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ORIGINAL ARTICLE

Identification of stress-related proteins in *Escherichia coli* using the pollutant *cis*-dichloroethylene

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Abstract

Aims: To complement our proteome study, whole-transcriptome analyses were utilized here to identify proteins related to degrading *cis*-1,2-dichloroethylene (*cis*-DCE).

Methods and Results: Metabolically engineered *Escherichia coli* strains were utilized expressing an evolved toluene *ortho*-monooxygenase along with either (i) glutathione *S*-transferase and altered γ-glutamylcysteine synthetase or (ii) a rationally engineered epoxide hydrolase. *cis*-DCE degradation induced 30 known stress genes and 32 uncharacterized genes. Because of the reactive *cis*-DCE epoxides formed, we hypothesized that some of these uncharacterized genes may be related to a variety of stresses. Using isogenic mutants, IbpB, YchH, YdeI, YeaR, YgiW, YoaG and YodD were related to hydrogen peroxide, cadmium and acid stress. Additional whole-transcriptome studies with hydrogen peroxide stress using the most hydrogen peroxide-sensitive mutants, *ygiW* and *ychH*, identified that FliS, GalS, HcaR, MglA, SufE, SufS, Tap, TnaB, YhcN and YjaA are also involved in the stress response of *E. coli* to hydrogen peroxide, cadmium and acid, as well as are involved in biofilm formation.

Conclusion: Seventeen proteins are involved in the stress network for this organism, and YhcN and YchH were shown to be important for the degradation of *cis*-DCE.

Significance and Impact of the Study: Six previously uncharacterized proteins (YchH, YdeI, YgiW, YhcN, YjaA and YodD) were shown to be stress proteins.

Introduction

Adaptive responses to diverse environmental stresses are central to the survival of micro-organisms (López-Maury *et al.* 2008). Bacterial cells are exposed to a variety of stresses including those related to nutrient limitation (López-Maury *et al.* 2008), temperature (Neidhardt and VanBogelen 1981), pH (Foster 2004), oxygen (Farr and Kogoma 1991), pressure (Ishii *et al.* 2005), organic solvents (Ramos *et al.* 2002), heavy metals (Stohs and Bagchi 1995), antibiotics (Viveiros *et al.* 2007), other microbial species (Ryan *et al.* 2008) and their own wastes (Wang *et al.* 2005). In response to these stresses, cells modulate their gene expression (López-Maury *et al.* 2008). This modulation has been observed in *Escherichia coli*; for example, a rapid shift from low to high temperatures induces the synthesis of heat shock proteins (Neidhardt and VanBogelen 1981) to protect proteins from thermal inactivation (Skowyra *et al.* 1990). A similar induction of proteins possessing antioxidant functions in response to reactive oxidative stress has also been observed (Pomposiello *et al.* 2001). Gastrointestinal (GI) bacteria face an extremely acidic environment (pH 2), and *E. coli* has developed several mechanisms of acid survival which allow it to colonize the GI tract (Foster 2004; Lee *et al.* 2007). Increased biofilm formation has also been demonstrated

as a mechanism for stress survival (Zhang *et al.* 2007). Recently, whole-transcriptome profiling via DNA microarray technology has helped to discover many new stress-related proteins (Pomposiello *et al.* 2001; Zheng *et al.* 2001; Weber *et al.* 2005; Blanchard *et al.* 2007).

Previously, we engineered *E. coli* cells to degrade aerobically *cis*-1,2-dichloroethylene (*cis*-DCE) (Rui *et al.* 2004a,b), a US EPA priority pollutant (Ryoo *et al.* 2000). Because anaerobic degradation can lead to the formation of toxic vinyl chloride, a human carcinogen, two aerobic degradation strategies with *E. coli* were developed. The strains expressed an evolved toluene *ortho*-monooxygenase from *Burkholderia cepacia* G4 (TOM-Green with TomA3 V106A which converts *cis*-DCE into the reactive *cis*-DCE epoxide) (Canada *et al.* 2002) and either (i) the glutathione *S*-transferase IsoILR1 from *Rhodococcus* AD45 (which adds glutathione to the reactive *cis*-DCE epoxide) and γ-glutamylcysteine synthetase GSHI* (which forms glutathione and is not limited by feedback inhibition) (Rui *et al.* 2004b) or (ii) with an epoxide hydrolase (EchA) from *Agrobacterium radiobacter* AD1 (EchA F108L/I219L/C248I rationally engineered to recognize *cis*-DCE epoxide as a substrate and convert it into a diol) (Rui *et al.* 2004a).

The reactive epoxides formed by monooxygenases are electrophilic and may covalently modify various cellular constituents including DNA (van Hylckama Vlieg and Janssen 2001), RNA (van Hylckama Vlieg and Janssen 2001), proteins (Oldenhuis *et al.* 1991) and other small molecules (Oldenhuis *et al.* 1991), and in turn cause turnover-dependent inactivation of the biocatalyst and death of the cell. Hence, the *cis*-DCE epoxide formed causes cell stress.

The impact of this metabolic engineering and cell stress on the proteome was quantified using a shotgun proteomics technique in an effort to guide future metabolic engineering for remediation (Lee *et al.* 2006). For example, the expression of an evolved toluene *ortho*-monooxygenase, glutathione *S*-transferase and γ-glutamylcysteine synthetase for enhanced aerobic degradation of *cis*-DCE-induced glutathione synthesis (CysK, GshA and GST) and stress response proteins (e.g. AhpF, Dps, KatG and HtpG), as well as repressed fatty acid synthesis, gluconeogenesis, the tricarboxylic acid cycle and indole synthesis.

In this study, a whole-transcriptome approach was utilized to complement the previous proteomics study of the metabolic engineering for *cis*-DCE degradation in an effort to characterize how *E. coli* copes with the stress associated with remediation as well as to identify proteins which may be beneficial for *cis*-DCE degradation. The whole-transcriptome data complement the proteomic data, while providing more insights into the gene expression changes. Notably, many uncharacterized genes were induced along with well-known stress genes during enhanced degradation of *cis*-DCE from the current whole-transcriptome approach. Hence, we hypothesized that some of these hypothetical proteins may also be important in response to oxidative and other stresses. Using the Keio Collection of isogenic single-gene knockout mutants (Baba *et al.* 2006), seven proteins (IbpB, YchH, YdeI, YeaR, YgiW, YoaG and YodD) were identified as reducing stress related to exposure to H_2O_2, cadmium and acid. A second set of whole-transcriptome studies was performed to further study the effect of H_2O_2 on the two most H_2O_2-sensitive mutants (*ychH* and *ygiW*), and ten additional proteins were identified (FliS, HcaR, GalS, MglA, SufE, SufS, Tap, TnaB, YhcN and YjaA) that are involved in the stress response associated with exposure to H_2O_2, cadmium and acid. Therefore, this study elucidates that 17 new proteins are crucial in stress response in *E. coli*, which provide important insights for new strategies of bioremediation as well as for understanding bacterial physiology. Furthermore, we show that these stress proteins are important for the degradation of *cis*-DCE.

Materials and methods

Bacterial strains and growth conditions

The strains and plasmids used are given in Supplementary Table S1. All experiments were performed at 37°C using Luria–Bertani medium (LB) for growth (Sambrook *et al.* 1989), whereas buffer was used for *cis*-DCE degradation. Strains were maintained at −80°C as glycerol stocks for long-term storage, cells were precultured overnight from 1-day-old fresh single colonies with appropriate antibiotics to maintain plasmids (Table S1), and for two-plasmid systems, multiple antibiotics were used at the same concentrations. Indole was purchased from Fisher Scientific Co. (Pittsburg, PA, USA) and (*S*)-4,5-dihydroxy-2,3-pentanedione [DPD, the autoinducer-2 (AI-2) precursor] was purchased from Omm Scientific (Dallas, TX, USA). *suf*S, *ychH*, *ygiW* and *yhcN* in the pCA24N constructs (Kitagawa *et al.* 2005) were tightly regulated with the T5-*lac* promoter and its repressor *lacI*^{q} and were induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma, St Louis, MO, USA). The specific growth rates of each strain were determined by measuring the cell turbidity at 600 nm of two independent cultures as a function of time using values <0·7.

