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Three Distinct-Type Glutathione *S*-Transferases from *Escherichia coli* Important for Defense against Oxidative Stress

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Previously, we characterized glutathione *S*-transferase (GST) B1-1 from *Escherichia coli* enzymologically and structurally. Besides GST B1-1, *E. coli* has seven genes that encode GST-like proteins, for which, except SspA, neither biological roles nor biochemical properties are known. Here we show that the GST-like YfcF and YfcG proteins have low but significant GSH-conjugating activity toward 1-chloro-2,4-dinitorobenzene and GSH-dependent peroxidase activity toward cumene hydroperoxide. Analysis involving site-directed mutagenesis suggested that Ser16 and Asn11 were important for the activities of YfcF and YfcG, respectively. On the contrary, no residue around the catalytic site of GST B1-1 has been demonstrated to be essential for catalytic activity. Deletions of the *gst*, *yfcF*, and *yfcG* genes each decreased the resistibility of the bacteria to hydrogen peroxide, which was recovered by transformation with the expression plasmid for the deleted enzyme. The inactive YfcF(S16G) and YfcG(N11A) mutants, however, could not rescue the knockout bacteria. Thus, *E. coli* has at least three GSTs of distinct classes, all of which are important for defense against oxidative stress in spite of the structural diversity. This seems consistent with the hypothesis that GSTs constitute a protein superfamily that has evolved from a thioredoxin-like ancestor in response to the development of oxidative stress.

Key words: *Escherichia coli*, glutathione *S*-transferase, hydrogen peroxide, oxidative stress, site-directed mutagenesis.

Abbreviations: CDNB, 1-Chloro-2,4-dinitrobenzene; DCM, dichlromethane; GST, glutathione *S*-transferase.

Glutathione *S*-transferases (GSTs) [EC 2.5.1.18] catalyze the conjugation of a large variety of electrophilic compounds (*1*) to reduced glutathione (GSH). In this way, GSTs contribute to metabolism of xenobiotics including drugs and pesticides. Some GSTs also participate in defense against oxidative stress through GSH-dependent reduction of organic peroxides (*2*). In eukaryotes, there are three major groups of GSTs: cytosolic GSTs, mitochondrial GSTs, and microsomal GSTs. The cytosolic GSTs are the largest group and have been studied most intensely. They are homo- or hetero-dimers of about 25 kDa subunits, which constitute a multigene superfamily and are divided into various classes designated Alpha, Delta, Epsilon, Zeta, Theta, Mu, Pi, Sigma, Tau, and Omega based mainly on primary structure (*3–11*). The hetero-dimers are formed only by GST monomers of the same class. The amino acid sequence identities between the molecules of the same class are more than 40%, while the inter-class sequence identities are less than 25%. Despite the low inter-class sequence identities, the overall polypeptide folds indicated on crystallographic analyses are very similar (*12*). They have a two-domain structure: the N-terminal thioredoxin-like domain and the C-terminal α-helices domain.

In the catalytic mechanism of GSH-conjugation by GST, enhancement of nucleophilicity of the GSH thiol by lowering its pK_a is essential. In the Alpha, Mu, Pi, and Sigma class GSTs, the hydroxyl group of the conserved Tyr residue on the loop between the first β-strand and the first α-helix plays an essential role in lowering the pK_a of GSH by stabilizing the thiolate form (*13*). The Delta, Zeta, Theta, and Tau class GSTs have Ser and the Omega class GSTs have Cys instead of the Tyr residue as a catalytic residue.

In some bacteria, cytosolic GSTs have been identified as a catabolic enzyme with an essential role in growth on recalcitrant chemicals such as dichloromethane (DCM), 1,2-dichloroepoxyethane, lignin compounds, and so on (*14–17*). For example, DCM is efficiently utilized as a carbon and energy source by facultative methylotrophic bacteria, and the first step of the DCM utilization is catalyzed by DCM dehalogenase, a member of the cytosolic GST superfamily, in a GSH-dependent substitution reaction and forms inorganic chloride and *S*-chloromethyl glutathione. In contrast to the eukaryotic GSTs functioning in drug metabolism, these bacterial enzymes are very specific to each substrate and have no activity toward 1-chloro-2,4-dinitrobenzene (CDNB) or other typical substrates for GST.

