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Involvement of catalase and superoxide dismutase in hydrophobic organic solvent tolerance of *Escherichia coli*

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Abstract

Escherichia coli strains are generally sensitive to hydrophobic organic solvents such as *n*-hexane and cyclohexane. Oxidative stress in *E. coli* by exposure to these hydrophobic organic solvents has been poorly understood. In the present study, we examined organic solvent tolerance and oxygen radical generation in *E. coli* mutants deficient in reactive oxygen species (ROS)-scavenging enzymes. The organic solvent tolerances in single gene mutants lacking genes encoding superoxide dismutase (*sodA*, *sodB*, and *sodC*), catalase (*katE* and *katG*), and alkyl hydroperoxide reductase (*ahpCF*) were similar to that of parent strain BW25113. We constructed a BW25113-based *katE katG* double mutant (BW25113Δ*katE*Δ*katG*) and *sodA sodB* double mutant (BW25113*sodA*Δ*sodB*). These double-gene mutants were more sensitive to hydrophobic organic solvents than BW25113. In addition, the intracellular ROS levels in *E. coli* strains increased by the addition of *n*-hexane or cyclohexane. The ROS levels in BW25113Δ*katE*Δ*katG* and BW25113Δ*sodA*Δ*sodB* induced by exposure to the solvents were higher than that in BW25113. These results suggested that ROS-scavenging enzymes contribute to the maintenance of organic solvent tolerance in *E. coli*. In addition, the promoter activities of *sodA* and *sodB* were significantly increased by exposure to *n*-hexane.

Keywords: *Escherichia coli*, Organic solvent tolerance, Catalase, Superoxide dismutase, Reactive oxygen species

Introduction

Efficient microbial production of valuable organic compounds including biofuels and fine chemicals from renewable biomass resources is one of the crucial challenges in the establishment of a sustainable society. Some of these valuable chemicals such as advanced biofuels and bulk chemicals, including organic solvents including 1-octanol and styrene, are toxic to various microorganisms such as *E. coli* and *Pseudomonas putida* (Akhtar et al. 2015; Lennen et al. 2013; Mukhopadhyay 2015). Accumulation of these toxic products can negatively impact the viability of microbes and impede their efficient mass production of organic compounds (Doukyu

and Iida 2020; Doukyu et al. 2003; Mukhopadhyay 2015). Bacterial growth and stress response have been studied in a variety of organic solvents (Heipieper et al. 2007; Inoue and Horikoshi 1989; Ramos et al. 2002). Solvents with higher hydrophobicity tend to increase in toxicity (Kabelitz et al. 2003). At saturated concentrations, the toxicity of the hydrophobic organic solvent is inversely correlated with the $\log P_{ow}$ of the solvent (common logarithm of partition coefficient of given solvent in a mixture of n-octanol and water) (Inoue and Horikoshi 1989). In the $\log P_{ow}$ range of 2 to 4, increasing the hydrophobicity of the solvent could enhance the level of toxicity (Kabelitz et al. 2003). The effects of organic solvents on membrane structure and fluidity differ depending on the polarity of the solvent (Griepernau et al. 2007).

It has been reported that reactive oxygen species (ROS) are generated in *Escherichia coli* during exposure to hydrophilic solvents such as ethanol and *n*-butanol

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(Cao et al. 2017; Chin et al. 2013; Rutherford et al. 2010). A ROS assay using a cell-permeant fluorescent dye showed a significant increase in intracellular ROS levels in *n*-butanol-exposed *E. coli* cells (Rutherford et al. 2010). In addition, an *E. coli* strain expressing metallothioneins, which function in ROS scavenging, exhibited an increased growth rate under *n*-butanol stress (Chin et al. 2013). Thus, ROS generation has been suggested to be attributable in part to the toxicity of hydrophilic organic solvents. On the other hand, various tolerance mechanisms in *E. coli* strains have been studied with hydrophobic organic solvents such as *n*-hexane and cyclohexane (Aono 1998). These findings are useful for improving the production of hydrophobic solvents and fatty acids (Akhtar et al. 2015; Lennen et al. 2013). However, little is known about the ROS generation in microbial cells by these hydrophobic organic solvents.

Various ROS, including superoxide, H_2O_2 and hydroxyl radical, are generated as by-products in cells grown aerobically. Specific enzymes such as catalase and superoxide dismutase (SOD) decrease the levels of cytotoxic ROS. *E. coli* harbors two different catalase genes: *katG*, which encodes hydroperoxidase I (HPI) (Triggs-Raine et al. 1988) and *katE*, which encodes HPII (Mulvey et al. 1988). HPI is induced by H_2O_2 in an OxyR-dependent manner, while expression of HPII is dependent on a sigma factor, RpoS (Mukhopadhyay and Schellhorn 1994). The activities of both catalases increase as the growing cells enter stationary phase. In addition, the genome of *E. coli* includes the alkyl hydroperoxide reductase gene (*ahpCF*), which scavenges H_2O_2 and organic hydroperoxides (Ferrante et al. 1995; Smillie et al. 1992). *E. coli* possesses three distinct SOD genes: *sodA*, which encodes a cytosolic manganese-containing SOD (Touati 1983), *sodB*, which encodes a cytosolic iron-containing SOD (Sakamoto and Touati 1984) and *sodC*, which encodes a periplasmic copper and zinc-containing SOD (Imlay and Imlay 1996). SodC is induced in stationary phase and seems to be important to protect the cell from exogenous ROS attacks (Gort Amy et al. 2002).

Both the *katE katG* double mutant and *sodA sodB* double mutant are sensitive to H_2O_2 due to DNA damage (Imlay and Linn 1987; Ruiz-Laguna and Pueyo 1999). In addition, ROS levels significantly increased in *sodA sodB* double mutant by the addition of *p*-nonylphenol (Okai et al. 2004). Thus, it was suggested that one of the primary actions of *p*-nonylphenol in cells is the generation of superoxide.

In this study, we examined the involvement of catalase and SOD genes in organic solvent-tolerance in *E. coli*. We found that two BW25113-based mutants, the Δ*katE*Δ*katG* mutant and Δ*sodA*Δ*sodB* mutant,

were highly sensitive to organic solvents. In addition, it was shown that the increases in intracellular ROS levels in these two mutants were larger than that in strain BW25113 when these strains were treated with hydrophobic organic solvents. These results showed that catalase and SOD are implicated in the organic solvent tolerance in *E. coli*.

Materials and methods

Media, culture conditions and materials

E. coli strains were grown aerobically at 37 °C in LBGMg medium consisting of 1% tryptone, 0.5% yeast extract, 1% NaCl, 0.1% glucose, and 10 mM $MgSO_4$ (Aono et al. 1991). The LBGMg medium was solidified with 1.5% (wt/vol) agar. Ampicillin (50 μg/ml) or kanamycin (50 μg/ml) was added to the medium when necessary. Lysogeny broth (LB) agar medium was used for measuring colony-forming units (Neidhardt et al. 1974). M9 medium was used for ROS assay (Neidhardt et al. 1974). Growth of cells in liquid culture was monitored by measuring the optical density at 660 nm (OD_{660}). The *tert*-butyl hydroperoxide was obtained from FUJIFILM Wako Pure Chemical Industries (Osaka, Japan). The organic solvents used were of the highest quality available (FUJIFILM Wako Pure Chemical Industries). The $\log P_{ow}$ values of the hydrophobic solvents used in this study were as follows: cyclooctane ($\log P_{ow}$, 4.1), *n*-hexane ($\log P_{ow}$, 3.9) and cyclohexane ($\log P_{ow}$, 3.4).