Verification of mutations

The isogenic *ychH* and *ygiW* mutations were confirmed via the DNA microarrays which showed no signal for these two strains for their respective loci. The *ydeI*, *yhcN*,

yjaA and *yodD* mutations were verified via PCR by two independent reactions using primers (Table S2) that flanked each locus as well as the upstream primer paired with a primer in the inserted kanamycin-resistance gene.

Total RNA isolation for DNA microarrays

A total of twelve planktonic RNA samples for the six sets of microarray experiments [set (i) was repeated] were prepared: (i) cells of *E. coli* TG1 with TOM-Green/IsoILR1/GSHI* *vs* no cloned genes were contacted for 2 h with 1 mmol l^{-1} *cis*-DCE together with 0·5 mmol l^{-1} IPTG (to induce the cloned genes) and 5 mmol l^{-1} succinate (as a carbon/energy source during the *cis*-DCE degradation) in 60 ml to determine the effect of expressing eight genes (encoding TOM-Green/IsoILR1/GSHI*) on *cis*-DCE mineralization as performed previously in the proteomics study (Lee *et al.* 2006), (ii) cells of TG1 with TOM-Green/EchA F108L/I219L/C248I *vs* TOM-Green contacted for 2 h with 1 mmol l^{-1} *cis*-DCE together with 0·5 mmol l^{-1} IPTG and 5 mmol l^{-1} succinate to determine the effect of the evolved epoxide hydrolase on *cis*-DCE mineralization (Lee *et al.* 2006), (iii) cells (turbidity of 1·0) of *E. coli* BW25113 with 2 mmol l^{-1} H_2O_2 *vs* no H_2O_2 contacted for 10 min with shaking to determine gene expression in response to H_2O_2 stress because *cis*-DCE mineralization induced many oxidative stress genes, (iv) cells (turbidity of 1·0) of the BW25113 *ygiW* strain with 2 mmol l^{-1} H_2O_2 *vs* no H_2O_2 contacted for 10 min to determine gene expression in response to H_2O_2 stress in the absence of YgiW and (v) cells (turbidity of 1·0) of the BW25113 *ychH* strain with 2 mmol l^{-1} H_2O_2 *vs* no H_2O_2 contacted for 10 min to determine gene expression in response to H_2O_2 stress in the absence of YchH.

For all samples, cells were immediately chilled with dry ice and 95% ethanol (to prevent RNA degradation) for 30 s before centrifuging in 1·8- ml centrifuge tubes at 13 000 ***g*** for 30 s; cell pellets were frozen immediately using dry ice and stored at −80°C. RNA was isolated as described previously using sonication and a bead beater (Ren *et al.* 2004a).

DNA microarray analysis

The *E. coli* Genechip antisense genome array (P/N 900381; Affymetrix, Santa Clara, CA, USA) and the *E. coli* GeneChip Genome 2.0 array (P/N 900551, a more cost effective format; Affymetrix) were used to study the differential gene expression profile of the K12 cells. The same type of microarray was used for each binary comparison. cDNA synthesis, fragmentation and hybridizations were as described previously (González Barrios *et al.* 2006). Hybridization was performed for 16 h, and the total cell intensity was scaled to an average value of 500. The probe array images were inspected for any image artefact. Background values, noise values and scaling factors of both arrays were examined and were comparable. The intensities of the polyadenosine RNA controls of *Bacillus subtilis* (*lys*, *phe*, *thr* and *dap*) at different concentrations were used to monitor the labelling and scanning process. For each binary microarray comparison of differential gene expression, if the gene with the larger transcription rate did not have a consistent transcription rate based on the 11–15 probe pairs (P-value < 0·05), these genes were discarded. A gene was considered differentially expressed when the P-value for comparing two chips was lower than 0·05 (to assure that the change in gene expression was statistically significant and that false positives arise <5%) and when the expression ratio was higher (two to fourfold) than the standard deviation for the whole microarray (Ren *et al.* 2004b) [1·5-fold for condition (i) described earlier, 1·2-fold for (ii), 1·8-fold for (iii), 3·0-fold for (iv) and 2·8-fold for (v)]. Gene functions were obtained from the Affymetrix–NetAffx Analysis Center (https://www.affymetrix.com/analysis/netaffx/index.affx).

Survival assays

The resistance assays (survival test) with hydrogen peroxide and cadmium were adapted (Zhang *et al.* 2007). Overnight cultures grown for 16 h in LB were regrown to mid-log phase in LB (turbidity at 600 nm of 1), and either (i) 1 ml of each culture was incubated with H_2O_2 at a final concentration of 34 mmol l^{-1} for 15 min without shaking or (ii) 1 ml of each culture was incubated with a final concentration of 4 mg ml^{-1} $CdCl_2$ for 40 min without shaking. The acid resistance assay (survival test) was also adapted (Masuda and Church 2003). Overnight precultures grown in LB for 19 h were regrown to mid-log phase (turbidity at 600 nm of 1) in LB, and the cultures were diluted 40-fold in phosphate-buffered saline (pH 7·2 to determine the initial number of cells) or in LB (pH 2·5); the cells at pH 2·5 were incubated for 60 min without shaking. The percentage of cells surviving the stresses was calculated as the number of colony forming units (CFU) per ml remaining after each stress divided by the initial CFU per ml. At least two independent experiments were conducted.

Crystal-violet biofilm assay

A static biofilm formation assay was performed in 96-well polystyrene plates as previously reported (Pratt and Kolter 1998). Briefly, cells were inoculated with an initial turbidity at 600 nm of 0·05 for 7 and 24 h without shaking. Cell

growth, biofilm at the liquid–plastic interface and total biofilm were measured using crystal-violet staining. Each data point was averaged from at least 12 replicate wells (six wells from each of two independent cultures).

Degradation of *cis*-DCE

The rate of *cis*-DCE degradation was determined from headspace analysis using gas chromatography as described previously (Rui *et al.* 2004b). Briefly, overnight cultures of BW25113, the *ychH* mutant, and the *yhcN* mutant harbouring pMMB206-TOM-Green were grown to a turbidity at 600 nm of 0·3, induced with 0·5 mmol l^{-1} IPTG for 2 h, washed, resuspended to a turbidity of 5 at 600 nm in Tris–HNO_3 buffer (50 mmol l^{-1}, pH 7·0) with 0·5 mmol l^{-1} IPTG and 5 mmol l^{-1} succinate (as a carbon/energy source during the *cis*-DCE degradation) in a 60- ml vial and contacted with 87 μmol l^{-1} *cis*-DCE based on Henry's Law (Washington and Weaver 2006). Parallel experiments were conducted to determine the extent of conversion of *cis*-DCE to chloride ions as described previously (Rui *et al.* 2004b) by inducing cells for 2 h at a turbidity at 600 nm of 0·2 to avoid interference from the indigoids produced. Two independent experiments were performed, and empty plasmid pMMB206 was used as a negative control.

Quantitative real-time reverse transcription PCR (qRT-PCR)

To validate the DNA microarray data, the transcription of *gshA*, *mglA*, *sufS*, *tauA* and *yfiD* was quantified with forward and reverse primers (Table S2) using qRT-PCR (Bansal *et al.* 2007). The ten RNA samples used for the whole-transcriptome experiments plus an additional independent culture were used for qRT-PCR experiments. The housekeeping gene *rpoA* (RNA polymerase α subunit) with primers *rpoA* forward and *rpoA* reverse (Table S2) was used to normalize the expression data. A total of 240 reactions were performed based on three replicate reactions for each of the three genes and the *rpoA* housekeeping gene for the 20 sets of RNA samples using the StepOne™ Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) and SuperScript™ III Platinum® SYBR® Green One-Step qRT-PCR kit (Invitrogen, Carlsbad, CA, USA).

Microarray accession numbers

The microarray data summarized in supplementary Tables S3–S7 have been deposited in the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) (Edgar *et al.* 2002) and are accessible through accession number GSE 13698.

