of the necessary substrate, GSH, proceeds by γ-glutamylcysteine synthetase (GSHI) with the conversion of glutamate and cysteine into γ-glutamylcysteine, which is then converted to GSH by GSH synthetase (Meister, 1995). Synthesis of γ-glutamylcysteine is rate limiting, and GSHI is feedback inhibited by glutathione (Kelly *et al*., 2002). A variant of GSHI, GSHI*, which is desensitized to feedback inhibition of glutathione, has been isolated by Murata and Kimura (1982) and was used here to overexpress GSH.

It was reasoned that a recombinant strain expressing GST should be less sensitive to the toxic effects resulting from the co-metabolism of chlorinated ethenes. In this study, metabolic engineering was used to construct a strain in which the evolved monooxygenase TOM-Green and a novel GST of *Rhodococcus* sp. strain AD45 were co-expressed on separate compatible plasmids to degrade *cis*-DCE (thus, this is the first report of a cloned GST with activity in *E. coli* towards *cis*-DCE epoxide). GSHI* was also cloned to overexpress the substrate for GST, GSH, to form a recombinant bacterium that expresses eight genes simultaneously creating an engineered pathway that degrades *cis*-DCE 3.5-fold faster.

Results

Cloning and activity of two novel glutathione S*-transferases*

Polymerase chain reaction (PCR) amplification from the ≈45 kb (results not shown) pHL8 (van Hylckama Vlieg *et al*., 2000a) with the primers originally designed for *isoI* resulted in the cloning of two new genes (*isoILR1* and *isoILR2*) putatively coding for GST as well as the intended *isoI* (Fig. 1). The three GST genes were isolated from three independent cloning experiments, and their differences were identified by DNA sequencing. Because of the flanking sequence similarity, the initial PCR appears to have amplified three similar genes. The two new GST genes were from the cosmid and are not a PCR artifact because the number of nucleotide differences between the genes far exceeds the errors that could be generated by the high-fidelity *Vent* DNA polymerase. IsoILR1 was isolated as a new GST and is functionally expressed in *E. coli* as indicated by its activity towards both epoxypropane and *cis*-DCE epoxide. The initial specific activity of IsoILR1 towards epoxypropane at 5 mM in the single-plasmid system (TG1/pMMB277-isoILR1) with only IsoILR1 expressed was 1170 ± 70 nmol min^{-1} mg^{-1} protein, which was much higher than that in dual-plasmid strains, TG1/pBS(Kan)TOM-Green/pMMB277-isoILR1 (237 ± 79 nmol min^{-1} mg^{-1} protein) and TG1/pBS(Kan)TOM-Green/pMMB277-Ptac-isoILR1-gshI*

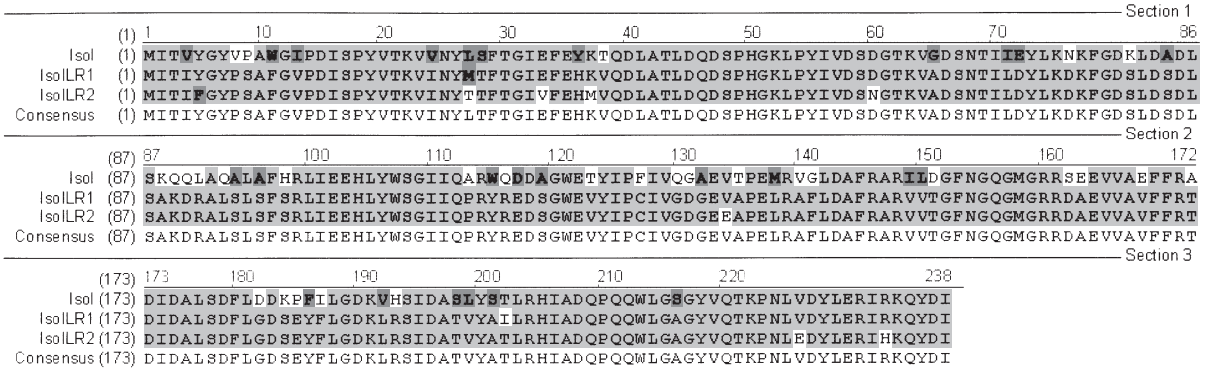

Fig. 1. Protein sequence alignment of the glutathione *S*-transferases IsoI, IsoILR1 (accession no. AY557370) and IsoILR2 (accession no. AY557371). Light shading indicates identical residues, darker shading indicates a conservative substitution, and white indicates non-conservative or weakly conservative substitutions.

