The L-Cysteine/L-Cystine Shuttle System Provides Reducing Equivalents to the Periplasm in Escherichia coli

Received for publication, November 2, 2009, and in revised form, March 26, 2010 Published, JBC Papers in Press, March 29, 2010, DOI 10.1074/jbc.M109.081356

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Intracellular thiols like L-cysteine and glutathione play a critical role in the regulation of cellular processes. Escherichia coli has multiple L-cysteine transporters, which export L-cysteine from the cytoplasm into the periplasm. However, the role of L-cysteine in the periplasm remains unknown. Here we show that an L-cysteine transporter, YdeD, is required for the tolerance of E. coli cells to hydrogen peroxide. We also present evidence that L-cysteine, a product from the oxidation of L-cystine by hydrogen peroxide, is imported back into the cytoplasm in a manner dependent on FliY, the periplasmic L-cystine-binding protein.Remarkably, this protein, which is involved in the recycling of the oxidized L-cysteine, is also found to be important for the hydrogen peroxide resistance of this organism. Furthermore, our analysis of the transcription of relevant genes revealed that the transcription of genes encoding FliY and YdeD is highly induced by hydrogen peroxide rather than by L-cysteine. These findings led us to propose that the inducible L-cysteine/L-cystine shuttle system plays an important role in oxidative stress tolerance through providing a reducing equivalent to the periplasm in E. coli.

A key building block of proteins, L-cysteine is an amino acid with a thiol side chain. Because of its high reactivity, L-cysteine is an important structural and functional component of many proteins. Although L-cysteine is pivotal for various protein functions, the molecule itself is toxic to cells even at low concentrations in both prokaryotes and eukaryotes (1–3). It has been reported that threonine deaminase, an enzyme in L-leucine biosynthesis, is inhibited by L-cysteine (4), which could be part of the reason for the cytotoxicity of L-cysteine in this organism. To maintain the L-cysteine concentrations below the threshold of cytotoxicity, the intracellular L-cysteine level is strictly controlled.

Serine acetyltransferase, a key enzyme in the L-cysteine synthesis pathway of E. coli, is under the control of feedback inhibition by L-cysteine. In addition, E. coli has five or more enzymes having L-cysteine desulphydrase activity (TnaA, CysK, CysM, MalY, and MetC). These systems may prevent the accumulation of excess L-cysteine in cells.

E. coli has L-cysteine transporters in the inner membrane (YdeD, Yfik, and Bcr) (5–7), and in the outer membrane (ToIC) (8). It is known that ToIC associates with the inner membrane and accessories, e.g. AcrAB or AcrEF, forming tripartite efflux pumps which export toxic compounds directly from the cytoplasm to the outside of the cells. However, in the L-cysteine export system, ToIC does not associate with the L-cysteine transporters in the inner membrane (YdeD, Yfik, and Bcr) (8). These findings suggest that L-cysteine, transported from the cytoplasm, is first pooled in the periplasm, and then exported through ToIC in the outer membrane. Despite this knowledge, the role of the periplasmic L-cysteine remained elusive.

The electron transport chain in the inner membrane of E. coli is thought to generate reactive oxygen species (ROS),3 such as superoxide and hydrogen peroxide (H$_2$O$_2$), due primarily to the leakage of electrons (9). H$_2$O$_2$ in the cytoplasm is eliminated by two catalases (KatE and KatG) and a peroxidase (AhpCF). An Hpx$^{-}$ mutant lacking all of these three major enzymes accumulates H$_2$O$_2$ in cells (10). However, these enzymes do not exist in the periplasm, but superoxide dismutase (SodC), which generates the H$_2$O$_2$, localizes in this compartment. This fact raises a question concerning how H$_2$O$_2$ generated in the periplasm is eliminated.

In addition, E. coli is exposed to H$_2$O$_2$, which is produced by phagocytes, in the environment. If the cells could detoxify H$_2$O$_2$ in the periplasm before its penetration into the cytoplasm, it would diminish its toxicity. Thus, the possession of such H$_2$O$_2$ removal ability in the periplasm may be beneficial for the cells. It is known that the sulphydryl group of L-cysteine can react with H$_2$O$_2$ to yield H$_2$O and L-cystine (11) as in Equation 1.

$$H_2O_2 + 2 \text{L-cysteine} \rightarrow 2H_2O + \text{L-cystine}$$

(Eq 1)

Thus, we speculated that an L-cysteine transporter such as YdeD exports L-cysteine as a scavenger of H$_2$O$_2$ into the periplasm. These considerations have led us to study the role of