Results

Whole-transcriptome analysis for *cis*-DCE mineralization

Previously, we performed a quantitative proteome study to determine the impact of engineering an *E. coli* strain for enhanced *cis*-DCE degradation (Lee *et al.* 2006). However, the number of identified proteins was much lower (maximum of 364 proteins identified) than the number of genes (more than 4200 transcripts in *E. coli*); hence, here, we performed two sets of whole-transcriptome studies [microarray conditions (i) and (ii)] to explore further the impact of degrading *cis*-DCE.

Degrading 1 mmol l^{-1} *cis*-DCE for 2 h while expressing the evolved toluene *ortho*-monooxygenase, glutathione *S*-transferase and γ-glutamylcysteine synthetase *vs* no cloned genes (microarray condition (i)] altered the gene expression of 60 genes more than threefold; 58 genes were induced, and two genes were repressed (Table S3). Because 73–79% of the cells were viable under these conditions (26), the observed transcriptomic response was considered to be reflective of a stress event rather than a survival response. Also, this altered gene expression pattern is the result of converting approx. 26% of the 1 mmol l^{-1} *cis*-DCE into chloride ions after 3 h (Rui *et al.* 2004b) and the stress this causes the cell. As expected, the expression of *gshA* (encodes GSHI) was induced (14-fold) compared to the control (Table S3) because of the cloning of GSHI* (Rui *et al.* 2004b), which matches well with an 8·6-fold induction of GshA in the proteome study (Lee *et al.* 2006). Of the 46 proteins that were identified as significantly up-regulated in the proteomic study (Lee *et al.* 2006), eight of those genes (*cysK*, *dps*, *gshA*, *htpG*, *iscS*, *rpmD*, *rpmE* and *rpmE*) were also induced in this study. Additionally, the whole-transcriptome analysis identified sulfate metabolism genes (*cbl*, *cysD* and *tauABCD*), 30 well-known stress genes (heat, osmosis, acid and oxidative stress), and 18 hypothetical genes as induced (Table S3), while the proteomic analysis identified only five stress response proteins (AhpF, Dps, HtpG, KatG and SodB) (Lee *et al.* 2006). Also, data from two independent whole-transcriptome experiments match very well each other (Table S3). Therefore, the whole-transcriptome approach complements the proteomic study, while providing more insights into the gene expression changes which occur during enhanced *cis*-DCE degradation.

Similarly, degrading 1 mmol l^{-1} *cis*-DCE while expressing the evolved toluene *ortho*-monooxygenase and evolved epoxide hydrolase *vs* only the evolved toluene *ortho*-monooxygenase (no evolved EchA) [microarray condition (ii)] altered the expression of 65 genes more than twofold; 56 genes were induced, and nine

genes were repressed (Table S3). This altered gene expression pattern is the result of converting approx. threefold more of the 1 mmol l^{-1} *cis*-DCE into chloride ions after 2 h (52 *vs* 18% mineralization) (Rui *et al.* 2004a) and the stress this causes the cell. To get a larger response, this experiment is different from the previous proteomic study in which we studied the effect of adding the evolved EchA compared to wild-type EchA (Lee *et al.* 2006); hence, we studied the impact of removing the toxic epoxide from the cell here. Among the induced genes, 21 genes are well-known stress-related genes involved in heat shock (*ibpAB*, *hsp15* and *htpX*), antibiotic resistance (*marRA*) and glyoxylate metabolism (*glxA23* and *gloA*), and 17 are hypothetical genes (Table S3). Therefore, we confirmed that *cis*-DCE degradation with TOM-Green and an evolved EchA (detoxifies epoxides) causes a strong stress response similar to heat shock and oxidative stress because of the greater amount of *cis*-DCE degraded (Rui *et al.* 2004a).

Confirmation of new stress proteins from *cis*-DCE stress

The majority of the induced genes in both systems for enhanced *cis*-DCE degradation was stress related. Fifteen genes (*clpB*, *dnaKJ*, *groSL*, *grpE*, *hslJ*, *hslO*, *hslV*, *hsp15*, *hspQ*, *htpG*, *htpX* and *ibpAB*) were associated with heat shock, and nine genes (*ariR*, *bshA*, *cysK*, *dps*, *grxA*, *ibpAB*, *sbp* and *soxS*) were associated with oxidative stress, because the same nine genes were most significantly induced with the treatment of H_2O_2 in a previous study (Zheng *et al.* 2001). Hence, exposure to *cis*-DCE leads to complex stress response. Along with these well-characterized stress genes, 32 hypothetical genes are induced from our whole-transcriptome studies with *cis*-DCE (Table S3). We hypothesized that some of these hypothetical genes may be involved in the stress response mechanism, and 15 isogenic *E. coli* K-12 BW25113 mutants (Table 1 marked with *) were tested for H_2O_2 resistance. We used H_2O_2 resistance because the whole-transcriptome response resembled that with H_2O_2 (Zheng *et al.* 2001) and because ethene epoxides are impractical because of their very limited stabilities; for example, the half lives of trichloroethene epoxide, vinyl chloride epoxide, *trans*-1,2-dichloroethene epoxide and *cis*-1,2-dichloroethene epoxide are 21, 78 s, 31 and 72 h, respectively (van Hylckama Vlieg *et al.* 1996).

Seven of the 15 mutants [*ibpB*, *ychH*, *ydeI* (a paralog of *ygiW*), *yeaR*, *ygiW*, *yoaG* and *yodD*] were at least fivefold more sensitive to H_2O_2 (34 mmol l^{-1} for 15 min) than the wild-type strain (Fig. 1). In particular, deletion mutants of *ygiW* and *ychH* had 35- and 47-fold lower survival than wild-type *E. coli*.

Differentially expressed genes in response to hydrogen peroxide

To probe the genetic basis of how the *ygiW* and *ychH* mutants respond to H_2O_2 stress, another whole-transcriptome study was performed for the wild-type strain and the isogenic *ygiW* and *ychH* mutants with H_2O_2 [microarray conditions (iii), (iv) and (v)]. Unlike the survival test (Fig. 1 with 34 mmol l^{-1} H_2O_2 for 15 min), cells were contacted with and without 2 mmol l^{-1} H_2O_2 for 10 min, in which cells grew well, to investigate the stress response. Overall, 71 genes were regulated more than fourfold in the wild-type strain; 56 genes were induced, and 15 genes were repressed (Tables S4 and S5). In the absence of YgiW, 328 genes were regulated more than fourfold; 192 genes were induced, and 136 genes were repressed (partial data given in Tables S4 and S5). In the absence of YchH, 249 genes were regulated more than fourfold; 114 genes were induced, and 135 genes were repressed (partial data given in Tables S4 and S5). Corroborating the deletion mutations, the microarray signals of the *ygiW* and *ychH* genes were very low in their respective microarray experiments (Table 1). Based on these results, it is clear that H_2O_2 regulates far more genes in the H_2O_2-sensitive *ygiW* and *ychH* mutants than in the wild-type strain.

Our whole-transcriptome data with the wild-type strain agree well with the previous report of transcriptional profiling with H_2O_2 treatment (Zheng *et al.* 2001), with 12 genes induced in common with the most significantly induced genes reported. For example, *ariR* [formerly *ymgB* (Lee *et al.* 2007)], *bshA* [formerly *ycfR* (Zhang *et al.* 2007)], *fpr*, *grxA*, *katG*, *sufESDCBA* and *ymgA* were highly induced in our whole-transcriptome study (Table S4) as well as in the previous study (Zheng *et al.* 2001), and gene expression of a master oxidative regulator *oxyR* was not changed in both studies. In addition, 11 genes (*dinD*, *dinF*, *dinI*, *dinP*, *oxyS*, *recN*, *recX*, *sbmC*, *sulA* and *umuDC*) were newly identified as induced stress genes with H_2O_2 treatment here (Table S4 marked with #). We detected induction of *oxyS* (another important regulator for oxidative stress), several genes encoding DNA repair proteins (*dinD*, *dinF*, *dinI*, *dinP*, *recN*, *recX* and *umuDC*), and other stress-inducible genes (*sbmC* and *sulA*) with the treatment of H_2O_2 (Table S4). Among the repressed genes, the maltose transport operon (*malEFG* and *malMBK*) was most repressed by H_2O_2 in the wild-type strain and in the *ygiW* and *ychH* mutants (Table S5). Therefore, our results corroborate the previous transcriptional profiling results with H_2O_2 (Zheng *et al.* 2001) and expand the list of oxidative stress genes in *E. coli*.