(237 nmol min^{-1} mg^{-1} protein) with TOM-Green co-expressed. Such results are intuitive as TOM-Green is a complex protein with six subunits totalling 1485 amino acids, and it was expressed from a high-copy-number plasmid; hence, the expression of TOM-Green requires substantial cellular resources and the expression of IsoILR1 is reduced. Although the activity of IsoILR1 in the dual-plasmid strain decreased to about one-third of that in the single-plasmid system, it is still high compared with the activity of TOM-Green towards *cis*-DCE (i.e. significantly higher than the rate of *cis*-DCE epoxide formation). In addition, the IsoILR1 enzymatic activity increased linearly with increasing GSH concentrations up to 10 mM (data not shown), which suggests that GSH functions as the cofactor and is limiting.

The original GST gene of pHL8 (van Hylckama Vlieg *et al*., 2000a) was confirmed by DNA sequencing to be *isoI* (accession no. AJ249207). However, in our experiments, IsoI lacked activity towards epoxypropane and showed no enhancement in *cis*-DCE degradation when co-expressed with TOM-Green, which may be because it was not functionally expressed in *E. coli*, as evidenced by the formation of inclusion bodies (insoluble fraction in SDS-PAGE; results not shown). Our efforts to solve the insolubility problem via chaperone GroEL co-expression on plasmid pKY206 (Ashiuchi *et al*., 1995) and fusing *isoI* to the maltose-binding protein (New England Biolabs) were not successful. Thus, IsoILR1, the newly discovered functional GST, replaced IsoI in our metabolic engineering effort to reduce toxicity during aerobic degradation of chlorinated ethenes. Although the active GST (IsoILR1) shares significant homology with IsoI (76% identity at the protein level, Fig. 1), it is a different enzyme and was named IsoILR1. IsoILR2, on the other hand, shares 96% identity with IsoILR1 (nine amino acids difference) but has no activity towards epoxypropane and shows no enhancement in *cis*-DCE degradation when co-expressed with TOM-Green (data not shown).

A sequence similarity search with IsoILR1 and IsoILR2 showed that the two proteins share the highest homology with IsoI of *Rhodococcus* sp. strain AD45 (76% and 74% respectively). Some other (putative) glutathione *S*-transferases, such as the *Caenorhabditis elegans* failed-axon-connections protein and the *blr6664*-glutathione *S*-transferase from *Bradyrhizobium japonicum* USDA 110, share a much lower sequence identity with IsoILR1 and IsoILR2 (20–30%), indicating that they are distantly related.

Increased cis*-DCE degradation by co-expression of glutathione* S*-transferase and TOM-Green*

In evaluating *cis*-DCE degradation, TOM-Green from pBS(Kan)TOM-Green (Canada *et al*., 2002) was always expressed to initiate the degradation reaction by forming *cis*-DCE epoxide. Naphthol activity assays were used to monitor TOM-Green activity in all the *cis*-DCE degradation experiments to ensure that the difference in *cis*-DCE degradation rates was not caused by differences in TOM-Green activity. It was assumed that GST should have no effect on naphthol formation in the assay by TOM-Green either because no naphthalene epoxide is formed during the TOM-Green transformation or because naphthalene epoxide (if formed) was not within the substrate range of IsoILR1. As shown in Table 1, TOM-Green activity was relatively constant for each strain (3.1 ± 0.6 nmol min^{-1} mg^{-1} protein for the $GST^{-}GSHI^{*-}$ strain compared with 2.9 ± 0.7 nmol min^{-1} mg^{-1} protein for the $GST^{+}GSHI^{*-}$ strain).

The enhancement in *cis*-DCE degradation and mineralization rate resulting from the cloning of IsoILR1 at different initial substrate concentrations is also listed in Table 1. There was a 1.9-fold improvement in mineralization rate (formation of Cl^{-} from *cis*-DCE) at 540 μM initial *cis*-DCE concentration and a 1.7-fold improvement when the substrate concentration increased to 5400 μM. The

Table 1. Effect of cloning GST and GSHI* on the intracellular glutathione concentrations, degradation rates for *cis*-DCE, *trans*-DCE and TCE, TOM-Green activity via naphthalene oxidation and specific growth rates.