3 The abbreviations used are: ROS, reactive oxygen species; ER, endoplasmic reticulum; SOD, superoxide dismutase.
l-cysteine transporters in *E. coli*. In this report, we show evidence that the l-cysteine transporter YdeD indeed functions against H₂O₂ stress in *E. coli*. We also provide evidence that the periplasmic l-cysteine-binding protein FliY is involved not only in the uptake of l-cysteine, a product of oxidation of l-cysteine, but also in the H₂O₂ tolerance of this organism. Further, our data show that H₂O₂ stress highly induces the expression of the genes encoding YdeD and FliY. From these findings, we propose that the inducible l-cysteine/l-cystine shuttle system plays an important role for the resistance of cells to H₂O₂ in the periplasm.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Oligonucleotides—** *E. coli* strains and plasmids used in this work are listed in Table 1, and oligonucleotides used are listed in supplemental Table S1. Gene cloning and DNA manipulation and the transformation of *E. coli* strains were performed according to standard methods (12). *E. coli* wild-type strain, BW25113, their derivatives (deletion mutants), and plasmids, pCA24N and pDsba were supplied by the National BioResource Project (NBRP). A Hpx-mutant strain lacking two catalases (KatE and KatG) and a peroxidase (AhpCF)(10) was kindly provided by James A. Imlay. High l-cysteine-producing plasmid pDES (supplied by Ajinomoto) is a derivative of pACYC184 containing the altered cysE gene encoding the l-cysteine feedback inhibition-insensitive mutant SAT (T167A), the wild-type ydeD gene encoding inner membrane l-cysteine transporters (5), and the altered serA gene encoding the l-serine feedback inhibition-insensitive mutant of d-3-phosphoglycerate dehydrogenase (T410stop). Each gene fragment is under the control of the constitutive promoter of the *E. coli* ompA gene encoding outer membrane protein A precursor (13). The medium copy number vector pSTV29 was purchased from Takara Bio Co. (Kyoto, Japan). The construction of pYedD has already been described (8).

**Media and Growth Conditions—** Luria-Bertani (LB) complete medium or SM1 medium that is supplemented with LB broth, l-methionine, and thiosulfate (8) was used for the general cultivations of *E. coli*. When appropriate, antibiotics were added at 50 μg/ml (for kanamycin), 30 μg/ml (for chloramphenicol), and 10 μg/ml (for tetracycline). Growth of cultures was monitored by measuring of the optical density at 660 nm (OD₆₆₀). H₂O₂ was added to the medium at the indicated concentration. For solid medium, 1.5% agar was added.

**l-Cysteine Uptake Assays—** Cells grown to mid-exponential phase were harvested by centrifugation, washed twice with cold KPM solution (10 mM MgSO₄, 0.1 mM KH₂PO₄; pH was adjusted to 6.5 with H₃PO₄), and suspended in cold KPM solution to a density of 10⁸ cells per ml. Portions of the cell suspension (6 ml each) were energized, by the addition of 0.57 ml of 40% d-glucose, followed by incubation for 10 min at 37 °C. The l-cysteine uptake assay was initiated by the addition of 2.5 μl of l-[¹⁴C]cysteine (291.3 mCi/mmol). Following the incubation of the cells at room temperature for the indicated time, the cells were collected by filtration through a GF/C filter (Whatman), and the cells collected on the filter were washed three times with KPM solution. Then, the radioactivity derived from l-[¹⁴C]cysteine was measured by liquid scintillation counter LS6500 (Beckman).

**Preparation of Intracellular l-Cysteine—** After *E. coli* cells were grown to stationary phase at 30 °C in LB medium or SM1 medium that was supplemented with L broth, l-methionine, and thiosulfate, 1 ml of the cell culture was harvested, washed with distilled water, and suspended in 0.2 ml of distilled water. The intracellular amino acids were then extracted from the cells by boiling the cell suspension for 10 min using a heat block. After centrifugation (1 min at 15,000 × g) of the heated sample, the supernatant was used as an intracellular amino acid extract (7).

**Quantification of l-Cysteine—** The amount of l-cysteine in culture supernatants was determined according to the method of Gaitonde (14). 100 μl of the sample was incubated with 200 μl of Gaitonde reagent (250 mg ninhydrin dissolved in a mixture of 4 ml of concentrated HCl and 16 ml of glacial acetic acid) at 100 °C for 15 min. Under the strongly acidic conditions, ninhydrin reacts specifically with l-cysteine even in the presence of other thiols, forming a pink-colored product (*E₄₅₅* 560 nm). The reaction product was immediately cooled on ice and diluted to 1.8 ml with 99.5% (v/v) ethanol. The concentration of l-cysteine in the original sample was then determined by measuring the absorbance at 560 nm of the diluted sample.