Table 1 List of 39 genes indentified with either *cis*-DCE or with H_2O_2 for further study. Genes were selected from five sets of whole-transcriptome studies. GST/GSH represents the TOM-Green/glutathione *S*-transferase (IsolLR1)/γ-glutamylcysteine synthetase (GSHI*) system, EH represents the TOM-Green/evolved epoxide hydrolase (EchA 108L/I219L/C248I), no EH represents the TOM-Green without epoxide hydrolase and $+H_2O_2$ represents treatment with 2 mmol l^{-1} H_2O_2 for 10 min. Asterisks (*) indicate the selection of possible stress-related genes from *cis*-DCE microarray analysis (Table S3). Triangle (Δ) indicates the selection of possible stress-related genes from microarray analysis with H_2O_2 treatment (Table S4 and S5). Delta (δ) indicates the selection of possible stress-related genes from comparison analysis of BW25113 with H_2O_2 treatment *vs* BW25113 *ygiW* with H_2O_2 treatment and BW25113 with H_2O_2 treatment *vs* BW25113 *ychH* with H_2O_2 treatment (Tables S6 and S7)

		Fold changes in DNA microarrays						
Gene	b#	GST/GSH *vs* no cloned gene	EH *vs* no EH	$WT^{+H_2O_2}$ *vs* WT	$WT^{+H_2O_2}$ *vs* $ygiW^{+H_2O_2}$	$WT^{+H_2O_2}$ *vs* $ychH^{+H_2O_2}$	μ, h^{-1}	Description
WT	NA	NA	NA	NA	NA	NA	1·5 ± 0·1	
Genes selected from two sets of DNA microarrays for *cis*-DCE mineralization								
*ychH**	b1205	3·8 ± 2·1	1·3	1·3	5·7	168·9	1·45 ± 0·07	Hypothetical protein
*ydeI**	b1536	−1·2 ± 0·4	−1·1	1·7	−1·3	−1·6	1·40 ± 0·01	YdeI and YgiW are paralogs
*ydhM**	b1649	3·2 ± 0·5	3·5	1·2	−1·1	−1·2	ND	Hypothetical protein
*yeaQ**	b1795	2·8 ± 1·1	2·8	4·0	−1·5	−1·6	ND	Hypothetical protein, nitric oxide stress responsive
*yoaG**	b1796	1·1 ± 0·3	3·0	1·1	−1·1	−2·6	1·49 ± 0·00	Hypothetical protein, nitric oxide stress responsive
*yeaR**	b1797	1·4 ± 0·1	3·5	1·2	−1·1	−3·2	1·59 ± 0·01	Hypothetical protein, nitric oxide stress responsive
*yebV**	b1836	2·3 ± 1·0	2·0	2·8	−1·4	−2·0	ND	Hypothetical protein
*yodD**	b1953	3·9 ± 2·6	1·5	2·5	1·1	−2·3	1·37 ± 0·02	Hypothetical protein
*yegP**	b2080	3·0 ± 1·5	1·4	3·2	−1·2	−1·9	ND	Hypothetical protein
*ygiW**	b3024	4·0 ± 1·3	1·7	2·0	477·7	1·2	1·45 ± 0·02	Hypothetical protein, b3024
*rpmG**	b3636	3·3 ± 1·4	2·6	−1·6	1·1	1·3	ND	50S ribosomal subunit protein L33
*rpmE**	b3936	3·2 ± 1·2	2	−1·2	1·2	1·2	ND	50S ribosomal subunit protein L31
*ibpB**	b3686	9·2 ± 0·9	2·0	−1·3	5·3	5·7	1·48 ± 0·05	Heat shock protein
*ibpA**	b3687	9·8 ± 0·0	2·3	1·0	3·0	2·0	ND	Heat shock protein
Genes selected from three sets of DNA microarrays with H_2O_2 treatment								
ybdB$^{\Delta}$	b0597	−1·2 ± 0·1	−1·1	7·5	1·7	−1·1	ND	Hypothetical protein
ybiI$^{\Delta}$	b0803	1·3 ± 0·4	1·1	5·3	2·1	−1·2	ND	Hypothetical protein
ybiX$^{\Delta}$	b0804	−1·4 ± 0·1	−1·3	8·6	2·6	−1·1	ND	Hypothetical protein
nhoA$^{\Delta}$	b1463	−1·3 ± 0·2	−1·4	12·1	−1·3	−2·5	ND	Putative *N*-hydroxyarylamine *O*-acetyltransferase
sufE$^{\Delta}$	b1679	−1·5 ± 0·1	−1·1	13·0	34·3	36·8	1·61 ± 0·02	Protein for Fe–S cluster assembly
sufS$^{\Delta}$	b1680	−1·8 ± 0·9	1·4	8·0	18·4	19·7	1·52 ± 0·01	Protein for Fe–S cluster assembly
sufC$^{\Delta}$	b1682	−1·3 ± 0·6	1·1	8·6	13·9	13·9	ND	Protein for Fe–S cluster assembly
yhcN$^{\Delta}$	b3238	1·7 ± 0·4	1·1	6·5	8·6	13	1·42 ± 0·04	YhcN family consisted of nine small uncharacterized genes
yjaA$^{\Delta}$	b4011	−1·4 ± 0·2	−1·1	−4·0	−5·7	2·6	1·44 ± 0·01	Hypothetical protein
feaR$^{\delta}$	b1384	−1·2 ± 0·0	1·5	1·6	11·3	1·7	ND	Regulatory protein for 2-phenylethylamine catabolism
tap$^{\delta}$	b1885	−1·5 ± 0·7	1·0	−1·1	−3·5	−222·9	1·37 ± 0·05	Chemotaxis protein IV, peptide sensor receptor
fliD$^{\delta}$	b1924	−1·5 ± 0·4	−1·2	−1·4	−3·0	−168·9	ND	Flagellar biosynthesis
fliS$^{\delta}$	b1925	−1·7 ± 0·6	1·4	−1·1	−3·0	−137·2	1·25 ± 0·09	Flagellar biosynthesis
yedF$^{\delta}$	b1930	−1·1 ± 0·1	1·0	−1·7	7·0	9·8	ND	Hypothetical protein
yeeF$^{\delta}$	b2014	−1·6 ± 0·5	−2·5	−1·9	−1·9	7·0	ND	Hypothetical protein
mglC$^{\delta}$	b2148	−1·4 ± 0·1	−1·1	−2·1	16	12·1	ND	Methyl-galactoside transport and galactose taxis
mglA$^{\delta}$	b2149	−1·8 ± 0·4	−1·2	−1·1	29·9	10·6	1·4 ± 0·2	ATP-binding component of methyl-galactoside transport
mglB$^{\delta}$	b2150	1·2 ± 0·1	−1·4	−1·1	24·3	3·7	ND	Galactose-binding transport protein; receptor for galactose taxis

Table 1 (*Continued*)

Gene	b#	Fold changes in DNA microarrays: GST/GSH *vs* no cloned gene	EH *vs* no EH	$WT^{+H_2O_2}$ *vs* WT	$WT^{+H_2O_2}$ *vs* $ygiW^{+H_2O_2}$	$WT^{+H_2O_2}$ *vs* $ychH^{+H_2O_2}$	μ, h^{-1}	Description
galS$^{\delta}$	b2151	−1·5 ± 0·2	1·0	2·6	10·6	4·3	1·46 ± 0·05	Mgl repressor, galactose operon inducer
hcaR$^{\delta}$	b2537	1·0 ± 0·1	1·0	1·5	10·6	1·6	1·35 ± 0·01	Transcriptional activator of hca cluster (LysR family)
luxS	b2687	1·3 ± 0·3	1·0	1·3	−1·0	1·1	ND	Autoinducer 2 synthesis
tnaA$^{\delta}$	b3708	−1·3 ± 0·3	−1·7	−1·1	4·3	1·6	ND	Tryptophanase
tnaB$^{\delta}$	b3709	−1·6 ± 0·1	−1·1	−1·7	24·3	6·1	1·24 ± 0·03	Low-affinity tryptophan permease
glnG$^{\delta}$	b3868	−1·4 ± 0·1	−1·1	−2·3	−6·1	−2·3	ND	Response regulator for glutamine
yjcB$^{\delta}$	b4060	1·5 ± 0·6	1·6	−1·9	−84·4	−9·2	ND	Hypothetical protein

cis-DCE, *cis*-1,2-dichloroethylene; NA, not applicable; ND, not determined; μ, specific growth rate; WT, wild-type strain.