Strain[f]	Intracellular glutathione	Mineralization rate of chlorinated ethenes[a,b,c] (nmol min^{-1} mg^{-1} protein)				*cis*-DCE degradation rate[b,c,d] (nmol min^{-1} mg^{-1} protein)	Naphthol formation rate[b,e] (nmol min^{-1} mg^{-1} protein)	Specific growth wet cell rate (h^{-1})
	μM GSH mg^{-1}	540 μM *cis*-DCE	5400 μM *cis*-DCE	345 μM *trans*-DCE	339 μM TCE	1620 μM *cis*-DCE	5 mM naphthalene	
GST$^-$ GSHI*$^-$	9.4 ± 0.3	0.59 ± 0.1	1.5 ± 1	0.71 ± 0.04	4.14 ± 0.16	7.7	3.1 ± 0.6	0.34 ± 0.05
GST$^+$ GSHI*$^-$	4.1 ± 0.2	1.1 ± 0.06	2.4 ± 1	1.03 ± 0.21	5.16	12.3	2.9 ± 0.7	0.54 ± 0.05
GST$^+$ GSHI*$^+$	31.5 ± 0.8	2.1 ± 0.1	4.7 ± 1	1.29 ± 0.03	6.82 ± 0.24	17.9	3.7 ± 0.4	0.60 ± 0.10

a. Determined via chloride ion release.
b. Total protein was 0.18 mg protein ml^{-1} OD^{-1}.
c. Initial *cis*-DCE concentrations were calculated based on Henry's law with Henry's constant 0.17 for *cis*-DCE, 0.38 for *trans*-DCE and 0.39 for TCE (Dolfing *et al.*, 1993) (1 mM and 10 mM *cis*-DCE, 1 mM *trans*-DCE and 1 mM TCE were added for the mineralization experiments, and 3 mM *cis*-DCE was added for the degradation experiment as if all the volatile organic was in the liquid phase).
d. Determined via gas chromatography monitoring *cis*-DCE degradation.
e. Naphthalene solubility is 0.27 mM in water (Dean, 1985).
f. GST$^-$GSHI*$^-$: TG1/pBS(Kan)TOM-Green/pMMB277; GST$^+$GSHI*$^-$: TG1/pBS(Kan)TOM-Green/pMMB277-isoILR1; GST$^+$GSHI*$^+$: TG1/pBS(Kan)TOM-Green/pMMB277-Ptac-isoILR1-gshI*.

cis-DCE degradation rate as monitored by gas chromatography (GC) increased 1.6-fold at 1621 μM initial *cis*-DCE concentration.

Enhanced intracellular glutathione by cloning γ-glutamylcysteine synthetase

The intracellular glutathione content increased upon cloning of GSHI* (Table 1). The GSH concentration in the GST$^+$GSHI*$^+$ strain is 31.5 μM mg^{-1} wet cell, which increased more than sevenfold compared with the GST$^+$GSHI*$^-$ strain (4.1 μM mg^{-1} wet cell) and increased 3.4-fold compared with the GST$^-$GSHI*$^-$ strain (9.4 μM mg^{-1} wet cell).

Further improvement in cis*-DCE degradation by adding γ-glutamylcysteine synthetase*

The increased glutathione level that resulted from cloning GSHI* increased the mineralization rate of *cis*-DCE (540 μM) by 3.5-fold versus the GST$^-$GSHI*$^-$ strain (Table 1). At 5.4 mM *cis*-DCE, the pattern was similar but with a 3.2-fold increase, which indicates that adding GST and GSH was beneficial over a wide substrate concentration range. These results were corroborated by the *cis*-DCE degradation (GC analysis), which was also improved 2.4-fold (Table 1). Although these degradation rates are of the order of a few nmol min^{-1} mg^{-1} protein and are low compared with anaerobic dechlorination of *cis*-DCE that proceeds at about 50 nmol min^{-1} mg^{-1} protein (Cupples *et al.*, 2003), the reductive dechlorination of *cis*-DCE has the risk of accumulating carcinogenic vinyl chloride (Coleman *et al.*, 2002b). Again, the naphthol synthesis assays revealed similar TOM-Green activity upon cloning GSHI* (2.9–3.7 nmol min^{-1} mg^{-1} protein at 5 mM naphthalene concentration), indicating that the enhanced degradation of *cis*-DCE resulted from the elevated levels of GSH from cloning GSHI*.