Bacterial strains and plasmids

The *E. coli* strains and plasmids used in this study are summarized in Tables 1 and 2, respectively. Strain BW25113 and its single-gene knockout mutants were obtained from the National Bio-Resource Project (NIG, Mishima, Japan) (Baba et al. 2006). The plasmid pCP20 was also supplied by NIG. pMC1403 contains a sequence downstream of the 10th codon of *lacZ*, but it does not contain the *lacZ* promoter, the Shine-Dalgarno sequence, and the start codon (Casadaban et al. 1980). The plasmid pMW119 was purchased from Nippon Gene (Tokyo).

Construction of BW25113Δ*katE*Δ*katG* and BW25113Δ*sodA*Δ*sodB*

The Km^R cassettes in BW25113Δ*sodB* and BW25113Δ*katE* were eliminated with pCP20 (Cherepanov and Wackernagel 1995). Elimination of the Km^R cassette was confirmed by PCR analysis using chromosomal DNA. The combination of primers for BW25113Δ*sodB* was sodB-S and sodB-AS, and that for BW25113Δ*katE* was katE-S and katE-AS (Table 3). BW25113Δ*sodA*Δ*sodB* and BW25113Δ*katE*Δ*katG* were constructed from the Km^R cassette-eliminated mutants BW25113Δ*sodB* and BW25113Δ*katE* by P1 transduction

Table 1 *Escheria coli* strains used in this study

Strain	JW ID[a]	Relevant characteristics	References
BW25113		$lacI^q$ $rrnB_{T14}$ $lacZ_{WJ16}$ $hsdR514$ $araBAD_{AH33}$ $rhaBAD_{LD78}$	(Baba et al. 2006)
BW25113Δ*katE*	JW1721	Same as BW25113, but with $katE::Km^R$	(Baba et al. 2006)
BW25113Δ*katG*	JW3914	Same as BW25113, but with $katG::Km^R$	(Baba et al. 2006)
BW25113Δ*sodA*	JW3879	Same as BW25113, but with $sodA::Km^R$	(Baba et al. 2006)
BW25113Δ*sodB*	JW1648	Same as BW25113, but with $sodB::Km^R$	(Baba et al. 2006)
BW25113Δ*sodC*	JW1638	Same as BW25113, but with $sodC::Km^R$	(Baba et al. 2006)
BW25113Δ*ahpF*	JW0599	Same as BW25113, but with $ahpF::Km^R$	(Baba et al. 2006)
BW25113Δ*katE*Δ*katG*		Same as BW25113, but with Δ*katE* and Δ*katG*	This study
BW25113Δ*sodA*Δ*sodB*		Same as BW25113, but with Δ*sodA* and Δ*sodB*	This study
BW25113(pMW119)		BW25113 harboring pMW119	This study
BW25113Δ*katE*Δ*katG*(pMW119)		BW25113Δ*katE*Δ*katG* harboring pMW119	This study
BW25113Δ*katE*Δ*katG*(pMWkatE)		BW25113Δ*katE*Δ*katG* harboring pMWkatE	This study
BW25113Δ*katE*Δ*katG*(pMWkatG)		BW25113Δ*katE*Δ*katG* harboring pMWkatG	This study
BW25113Δ*katE*Δ*katG*(pMWkatEkatG)		BW25113Δ*katE*Δ*katG* harboring pMWkatEkatG	This study
BW25113Δ*sodA*Δ*sodB*(pMW119)		BW25113Δ*sodA*Δ*sodB* harboring pMW119	This study
BW25113Δ*sodA*Δ*sodB*(pMWsodA)		BW25113Δ*sodA*Δ*sodB* harboring pMWsodA	This study
BW25113Δ*sodA*Δ*sodB*(pMWsodB)		BW25113Δ*sodA*Δ*sodB* harboring pMWsodB	This study
BW25113Δ*sodA*Δ*sodB*(pMWsodAsodB)		BW25113Δ*sodA*Δ*sodB* harboring pMWsodAsodB	This study
BW25113(pMCkatEp)		BW25113 harboring pMCkatEp	This study
BW25113(pMCkatGp)		BW25113 harboring pMCkatGp	This study
BW25113(pMCsodAp)		BW25113 harboring pMCsodAp	This study
BW25113(pMCsodBp)		BW25113 harboring pMCsodBp	This study

[a] JW ID of the Keio Collection by the National Bio-Resource Project (NIG, Mishima, Japan): *E. coli* (Baba et al. 2006)

Table 2 Plasmids used in this study

Plasmids	Relevant characteristics	References
pCP20	pSC101-based vector expressing the Flp recombinase with $repA$(Ts), Amp^r, Cm^r	(Cherepanov and Wackernagel 1995)
pMW119	Expression vector with the replication origin of pSC101, Amp^r	Nippon Gene
pMWkatE	pMW119-based plasmid carrying *katE*	This study
pMWkatG	pMW119-based plasmid carrying *katG*	This study
pMWkatEkatG	pMW119-based plasmid carrying *katE* and *katG*	This study
pMWsodA	pMW119-based plasmid carrying *sodA*	This study
pMWsodB	pMW119-based plasmid carrying *sodB*	This study
pMWsodAsodB	pMW119-based plasmid carrying *sodA* and *sodB*	This study
pMC1403	Cloning vector for the *lacZ* reporter system with the replication origin of pMB1, Amp^r	(Casadaban et al. 1980)
pMCkatEp	pMC1403-based plasmid carrying the *katE* promoter region	This study
pMCkatGp	pMC1403-based plasmid carrying the *katG* promoter region	This study
pMCsodAp	pMC1403-based plasmid carrying the *sodA* promoter region	This study
pMCsodBp	pMC1403-based plasmid carrying the *soxB* promoter region	This study

of kanamycin-resistance with BW25113Δ*sodA* and BW25113Δ*katG* as the donor, respectively. The Km^R cassettes in BW25113Δ*sodA*Δ*sodB* and BW25113Δ*katE*Δ*katG* were also eliminated with pCP20. Elimination of the Km^R cassette in the *sodA* and *katG* region was confirmed by PCR analysis. The combination of primers for the *sodA* disruption was sodA-S and sodA-AS, and that for the Δ*katG* disruption was katG-S and katG-AS.

Measurement of organic solvent-tolerance in *E. coli*

Cultures of *E. coli* strains in LBGMg medium after 16 h of incubation (OD$_{660}$, 4 to 5) at 30 °C were diluted with