**Quantification of Total Free l-Cysteine (l-Cysteine Plus l-Cystine)—** To determine the amount of the total l-cysteine, l-cysteine in the sample was reduced by incubation with 5 mM

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**TABLE 1**

Bacterial strains and plasmids used

| E. coli strain or plasmid | Genotype | Ref. or source |
|--------------------------|----------|---------------|
| BW25113                  | lacZΔ105, rpsBΔ25, lacZΔ105, hsdRΔ314 araBADΔ315, rhaBADΔ315 | (35) |
| BW25113                  | pYedD (Enhancing periplasmic l-cysteine excretion) | (35) |
| BW25113                  | pDE, pCA24N (l-Cystine overproducer) | (35) |
| JW3832                   | BW25113 dsbA::Km² | (35) |
| JW5182                   | BW25113 dsbB::Km² | (35) |
| JW5250                   | BW25113 ydeD::Km² | (35) |
| JW2663                   | BW25113 gshA::Km² | (35) |
| JW2562                   | BW25113 yfiK::Km² | (35) |
| JW5363                   | BW25113 cysE::Km² | (35) |
| JW1905                   | BW25113 flJ::Km² | (35) |
| JW1905                   | pCA24N T3–lac promoter, pQB2-based, Cm² | (36) |
| JW1905                   | pFHY pCA24N, flJ::Km² on 0.8 kb DNA fragment | (36) |
| JW1905                   | pDsba pCA24N, dsbA::Km² on 0.6 kb DNA fragment | (36) |
| JW1905                   | pSTV29 lac promoter, pACYC184-based, Cm² | (8) |
| JW1905                   | pYdeD pSTV29, ydeD::Km² | (8) |
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Dithiothreitol in 200 mM Tris–HCl buffer (pH 8.6) for 10 min. Then the amount of l-cysteine in the reduced sample was determined using the method by Gaitonde.

**Determination of l-Cystine**—The amount of l-cystine was calculated by subtracting the amount of l-cysteine from that of total free l-cysteine.

**Real-time Quantitative Polymerase Chain Reaction Analysis**—Primers used in this study were designed using the Primer Express (Applied Biosystems, Foster City, CA; supplemental Table S1). *E. coli* cells were lysed with 1 mg/ml lysozyme in 10 mM Tris–HCl (pH 8.0) containing 1 mM EDTA buffer. Total RNA was prepared using the RNeasy Mini Kit (Qiagen, Valencia, CA) and the RNase-Free DNase Set (Qiagen). Complementary DNA was synthesized from 1 μg of total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). For real-time quantitative polymerase chain reaction (qPCR), cDNA was amplified with oligonucleotide primers specific to each target gene using the 7300 Real-Time PCR System (Applied Biosystems). Reactions contained Power SYBR PCR Master Mix (Applied Biosystems), forward and reverse primers (0.1 μM each), and a cDNA template (20 ng). For the dissociation curve analysis, the following conditions were used: initial steps at 50 °C for 2 min, 95 °C for 10 min; 40 cycles of PCR at 95 °C for 15 s, 60 °C for 1 min; and final steps at 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s (15). The melting curve for each PCR product was determined according to the supplier’s guidelines, ensuring specific amplification of the target gene. Quantitative values were obtained as the threshold PCR cycle number (Ct) when the increase in the fluorescent signal of the PCR product showed exponential amplification. The mRNA level of each gene was normalized to that of *rrsH* in the same sample. The cycle threshold (Ct) value for each reaction was determined using the 7300 Real-time PCR System software package (Applied Biosystems). The Ct values were used to calculate the mean-fold change of the reactions via the 2^−ΔΔCt method for each sample in triplicate, in which ΔΔCt indicates no change in abundance (16).

**Hydrogen Peroxide Measurements**—The concentration of H$_2$O$_2$ in the sample was measured using color reagent mixture containing peroxidase, 4-aminophenyl, and phenol as a specific pink product from oxidative condensation of phenol, 4-aminophenyl, and H$_2$O$_2$. 5 μl of sample was incubated with 1 ml of the coloring reagent mixture (Wako) at room temperature for 10 min. Then, the amount of the pink product derived from H$_2$O$_2$ in the original sample was determined by measuring absorbance at 505 nm (A$_{505}$).

**Redox State Analysis on DsbA**—To determine the *in vivo* redox states of DsbA, the free cysteine residues of the protein were acid trapped and alkylated with the high molecular mass reagent AMS (Invitrogen) as described (17). Alkylated samples were separated by SDS-PAGE, and detected by Western blot analysis with anti-DsbA that has been described (18).

**Microscopic Analysis**—Cultures, grown in LB complete medium at 37 °C overnight, were diluted 1:100-fold into the same medium with or without 100 μM H$_2$O$_2$. After growth was continued for 8 h, cells were harvested for microscopic analyses. The pictures of the cells were taken with the Axiovert 200M microscope (ZEISS, Osaka, Japan). Images were collected and processed using the AxioVision 4.5 software (ZEISS, Osaka, Japan).