GST and TOM-Green kinetics

The Monod constants K_m and V_{max} for IsoILR1 with epoxypropane as the substrate when IsoLR1 was co-expressed with TOM-Green in the dual-plasmid system TG1/pBS(Kan)TOM-Green/pMMB277-isoILR1 were V_{max} = 294 nmol min^{-1} mg^{-1} protein and K_m = 3 mM. The Monod constants for TOM-Green on *cis*-DCE when it was expressed with both IsoILR1 and GSHI* were V_{max} = 27 nmol min^{-1} mg^{-1} protein and K_m = 0.51 mM for TG1/pBS(Kan)TOM-Green/pMMB277-Ptac-isoILR1-gshI* compared with V_{max} = 10 nmol min^{-1} mg^{-1} protein and K_m = 0.34 mM for the GST$^-$GSHI*$^-$ strain (TG1/pBS(Kan)Tom-Green/pMMB277). Hence, the addition of glutathione *S*-transferase and γ-glutamylcysteine synthetase increased V_{max} 2.7-fold; these results corroborate the initial degradation rates as well as the GC results.

*Enhanced degradation of other chlorinated ethenes by cloning of GST and GSHI**

Cloning of GST and GSHI* also increased the mineralization rate of *trans*-DCE (initial concentration 345 μM) and TCE (339 μM) by 1.8- and 1.7-fold, respectively, versus the GST$^-$GSHI*$^-$ strain (Table 1). This indicates that the engineered metabolic pathway is beneficial for several chlorinate ethenes. No appreciable enhancement was detected for the degradation of vinyl chloride or 1,1-DCE. The absence of improvement in 1,1-DCE degradation was expected as its epoxide was very unstable and has never

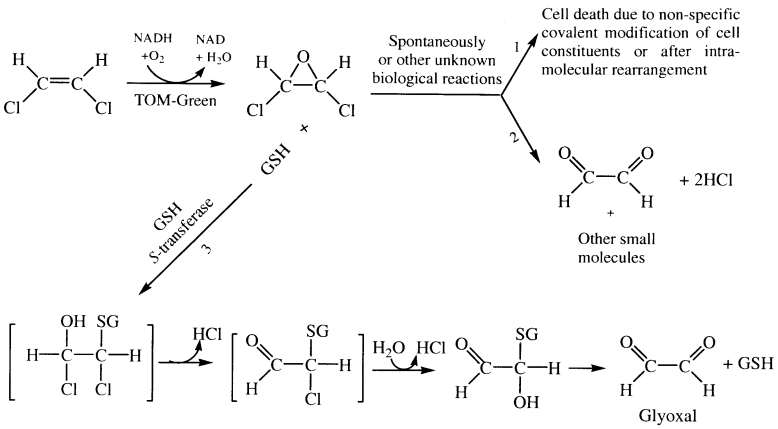

Fig. 2. Enhanced *cis*-DCE mineralization resulting from the cloning of GST and GSHI* (derived from van Hylckama Vlieg *et al*., 1998). Steps 1, 2 and 3 are the three possible transformation pathways for *cis*-DCE epoxide.

been detected in biological systems (van Hylckama Vlieg *et al*., 1996).

Growth advantage with IsoILR1

Cloning of IsoILR1 (GST^+GSHI^{*-}) increased the specific growth rate of *E. coli* TG1 by 60% compared with the GST^- $GSHI^{*-}$ strain, whereas cloning of GSHI* along with GST brought an additional 10% faster growth (Table 1). All strains containing dual plasmids and cultures for the growth rate measurement were started from 13 h precultures in Luria–Bertani (LB) medium supplemented with 100 μg ml^{-1} kanamycin (Kan) and 50 μg ml^{-1} chloramphenicol (Cam). The growth enhancement of the GST^+ strain may be ascribed to GST ensuring the smooth synthesis and correct folding of enzymes in the presence of antibiotics as GSTs are involved in a variety of metabolic processes including ensuring the correct folding, synthesis, regulation and degradation of enzymes, and defending against many toxic insults including some antibiotics (Apontoweil and Berends, 1975; Alonso-Moraga *et al*., 1987; Penninckx and Taylor, 1992; Koonin *et al*., 1994).