Table 3 Primers used in this study

Primer	Sequence (5′ to 3′)	Positions
katE-S	TACTCAGTCACTTCCCCTTC	426–445 bp upstream of the initiation codon of *katE*
katE-AS	AACTACGGCATTATCGAGGC	934–953 bp downstream of the stop codon of *katE*
katG-S	GGGGCAGATTAACGTTTCGT	1141–1160 bp upstream of the initiation codon of *katG*
katG-AS	GCCAGCACAATCAGCACAAT	924–943 bp downstream of the stop codon of *katG*
sodA-S	CGATGTTAGCGGCGACAATA	1277–1296 bp upstream of the initiation codon of *sodA*
sodA-AS	GCTCTGGCTTTGACTTTACG	1190–1209 bp downstream of the stop codon of *sodA*
sodB-S	TTCGATCACGCTCTGTGCTT	904–923 bp upstream of the initiation codon of *sodB*
sodB-AS	TTAACTATCCGTTGCTGGCG	1305–1324 bp downstream of the stop codon of *sodB*
katEc-S	AAA<u>GTCGAC</u>ATTTGCCACGCAGCATCCAG	311–330 bp upstream of the initiation codon of *katE*, SalI site underlined
katEc-AS	TTT<u>GGTACC</u>AGGCCGGATAAGGCGTTCAC	69–88 bp downstream of the stop codon of *katE*, KpnI site underlined
katGc-S	AAA<u>GGTACC</u>TTACGCGATTTGCCATACGC	352–371 bp upstream of the initiation codon of *katG*, KpnI site underlined
katGc-AS	TTT<u>GAGCTC</u>GTGTGTAGTTTTCGTTCGCC	63–82 bp downstream of the stop codon of *katG*, SacI site underlined
sodAc-S	AAA<u>GCATGC</u>TAAAAACAGGCTGCACTGGC	333–352 bp upstream of the initiation codon of *sodA*, SphI site underlined
sodAc-AS	TTT<u>GTCGAC</u>TTTTTTAAGCTGATATGCGGCC	32–53 bp downstream of the stop codon of *sodA*, SalI site underlined
sodBc-S	AAA<u>GTCGAC</u>CTCTCAGTGAAGACTACTGG	182–201 bp upstream of the initiation codon of *sodB*, SalI site underlined
sodBc-AS	TTT<u>GGATCC</u>TGCCTTATCCGACCTACATC	63–82 bp downstream of the stop codon of *sodB*, BamHI site underlined
katEp-S	AAA<u>GAATTC</u>AACCGGGAGGTATGTAATCC	446–466 bp upstream of the initiation codon of *katE*, EcoRI site underlined
katEp-AS	TTT<u>GGATCC</u>TGCTGATGTGGGTTCTTTTCG	15–35 bp downstream of the initiation codon of *katE*, BamHI site underlined
katGp-S	AAA<u>CCCGGG</u>CGAATATTGCCATGGGATATGG	439–459 bp upstream of the initiation codon of *katG*, SmaI site underlined
katGp-AS	TTT<u>GGATCC</u>GTGGTGTTATGGATATCGTCTG	11–32 bp downstream of the initiation codon of *katG*, BamHI site underlined
sodAp-S	AAA<u>GAATTC</u>GCCCAGAAATTCGGTAGTAAC	451–472 bp upstream of the initiation codon of *sodA*, EcoRI site underlined
sodAp-AS	TTT<u>GGATCC</u>TCCAGGGCATCGTAAGCATACG	26–47 bp downstream of the initiation codon of *sodA*, BamHI site underlined
sodBp-S	AAA<u>GAATTC</u>GTACCGGTTTTGATTGCAGC	434–453 bp upstream of the initiation codon of *sodB*, EcoRI site underlined
sodBp-AS	TTT<u>GGATCC</u>GCCAGAGCATCTTTAGCATAT	27–47 bp downstream of the initiation codon of *sodA*, BamHI site underlined

0.8% saline by serial tenfold dilutions. Five microliters of each suspension was spotted on LBGMg agar medium. The agar surface was overlaid with organic solvents (Tsukagoshi and Aono 2000). The formation of colonies on the agar was observed after 48 h of incubation at 25 °C.

Cloning of the *katE*, *katG*, *sodA* and *sodB* genes

The regions of *katE*, *katG*, *sodA* and *sodB* were amplified by PCR using AccuPrime Taq DNA Polymerase (Thermo Fisher Scientific Inc.) with high fidelity and BW25113 chromosomal DNA as the template. The primers used were designed according to the genome sequence of BW25113 deposited in GenBank (accession number CP009273). The combination of primers for *katE* was katEc-S and katEc-AS, that for *katG* was katGc-S and katGc-AS, that for *sodA* was sodAc-S and sodAc-AS, and that for *sodB* was sodBc-S and sodBc-AS (Table 3). A restriction endonuclease cleavage site was introduced into all primer sequences. The amplified fragments were digested with the relevant restriction enzymes and ligated into the cloning site of pMW119 under the same direction as the *lac* promoter to construct plasmids pMWkatE, pMWkatG, pMWsodA, and pMWsodB, respectively. The pMWkatE was digested with KpnI and

SacI, and then the fragments containing *katE* were ligated between KpnI and SacI sites of pMWkatG. The resulting plasmid was designated pMWkatEkatG. In addition, the pMWsodA was digested with SphI and SalI, and then the fragments containing *sodA* were ligated between SphI and SalI sites of pMWsodB. The resulting plasmid was designated pMWsodAsodB.

Enzyme activity assay

E. coli cells grown in LBGMg medium after incubation at 30 °C for 16 h were harvested by centrifugation ($4400 \times g$ for 10 min at 4 °C) and suspended in 10 mM of Tris–HCl buffer (pH 8.0). The cell suspension was sonicated on ice and centrifuged ($10,000 \times g$, 10 min at 4 °C). The supernatant was used for the enzyme activity assay.

Catalase activity was determined by following the rate of H_2O_2 consumption at 240 nm (Claiborne et al. 1979). The enzyme activity was calculated from the molar adsorption coefficient of the H_2O_2 ($\varepsilon = 43.6$ M^{-1} cm^{-1}). The reaction mixture (1 mL) contained 50 mM potassium phosphate buffer at pH 7.0 and 10 mM H_2O_2. The reaction was initiated by adding the enzyme solution (50 μL) to the reaction mixture and the initial velocity of H_2O_2 disappearance was measured at 30 °C. One unit

of enzyme activity was defined as the amount of enzyme that decomposes 1 μmol of H_2O_2 per min.

SOD activity was measured by following the rate of pyrogallol autoxidation (Marklund and Marklund 1974). The enzyme solution (10 μL) was mixed with 2.48 mL of 50 mM Tris–HCl and 1 mM EDTA at pH 8.2. After pre-incubation for 5 min at 25 °C, the reaction was started by adding 10 μL of 50 mM pyrogallol solution in 10 mM HCl. The change of absorbance was monitored at 325 nm. One unit of enzyme is defined as the amount of enzyme that inhibits the autoxidation rate of pyrogallol by 50%.

Protein content

The protein concentration was determined by the method of Bradford (Bradford 1976) using bovine serum albumin as the standard.

Sensitivities of *E. coli* strains to H_2O_2 and menadione

E. coli cells grown in LBGMg medium (10 mL) at 37 °C to an OD_{660} of about 0.6 (approximately 4 to 5×10^8 cells/ml) were harvested by centrifugation (4400×g for 10 min at 4 °C), and suspended in PBS buffer (10 mL) consisting of 140 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, and 1.5 mM KH_2PO_4 (pH 7.4). The cell suspension was washed once by centrifugation, resuspended in PBS buffer with H_2O_2 (1 to 4 mM) or menadione (15 to 60 mM), and further incubated with shaking at 37 °C. After incubation for 1 h, each suspension was plated on LB agar medium. The number of colonies formed on the agar plate was counted after 24 h of incubation at 30 °C.

Detection of reactive oxygen species

The ROS were detected with the 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA) (Molecular Probes, Eugene, OR) as reported previously with a slight modification (Rutherford et al. 2010). Carboxy-H_2DCFDA is a cell-permeable indicator for ROS that does not fluoresce until it is hydrolyzed by esterases and oxidation occurs within cells. A 100 μl culture of overnight-grown *E. coli* cells was inoculated onto 10 ml of fresh LBGMg medium and incubated at 37 °C with shaking. After incubation for 3 h, 0.5 mL of 7.78 M *tert*-butyl hydroperoxide (TBHP; used as a positive control for oxidative stress) or 1 mL of an organic solvent (*n*-hexane or cyclohexane) was added to the culture. The culture was further incubated at 37 °C for 3 h with shaking. Twenty microliters of each culture was added to 1 ml of M9 medium. After incubation at 37 °C for 45 min, 50 μl of 25 mM carboxy-H_2DCFDA samples was added to the medium. After incubation at 37 °C for 10 min, the OD_{660} and the fluorescence excitation/emission at 485/535 nm of each sample were measured by spectro-fluorometer (RF-6000; Shimadzu Co., Kyoto, Japan).

Specific fluorescence was calculated as fluorescence/ OD_{660}.