**RESULTS**

The l-Cysteine Transporter YdeD Contributes to Hydrogen Peroxide Tolerance in *E. coli*—To address our hypothesis concerning the role of l-cysteine transporters in the detoxification of H$_2$O$_2$, we first investigated whether YdeD is involved in H$_2$O$_2$ resistance in *E. coli*. For this purpose, ΔydeD mutant and wild-type cells were transformed with either pYdeD, a middle copy plasmid carrying the ydeD gene with the original promoter, or the empty vector pSTV29. Serial dilutions of the overnight culture in LB medium were spotted onto the surface of a H$_2$O$_2$-containing LB agar plate. The cells were then grown at 30 °C for 24 h. The ydeD deletion mutant cells displayed increased H$_2$O$_2$ sensitivity (Fig. 1A). In contrast, mutants missing either one of the other l-cysteine transporter genes, *yfiK* or *bcr*, did not show an increased H$_2$O$_2$ sensitivity.
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(supplemental Fig. S1). Remarkably, both wild-type cells and ΔydeD cells overexpressing YdeD showed higher levels of \( \text{H}_2\text{O}_2 \) tolerance than that of wild-type cells (Fig. 1A). These results demonstrate that YdeD contributes to \( \text{H}_2\text{O}_2 \) resistance in *E. coli*.

The levels of \( \text{H}_2\text{O}_2 \) tolerance were clearly elevated by \( \text{H}_2\text{O}_2 \) pretreatment (Fig. 1B), suggesting that *E. coli* has an inducible \( \text{H}_2\text{O}_2 \) tolerance system. Interestingly, qPCR showed an 11.2-fold increase in the *ydeD* gene expression after treatment with 0.88 mM \( \text{H}_2\text{O}_2 \) for 30 min (Fig. 1C).

Glutathione is a redox-active tripeptide (\( \text{l}-\gamma-\text{glutamyl}-\text{l}-\text{cysteinylglycine; GSH} \) that is present in the cytoplasm of many organisms. It is reported that GSH also exists in the periplasm of *E. coli* (19). To examine whether GSH plays a role in the \( \text{H}_2\text{O}_2 \) resistance of *E. coli* cells, we tested the effects of the deletion of the *gshA* gene on the \( \text{H}_2\text{O}_2 \) resistance of *E. coli*. This gene encodes \( \gamma-\text{glutamylcysteine} \) synthetase, a key enzyme in GSH synthesis. We found that \( \text{H}_2\text{O}_2 \) tolerance was increased by YdeD overexpression even in the \( \Delta\text{gshA} \) mutant (Fig. 1D). Importantly, no significant difference in \( \text{H}_2\text{O}_2 \) tolerance was observed between the wild-type cells and the \( \Delta\text{gshA} \) mutant. These findings suggest that, in contrast to \( \text{l}-\text{cysteine}, \) GSH may not contribute to the \( \text{H}_2\text{O}_2 \) resistance of *E. coli* cells.

**l-Cysteine Is Oxidized to l-Cystine in the Periplasm**—As the periplasm is under oxidative conditions, it is inferred that \( \text{l}-\text{cysteine} \) is oxidized into \( \text{l}-\text{cystine} \) in this oxidative cellular compartment. Thus, we investigated whether \( \text{l}-\text{cystine} \) is, in fact, produced in the periplasm. Because *E. coli* cells maintain an extremely low cellular \( \text{l}-\text{cystine} \) level, we could not determine the cellular contents of \( \text{l}-\text{cysteine} \) and \( \text{l}-\text{cystine} \). Therefore, we used cells transformed with pDES, which allows the cells to produce a large amount of \( \text{l}-\text{cysteine} \) and effectively export it into the periplasm. The pDES plasmid encodes serine acetyltransferase (T167A) and \( \text{d-3-phosphoglycerate} \) dehydrogenase (T410stop), which are released from feedback inhibition by \( \text{l}-\text{cysteine} \) and \( \text{l}-\text{serine} \), respectively, and YdeD (8).

As shown in Fig. 2A, \( \text{l}-\text{cystine} \) accounted for about 30% of the total free cysteine (\( \text{l}-\text{cysteine} \) plus \( \text{l}-\text{cystine} \)) in *E. coli* cells carrying the pDES plasmid. The further overexpression of DsbA, which oxidizes peptidyl-\( \text{l}-\text{cysteine} \) residues into a disulfide bridge in the periplasm (20), facilitated \( \text{l}-\text{cystine} \) formation: the ratio of \( \text{l}-\text{cysteine} \) to the total cellular \( \text{l}-\text{cystine} \) reached about 50% (Fig. 2A). These findings suggest that a significant fraction of endogenously produced \( \text{l}-\text{cysteine} \) is in fact exported into the periplasm, where it is oxidized into \( \text{l}-\text{cystine} \) even in the absence of a specific exogenous oxidative stressor. These observations are consistent with the proposed role of \( \text{l}-\text{cysteine} \) as a reducing equivalent in the periplasm.