Discussion

Two genes encoding new glutathione *S*-transferases (*isoILR1* and *isoILR2*) from *Rhodococcus* sp. strain AD45 were identified, and the sequences were characterized. Fifty-seven amino acids out of 238 are different for IsoI and IsoILR1. In the N-terminal domain, which is involved in glutathione binding (Armstrong, 1997), there are 11 conservative substitutions as well as five non-conservative mutations including V8P, P9S, T38V, N76D and K81S (Fig. 1). Two non-conservative substitutions of IsoILR1 in the extreme N-terminus (relative to IsoI), V8P and P9S, may imply some important functional change. In the theta class of GSTs, to which all known bacterial GSTs belong (Vuilleumier, 1997), a serine located in the N-terminal part of the protein has been hypothesized to enhance the nucleophilicity of the glutathione sulphydryl group (Board *et al*., 1995; Vuilleumier and Leisinger, 1996). There is a serine at position 17 of IsoI and of IsoILR1; however, the addition of one serine at the more N-terminal position (P9S) of IsoILR1 could be responsible for its capability for tackling *cis*-DCE epoxide. Site-specific mutagenesis studies may be able to assess whether the substitution of this serine plays an important catalytic role.

As for IsoILR2, no activity towards epoxypropane or *cis*-DCE epoxide could be detected although it shares 96% identity with IsoILR1 with only nine different amino acids. The lack of activity of IsoILR2 could result from the non-conserved substitutions at the N-terminus, M27T, E32V, K37M and D60N, and also the change in the tyrosine residue at position 5 to phenylalanine, as the N-terminal tyrosine plays the role of enhancing the nucleophilicity of the thiol group in some GSTs (Armstrong, 1997).

Cloning of γ-glutamylcysteine synthetase of the GSH biosynthesis pathway of *E. coli* increased the cellular GSH concentration by sevenfold. One seemingly counterintuitive observation was that the GSH level in the GST^+GSHI^{*-} strain was only 44% of that in the GST^- $GSHI^{*-}$ strain. It is hypothesized that free GSH was turned over non-specifically by the cloned GST, which was involved in various intracellular processes.

The enhancement in *cis*-DCE degradation rate upon addition of GSHI* and IsoILR1 determined by monitoring substrate depletion via the GC was not as great as the mineralization rate indicated by the amount of Cl^- liberated (2.3-fold versus 3.5-fold enhancement). This is not a discrepancy, however, as the enhancement in *cis*-DCE mineralization rate is the addition of two parts: one results from the improved TOM-Green activity as a result of GST preventing *cis*-DCE epoxide from inactivating the enzyme and killing the cell, and the other is the additional amount of Cl^- formed from the enzymatic transformation by IsoILR1 (Fig. 2). The increase in *cis*-DCE degradation rate

as measured by GC is due to the overall increase in TOM-Green activity.

As the toxicity of transformation intermediates is the main limiting factor for the application of monooxygenase-expressing organisms for the degradation of chlorinated ethenes, the detoxification by the rational combination of catabolic segments from different organisms sheds light on the power of metabolic engineering in bioremediation of environmental pollution. Based on the Gibbs free energy calculation of aerobic degradation of chlorinated ethenes to water, carbon dioxide and HCl, growth on nearly all chlorinated aliphatics is thermodynamically possible (e.g. the Gibbs free energy change for the aerobic mineralization of *cis*-DCE is -1143 kJ mol^{-1}) (van Hylckama Vlieg and Janssen, 2001). If the reactive intermediates may be effectively detoxified, the main biochemical factor that hampers chlorinated ethenes from supporting cell growth is the lack of appropriate enzymes to harvest their energy. This may be solved by selecting or evolving an enzyme that can convert glyoxal to a product capable of entering central metabolism cycles, as possibly with a recently isolated β-proteobacterium strain that uses *cis*-DCE for carbon and energy (Coleman *et al*., 2002a). Then, through metabolic engineering, the reactive intermediate, *cis*-DCE epoxide, may be channelled into a productive metabolic pathway so that *cis*-DCE can be used as the sole carbon and energy source (van Hylckama Vlieg *et al*., 2000b).

As various directed evolution techniques have greatly increased the process by which different genes are adapted (Arnold, 1998), glutathione *S*-transferases and various other enzymes involved in the metabolism of epoxides can be tuned to accept the epoxides of other chloroethenes within their substrate range, including 1,1-dichloroethylene, *trans*-DCE, TCE and PCE. An alternative epoxide detoxification strategy using epoxide hydrolases is even more attractive as no additional cofactors or prosthetic groups are required for enzymatic activity (Steinreiber and Faber, 2001); however, no known epoxide hydrolase with activity towards chlorinated epoxides has been discovered, so it may be necessary to evolve one to recognize these substrates.