Selection of possible stress-related genes from whole-transcriptome analysis

From the three sets of whole-transcriptome studies with H_2O_2 (Tables S4 and S5 marked with a Δ), several induced genes of hypothetical proteins (NhoA, YbdB, YbiI, YbiX, YhcN and YjaA) and *suf* operon proteins (SufC, SufE and SufS) were selected for further phenotypic study (Table 1 marked with a Δ). An additional comparison of the whole-transcriptome data from the three sets of whole-transcriptome studies with H_2O_2 was performed to understand how the stress sensitive *ygiW* and *ychH* mutants respond to H_2O_2 stress relative to the wild-type strain (BW25113 with H_2O_2 treatment *vs* BW25113 *ygiW* with H_2O_2 treatment and BW25113 with H_2O_2 treatment *vs* BW25113 *ychH* with H_2O_2 treatment) (Tables S6 and S7). Although the gene expression patterns (induction or repression) of many genes responding to H_2O_2 was similar for the three strains (Tables S4 and S5), many genes were distinctively regulated in the absence of YgiW and YchH. Among the induced or repressed genes (Tables S6 and S7 marked with δ), 13 genes exhibiting a

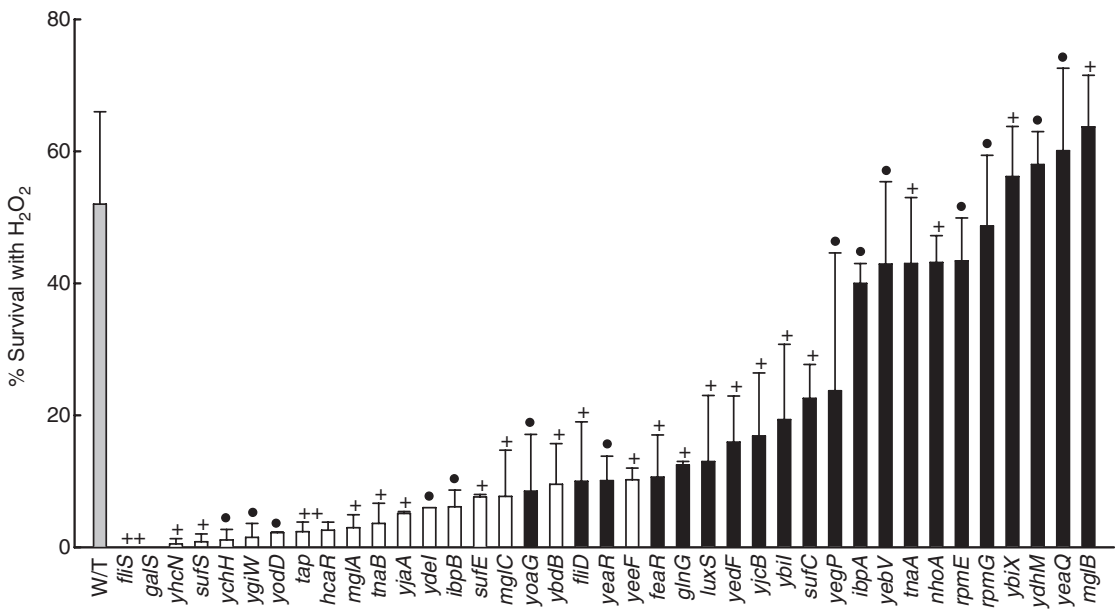

Figure 1 Survival of *Escherichia coli* BW25113 and its isogenic mutants after treatment with H_2O_2 (34 mmol l^{-1}) for 15 min in Luria–Bertani medium at 37°C. Mutants are indicated that were selected from the whole-transcriptome studies with *cis*-1,2-dichloroethylene stress (●) and H_2O_2 stress (+); mutants indicated with a solid black bar were not selected for further phenotype analysis because they had similar rates compared to the wild-type strain. The experiments were repeated at least twice, and error bars represent one standard deviation.

distinctive change in expression [*galS*, *mglA*, *mglB*, *mglC*, *tnaA*, *tnaB*, *yedF* and *yeeF* (Table S6), and *fliD*, *fliS*, *glnG*, *tap* and *yjcB* (Table S7)], and two genes encoding regulator proteins (FeaR and HcaR) were selected for the further study (Table 1 marked with a δ). Also, the AI-2 synthesis gene *luxS* was chosen because AI-2 triggers oxidative stress response in *Mycobacterium avium* leading to biofilm formation (Geier *et al.* 2008). Note that 19 genes in Table 1 are hypothetical genes; hence, they are poorly characterized in terms of stress response. Therefore, 25 genes were chosen for further study from these three sets of hydrogen peroxide arrays.

Confirmation of new stress proteins from H_2O_2 stress

In addition to the eight H_2O_2-sensitive proteins identified in the whole-transcriptome studies with *cis*-DCE (Fig. 1), ten mutants (*fliS*, *galS*, *hcaR*, *mglA*, *sufE*, *sufS*, *tap*, *tnaB*, *yhcN* and *yjaA*) were found to be more sensitive (above sevenfold) to H_2O_2 (Fig. 1) out of the 25 mutants studied. Of the ten, *fliS*, *galS*, *sufS* and *yhcN* mutants were most sensitive to H_2O_2 (more than 60-fold decreased survival). The two quorum-sensing mutants (*luxS* and *tnaA*) were less sensitive to H_2O_2 (Fig. 1); however, the addition of indole (1 mmol l^{-1}) to the *tnaA* mutant and addition of the AI-2 precursor DPD (100 μmol l^{-1}) to the *luxS* mutant did not significantly affect survival with H_2O_2 (data not shown). Therefore, quorum-sensing molecules do not appear to be directly involved in response to H_2O_2 stress in *E. coli*.

Cadmium and acid sensitivity

Because the regulation of stress responses overlap (Weber *et al.* 2005; Chung *et al.* 2006) based on shared sigma factors and interactions with the cyclic AMP receptor protein (CRP), the network of stress response may be highly interwoven. Thus, all 17 H_2O_2-sensitive mutants (eight mutants out of *cis*-DCE microarrays and ten mutants out of hydrogen peroxide microarrays) were investigated for resistance to both cadmium (4 mg ml^{-1} for 40 min) and acid (pH 2·5 for 60 min) treatments. In general, the mutants were more sensitive to cadmium (Fig. 2a) which indicates that cadmium stress is primarily because of oxidative stress (Stohs and Bagchi 1995). Furthermore, four mutants (*hcaR*, *sufE*, *sufS* and *yodD*) were very sensitive to acid (more than 60-fold lower survival) (Fig. 2b).

Biofilm formation

Because stress (H_2O_2, cadmium, HCl and low temperature) can increase the biofilm formation of *E. coli* through BhsA (Zhang *et al.* 2007), biofilm formation of the 17 mutants was also measured. Most of the mutants (except *fliS*, *sufE* and *tnaB*) produced more (up to 22-fold) biofilm than the wild-type strain (Fig. 2c). The results confirm that stress increases biofilm formation (Zhang *et al.* 2007).