The Intracellular l-Cysteine/l-Cystine Ratio Increases Upon Treatment of the Cells with Hydrogen Peroxide—If \( \text{l}-\text{cysteine} \) is used as a reducing equivalent to detoxify \( \text{H}_2\text{O}_2 \) in *in vivo*, a portion of \( \text{l}-\text{cysteine} \) will be oxidized after treatment of the cells with \( \text{H}_2\text{O}_2 \). To test this, we also determined the intracellular contents of \( \text{l}-\text{cysteine} \) and \( \text{l}-\text{cystine} \) after treatment of cells with \( \text{H}_2\text{O}_2 \). To detect the changes in the intracellular \( \text{l}-\text{cysteine} \)/\( \text{l}-\text{cystine} \) ratio before and after \( \text{H}_2\text{O}_2 \) treatment, we used *E. coli* cells carrying the pDES plasmid. As shown in Fig. 2B, the ratio of \( \text{l}-\text{cysteine} \) to the total \( \text{l}-\text{cysteine} \) of the cells after treatment with 0.88 mM \( \text{H}_2\text{O}_2 \) (52%) was higher than that of the cells before treatment with \( \text{H}_2\text{O}_2 \) (41%). This ratio reached 73% after treatment with 8.8 mM \( \text{H}_2\text{O}_2 \) (data not shown). These results are consistent with the finding that endogenous \( \text{l}-\text{cysteine} \) can detoxify exogenous \( \text{H}_2\text{O}_2 \) in the periplasm.

**Overexpression of the l-Cysteine Transporter YdeD Can Affect the Redox Environment of the Periplasm**—We showed evidence that the overexpression of the \( \text{l}-\text{cysteine} \) transporter, YdeD, confers \( \text{H}_2\text{O}_2 \) resistance to *E. coli* (see above). We next wanted to examine the influence of the \( \text{l}-\text{cysteine} \) transporter, YdeD, on the redox environment of the periplasm. To this end, we investigated the redox state of DsbA after the overexpression of the \( \text{l}-\text{cysteine} \) transporter from the pYdeD plasmid because DsbA is a periplasmic protein with a pair of redox-active \( \text{l}-\text{cysteines} \) that can alternate between the oxidized and the reduced states. Importantly, these two \( \text{l}-\text{cysteines} \) are normally maintained in the fully oxidized state *in vivo* by the membrane protein DsbB, which passes electrons from DsbA to quinones in the respiratory chain (17). To study the oxidative state of DsbA *in vivo*, we used an alkyllating reagent, AMS, which modifies free \( \text{l}-\text{cysteines} \). The modification of
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FIGURE 3. The roles of FliY in E. coli resistance to H₂O₂. A, serial dilutions of the overnight cultures of the wild-type strain BW2513 (WT), a ΔfliY mutant JW1905 (ΔfliY), and a ΔydeD mutant JW5250 (ΔydeD) were spotted on a LB solid medium plate containing 0 mM or 0.75 mM H₂O₂. The sensitivity of each mutant to H₂O₂ was then examined by incubating the plates at 30 °C for 1 day. B, overnight cultures of various E. coli strains were inoculated in LB liquid medium and grown in the presence (closed) or the absence (open) of 0.5 mM H₂O₂ at 30 °C. Growth was monitored by measuring the optical density at 660 nm (OD₆₆₀). The strains used are: the wild-type strain BW25113 carrying an empty vector pCA24N (circles), and the ΔfliY mutant JW1905 carrying pCA24N (triangles) or pFliY encoding FliY (diamonds).

free L-cysteines with AMS allows the separation of the oxidized and the reduced form of DsbA on a gel (17). Upon overexpression of the L-cysteine transporter, a fraction of DsbA was indeed reduced in the cells (supplemental Fig. S2). This observation is consistent with the hypothesis that L-cysteine exported into the periplasm by YdeD can function as a reducing equivalent in the periplasm.

L-Cysteine Is Imported Dependentely of the Periplasmic L-Cysteine-Binding Protein, FliY—In Lactobacillus fermentum BR11, the L-cysteine binding protein BspA is responsible for L-cysteine uptake. FliY of E. coli was identified as a BspA ortholog and has been shown to bind L-cysteine (21). However, the mechanism of the L-cystine import remained unclear in this organism. To examine whether the uptake of L-cysteine is dependent on FliY, the rate of uptake of L-[¹⁴C]cysteine in a ΔfliY strain was compared with that of E. coli wild-type strain. It was found that the intracellular uptake of L-[¹⁴C]cysteine was significantly impaired in the ΔfliY mutant, only 37% of that in wild-type cells (Fig. 2C), indicating that FliY is required for the efficient uptake of L-cysteine by E. coli.