Construction of the *lacZ* reporter fusions

The promoter regions of *katE*, *katG*, *sodA* and *sodB* were amplified by PCR using AccuPrime Taq DNA Polymerase and BW25113 chromosomal DNA as the template. The combination of primers for the *katE* promoter region was katEp-S and katEp-AS, that for the *katG* promoter region was katGp-S and katGp-AS, that for the *sodA* promoter region was sodAp-S and sodAp-AS, and that for the *sodB* promoter region was sodBp-S and sodBp-AS (Table 3). The amplified fragments were digested with the relevant restriction enzymes and ligated into the cloning site of pMC1403 to construct plasmids pMCkatEp, pMCkatGp, pMCsodAp, and pMCsodBp, respectively.

Assay for plasmid-borne β-galactosidase activity

E. coli strains were grown in LBGMg containing 50 μg/ ml ampicillin at 37 °C. Cells in the exponential phase of growth were treated with a small volume of chloroform and assayed for β-galactosidase activity as described previously (Miller 1972).

Results

Organic solvent-tolerances of *E. coli* mutants deficient in ROS-scavenging enzymes

The colony-forming efficiency of the BW25113-based Δ*katE*, Δ*katG*, Δ*ahpF*, Δ*sodA*, Δ*sodB*, Δ*sodC*, Δ*katE*Δ*katG* and Δ*sodA*Δ*sodB* mutants was investigated using an LBGMg agar plate in the presence of *n*-hexane (Fig. 1). All strains formed colonies in all spots on the plate without any solvent. The colony-forming
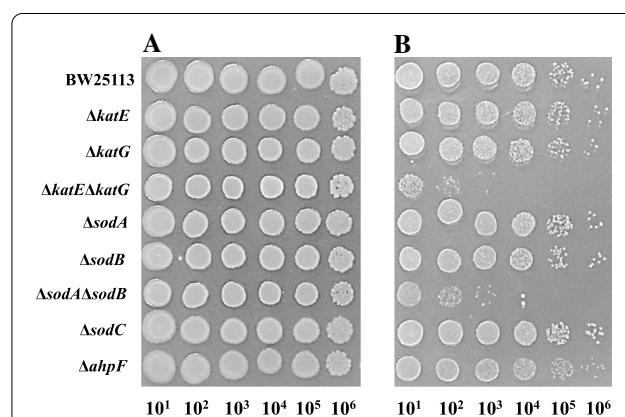

Fig. 1 Colony-forming efficiency of *E. coli* BW25113 and its mutants deficient in ROS-scavenging enzymes on LBGMg agar medium. Each strain was grown in the absence of an organic solvent (**A**) and in the presence of *n*-hexane (**B**). Each strain was spotted at a tenfold dilution and incubated at 25 °C for 48 h

efficiencies in the single gene mutants were similar to that in the parent strain BW25113 in the presence of *n*-hexane. In contrast, the double gene mutants, BW25113Δ*katE*Δ*katG* and BW25113Δ*sodA*Δ*sodB*, were highly sensitive to *n*-hexane, exhibiting 10^2- or 10^3-fold lower colony-forming efficiencies than the parent strain in the presence of the solvent. These results indicated that catalase and SOD were involved in the maintenance of organic solvent-tolerance in *E. coli*.

Complementation of organic solvent-tolerances of BW25 113Δ*katE*Δ*katG* and BW25113Δ*sodA*Δ*sodB* by transformation of the catalase- or SOD-coding gene was investigated (Fig. 2). The colony-forming efficiencies in BW25113Δ*katE*Δ*ka tG*(pMWkatE), BW25113Δ*katE*Δ*katG*(pMWkatG), and BW25113Δ*katE*Δ*katG*(pMWkatEkatG) were about 10-, 10^2-, and 10^3-fold higher than that in BW25113(pMW119) in the presence of *n*-hexane, respectively. On the other hand, the colony-forming efficiencies in BW25113Δ*sodA*Δ*sodB*(pMWsodA) and BW25113Δ*sodA*Δ*sodB*(pMW sodB) were both about tenfold higher than that in BW25 113(pMW119). In addition, the efficiency in BW25113 Δ*sodA*Δ*sodB*(pMWsodAsodB) was about 10^3-fold higher than that in BW25113(pMW119). Thus, it was shown that *katE*, *katG*, *sodA*, and *sodB* genes contribute to the maintenance of *n*-hexane-tolerance in *E. coli*.

Activities of ROS-scavenging enzymes and susceptibility to H_2O_2 and menadione in BW25113Δ*katE*Δ*katG* and BW25113Δ*sodA*Δ*sodB*

The levels of catalase and SOD activities in BW25113Δ*katE*Δ*katG* and BW25113Δ*sodA*Δ*sodB* were compared with the parent strain BW25113 (Table 4). Most of the catalase and SOD activities were eliminated in BW25113Δ*katE*Δ*katG* and BW25113Δ*sodA*Δ*sodB*,

Table 4 Catalase and SOD activities of *E. coli* strains

Strain	Enzymatic activity	
	Catalase (U/mg)	SOD (U/mg)
BW25113	1.41 ± 0.07	0.18 ± 0.01
BW25113Δ*katE*Δ*katG*	0.06 ± 0.07	0.20 ± 0.03
BW25113Δ*sodA*Δ*sodB*	1.55 ± 0.15	0.01 ± 0.01

Data represent the mean ± SD of triplicate experiments

respectively. The slight remaining activities of catalase and SOD seemed to be attributable to AhpCF and SodC, respectively. These results showed good agreement with a previous report (Alhama et al. 1998).

Susceptibility of the mutants to ROS was also confirmed by measuring the cell viability after exposure to H_2O_2 and a redox-cycling agent, menadione (Additional file 1: Fig. S1). The survival fraction of BW25113Δ*katE*Δ*katG* in 4 mM H_2O_2 and BW25113Δ*sodA*Δ*sodB* in 60 mM menadione were 40% and 0.3% of those of the parent strain BW25113, respectively.

Growth of the *E. coli* mutants in liquid medium in the presence of organic solvents