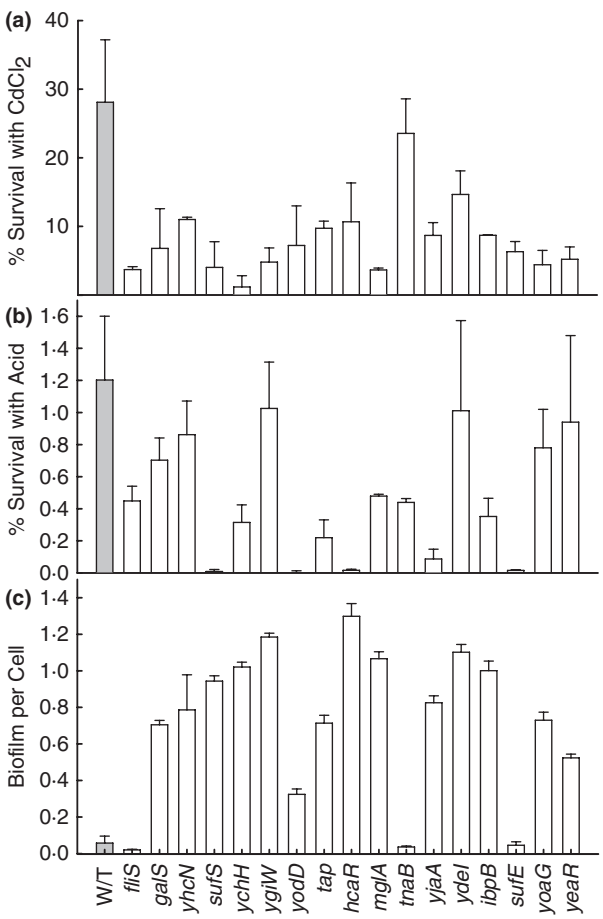

Figure 2 Survival of BW25113 and its isogenic mutants after treatment with $CdCl_2$ (4 mg ml^{-1}) for 40 min (a) and acid (pH 2·5) for 60 min (b). Biofilm formation normalized by bacterial growth (turbidity at 620 nm) is shown after 7 h (c). All experiments were performed in Luria–Bertani at 37°C and were repeated at least twice. Error bars represent one standard deviation.

Specific growth rates

The specific growth rates of the 17 most important mutants were similar to that of wild-type strain (Table 1). Hence, the phenotypic changes (sensitivity to H_2O_2, cadmium and acid) were not the result of altered growth rates.

Complementation of biofilm formation and hydrogen peroxide stress

To verify that the genes of interest are responsible for the altered phenotypes such as biofilm formation and H_2O_2

stress resistance, YgiW, YchH, YhcN, GalS were added in *trans* using the complementation plasmids pCA24N-*ygiW*, pCA24N-*ychH*, pCA24N-*yhcN* and pCA24N-*galS* (Kitagawa *et al.* 2005). Biofilm formation was complemented by overexpressing YgiW, YchH and YhcN via pCA24N-*ygiW*, pCA24N-*ychH* and pCA24N-*yhcN* (Fig. 3a). Survival with H_2O_2 was partially complemented by overexpressing GalS with pCA24N-*galS* (Fig. 3b), while biofilm formation was partially complemented with pCA24N-*galS* (Fig. 3a). The results demonstrate that these genes are responsible for the altered phenotypes.

Verification of whole-transcriptome results by qRT-PCR

For the five sets of whole-transcriptome experiments, qRT-PCR was used to verify gene expression for the highly differentially expressed genes (*gshA*, *ibpB*, *mglA*, *sufS*, *tauA*, *tnaB* and *yfiD*) by using the housekeeping gene *rpoA*. The changes in gene expression obtained with qRT-PCR were comparable to the whole-transcriptome studies (Table 2). Notably, high induction of *sufS* was verified for treatment with H_2O_2 for the wild-type strain and the *ygiW* and *ychH* mutants (Table 2 and Table S4); the *sufS* mutant is sensitive to all stresses such as H_2O_2, cadmium and acid (Fig. 4).

Reduced *cis*-DCE degradation by deletion of *ychH* and *yhcN*

We reasoned that the stress genes identified here should be important for the degradation of *cis*-DCE. Deletion of *ychH* and *yhcN* in *E. coli* expressing TOM-Green resulted in reduced *cis*-DCE degradation rates (0·47 ± 0·05 for

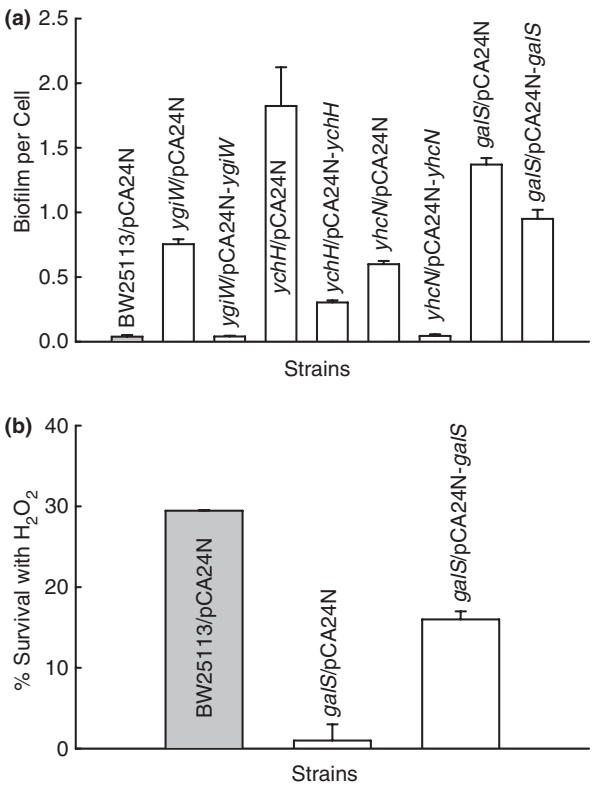

Figure 3 Complementation of biofilm formation and response to H_2O_2 stress. Biofilm formation normalized by bacterial growth (turbidity at 620 nm) is shown after 7 h (a) and survival with H_2O_2 (34 mmol l^{-1}) is shown after 15 min (b). For the pCA24N-derived plasmids, 1 mmol l^{-1} isopropyl-β-D-thiogalactopyranoside was used. All experiments were performed in Luria–Bertani medium at 37°C and were repeated at least twice. Error bars represent one standard deviation.

Table 2 Confirmation of the whole-transcriptome results via qRT-PCR. GST/GSH represents the TOM-Green/glutathione *S*-transferase (IsolLR1)/γ-glutamylcysteine synthetase (GSHI*) system, EH represents the TOM-Green/evolved epoxide hydrolase (EchA 108L/I219L/C248I), no EH represents the TOM-Green without epoxide hydrolase and $^{+H_2O_2}$ represents treatment with 2 mmol l^{-1} H_2O_2 for 10 min. The housekeeping gene *rpoA* (RNA polymerase α subunit) was used to normalize qRT-PCR data. Two independent qRT-PCR experiments were performed and the average of the fold changes are shown

	cis-1,2-dichloroethylene degradation				H_2O_2 treatment					
	GST/GSH *vs* no cloned gene		EH *vs* no EH		WT$^{+H_2O_2}$ *vs* WT		*ygiW*$^{+H_2O_2}$ *vs ygiW*		*ychH*$^{+H_2O_2}$ *vs ychH*	
Gene	RT-PCR	Microarray	RT-PCR	Microarray	RT-PCR	Microarray	RT-PCR	Microarray	RT-PCR	Microarray
gshA	59·5	17·5	ND	1·4	ND	1·1	ND	1·0	ND	1·0
tauA	2·1	9·6	1·0	1·0	ND	1·1	ND	1·4	ND	1·1
yfiD	ND	2·6	4·4	4·6	ND	1·4	ND	2·3	ND	1·9
ibpB	3·5	9·2	2·5	2·0	ND	−1·3	ND	3·0	ND	−6·1
sufS	ND	−1·8	ND	1·4	57·7	8·0	113·4	18·4	46·7	19·7
mglA	ND	−1·8	ND	−1·2	−4·3	−1·1	−9·7	−34·3	−28·8	−19·7
tnaB	ND	−1·6	ND	−1·2	−5·2	−1·7	−5·8	−36·8	−51·2	−13·9

qRT-PCR, quantitative real-time reverse transcription-PCR; ND, not determined.