Consistent with the role of FliY in the uptake of L-cysteine, the disruption of the fliY gene increased the ratio of intracellular L-cysteine to L-cysteine plus L-cystine by about 50% (Fig. 2A). Remarkably, the ratio reached 96% in the ΔfliY cells that overexpress DsbA from the pDSbA plasmid. It should be noted that, in these experiments, we used an E. coli strain that exports a large amount of L-cysteine so that we could detect L-cysteine and L-cystine in the periplasm (see above). These results led us to draw a model in which endogenous L-cysteine is exported to the periplasm by YdeD and oxidized to L-cystine, which is then imported back into the cytoplasm in a FliY-dependent manner.

FliY Is Also Involved in the H₂O₂ Resistance of E. coli—We envisaged that, in addition to YdeD, FliY may also contribute to H₂O₂ resistance as it can be expected that L-cysteine uptake will promote the recycling of the l-cystine into l-cysteine (22). To test this possibility, serial dilutions of ΔfliY or ΔydeD mutant cells were spotted onto the surface of an H2O2-containing LB agar plate. The cells were then grown at 30 °C for 24 h. The ΔfliY and ΔydeD mutants showed a higher sensitivity to H₂O₂ than wild-type cells (Fig. 3A). Moreover, in liquid medium, the growth of the ΔfliY cells was completely inhibited in the presence of 0.5 mM H₂O₂, though the mutant would normally grow in the same medium without H₂O₂ (Fig. 3B). The growth defect of ΔfliY was partially restored by the transformation of the mutant cells with the pFliY plasmid. These results demonstrate that FliY contributes to growth in liquid medium with H₂O₂.

It should be noted that, as we showed before, overexpression of YdeD conferred an increased H₂O₂ resistance to the wild-type cells. However, such an increased resistance was not observed when FliY was overproduced in the wild-type cells (not shown and Fig. 3D). The difference in the effect of protein overexpression between YdeD and FliY may reflect the fact that, while YdeD directly promotes the export of L-cysteine into the periplasm, FliY is involved in the uptake of L-cystine that has been exported in the form of L-cysteine and is then oxidized in the periplasm. Nevertheless, the increased sensitivity of the ΔfliY mutant to H₂O₂ indicates the importance of the L-cystine uptake system in the H₂O₂ defense mechanism of E. coli. Taken together, we conclude that YdeD and FliY are the key components in the L-cysteine/L-cystine shuttle system, which confers H₂O₂ resistance to E. coli cells.

The L-Cysteine/L-Cystine Shuttle System Is Induced by H₂O₂—Next, we elucidated whether this system is inducible under oxidative stress. For this purpose, we quantified the expression level of related genes using real-time qPCR. As positive controls for the H₂O₂ treatment of the cells, we also examined the expression of the katG and ahpC genes, which are known to be up-regulated in response to H₂O₂ in an oxyR-dependent manner. The levels of katG and ahpC expression increased significantly under the stress conditions (Fig. 4), consistent with the observation by Storz et al. (23). Among the genes encoding L-cysteine/L-cystine transporters, ydeD, yfiK, and fliY were highly induced, at 11.2-, 4.0-, and 10.2-fold, respectively, after treatment of the cells with 0.88 mM H₂O₂ (Fig. 4). Both YdeD and FliY are key components involved in H₂O₂ resistance of E. coli. The dramatic increases in the expression of these genes are consistent with our observation in Fig. 1B that the levels of tolerance of E. coli to H₂O₂ were elevated by pretreatment of the cells with this reagent.

Curiously, in addition to H₂O₂, exogenous L-cysteine also induced the expression of ydeD, yfiK, and fliY genes (Fig. 5). However, the induction of these genes by exogenous L-cysteine was not as significant as that by H₂O₂ (Fig. 4). Further, in contrast to the katG and ahpC genes whose H₂O₂-dependent induction was abolished in an ΔoxyR mutant, expression of the
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![Graph](image)

**FIGURE 4. Induction of the ydeD and fliY gene expression by H₂O₂.** Induction of the transcription of relevant genes was analyzed after treatment of the cells with 1 mM H₂O₂ for 20 min. Relative expression (y-axis) represents fold change of each mRNA level after treatment of the cells with H₂O₂ as compared with that of the untreated cells (dotted line).

**FIGURE 5. Induction of the ydeD and fliY gene expression by L-cysteine.** Induction of the transcription of relevant genes in cells 20 min after addition of 10 mM L-cysteine to the culture. Relative expression represents the fold change of each mRNA level in cells 20 min after incubation with L-cysteine as compared with that of the cells without stress treatment (dotted line).