Experimental procedures

Chemicals, organisms and growth conditions

All materials were purchased from Fisher Scientific unless otherwise stated. Glutathione, glutathione reductase and vinyl chloride were purchased from Sigma Chemical, and *cis*-DCE and *trans*-DCE were purchased from TCI America. *Escherichia coli* TG1 (Gibson, 1984) was used as the host for plasmid construction and functional expression of the genes and was grown routinely at 37°C LB broth (Sambrook *et al*., 1989) supplemented with Kan (100 μg ml^{-1}) and Cam (50 μg ml^{-1}) to maintain plasmids. All experiments were conducted using exponential phase cultures obtained by diluting overnight cultures in LB + Kan + Cam medium to an optical density at 600 nm (OD) of 0.05–0.10 and growing to an OD of 1.5. At an OD of 0.3–0.4, 0.5 mM isopropyl-β-thiogalactopyoside (IPTG) was added to induce *isoILR1* and *gshI** under the control of the *tac* promoter and TOM-Green under the *lac* promoter.

Cell preparation for whole-cell assays

The exponentially grown cells were washed once with one volume of Tris-HCl buffer (50 mM, pH 7.4) to determine TOM-Green activity via a naphthol formation assay or three times with one volume of Tris-HNO_3 (50 mM, pH 7.0) buffer for the *cis*-DCE experiments to remove interfering metabolic byproducts and trace chloride.

Protein analysis and molecular techniques

Total cellular protein for the exponentially grown culture was determined with the Total Protein kit (Sigma Chemical). Expression of recombinant proteins was analysed on standard 12% Laemmli discontinuous sodium dodecyl sulphate (SDS)-polyacrylamide gels (Sambrook *et al*., 1989). Plasmid DNA was isolated using a Midi or Mini kit (Qiagen). Polymerase chain reaction products were purified with a Wizard® PCR Preps DNA purification system (Promega). DNA fragments were isolated from agarose gels using a QIAquick gel extraction kit (Qiagen). Transformation of *E. coli* was carried out by electroporation using a Bio-Rad gene pulser/pulse controller at 1.5 kV, 25 μF and 200 Ω.

PCR amplification and plasmid construction

The high-copy-number plasmid pBS(Kan)TOM-Green (Canada *et al*., 2002) was used (Fig. 3) for the expression of TOM-Green. For expression of IsoILR1 and GSHI*, the low-copy-number, broad-host-range plasmid pMMB277 (Morales *et al*., 1991) was chosen. The *isoILR1* gene was amplified by PCR using the cosmid pHL8 of *Rhodococcus* sp. strain AD45 (van Hylckama Vlieg *et al*., 2000a) as a template with forward primer 5′-CGAGAATTCAACCTTCCTCCAATGGTGAGC-3′ and reverse primer 5′-GAGAATCTTGCGAAGCTTAGGGCT TGTG-3′ where underlining indicates the *Eco*RI and *Hin*dIII restriction enzyme sites respectively. The PCR fragment was double digested with *Eco*RI and *Hin*dIII and ligated into pMMB277 at the same restriction sites, yielding pMMB277-isoILR1. The *gshI** gene was placed after *isoILR1* so both shared the same *tac* promoter; *gshI** from *E. coli* B was obtained from plasmid pKUGS-AB (Murata and Kimura, 1982) using PCR amplification with forward primer GF (5′-ATTTTAAGCTTCGGGAGGTCAATATGATCCCGGACG-3′) and reverse primer GR (5′-CTCTCATCCGCCAAAACAG AAGCTTGG CTG-3′) where the *Hin*dIII sites are underlined. The PCR product was digested with *Hin*dIII and ligated into *Hin*dIII-digested pMMB277-isoILR1, resulting in pMMB277-Ptac-isoILR1-gshI* (Fig. 3). All PCR amplifications were performed with *Vent* DNA polymerase (New England Biolabs) using a PCR programme of 30 cycles of 94°C for 45 s, 55°C

