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 (BW25113ΔkatEΔkatG) and *sodA* *sodB* double mutant (BW25113ΔsodAΔsodB). 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 BW25113ΔkatEΔkatG and BW25113ΔsodAΔsodB 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.
A ROS assay using a cell-permeant fluorescent dye showed a significant increase in intracellular ROS levels in \textit{n}-butanol-exposed \textit{E. coli} cells (Rutherford et al. 2010). In addition, an \textit{E. coli} strain expressing metallothioneins, which function in ROS scavenging, exhibited an increased growth rate under \textit{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 \textit{E. coli} strains have been studied with hydrophobic organic solvents such as \textit{n}-hexane and cyclohexane (Aono 1998). These findings are useful for improving the production of hydrophilic 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, \(\text{H}_2\text{O}_2\) 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. \textit{E. coli} harbors two different catalase genes: \textit{katG}, which encodes hydroperoxidase 1 (HPI) (Triggs-Raine et al. 1988) and \textit{katE}, which encodes HPII (Mulvey et al. 1988). HPI is induced by \(\text{H}_2\text{O}_2\) 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 \textit{E. coli} includes the alkyl hydroperoxide reductase gene (\textit{ahpCF}), which scavenges \(\text{H}_2\text{O}_2\) and organic hydroperoxides (Ferrante et al. 1995; Smillie et al. 1992). \textit{E. coli} possesses three distinct SOD genes: \textit{sodA}, which encodes a cytosolic manganese-containing SOD (Touati 1983), \textit{sodB}, which encodes a cytosolic iron-containing SOD (Sakamoto and Touati 1984) and \textit{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 \textit{katE}\textit{katG} double mutant and \textit{sodA}\textit{sodB} double mutant are sensitive to \(\text{H}_2\text{O}_2\) due to DNA damage (Imlay and Linn 1987; Ruiz-Laguna and Pueyo 1999). In addition, ROS levels significantly increased in \textit{sodA}\textit{sodB} double mutant by the addition of \textit{p}-nonylphenol (Okai et al. 2004). Thus, it was suggested that one of the primary actions of \textit{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 \textit{E. coli}. We found that two BW25113-based mutants, the \(\Delta\text{katE}\Delta\text{katG}\) mutant and \(\Delta\text{sodA}\Delta\text{sodB}\) 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 \textit{E. coli}.

Materials and methods

Media, culture conditions and materials

\textit{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 \(\mu\)g/ml) or kanamycin (50 \(\mu\)g/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 (\(\text{OD}_{660}\)). 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), \textit{n}-hexane (\(\log P_{ow}\), 3.9) and cyclohexane (\(\log P_{ow}\), 3.4).

Bacterial strains and plasmids

The \textit{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 BW25113\(\Delta\text{katE}\Delta\text{katG}\) and BW25113\(\Delta\text{sodA}\Delta\text{sodB}\)

The \(\text{Km}^R\) cassettes in BW25113\(\Delta\text{sodB}\) and BW25113\(\Delta\text{katE}\) were eliminated with pCP20 (Cherepanov and Wackernagel 1995). Elimination of the \(\text{Km}^R\) cassette was confirmed by PCR analysis using chromosomal DNA. The combination of primers for BW25113\(\Delta\text{sodB}\) was sodB-S and sodB-AS, and that for BW25113\(\Delta\text{katE}\) was katE-S and katE-AS (Table 3). BW25113\(\Delta\text{sodA}\Delta\text{sodB}\) and BW25113\(\Delta\text{katE}\Delta\text{katG}\) were constructed from the \(\text{Km}^R\) cassette-eliminated mutants BW25113\(\Delta\text{sodB}\) and BW25113\(\Delta\text{katE}\) by P1 transduction.
of kanamycin-resistance with BW25113ΔsodA and BW25113ΔkatG as the donor, respectively. The KmR cassettes in BW25113ΔsodAΔsodB and BW25113ΔkatEΔkatG were also eliminated with pCP20. Elimination of the KmR 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 ΔkatG 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 ºC were diluted with...
Table 3 Primers used in this study