...dative stress conditions. These findings indicate that, under oxidative stress conditions, *E. coli* up-regulates the expression of the genes involved in the export of L-cysteine or the uptake of L-cystine but not the expression of the genes involved in the synthesis of L-cysteine. Thus, to resist oxidative stress, *E. coli* appears to promote the L-cysteine/L-cystine shuttle system rather than enhancing the synthesis of L-cysteine.

The Growth Defect of *Hpx*⁻ (katG katE ahpCF) Mutants under the Presence of 100 μM H₂O₂ Is Suppressed by Overexpression of the L-Cysteine Transporter YdeD—It has been reported that an *Hpx*⁻ mutant, which lacks alkyl hydroperoxide (Ahp) and two catalases (KatE and KatG), has very little H₂O₂-scavenging activity (10). Consistent with its decreased H₂O₂-scavenging ability, the growth of this *Hpx*⁻ mutant was impaired for 12 h in LB liquid medium containing 100 μM H₂O₂ at 37 °C (Fig. 6). It should be noted here that the same *Hpx*⁻ mutant grew rather normally with 50 μM H₂O₂ (not shown). This finding suggests that the presence of 100 μM or higher concentrations of H₂O₂ is required even for the H₂O₂-sensitive mutant to exhibit a clear growth defect.

We showed that overexpression of YdeD but not of FliY conferred increased H₂O₂ resistance to the wild-type cells. We envisaged that, if overexpression of YdeD leads to the enhanced detoxication of H₂O₂, the overexpression may also suppress the H₂O₂-hypersensitive growth phenotype of the *Hpx*⁻ mutant grown in the presence of 100 μM H₂O₂. To test this, we compared the growth of the *Hpx*⁻ mutant with that of the same mutant overexpressing YdeD in LB liquid medium containing 100 μM H₂O₂. As shown in Fig. 6, the growth of the *Hpx*⁻ mutant was partially but clearly restored by the transformation of the mutant cells with the pYdeD plasmid. In addition, overexpression of YdeD in the *Hpx*⁻ mutant decreased the number of the cells forming filaments. These finding further supports the protective role of YdeD against H₂O₂.

**DISCUSSION**

Mutants missing the L-cysteine transporter, YdeD, or the L-cystine-binding protein, FliY, exhibited an increased sensitivity to H₂O₂. Further, our data indicated that *E. coli* FliY is involved in the uptake of L-cystine from the periplasm to the cytoplasm. Interestingly, the expressions of the genes encoding these proteins were dramatically increased upon treatment of the cells with H₂O₂. These findings led us to propose that *E. coli* removes periplasmic H₂O₂ using L-cysteine supplied to the periplasm from the cytoplasm by a L-cysteine/L-cystine shuttle system (Fig. 7).

The question then is why does *E. coli* require such a L-cysteine/L-cystine shuttle system? The inner membrane is the place where *E. coli* produces ATP via the respiratory chain. The process often generates superoxide (O₂⁻) due primarily to the leakage of electrons (Fig. 7) (9). The respiratory electron

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transport chain-linked ROS generation accounts for as much as 87% of the total H₂O₂ production in aerobic growth (26). Superoxide dismutases (SODs) convert O₂⁻ to H₂O₂. SODs exist in the periplasm (SodC) as well as the cytoplasm (SodA and SodB). Thus, as a result of the reaction to destroy the superoxide, H₂O₂ would be generated. In addition, E. coli cells are exposed to H₂O₂, which is produced by phagocytes, in the environment. Local H₂O₂ concentrations may rise up to 100 μM inside phagocytes and even more than 1 mM near H₂O₂-generating lactic acid bacteria (10). As H₂O₂ also can damage molecules in the cells, it needs to be removed. However, no H₂O₂ scavenger has been identified in the periplasm, in contrast to the cytoplasm, where multiple H₂O₂ scavengers (KatE, KatG, AhpC) exist. It should be noted that thiol peroxidase (Tpx), which was initially characterized as a periplasmic H₂O₂ scavenger, actually localizes in the cytoplasm (27). We suggest that the absence of an efficient enzyme catalyst to remove H₂O₂ from the periplasm may be one of the reasons why l-cysteine is exported to the periplasm.