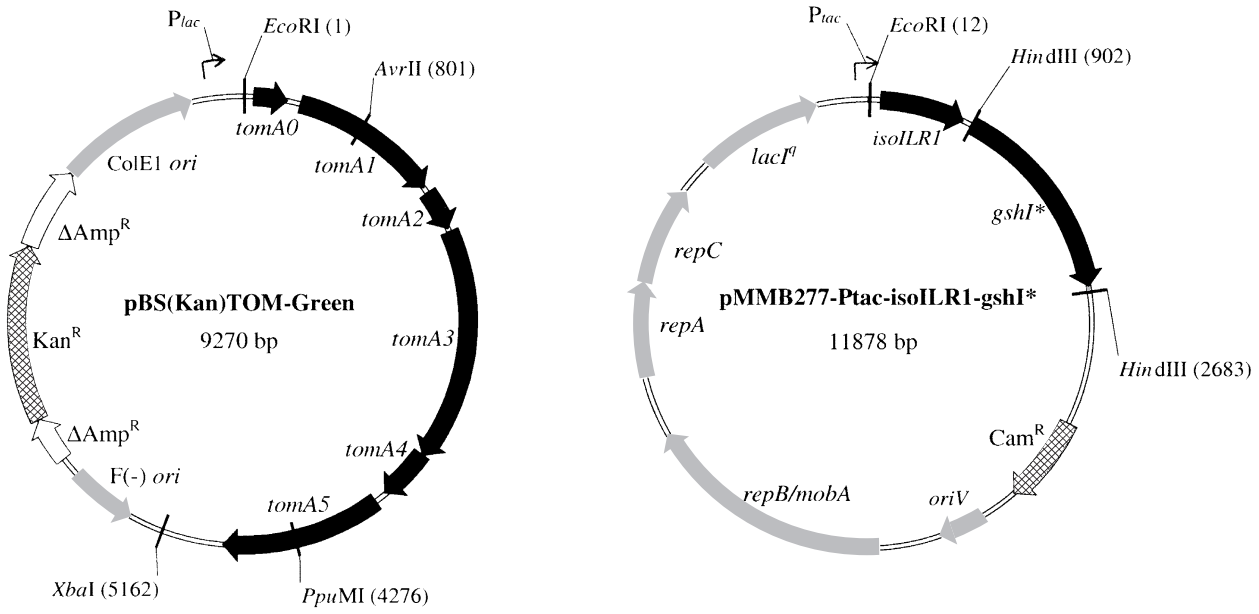

Fig. 3. Plasmid maps of pBS(Kan)TOM-Green and pMMB277-ptac-isoILR1-gshI*. Cloning restriction enzyme sites are listed. pBS(Kan)TOM-Green is a variant of pBS(Kan)TOM from DNA shuffling with the substitution V106A in *tomA3* (Canada *et al*., 2002).

for 45 s and 72°C for 1–2 min, with a final extension of 72°C for 7 min. The presence of *isoILR1* and *gshI** was confirmed by DNA sequencing.

GST activity

Cell extracts were prepared as described by van Hylckama Vlieg *et al*. (1998). The cells were disrupted by sonication with a F60 sonic dismembrator (Fisher Scientific) 10 times for 10 s at 10 W (with a 1 min pause on ice), and the supernatants of the sonicated cells, which contained soluble GST, were used for the enzyme assay. The specific activity of GST towards epoxypropane (5 mM) was assayed in the presence of 5 mM glutathione at 25°C with 2.5 ml of disrupted cell supernatant in a 15 ml serum vial sealed with a Teflon-coated septum and aluminium crimp. The degradation rate of epoxypropane was determined by headspace concentrations and was monitored using GC. The GC (Agilent 6890 N 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^{-1}) and air (300 ml min^{-1}), and nitrogen was used as the carrier gas (20 ml min^{-1}). Headspace samples injected into the GC were analysed isothermally at 80°C (retention time of epoxypropane was 0.9 min). For the kinetic studies, 0.2–5 mM epoxypropane was added to the cell extracts that were supplemented with 5 mM glutathione, and substrate depletion was monitored using GC.

TOM-Green activity

TOM-Green activity was measured using the same culture that was used for *cis*-DCE degradation by splitting the cells and incubating with naphthalene in the absence of *cis*-DCE as described previously (Canada *et al*., 2002). In this way, relatively constant TOM-Green activity was checked during *cis*-DCE degradation with the various plasmid constructs. The assay was conducted in a 15 ml serum vial by contacting 2.5 ml of exponentially grown, washed cells at an OD of 2.0 with 5 mM naphthalene for 1 h. The concentration of naphthols formed was reacted with tetrazotized *o*-dianisidine and measured spectrophotometrically at 540 nm.