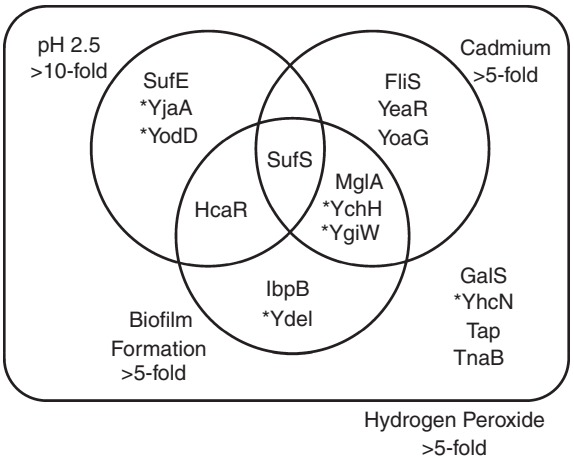

Figure 4 Grouping of the biofilm formation and stress responses to cadmium and acid of the 17 BW25113 isogenic mutants demonstrating sensitivity to hydrogen peroxide stress (Fig. 1). The overlapping response behaviour suggests an interwoven stress response network. Fold changes are relative to the BW25113 wild-type strain. The six previously uncharacterized proteins are denoted with an asterisks (*).

ychH and 0·37 ± 0·03 for *ychN vs* 0·9 ± 0·2 nmol min^{-1} mg^{-1} protein for the wild-type strain). Corroborating this result, less chloride was measured with these two stress proteins removed (32 ± 5% for *ychH* and 21 ± 9% for *ychN vs*48 ± 24% mineralization for the wild-type strain). Hence, YchH and YhcN are important for *cis*-DCE degradation.

Discussion

The two sets of whole-transcriptome analysis corroborated the quantitative proteomic study for the enhanced *cis*-DCE mineralization and identified six new stress-related proteins (YchH, YdeI, YgiW, YhcN, YjaA and YodD) that are important in stress responses including resistance to H_2O_2, cadmium and acid and that are important in biofilm formation. In addition, it was demonstrated that *cis*-DCE degradation with engineered *E. coli* strains expressing an evolved toluene *ortho*-monooxygenase, glutathione *S*-transferase and γ-glutamylcysteine synthetase or expressing an evolved toluene *ortho*-monooxygenase and evolved epoxide hydrolase cause a strong stress response similar to heat shock and/or oxidative stress. In addition, several new loci have been identified that are related to H_2O_2 stress.

It has been proposed that aerobic degradation of *cis*-DCE with a monooxygenase and glutathione *S*-transferase or epoxide hydrolase may produce the toxic intermediate glyoxal (similar structure to glyoxylate), which prevents complete mineralization of *cis*-DCE (Lee *et al.* 2006). The current whole-transcriptome data indicate the induction of three genes (*gloA* and *glxA23*) involved in glyoxylate conversion during enhanced *cis*-DCE degradation with the evolved epoxide (Table S3), which partially supports the hypothesis of glyoxal production. Thus, we speculate that the induction or overexpression of glyoxalase I and glyoxalase II, which catalyse the conversion of methylglyoxal into D-lactate (Clugston *et al.* 1998), may be beneficial for channelling glyoxal into a central metabolic cycle (van Hylckama Vlieg and Janssen 2001).

From the whole-transcriptome analyses, 40 additional genes were selected for further study for stress resistance using single-gene knockout strains [Kieo Collection (Baba *et al.* 2006)]. Seventeen of these (Figs 1 and 4) were identified as important in the stress responses to H_2O_2, cadmium or acid, and in biofilm formation, with six being previously uncharacterized proteins (YchH, YdeI, YgiW, YhcN, YjaA and YodD). These seven uncharacterized proteins have now been related clearly to a cellular function, stress response. In addition, YchH and YhcN were shown to be directly connected to *cis*-DCE degradation.

Of the 17 proteins related to H_2O_2, cadmium and acid stress, 11 of these proteins (FliS, GalS, HcaR, IbpB, MglA, SufE, SufS, Tap, TnaB, YeaR and YoaG) have been linked previously to a stress response via whole-transcriptome analysis, proteome analysis or chemotactic response in *E. coli*, but this is the first report where deletion mutants have been used to confirm their relation to a stress response with *cis*-DCE, H_2O_2, cadmium and acid (previous authors used heat and other chemicals). For example, gene expression of *sufABCDSE* is induced by H_2O_2 (Zheng *et al.* 2001), and oxidant induction of the *suf* operon is regulated by OxyR, IHF, Fur and IscR (Lee *et al.* 2008). Our results indicate that the *sufE* and *sufS* mutants are stress related, and SufS was identified as a general stress response protein because of its high level of sensitivity to all of the stressors evaluated (Fig. 4). Characterization of the *suf* operon (*sufABCDSE*) indicates that the Suf proteins constitute an alternative pathway for Fe–S cluster assembly in *E. coli* (Takahashi and Tokumoto 2002). Also, a component of toluene *ortho*-monooxygenase, TomA5 (40-kDa NADH-oxidoreductase) contains a [2Fe2S] cluster (Newman and Wackett 1995). Thus, sulfur metabolism may be important for the enhanced *cis*-DCE mineralization because the induction of many proteins (Cbl, CysADKP, IscS, Sbp and TauABCD) involved in the sulfur metabolism was observed in the proteomic (Lee *et al.* 2006) and transcriptomic data (Table S6). Note IscS plays a major role in providing sulfur for Fe–S cluster synthesis (Schwartz *et al.* 2000).

Tap and FliS may also be involved in an evasion stress response as both are involved in flagellar biosynthesis and chemotaxis, and both proteins were sensitive to the oxidative stressors H_2O_2 (Fig. 1) and cadmium

(Fig. 2a). Tap allows the cell to respond chemotactically to dipeptides (Manson *et al.* 1986), low pH (Yamamoto *et al.* 1990) and thermal stimuli (Nara *et al.* 1991). FliS binds to the FliC C-terminal helical domain and acts as a substrate-specific chaperone (Auvray *et al.* 2001). MglBAC is involved in the galactose transport, GalR and GalS negatively regulate MglBAC, and GalR and GalS are regulated by the global regulator CRP (Semsey *et al.* 2007). Also, we found *mglBAC* and *galS* are induced to the largest extent upon the addition of H_2O_2 for BW25113 *vs* BW25113 *ygiW* and BW25113 *vs* BW25113 *ychH* (Table S6). The *yeaR–yoaG* operon is induced in response to nitric oxide stress (Justino *et al.* 2005), and transcription of the *yeaR–yoaG* operon is regulated by nitrate-responsive regulator NarL (Lin *et al.* 2007). HcaR is a transcriptional activator for the *hca* cluster and is responsible for the initial catabolism of 3-phenylpropionic acid (Díaz *et al.* 1998). IbpB, a heat shock protein, along with IbpA participates in the defence against copper-induced stress (Matuszewska *et al.* 2008), and *ibpB* (alternative name *hslS*) is one of the most induced genes after contact with H_2O_2 (Zheng *et al.* 2001) and in *E. coli* biofilm cells (Ren *et al.* 2004a). TnaB (tryptophan permease) is critical for tryptophan uptake (Yanofsky *et al.* 1991), and TnaB along with TnaA (tryptophanase) contributes to drug resistance in *E. coli* (Kobayashi *et al.* 2006).