The cell growth of BW25113, BW25113Δ*katE*Δ*katG* and BW25113Δ*sodA*Δ*sodB* in the LBGMg liquid medium in the presence of a hydrophobic solvent including cyclooctane, *n*-hexane, or a hydrophobic solvent mixture of *n*-hexane and cyclohexane (9:1 vol/vol) was examined by measuring the turbidity (Fig. 3). In the absence of the solvent, the specific growth rates of BW25113, BW25113Δ*katE*Δ*katG* and BW25113Δ*sodA*Δ*sodB* during the exponential growth phase were 1.6 h^{-1}, 1.5 h^{-1} and 1.3 h^{-1}, respectively. In the presence of cyclooctane, the growth rates of BW25113, BW25113Δ*katE*Δ*katG* and BW25113Δ*sodA*Δ*sodB* were 1.5 h^{-1}, 1.2 h^{-1} and 1.0 h^{-1}, respectively. Thus, the growth of these mutants without any organic solvents and in the presence of cyclooctane was slower than that of the parent strain BW25113. The growth of BW25113Δ*katE*Δ*katG* and BW25113Δ*sodA*Δ*sodB* was highly suppressed compared to that of BW25113 in the presence of *n*-hexane or the mixture of *n*-hexane and cyclohexane. These results suggested that each of catalase and SOD is involved in the maintenance of tolerance to hydrophobic organic solvents. BW25113Δ*sodA*Δ*sodB* exhibited an extended lag phase and BW25113Δ*katE*Δ*katG* did not grow during 8 h cultivation in the presence of *n*-hexane. Thus, BW25113Δ*katE*Δ*katG* was more sensitive to *n*-hexane than BW25113Δ*sodA*Δ*sodB*.
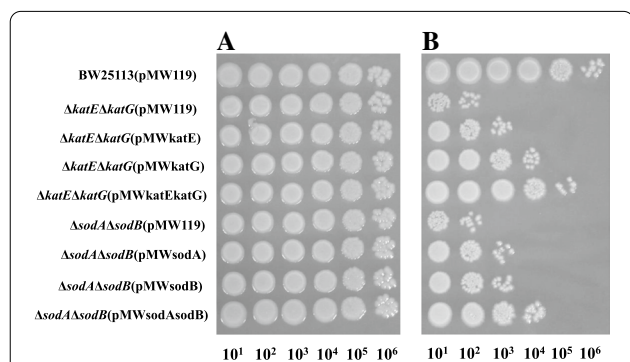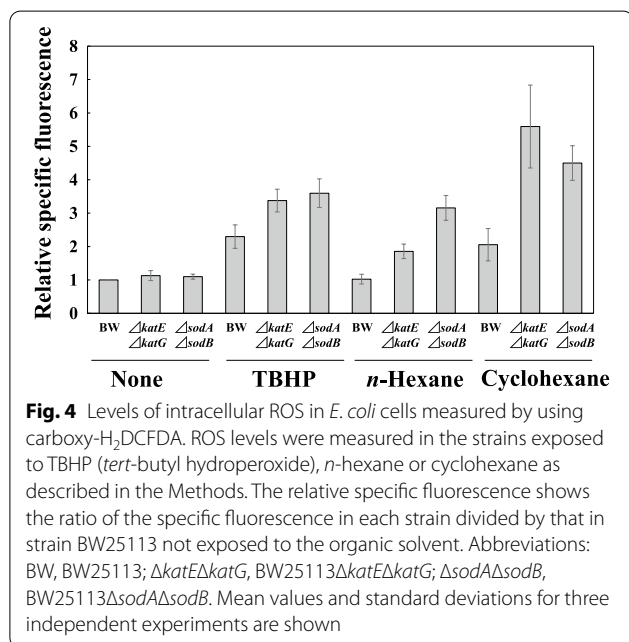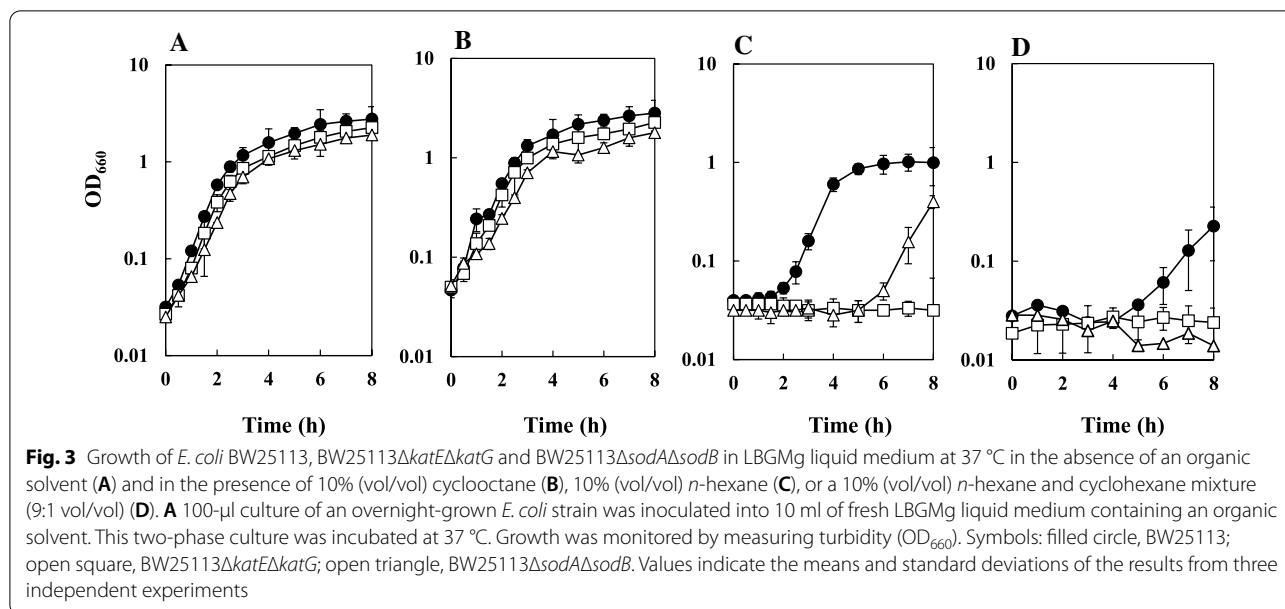

Fig. 2 Colony-forming efficiency of BW25113-based recombinant *E. coli* strains. Each strain was grown on LBGMg agar medium containing ampicillin (50 μg/ml) and isopropyl-β-D-thiogalactopyra noside (IPTG; 0.5 mM) in the absence of an organic solvent (**A**) and in the presence of *n*-hexane (**B**). Each strain was spotted at a tenfold dilution and incubated at 25 °C for 48 h

Fig. 3 Growth of *E. coli* BW25113, BW25113Δ*katE*Δ*katG* and BW25113Δ*sodA*Δ*sodB* in LBGMg liquid medium at 37 ℃ in the absence of an organic solvent (**A**) and in the presence of 10% (vol/vol) cyclooctane (**B**), 10% (vol/vol) *n*-hexane (**C**), or a 10% (vol/vol) *n*-hexane and cyclohexane mixture (9:1 vol/vol) (**D**). A 100-μl culture of an overnight-grown *E. coli* strain was inoculated into 10 ml of fresh LBGMg liquid medium containing an organic solvent. This two-phase culture was incubated at 37 ℃. Growth was monitored by measuring turbidity (OD$_{660}$). Symbols: filled circle, BW25113; open square, BW25113Δ*katE*Δ*katG*; open triangle, BW25113Δ*sodA*Δ*sodB*. Values indicate the means and standard deviations of the results from three independent experiments

Fig. 4 Levels of intracellular ROS in *E. coli* cells measured by using carboxy-H$_2$DCFDA. ROS levels were measured in the strains exposed to TBHP (*tert*-butyl hydroperoxide), *n*-hexane or cyclohexane as described in the Methods. The relative specific fluorescence shows the ratio of the specific fluorescence in each strain divided by that in strain BW25113 not exposed to the organic solvent. Abbreviations: BW, BW25113; Δ*katE*Δ*katG*, BW25113Δ*katE*Δ*katG*; Δ*sodA*Δ*sodB*, BW25113Δ*sodA*Δ*sodB*. Mean values and standard deviations for three independent experiments are shown

Detection of ROS in *E. coli* cells exposed to hydrophobic organic solvents

We examined the ROS levels in BW25113, BW25113Δ*katE*Δ*katG* and BW25113Δ*sodA*Δ*sodB* in the presence of *n*-hexane or cyclohexane with carboxy-H$_2$DCFDA, a fluorescent indicator for ROS in cells (Fig. 4). No significant difference in ROS levels was observed among these three strains in the absence of TBHP (a known inducer for ROS) or organic solvents. Addition of TBHP enhanced the ROS levels in these three strains. Treatment with *n*-hexane had little impact on ROS levels in BW25113 but markedly elevated ROS levels in BW25113Δ*katE*Δ*katG* and BW25113Δ*sodA*Δ*sodB*. The ROS levels by exposure to *n*-hexane in BW25113Δ*katE*Δ*katG* and BW25113Δ*sodA*Δ*sodB* were 1.9- and 3.2-fold higher than that in BW25113, respectively. Cyclohexane-exposure significantly increased ROS levels in all strains. The ROS levels by exposure to cyclohexane in BW25113Δ*katE*Δ*katG* and BW25113Δ*sodA*Δ*sodB* were 2.8- and 2.3-fold higher than that in BW25113, respectively. The ROS levels in the three strains by the addition of cyclohexane were higher than those by addition of *n*-hexane, respectively.