Extents of mineralization of cis*-DCE and rates of degradation*

For extents of mineralization of *cis*-DCE as indicated by Cl^- production, the exponentially grown, washed cell suspension (2.5 ml) was adjusted to an OD of 3.5, sealed in 15 ml glass serum vials and contacted with *cis*-DCE at an initial liquid concentration of either 540 μM or 5.4 mM [based on a Henry's law constant of 0.17 (Dolfing *et al*., 1993), 2.5 or 25 μmol of *cis*-DCE were injected to the cells in 5 μl of N,N-dimethylformamide (DMF) at 0.2 vol%]. Isopropyl-β-thiogalactopyoside (0.5 mM) was added along with sodium succinate at a final concentration of 5 mM (as a substrate to produce NADH). After 2 or 3 h of incubation at 37°C and 250 r.p.m., the whole-cell reaction was quenched by heating the vials in boiling water for 90 s and centrifuging (16 000 *g*, 4 min) to remove the cells. Chloride concentrations were determined spectrophotometrically at 460 nm according to the method of Bergmann and Sanik (1957).

Parallel experiments to determine the *cis*-DCE degradation rate were conducted using GC to monitor *cis*-DCE depletion as described previously (Shim and Wood, 2000). The exponentially grown cells were washed, sealed in 15 ml glass serum vials (2.5 ml, OD 3.5) and contacted with *cis*-DCE at initial liquid concentrations of 270 μM and 1621 μM (in total,

1.25 μmol or 7.5 μmol of *cis*-DCE was injected to the contacting cell) at 25°C and 250 r.p.m. for 1–4 h. Under such conditions, the activity of the cells remained constant for at least 2 h. Cell suspensions of TG1/pBS(Kan) (Canada *et al*., 2002) were used as the negative control, and at least three independent experiments were analysed.

To determine the enzyme kinetics, 2.5 ml of washed, exponentially grown cells (OD ≈3.0) were sealed in 15 ml glass vials, and 0.25–12.5 μmol of *cis*-DCE was added from different stock solutions (50 μM, 250 μM and 1000 μM in DMF at 0.2–0.4 vol%). Substrate depletion was monitored by GC, and the measurements for the signals for each concentration were taken within 40 min. K_m and V_{max} were determined from the initial rate data.

Extents of mineralization of other chlorinated ethenes

Washed, exponentially grown cell suspensions (2.5 ml with contact OD ≈3.5) sealed in 15 ml glass vials were used for the assays. The supernatant chloride ion concentrations generated from mineralizing the chlorinated ethenes (TCE, *trans*-DCE, vinyl chloride and 1,1-DCE) were measured after 3 h incubation for *trans*-DCE and 1 h for the other substrates. For each substrate, 2.5 μmol was added to the cell suspension with the exception of vinyl chloride (0.32 μmol was injected). The actual concentrations were calculated based on the reported Henry's law constant (Dolfing *et al*., 1993).

Determination of glutathione content

To measure the intracellular glutathione concentration, the method of Murata *et al*. (1980) was adopted with modifications. Exponentially grown cells were cooled and centrifuged at 5000 *g*, 4°C for 5 min. The cell pellet was then resuspended in a suitable amount of water to adjust the cell density to 10 mg wet cells ml^{-1}; 0.5 ml of the cell suspension was then heated at 100°C for 90 s, immediately cooled and then centrifuged at 16 000 *g* for 10 min to remove the cells. Total glutathione concentrations (both reduced and oxidized forms) in the supernatant were determined by the 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB)-oxidized glutathione reductase recycling assay of Tietze (1969). Oxidized glutathione was first reduced to GSH by the action of the highly specific yeast glutathione reductase, and then the total GSH was oxidized by DTNB to form 2-nitro-5-thiobenzoic acid, the concentration of which could be determined spectrophotometrically at 412 nm (Anderson, 1985).

DNA sequencing and analysis

A dye terminator cycle sequencing protocol based on the dideoxy method of sequencing DNA developed by Sanger *et al*. (1977) was used to sequence both strands of *isoI*, *isoILR1* and *isoILR2*. The ABI™ Prism BigDye Terminator cycle sequencing ready reaction kit (PerkinElmer) was used to perform the sequencing reactions, and a PE Biosystems ABI™ 373 DNA sequencer (Perkin-Elmer) was used to analyse the fluorescently labelled DNA fragments by gel electrophoresis. The BLAST program (Altschul *et al*., 1997) was used to search for GST proteins that share sequence similarity in various protein and gene databases, including DDBJ, EMBL and GenBank. Sequence data and pairwise similarity were analysed using the VECTORNTI program suite (InforMax) with default parameters.

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