| Primer   | Sequence (5’ to 3’) | Positions                      |
|----------|---------------------|--------------------------------|
| katE-S   | TACTCATGTCCTCCCCCTTC | 426–445 bp upstream of the initiation codon of katE |
| katE-AS  | AACTCCGCTATCCGAGGC   | 934–953 bp downstream of the stop codon of katE |
| katG-S   | GGGCGAGATTAAGGTTTGTG | 1141–1160 bp upstream of the initiation codon of katG |
| katG-AS  | GCCAGCACAATCAGCCAAAT | 924–943 bp downstream of the stop codon of katG |
| sodA-S   | CGATGTTAGCGGCAGCAATA | 1277–1296 bp upstream of the initiation codon of sodA |
| sodA-AS  | GCTCCTGCTTATGCTTACG  | 1190–1209 bp downstream of the stop codon of sodA |
| sodB-S   | TTCCATACGCGGCTTGCCTT | 904–923 bp upstream of the initiation codon of sodB |
| sodB-AS  | TTAATCTACGTTGCTGCGG | 1305–1324 bp downstream of the stop codon of sodB |
| katEc-S  | AAAGTCGACATTTCACGCG | 311–330 bp upstream of the initiation codon of katE, Sall site underlined |
| katEc-AS | TTTGACAGGGCGGATACGAG | 69–88 bp downstream of the stop codon of katE, Kpnl site underlined |
| katGc-S  | AAAGTACACTCGAGGTTTGTG | 352–371 bp upstream of the initiation codon of katG, Kpnl site underlined |
| katGc-AS | TTTGACGCTGTTGGTTTTCTTGCAG | 63–82 bp downstream of the stop codon of katG, Sall site underlined |
| sodAc-S  | AAAGCAGTTTAAAACACGCGTGACTGGG | 333–352 bp upstream of the initiation codon of sodA, SpfI site underlined |
| sodAc-AS | TTTGACGCTTTTTTACAGTATGCGGCGG | 32–53 bp downstream of the stop codon of sodA, Sall site underlined |
| sodBc-S  | AAATCGAAGCGTTCAGGGCAGAT | 182–201 bp upstream of the initiation codon of sodB, Sall site underlined |
| sodBc-AS | TTTGACGCTTCCTTACGTGATGAAAGTACTTGG | 63–82 bp downstream of the stop codon of sodB, BamHl site underlined |
| katEp-S  | AAAGAATTGAACCGGGAGGTATGGTATC | 446–466 bp upstream of the initiation codon of katE, EcoRI site underlined |
| katEp-AS | TTTGACGCTGCTGATGTTGTTGGTCTTTC | 15–35 bp downstream of the stop codon of katE, BamHl site underlined |
| katGp-S  | AAAAAACCCGGCGAATATTGGCAGGATTGAT | 439–459 bp upstream of the initiation codon of katG, Smal site underlined |
| katGp-AS | TTTGACGCTGCTGATGTTGTTGGTCTTTC | 11–32 bp downstream of the stop codon of katG, BamHl site underlined |
| sodAp-S  | AAAGAATTGAACCGGGAGGTATGGTATC | 451–472 bp upstream of the initiation codon of sodA, EcoRI site underlined |
| sodAp-AS | TTTGACGCTTCCTCAGGGCGTCAAGTACAG | 26–47 bp downstream of the initiation codon of sodA, BamHl site underlined |
| sodBp-S  | AAAGAATTGAACCGGGAGGTATGGTATC | 434–453 bp upstream of the initiation codon of sodB, EcoRI site underlined |
| sodBp-AS | TTTGACGCTTCCTCAGGGCGTCAAGTACAG | 27–47 bp downstream of the stop codon of sodB, BamHl 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 (Tsuchikoshi 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 Kpnl and SacI, and then the fragments containing katE were ligated between Kpnl and SacI sites of pMWkatG. The resulting plasmid was designated pMWkatEkatG. In addition, the pMWsodA was digested with SpfI and Sall, and then the fragments containing sodA were ligated between SpfI and Sall 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 (4000×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×g, 10 min at 4 °C). The supernatant was used for the enzyme activity assay.

Catalase activity was determined by following the rate of H2O2 consumption at 240 nm (Claiiborne et al. 1979). The enzyme activity was calculated from the molar adsorption coefficient of the H2O2 (ε = 43.6 M−1 cm−1). The reaction mixture (1 mL) contained 50 mM potassium phosphate buffer at pH 7.0 and 10 mM H2O2. The reaction was initiated by adding the enzyme solution (50 μL) to the reaction mixture and the initial velocity of H2O2 disappearance was measured at 30 °C. One unit
of enzyme activity was defined as the amount of enzyme that decomposes 1 μmol of H2O2 per min.

SOD activity was measured by following the rate of pyrogallol autooxidation (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 H2O2 and menadione**

_E. coli_ cells grown in LBGMg medium (10 mL) at 37 °C to an OD660 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 Na2HPO4, 2.7 mM KCl, and 1.5 mM KH2PO4 (pH 7.4). The cell suspension was washed once by centrifugation, resuspended in PBS buffer with H2O2 (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-H2DCFDA) (Molecular Probes, Eugene, OR) as reported previously with a slight modification (Rutherford et al. 2010). Carboxy-H2DCFDA 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-H2DCFDA samples was added to the medium. After incubation at 37 °C for 10 min, the OD660 and the fluorescence excitation/emission at 485/535 nm of each sample were measured by spectrofluorometer (RF-6000; Shimadzu Co., Kyoto, Japan).