Induction of *katE*, *katG*, *sodA* and *sodB* by *n*-hexane

We constructed plasmids pMCkatEp, pMCkatGp, pMC-sodAp and pMCsodBp containing *katE–lacZ*, *katG–lacZ*, *sodA–lacZ*, and *sodB–lacZ*-fused genes, respectively. BW25113 harboring one of the plasmids was assayed for plasmid-borne β-galactosidase activity (Fig. 5). Promoter activities of *katE*, *katG*, *soda*, and *sodB* with *n*-hexane were 1.2-, 1.3-, 3.9-, and 2.6-fold compared to those without any solvent, respectively. In particular, the promoter activities of *sodA* and *sodB* were significantly increased by exposure to *n*-hexane.

Discussion

The cell membrane is the main target of organic solvents (Aono et al. 1994). The cytoplasmic membrane of bacterial cells plays a crucial role in various cell functions such as regulation of solutes (nutrients and ions) passage, energy generation, synthesis of membrane lipids and cell
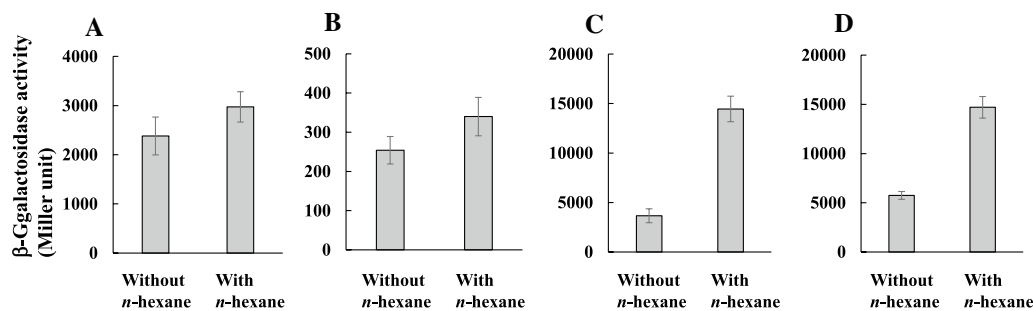
Fig. 5 Effect of *n*-hexane on the promoter activity of *katE, katG, sodA,* or *sodB*. **A** 100-μl culture of the overnight-grown strain BW25113(pMCkatEp) (**A**), BW25113(pMCkatGp) (**B**), BW25113(pMCsodAp) (**C**), or BW25113(pMCsodBp) (**D**) was inoculated into 10 ml of fresh LBGMg liquid medium containing 50 μg/ml ampicillin without or with 1 ml of *n*-hexane. The culture was incubated at 37 ℃ until reaching an OD_{600} of approximately 0.6. Cells were treated with chloroform and assayed for β-galactosidase activity. Values indicate the means and standard deviations of the results from three independent experiments

wall, secretion of extracytoplasmic proteins, and turgor pressure. Organic solvent molecules intercalate into the lipid bilayer of the membrane. Accumulation of organic solvent in the membrane disturbs a variety of vital cell processes, including those described above. The inner membrane in *E. coli* contains an electron transport chain that is the major site of the premature electron leakage to oxygen that generates superoxide (O_2^-). Disturbance of the electron transport chain by organic solvent can increase the level of ROS. ROS have the capacity to damage various biomolecules, including proteins, ribosomes and DNA, and to reduce cellular culturability (Imlay 2003).

In the present study, we investigated the involvement of ROS-scavenging enzymes in hydrophobic organic solvent-tolerance. The colony-forming efficiency of *E. coli* strains on LBGMg agar showed that the tolerances to *n*-hexane in single gene-knockout mutants lacking the *katE, katG, ahpF, sodA, sodB,* or *sodC* gene were similar to that in the parent strain BW25113 (Fig. 1). AhpF is the peroxiredoxin reductase component of alkyl hydroperoxide reductase (AhpCF) that has been reported to be involved in tolerance to organic solvents such as 1,2,3,4-tetrahydronaphthalene (tetralin), cyclohexane, propylbenzene, and 1,2-dihydronaphthalene (Ferrante et al. 1995). AhpF and AhpC proteins act together (Li Calzi and Poole 1997). AhpF utilizes NADH or NADPH as electron donor to AhpC, which converts alkylhydroperoxides to their respective alcohol forms. AhpC is specifically reduced by AhpF and cannot be reduced by other electron transfer systems such as thioredoxin reductase. However, our results showed that deficiency of *ahpF* did not influence the tolerance to *n*-hexane. It has been reported that a *sodA sodB* double mutant was much more sensitive to paraquat than the wild type, although the absence of only the *sodA* gene or only the *sodB* gene

had no effect on the sensitivity to paraquat (a superoxide generator) (Carlioz and Touati 1986). Both the *sodA sodB* double mutant and *katE katG* double mutant were more sensitive to *p*-nonylphenol (an endocrine disruptor) than the parent strain (Okai et al. 2004). In particular, the *sodA sodB* double mutant was highly sensitive to *p*-nonylphenol. These findings prompted us to construct BW25113Δ*katE*Δ*katG* and BW25113Δ*sodA*Δ*sodB* and then examine their organic solvent-tolerances. BW25113Δ*katE*Δ*katG* and BW25113Δ*sodA*Δ*sodB* lost most of their catalase and SOD activities, respectively (Table 4). In addition, we confirmed that BW25113Δ*katE*Δ*katG* and BW25113Δ*sodA*Δ*sodB* became sensitive to H_2O_2 and menadione (an O_2^- generator), respectively (Additional file 1: Fig. S1). Both BW25113Δ*katE*Δ*katG* and BW25113Δ*sodA*Δ*sodB* were highly sensitive to *n*-hexane and a mixture of *n*-hexane and cyclohexane (Figs. 1 and 3). These results showed that accumulation of either H_2O_2 or O_2^- in *E. coli* can exhibit an inhibitory effect on the cell growth. H_2O_2 and O_2^- are relatively weak cytotoxic radical oxygens compared to other radical oxygens such as hydroxyl radicals (Bruno-Barcena et al. 2010; Fridovich 1986). Therefore, the hydroxyl radical produced via Fenton reaction and Haber–Weiss reaction from H_2O_2 and O_2^- might be a main cause of the cytotoxicity by addition of hydrophobic organic solvents. Our assays using the fluorescent probe carboxy-H_2DCFDA showed an increase in ROS after solvent stress (Fig. 4). In addition, the ROS levels in *E. coli* cells induced by exposure to cyclohexane were higher than those observed by exposure to *n*-hexane. Thus, the ROS level in *E. coli* cells is likely to depend on the amount of organic solvents accumulated in the cells, since a larger amount of cyclohexane than *n*-hexane is accumulated in cells in an organic-aqueous two-liquid-phase system (Tsukagoshi and Aono 2000).

We found that the promoter activities of *sodA* and *sodB* were significantly increased by *n*-hexane (Fig. 5). Expression of SodA is regulated by several global transcription regulators, including the MarA/SoxS/Rob system and Fur (Ferric uptake regulator), and responds to changes in oxygen concentration, redox active compounds, and iron concentration (Fee 1991; Semsey 2014). SodB levels were relatively insensitive to changes in these conditions. SodB seems to be responsible for protection of a cytoplasmic superoxide-sensitive enzyme, while SodA is more effective in preventing DNA damage (Hopkin et al. 1992). Oxidative stress response genes in bacteria are often upregulated during exposure to solvents. The *sodA* gene in *E. coli* strains was upregulated by ethanol- or *n*-butanol-induced stress (Cao et al. 2017; Rutherford et al. 2010). Antioxidant enzymes such as catalase and superoxide dismutase in *Pseudomonas putida* showed increased activity upon exposure to toluene (Choi et al. 2014).