Genome-wide transcriptome approaches have been utilized extensively to investigate the global gene expression in response to oxidant stresses (Pomposiello *et al.* 2001; Zheng *et al.* 2001; Blanchard *et al.* 2007), acid (Tucker *et al.* 2002), cadmium (Wang and Crowley 2005), osmotic and heat (Gunasekera *et al.* 2008) and general stress via σ^s in *E. coli* (Lacour and Landini 2004; Weber *et al.* 2005). It was widely observed that the regulation of gene expression upon adding stress is interconnected in *E. coli*, which was also observed in this work (Fig. 4). For example, the oxidative acid resistance system is regulated by σ^s and CRP (Foster 2004), and σ^s regulates both gene expression for osmotic stress (Hengge-Aronis 1996) and regulates many acid resistance genes including the uncharacterized *ygiW* and *yodD* genes (Weber *et al.* 2005) identified in this study. Similarly, CRP is a global transcription factor regulating transcription at over 200 promoters (Grainger *et al.* 2005) including those for *bhsA*, *galS*, *ychH* and *yhnC* (Brown and Callan 2004) identified in this study. Furthermore, many heat shock genes overlap with oxidative stress genes (Farr and Kogoma 1991), and *cis*-DCE induced both heat shock and oxidative genes in this study. The toxicity of various heavy metals is because of the production of reactive oxygen species (Stohs and Bagchi 1995). Also, stress (H_2O_2, cadmium, HCl and low temperature) overlaps (increases) biofilm formation (Zhang *et al.* 2007), and 14 deletion mutants formed more biofilm in this study (Fig. 2c).

Many hypothetical proteins have also been investigated recently with respect to stress responses, including YhbO for multiple stresses (Abdallah *et al.* 2007), BhsA for multiple stresses and biofilm formation (Zhang *et al.* 2007) and YtfE for the repair of oxidative stress-damaged Fe–S clusters (Justino *et al.* 2007). Of the six uncharacterized proteins evaluated under multiple stressors in this study (Fig. 4), two (YodD and YjaA) were very sensitive to both H_2O_2 and acid, and four (YchH, YeaR, YgiW and YoaG) were very sensitive to H_2O_2 and cadmium, suggesting an interconnectedness in response to various stresses for these new proteins. Additionally, the YhcN family, which consists of nine, small, mostly uncharacterized proteins (Rudd *et al.* 1998) including BhsA, whose promoter also has a CRP binding site (Zhang *et al.* 2007). BhsA influences *E. coli* biofilm formation through stress response and surface hydrophobicity, and the BhsA mutant was much more sensitive to H_2O_2, cadmium, acid and heat stress (Zhang *et al.* 2007). Analogously, the YhcN mutant was sensitive to H_2O_2 (Fig. 1) and cadmium (Fig. 2a), forms more biofilm than the wild-type (Fig. 2c) and was shown to degrade less *cis*-DCE than the wild-type strain in this study.

Other proteins such as YgiW encoded by *ygiW* (b3024), which lies between two important quorum-sensing regulators, *qseB* (b3025) (Sperandio *et al.* 2002) and *mqsR* (b3022) (González Barrios *et al.* 2006), have their own promoter but still exhibit sensitivity to multiple stresses. In this work, the *ygiW* mutant was sensitive to H_2O_2 and cadmium and also produced elevated levels of biofilm (Fig. 4). From the whole-transcriptome data with the *ygiW* and *ychH* mutants (Tables S6 and S7), many flagellar biosynthesis genes were induced, which may partially explain the elevated biofilm observed (Fig. 2c), because flagellar motility facilitates adherence and development of biofilms (Pratt and Kolter 1998), and flagella genes are induced in biofilms (Domka *et al.* 2007). The *ygiW* gene is also induced by the indole-related, acid-resistance protein AriR (Lee *et al.* 2007) and by H_2O_2 (Zheng *et al.* 2001), further suggesting a possible role of YgiW in response to multiple stresses.

From the current whole-transcriptome study, 17 proteins have been identified as involved in a complex stress response network (Fig. 4), with six of those characterized for the first time here (YchH, YdeI, YgiW, YhcN, YjaA and YodD). The overlapping response behaviour suggests an interwoven stress response network. Furthermore, we provide more extensive lists of possible stress proteins involved in the response to H_2O_2 (Tables S4–S7), and many genes still need to be investigated further. It is hoped a more detailed knowledge of the stress response will aid the development of new strategies for bioremediation.

Acknowledgements

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 *Escherichia coli* strains and plasmids used. Kanamycin (Km^R) was used at 50 μg ml^{-1} for culturing the *E. coli* BW25113 isogenic mutants and at 100 μg ml^{-1} to maintain the pBS(Kan)-derived plasmids. Chloramphenicol (Cm^R) at 30 μg ml^{-1} was used for the pCA24N-derived plasmids and 50 μg ml^{-1} was used for pMMB206 or pMMB277.

Table S2 Primers used for quantitative real-time reverse transcription-PCR and verification of isogenic mutants.

Table S3 *Escherichia coli* genes significantly induced or repressed ($P < 0{\cdot}05$) with 1 mmol l^{-1} *cis*-DCE mineralization for 2 h by whole *E. coli* cells expressing (i) eight genes encoding evolved toluene *ortho*-monooxygenase, glutathione *S*-transferase and γ-glutamylcysteine synthetase *vs* no cloned genes (two independent experiments were performed using two independent biological replicates) and (ii) six genes encoding evolved toluene *ortho*-monooxygenase and evolved epoxide hydrolase *vs* evolved toluene *ortho*-monooxygenase (no evolved EH). Raw data for the four DNA microarrays are available using GEO series accession number GSE 13698. Primarily, genes differentially expressed above threefold for the eight-gene microarrays and above twofold for the EH microarrays are shown although some related genes are shown for completeness. ND, not detected. Asterisks (*) indicate the selection of possible stress-related genes for further phenotypic study.

Table S4 *Escherichia coli* genes induced in exponentially growing cells (turbidity at 600 nm of 1) upon addition of 2 mmol l^{-1} H_2O_2 for 10 min to K12 BW25113 (more than fourfold), to BW25113 *ygiW* (more than tenfold), and to BW25113 *ychH* (more than tenfold) in Luria–Bertani medium at 37°C. Some genes with less significant fold changes are shown for completeness. +H_2O_2 represents treatment with H_2O_2. Raw data for the six DNA microarrays are available using GEO series accession number GSE 13698. Pounds (#) indicate newly identified induced stress genes with H_2O_2 treatment. Triangles (Δ) indicate the selection of possible stress-related genes for further phenotypic study.

Table S5 *Escherichia coli* genes repressed in exponentially growing cells (turbidity at 600 nm of 1) upon addition of 2 mmol $^{-1}$ H_2O_2 for 10 min to BW25113 (more than fourfold), to BW25113 *ygiW* (more than tenfold), and to BW25113 *ychH* (more than tenfold) in Luria–Bertani medium at 37°C. Some genes with less significant fold changes are shown for completeness. +H_2O_2 represents treatment with H_2O_2. Raw data for the six DNA microarrays are available using GEO series accession number GSE 13698. Triangles (Δ) indicate the selection of possible stress-related genes for further phenotypic study.

Table S6 *Escherichia coli* genes induced more than tenfold ($P < 0{\cdot}05$) in exponentially growing cells (turbidity at 600 nm of 1) upon addition of 2 mmol l^{-1} H_2O_2 for

10 min in BW25113 *vs* BW25113 *ygiW* and BW25113 *vs* BW25113 *ychH* in Luria–Bertani medium at 37°C. Some genes with less significant fold changes are shown for completeness. $+H_2O_2$ represents treatment with H_2O_2. Raw data for the six DNA microarrays are available using GEO series accession number GSE 13698. Deltas (δ) indicate the selection of possible stress-related genes for further phenotypic study.

Table S7 *Escherichia coli* genes repressed more than ten-fold ($P < 0{\cdot}05$) in exponentially growing cells (turbidity at 600 nm of 1) upon 2 mmol l^{-1} H_2O_2 for 10 min in BW25113 *vs* BW25113 *ygiW* and BW25113 *vs* BW25113 *ychH* in Luria–Bertani medium at 37°C. Some genes with less significant fold changes are shown for completeness. $+H_2O_2$ represents treatment with H_2O_2. Raw data for the six DNA microarrays are available using GEO series accession number GSE 13698. Deltas ($^{\delta}$) indicate the selection of possible stress-related genes for further phenotypic study.

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