Specific fluorescence was calculated as fluorescence/OD660.

**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 ∆katE, ∆katG, ∆ahpF, ∆sodA, ∆sodB, ∆sodC, ∆katE∆katG and ∆sodA∆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
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, BW25113ΔkatEΔkatG and BW25113ΔsodAΔsodB, were highly sensitive to n-hexane, exhibiting 10²- or 10³-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 BW25113ΔkatEΔkatG and BW25113ΔsodAΔsodB by transformation of the catalase- or SOD-coding gene was investigated (Fig. 2). The colony-forming efficiencies in BW25113ΔkatEΔkatG(pMWkatE), BW25113ΔkatEΔkatG(pMWkatG), and BW25113ΔkatEΔkatG(pMWkatEkatG) were about 10⁻¹, 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 BW25113ΔsodAΔsodB(pMWsodA) and BW25113ΔsodAΔsodB(pMWsodB) were both about tenfold higher than that in BW25113(pMW119). In addition, the efficiency in BW25113ΔsodAΔsodB(pMWsodAsodB) was about 10³-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 H₂O₂ and menadione in BW25113ΔkatEΔkatG and BW25113ΔsodAΔsodB

The levels of catalase and SOD activities in BW25113ΔkatEΔkatG and BW25113ΔsodAΔsodB were compared with the parent strain BW25113 (Table 4). Most of the catalase and SOD activities were eliminated in BW25113ΔkatEΔkatG and BW25113ΔsodAΔsodB, 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 H₂O₂ and a redox-cycling agent, menadione (Additional file 1: Fig. S1). The survival fraction of BW25113ΔkatEΔkatG in 4 mM H₂O₂ and BW25113ΔsodAΔsodB 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, BW25113ΔkatEΔkatG and BW25113ΔsodAΔsodB 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, BW25113ΔkatEΔkatG and BW25113ΔsodAΔsodB 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, BW25113ΔkatEΔkatG and BW25113ΔsodAΔsodB 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 BW25113ΔkatEΔkatG and BW25113ΔsodAΔsodB 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. BW25113ΔsodAΔsodB exhibited an extended lag phase and BW25113ΔkatEΔkatG did not grow during 8 h cultivation in the presence of n-hexane. Thus, BW25113ΔkatEΔkatG was more sensitive to n-hexane than BW25113ΔsodAΔsodB.

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   |
| BW25113ΔkatEΔkatG | 0.06±0.07         | 0.20±0.03   |
| BW25113ΔsodAΔsodB | 1.55±0.15         | 0.01±0.01   |

Data represent the mean ± SD of triplicate experiments.

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

We examined the ROS levels in BW25113, BW25113ΔkatEΔkatG and BW25113ΔsodAΔsodB 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 BW25113ΔkatEΔkatG and BW25113ΔsodAΔsodB. The ROS levels by exposure to n-hexane in BW25113ΔkatEΔkatG and BW25113ΔsodAΔsodB 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 BW25113ΔkatEΔkatG and BW25113ΔsodAΔsodB 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, pMcSodAp 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
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 \( \left( O_2^- \right) \). 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-tetrahydropanthalethane (tetralin), cyclohexane, propylenebenzene, and 1,2-dihydropanthalethane (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) (Carliz 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 BW25113\( \Delta katE \Delta katG \) and BW25113\( \Delta sodA \Delta sodB \) and then examine their organic solvent-tolerances. BW25113\( \Delta katE \Delta katG \) and BW25113\( \Delta sodA \Delta sodB \) lost most of their catalase and SOD activities, respectively (Table 4). In addition, we confirmed that BW25113\( \Delta katE \Delta katG \) and BW25113\( \Delta sodA \Delta sodB \) became sensitive to \( H_2O_2 \) and menadione (an \( O_2^- \) generator), respectively (Additional file 1: Fig. S1). Both BW25113\( \Delta katE \Delta katG \) and BW25113\( \Delta sodA \Delta sodB \) 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/Fur system and Rob (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. 2012, 2014), maintenance of the proton motive force (Tsukagoshi and Aono 2000; Watanabe and Doukyu 2012, 2014), 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. 2010). Antioxidant enzymes such as catalase and superoxide dismutase 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. 2012, 2014), maintenance of the proton motive force (Tsukagoshi and Aono 2000; Watanabe and Doukyu 2012, 2014), 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. 2010). 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-H2DCFDA: 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 H2O2 and menadione on the cell viability of E. coli BW25113 and its mutants deficient in ROS-scavenging enzymes. Each strain was exposed to H2O2 (A) and menadione (B). After incubation with H2O2 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 H2O2 or menadione divided by that of untreated cells. Symbols: filled circles, BW25113; open squares, BW25113Δkat/EΔkatG; open triangles, BW25113ΔsodAΔsodB. Values indicate the means and standard deviations of the results from three independent experiments.