In this study, we showed that ROS-scavenging enzymes significantly contributed to the maintenance of tolerance to hydrophobic organic solvents in *E. coli*. Various mechanisms of organic solvent-tolerance in *E. coli* have been reported so far. These include the multidrug efflux pump (Tsukagoshi and Aono 2000; Watanabe and Doukyu 2012, 2014), maintenance of the proton motive force (Kobayashi et al. 1998), lipopolysaccharides (Abe et al. 2003), fatty acids synthesis (Oh et al. 2012), metabolic pathway for carbon catabolism (Shimizu et al. 2005), reduction of alkylhydroperoxide (Ferrante et al. 1995) and osmoprotectant transport (Doukyu et al. 2012). However, the involvement of catalase and SOD in hydrophobic organic solvent tolerance in *E. coli* has not been reported so far. Thus, the present study provides valuable new knowledge of the organic solvent-tolerance mechanisms in *E. coli*.

Abbreviations
ROS: Reactive oxygen species; SOD: Superoxide dismutase; HP: Hydroperoxidase; Carboxy-H_2DCFDA: 5-(And-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate; Km: Kanamycin; EDTA: Ethylenediaminetetraacetic acid; TBHP: *tert*-Butyl hydroperoxide.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s13568-021-01258-w.

Additional file 1: Figure S1. Effects of H_2O_2 and menadione on the cell viability of *E. coli* BW25113 and its mutants deficient in ROS-scavenging enzymes. Each strain was exposed to H_2O_2 (A) and menadione (B). After incubation with H_2O_2 and menadione for 1 h, viable cells were measured by examining the formation of colonies on LB agar medium. The survival fraction was calculated as the number of colonies treated with H_2O_2 or menadione divided by that of untreated cells. Symbols: filled circles, BW25113; open squares, BW25113Δ*katE*Δ*katG*; open triangles,

BW25113Δ*sodA*Δ*sodB*. Values indicate the means and standard deviations of the results from three independent experiments.

Availability of data and materials
All discussed data have been included into the manuscript or in the Additional file 1. Please turn to the corresponding author for all other requests.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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References
Abe S, Okutsu T, Nakajima H, Kakuda N, Ohtsu I, Aono R (2003) *n*-Hexane sensitivity of *Escherichia coli* due to low expression of *imp/ostA* encoding an 87 kDa minor protein associated with the outer membrane. Microbiology 149(Pt 5):1265–1273. https://doi.org/10.1099/mic.0.25927-0

Akhtar MK, Dandapani H, Thiel K, Jones PR (2015) Microbial production of 1-octanol: a naturally excreted biofuel with diesel-like properties. Metab Eng Commun 2:1–5. https://doi.org/10.1016/j.meteno.2014.11.001

Alhama J, Ruiz-Laguna J, Rodriguez-Ariza A, Toribio F, Lopez-Barea J, Pueyo C (1998) Formation of 8-oxoguanine in cellular DNA of *Escherichia coli* strains defective in different antioxidant defences. Mutagenesis 13(6):589–594. https://doi.org/10.1093/mutage/13.6.589

Aono R (1998) Improvement of organic solvent tolerance level of *Escherichia coli* by overexpression of stress-responsive genes. Extremophiles 2(3):239–248. https://doi.org/10.1007/s007920050066

Aono R, Aibe K, Inoue A, Horikoshi K (1991) Preparation of organic solvent-tolerant mutants from *Escherichia coli* K-12. Agric Biol Chem 55(7):1935–1938. https://doi.org/10.1080/00021369.1991.10870883

Aono R, Kobayashi H, Joblin KN, Horikoshi K (1994) Effects of organic solvents on growth of *Escherichia coli* K-12. Biosci Biotechnol Biochem 58(11):2009–2014. https://doi.org/10.1271/bbb.58.2009

Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, Mori H (2006) Construction of *Escherichia coli* K-12 in-frame,

single-gene knockout mutants: the Keio collection. Mol Syst Biol 2(2006):0008. https://doi.org/10.1038/msb4100050

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochemi 72(1):248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Bruno-Barcena JM, Azcarate-Peril MA, Hassan HM (2010) Role of antioxidant enzymes in bacterial resistance to organic acids. Appl Environ Microbiol 76(9):2747–2753. https://doi.org/10.1128/aem.02718-09

Cao H, Wei D, Yang Y, Shang Y, Li G, Zhou Y, Ma Q, Xu Y (2017) Systems-level understanding of ethanol-induced stresses and adaptation in *E. coli*. Sci Rep 7:44150. https://doi.org/10.1038/srep44150

Carlioz A, Touati D (1986) Isolation of superoxide dismutase mutants in *Escherichia coli*: is superoxide dismutase necessary for aerobic life? EMBO J 5(3):623–630. https://doi.org/10.1002/j.1460-2075.1986.tb04256.x

Casadaban MJ, Chou J, Cohen SN (1980) In vitro gene fusions that join an enzymatically active beta-galactosidase segment to amino-terminal fragments of exogenous proteins: *Escherichia coli* plasmid vectors for the detection and cloning of translational initiation signals. J Bacteriol 143(2):971. https://doi.org/10.1128/JB.143.2.971-980.1980

Cherepanov PP, Wackernagel W (1995) Gene disruption in *Escherichia coli*: Tc^R and Km^R cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. Gene 158(1):9–14. https://doi.org/10.1016/0378-1119(95)00193-A

Chin W-C, Lin K-H, Chang J-J, Huang C-C (2013) Improvement of *n*-butanol tolerance in *Escherichia coli* by membrane-targeted tilapia metallothionein. Biotechnol Biofuels 6(1):130. https://doi.org/10.1186/1754-6834-6-130

Choi HJ, Yoo J-S, Jeong YK, Joo WH (2014) Involvement of antioxidant defense system in solvent tolerance of Pseudomonas putida BCNU 106. J Basic Microbiol 54(9):945–950. https://doi.org/10.1002/jobm.201300176

Claiborne A, Malinowski DP, Fridovich I (1979) Purification and characterization of hydroperoxidase II of *Escherichia coli* B. J Biol Chem 254(22):11664–11668. https://doi.org/10.1016/S0021-9258(19)86535-3

Doukyu N, Toyoda K, Aono R (2003) Indigo production by *Escherichia coli* carrying the phenol hydroxylase gene from Acinetobacter sp. strain ST-550 in a water–organic solvent two-phase system. Appl Microbiol Biotechnol 60(6):720–725. https://doi.org/10.1007/s00253-002-1187-1

Doukyu N, Ishikawa K, Watanabe R, Ogino H (2012) Improvement in organic solvent tolerance by double disruptions of *proV* and *marR* genes in *Escherichia coli*. J Appl Microbiol 112(3):464–474. https://doi.org/10.1111/j.1365-2672.2012.05236.x

Doukyu N, Iida S (2020) Production of styrene oxide from styrene by a recombinant *Escherichia coli* with enhanced AcrAB-TolC efflux pump level in an aqueous-organic solvent two-phase system. Biosci Biotechnol Biochem 84(7):1513–1520. https://doi.org/10.1080/09168451.2020.1755219

Fee JA (1991) Regulation of sod genes in *Escherichia coli*: relevance to superoxide dismutase function. Mol Microbiol 5(11):2599–2610. https://doi.org/10.1111/j.1365-2958.1991.tb01968.x

Ferrante AA, Augliera J, Lewis K, Klibanov AM (1995) Cloning of an organic solvent-resistance gene in *Escherichia coli*: the unexpected role of alkylhydroperoxide reductase. Proc Natl Acad Sci U S A 92(17):7617–7621. https://doi.org/10.1073/pnas.92.17.7617

Fridovich I (1986) Biological effects of the superoxide radical. Arch Biochem Biophys 247(1):1–11. https://doi.org/10.1016/0003-9861(86)90526-6

Gort Amy S, Ferber Daniel M, Imlay James A (2002) The regulation and role of the periplasmic copper, zinc superoxide dismutase of *Escherichia coli*. Mol Microbiol 32(1):179–191. https://doi.org/10.1046/j.1365-2958.1999.01343.x

Griepernau B, Leis S, Schneider MF, Sikor M, Steppich D, Bockmann RA (2007) 1-Alkanols and membranes: a story of attraction. Biochim Biophys Acta 1768(11):2899–2913. https://doi.org/10.1016/j.bbamem.2007.08.002