Interestingly, H₂O₂ but not exogenous l-cysteine (Fig. 5) significantly induced the expression of the ydeD gene (Figs. 1C, 4, and 5). Thus, YdeD seems to be intrinsically used to protect cells from oxidative damages such as those caused by H₂O₂ and ionizing radiations (28). However, it has been reported that a ΔgshA mutation does not cause increased sensitivities to H₂O₂ or cumene hydroperoxide (29). In addition, we, ourselves, observed here that there was no detectable change in the levels of resistance to H₂O₂ between the wild-type cells and the ΔgshA mutant whether or not YdeD is overproduced (Fig. 1D). Thus, we propose that E. coli uses l-cysteine to protect cells from periplasmic H₂O₂ even though GSH exists in the periplasm as well as in the cytoplasm (17). Interestingly, under H₂O₂ stress, genes involved in the utilization of periplasmic GSH as a source of l-cysteine were induced (Fig. 4). This finding may imply that the periplasmic GSH is also used as a source of l-cysteine that is to be provided to the periplasm to reduce oxidative stress.

Furthermore, we showed evidence that FliY is involved in l-cysteine uptake in E. coli (Fig. 2C). FliY is a homologue of BspA, an L. fermentum BR11 l-cysteine binding protein. BspA is a component of the l-cysteine transport system in this organism. Here we showed that FliY is an important component of l-cysteine import and that this protein is necessary for resistance of E. coli to oxidative stress (Fig. 3, A and B). The latter finding is consistent with the previous observation in L. fermentum (30). It should be noted that, as FliY is a periplasmic solute-binding protein, FliY probably cooperates with a certain transporter for the uptake of l-cysteine (Fig. 7).
Interestingly, $\text{H}_2\text{O}_2$ induced the $ydeD$ gene as well as the $ydeI$ gene. These findings led us to propose that the $\ell$-cysteine exporter, $YdeD$, and the $\ell$-cystine-binding protein, $FliY$, are the components of the $\ell$-cysteine/$\ell$-cystine shuttle system in the inner membrane that contribute to $\text{H}_2\text{O}_2$ scavenging.

As shown in Fig. 4, $\text{H}_2\text{O}_2$ stress did not cause any detectable increase in the expression of genes involved in the production of intracellular thiols. This finding is consistent with the finding that the intracellular $\ell$-cysteine level is strictly controlled even under oxidative stress conditions (31). If $\text{H}_2\text{O}_2$ is detoxified by $\text{de novo}$ synthesized $\ell$-cysteine, cells will again face growth inhibition by the increase in $\ell$-cysteine toxicity. Thus, the $\ell$-cysteine recycle system may be beneficial for the growth of $E.\ coli$ cells, because the system allows cells to scavenge $\text{H}_2\text{O}_2$ without growth inhibition from the presence of a large amount of $\text{de novo}$ synthesized $\ell$-cysteine.

An intermembrane ROS defense system that is analogous to the $E.\ coli YdeD/FliY$ system may also exist in the cellular compartments of eukaryotes. In fact, in lung cancer cells, an $\text{xCT}$ and Multi-drug Resistant Proteins (MRP) are the components of the $\ell$-cysteine/$\ell$-cystine cycle system and are involved in selenite reduction in the extracellular space (supplemental Fig. S3A) (32). In addition, Burkitt’s Lymphoma cells overexpressing xCT were reported to become highly resistant to oxidative stress. Importantly, the cells exhibited $\text{H}_2\text{O}_2$ resistance even in the absence of GSH synthesis (33), implying the importance of $\ell$-cysteine in the resistance to $\text{H}_2\text{O}_2$.

Protein-disulfide isomerase, PDI, catalyzes the formation of protein disulfide bonds in the ER of eukaryotes. After the oxidation of its substrates, PDI is reduced. The reduced PDI, in turn, is oxidized by Ero1p, which then passes electrons to molecular oxygen. The latter reaction results in the generation of $\text{H}_2\text{O}_2$, which probably damages cells (34). However, it is not understood how cells are protected from the damage caused by the generation of ROS in the ER (supplemental Fig. S3B). An $\ell$-cysteine/$\ell$-cystine shuttle system that scavenges $\text{H}_2\text{O}_2$ instead of catalase might also exist in the ER lumen. Indeed, Yct1p, an $\ell$-cysteine-specific transporter from the Saccharomyces cerevisiae, was found to transport $\ell$-cysteine into the ER (35). Thus, it may be possible that, in addition to the plasma membrane, the ER membrane might also utilize a $\ell$-cysteine/$\ell$-cystine shuttle system to supply $\ell$-cysteine as a reducing equivalent to the ER lumen. Finally, these findings including our results suggest that an analogous $\ell$-cysteine/$\ell$-cystine shuttle system may be conserved from bacteria to mammals and may play important roles by supplying a reducing equivalent to the oxidative cellular compartments.

Acknowledgments—We thank N. Yoshida at Nara Institute of Science and Technology (NAIST), Japan, for his helpful discussion and assistance of this work, respectively. We thank H. Mori (NAIST, Japan), James A. Imlay (University of Illinois at Urbana-Champaign), and Ajinomoto, Co., Inc. (Tokyo, Japan) for providing strains and/or plasmids.

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