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**Authors' contributions**

ND designed the experiments and wrote the paper; ND and KT performed the experiments and analyzed the data. All authors read and approved the final manuscript.

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**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/osaA 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

Ahktar 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.metedeno.2014.11.001

Alhamra J, Ruiz-Laguna J, Rodriguez-Ariza A, Toribio F, Lopez-Barea J, Pueyo C (1998) Formation of 8-oxoquanine 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, Abe 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, Joibin 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/msb410050

Bradford KM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 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

Carloz& 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/10.1002/1460-2075.1986.tb04256.x

Casadaban MJ, Chou J, Cohen SN (1980) In vitro gene fusions that join an allozyme-active beta-galactosidase segment to amino-termini of exogenous proteins: Escherichia coli plasmid vectors for the detection and cloning of translational initiation signals. J Bacteriol 142(2):971. https://doi.org/10.1128/JB.142.2.971-980.1980

Cherepanov PP, Wackernagel W (1995) Gene disruption in Escherichia coli: the unexpected role of marR-proV genes required for catalase HPII expression in Escherichia coli. J Bacteriol 178(9):2564–2571. https://doi.org/10.1128/JB.178.9.2564-2571.1996

Chin W-C, Lin K-H, Chang J-J, Huang C-C (2013) Improvement of 1-Alkanols and membranes: a story of attraction. Biochim Biophys Acta 1830(7):1513–1520. https://doi.org/10.1016/j.bbapap.2013.01.016

Claiborne A, Malinowski DP, Fridovich I (1979) Purification and characterization of the periplasmic copper, zinc superoxide dismutase of Escherichia coli. J Biol Chem 254(22):11664–11668. https://doi.org/10.1016/S0021-9258(19)86535-3

Clonet S, Weimer J, Klaenov AV (1999) Cloning of an organic solvent tolerance gene from Acinetobacter sp. strain ST-550 in the autodissociation of pyrogallol and a convenient assay for superoxide dismutase. Eur J Biochem 247(3):469–474. https://doi.org/10.1111/j.1432-1033.1997.tb03714.x

Dougherty MJ, Politz MG, Kuziuk 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.02147-12

Di Fabbro GD, Lucy MA, Poole LB (1997) Requirement for the two AhpC cystine disulfide centers in catalysis of peroxide reduction by alkyl hydroperoxide reductase. Biochemistry 36(43):13357–13364. https://doi.org/10.1021/bi9736560

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

Ishikawa K, Ogino H (1999) A Pseudomonas thrives in high concentrations of toluene. Nature 338:264. https://doi.org/10.1038/338264a0

Kabeltz 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):225–227. 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

Lennon RM, Politz MG, Kuziuk 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.02147-12

Li Calzi M, Poole LB (1997) Requirement for the two AhpC cystine disulfide centers in catalysis of peroxide reduction by alkyl hydroperoxide reductase. Biochemistry 36(43):13357–13364. https://doi.org/10.1021/bi9736560

Marklund S, Marklund G (1974) Induction 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 (1998) Cloning and physical mapping of marR prophages in the forward and Escherichia coli strain K12. J Bacteriol 180(13):4184–4190. https://doi.org/10.1128/JB.180.13.4184-4190.1998

Mukhopadhyay S, Schellhorn HE (1994) Induction of Escherichia coli hydperoxide I by acetate and other weak acids. J Bacteriol 176(8):2300–2307.

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 39(9):1412–1418. https://doi.org/10.1016/j.freeradbiomed.2004.07.001

Ramos J, 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→A:T 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, Smidt HL, Benke P, Mukhopadhyay A, Keasing 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 155(1):418–420. https://doi.org/10.1128/JB.155.1.418-420.1984

Semsey S (2014) A mixed incoherent feed-forward loop allows conditional gene regulation of response dynamics. PLoS ONE 9(3):e91243. https://doi.org/10.1371/journal.pone.0091243
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 dismutase 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) Nucleotide sequence of katG, encoding catalase HPI of Escherichia coli. J Bacteriol 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-ToIC 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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