Heipieper HJ, Neumann G, Cornelissen S, Meinhardt F (2007) Solvent-tolerant bacteria for biotransformations in two-phase fermentation systems. Appl Microbiol Biotechnol 74(5):961–973. https://doi.org/10.1007/s00253-006-0833-4

Hopkin KA, Papazian MA, Steinman HM (1992) Functional differences between manganese and iron superoxide dismutases in *Escherichia coli* K-12. J Biol Chem 267(34):24253–24258. https://doi.org/10.1016/S0021-9258(18)35758-2

Imlay JA (2003) Pathways of oxidative damage. Annu Rev Microbiol 57(1):395–418. https://doi.org/10.1146/annurev.micro.57.030502.090938

Imlay JA, Linn S (1987) Mutagenesis and stress responses induced in *Escherichia coli* by hydrogen peroxide. J Bacteriol 169(7):2967–2976. https://doi.org/10.1128/jb.169.7.2967-2976.1987

Imlay KR, Imlay JA (1996) Cloning and analysis of *sodC*, encoding the copper-zinc superoxide dismutase of *Escherichia coli*. J Bacteriol 178(9):2564–2571. https://doi.org/10.1128/jb.178.9.2564-2571.1996

Inoue A, Horikoshi K (1989) A *Pseudomonas* thrives in high concentrations of toluene. Nature 338:264. https://doi.org/10.1038/338264a0

Kabelitz N, Santos PM, Heipieper HJ (2003) Effect of aliphatic alcohols on growth and degree of saturation of membrane lipids in *Acinetobacter calcoaceticus*. FEMS Microbiol Lett 220(2):223–227. https://doi.org/10.1016/S0378-1097(03)00103-4

Kobayashi H, Yamamoto M, Aono R (1998) Appearance of a stress-response protein, phage-shock protein A, in *Escherichia coli* exposed to hydrophobic organic solvents. Microbiology 144(Pt 2):353–359. https://doi.org/10.1099/00221287-144-2-353

Lennen RM, Politz MG, Kruziki MA, Pfleger BF (2013) Identification of transport proteins involved in free fatty acid efflux in *Escherichia coli*. J Bacteriol 195(1):135–144. https://doi.org/10.1128/JB.01477-12

Li Calzi M, Poole LB (1997) Requirement for the two AhpF cystine disulfide centers in catalysis of peroxide reduction by alkyl hydroperoxide reductase. Biochemistry 36(43):13357–13364. https://doi.org/10.1021/bi9713660

Marklund S, Marklund G (1974) Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur J Biochem 47(3):469–474. https://doi.org/10.1111/j.1432-1033.1974.tb03714.x

Miller JH (1972) Experiments in molecular genetics. ColdSpring Harbor Laboratory, Cold Spring Harbor, NY; pp. 352–355.

Mukhopadhyay A (2015) Tolerance engineering in bacteria for the production of advanced biofuels and chemicals. Trends Microbiol 23(8):498–508. https://doi.org/10.1016/j.tim.2015.04.008

Mulvey MR, Sorby PA, Triggs-Raine BL, Loewen PC (1988) Cloning and physical characterization of *katE* and *katF* required for catalase HPII expression in *Escherichia coli*. Gene 73(2):337–345. https://doi.org/10.1016/0378-1119(88)90498-2

Mukhopadhyay S, Schellhorn HE (1994) Induction of *Escherichia coli* hydroperoxidase I by acetate and other weak acids. J Bacteriol 176(8):2300–2307. https://doi.org/10.1128/jb.176.8.2300-2307.1994

Neidhardt FC, Bloch PL, Smith DF (1974) Culture medium for enterobacteria. J Bacteriol 119(3):736–747. https://doi.org/10.1128/JB.119.3.736-747.1974

Oh HY, Lee JO, Kim OB (2012) Increase of organic solvent tolerance of *Escherichia coli* by the deletion of two regulator genes, *fadR* and *marR*. Appl Microbiol Biotechnol 96(6):1619–1627. https://doi.org/10.1007/s00253-012-4463-8

Okai Y, Sato EF, Higashi-Okai K, Inoue M (2004) Effect of endocrine disruptor para-nonylphenol on the cell growth and oxygen radical generation in *Escherichia coli* mutant cells deficient in catalase and superoxide dismutase. Free Radic Biol Med 37(9):1412–1418. https://doi.org/10.1016/j.freeradbiomed.2004.07.001

Ramos JL, Duque E, Gallegos MT, Godoy P, Ramos-Gonzalez MI, Rojas A, Teran W, Segura A (2002) Mechanisms of solvent tolerance in gram-negative bacteria. Annu Rev Microbiol 56:743–768. https://doi.org/10.1146/annurev.micro.56.012302.161038

Ruiz-Laguna J, Pueyo C (1999) Hydrogen peroxide and coffee induce G:C→T: a transversions in the lacI gene of catalase-defective *Escherichia coli*. Mutagenesis 14:95–102. https://doi.org/10.1093/mutage/14.1.95

Rutherford BJ, Dahl RH, Price RE, Szmidt HL, Benke PI, Mukhopadhyay A, Keasling JD (2010) Functional genomic study of exogenous *n*-butanol stress in *Escherichia coli*. Appl Environ Microbiol 76(6):1935–1945. https://doi.org/10.1128/AEM.02323-09

Sakamoto H, Touati D (1984) Cloning of the iron superoxide dismutase gene (*sodB*) in *Escherichia coli* K-12. J Bacteriol 159(1):418–420. https://doi.org/10.1128/JB.159.1.418-420.1984

Semsey S (2014) A mixed incoherent feed-forward loop allows conditional regulation of response dynamics. PLoS ONE 9(3):e91243. https://doi.org/10.1371/journal.pone.0091243

Shimizu K, Hayashi S, Kako T, Suzuki M, Tsukagoshi N, Doukyu N, Kobayashi T, Honda H (2005) Discovery of *glpC*, an organic solvent tolerance-related gene in *Escherichia coli*, using gene expression profiles from DNA

microarrays. Appl Environ Microbiol 71(2):1093–1096. https://doi.org/10.1128/aem.71.2.1093-1096.2005

Smillie DA, Hayward RS, Suzuki T, Fujita N, Ishihama A (1992) Locations of genes encoding alkyl hydroperoxide reductase on the physical map of the *Escherichia coli* K-12 genome. J Bacteriol 174(11):3826–3827. https://doi.org/10.1128/jb.174.11.3826-3827.1992

Touati D (1983) Cloning and mapping of the manganese superoxide dis-mutase gene (*sodA*) of *Escherichia coli* K-12. J Bacteriol 155(3):1078–1087

Triggs-Raine BL, Doble BW, Mulvey MR, Sorby PA, Loewen PC (1988) Nucleo-tide sequence of katG, encoding catalase HPI of *Escherichia coli*. J Bacte-riol 170(9):4415–4419. https://doi.org/10.1128/jb.170.9.4415-4419.1988

Tsukagoshi N, Aono R (2000) Entry into and release of solvents by *Escherichia coli* in an organic-aqueous two-liquid-phase system and substrate

specificity of the AcrAB-TolC solvent-extruding pump. J Bacteriol 182(17):4803–4810. https://doi.org/10.1128/JB.182.17.4803-4810.2000

Watanabe R, Doukyu N (2012) Contributions of mutations in *acrR* and *marR* genes to organic solvent tolerance in *Escherichia coli*. AMB Express 2(1):58. https://doi.org/10.1186/2191-0855-2-58

Watanabe R, Doukyu N (2014) Improvement of organic solvent tolerance by disruption of the *lon* gene in *Escherichia coli*. J Biosci Bioeng 118(2):139–144. https://doi.org/10.1016/j.jbiosc.2014.01.011

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