Besides the catabolic GSTs with strict substrate specificity, those that have activity toward typical substrates such as CDNB have been identified from *Proteus mirabilis*

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(*18*, *19*), *Escherichia coli* (*20*), and so on. The GSTs from *P. mirabilis* and *E. coli* are 53% identical in primary structure, but have low homology with those from eukaryotes and the catabolic GSTs from bacteria. Thus, they are classified into the Beta class. Crystallographic analysis revealed that the GSTs from *E. coli* and *P. mirabilis* have a folding structure common among the cytosolic GSTs (*21*, *22*). These bacterial enzymes have not only GSH-conjugating activity toward typical substrates such as CDNB and ethacrynic acid but also GSH-dependent peroxidase activity toward cumene hydroperoxide (*19*, *20*, *23*). Allocati *et al*. (*24*) reported that *P. mirabilis* GST B1-1 has active roles in protection against oxidative stress generated by hydrogen peroxide and in detoxification of antimicrobial agents. Our knowledge of the biological roles of the class Beta GSTs, however, is limited.

In addition to GST B1-1, there are seven genes that code for GST-like proteins with divergent sequences in the *E. coli* genome (*25*). However, no biological role is known for them except stringent starvation protein A (SspA). SspA is an RNA-polymerase–associated transcriptional activator with the GST-fold structure but exhibits no GST activity (*26–28*). Similar to SspA, several proteins from various organisms have been reported to use the GST fold for functions other than catalyzing GSH-conjugation (*29*). In this study, we examined whether the GST-like gene products of *E. coli* have GST activity and found that the *yfcF* and *yfcG* gene products exhibit GST activity toward CDNB and GSH-dependent peroxidase activity toward cumene hydroperoxide. Primary structural comparison and analysis involving site-directed mutagenesis suggest that GST B1-1, YfcF, and YfcG are GSTs of distinct classes. Furthermore, we analyzed the knockout mutants of the GST genes, and found that all of them are important for resistibility to hydrogen peroxide.

MATERIALS AND METHODS

Materials—Restriction enzymes were purchased from New England Biolab (Ipswich, MA, USA). GSH was obtained from Kojin Co., Ltd. (Tokyo, Japan). Ethacrynic acid and *S*-hexyl-GSH were from Sigma (St. Louis, MO, USA). Cumene hydroperoxide and CDNB were from Nakalai Tesque (Kyoto, Japan). DE52 was from Whatman (Kent, UK). Oligonucleotides were synthesized by Hokkaido System Science Co. (Sapporo, Japan). Other reagents were obtained from Wako Pure Chemicals Ind. (Osaka, Japan). The *E. coli* strains JW1627 (Δ*gst*), JW2298 (Δ*yfcF*), and JW2299 (Δ*yfcG*) were obtained from National Bioresource Project for *E. coli* (National Institute of Genetics, Japan), and their parent strain BW25113 ($lacI^q$ $rrnB_{T14}$ $\Delta lacZ_{WJ16}$ *hsdR514* $\Delta araBAD_{AH33}$ $\Delta rhaBAD_{LD78}$) was obtained from Nara Institute of Science and Technology (Japan).

Construction of Expression Plasmids—Coding region of the *yfcF*, *yfcG*, *yibF*, *yliJ*, *yncG*, *yghU*, and *sspA* genes were amplified from genomic DNA by PCR using the following pairs of synthetic primers: 5′-GGC CGC ATA TGA GTA AAC CCG CTA TC-3′ (YfcF-N) and 5′-GCC GGG ATC CTA TCA GCC AGA TTG CTT C-3′ (YfcF-C) for YfcF; 5′-GCC GCC ATA TGA TCG ATC TCT ATT TCG-3′ (YfcG-N) and 5′-GGC CAA GCT TCT ATT AAC TAT CCG AAC GC-3′ (YfcG-C) for YfcG; 5′-TTA ACA TAT GAA ACT CGT CGG TAG C-3′ and 5′-ATA TGG ATC CTC ATC AAG CCT TTG GCG GT-3′ for YibF; 5′-GGC GCC ATA TGA TTA CGC TGT GGG G-3′ and 5′-GCG CGA ATT CAT TAG CTA ACG GGA ATC ATC-3′ for YliJ; 5′-AGG GGC ATA TGA TTA AGG-3′ and 5′-CCG GAA GCT TAT CAA ATA ATT TCA TTC CTT-3′ for YncG; 5′-CGG CAT ATG ACA GAC AAT ACT T-3′ and 5′- CCC GGA ATT CTT ACC CCT GAC GCT T-3′ for YghU; and 5′-AAT TCA TAT GGC TGT CGC TGC CAA C-3′ and 5′-TAT TGG ATC CTA TTA ACT CCG GCC CAG AC-3′ for SspA. After each amplified DNA had been digested at the restriction sites designed in the primers, it was inserted into the *Nde*I/*Bam*HI site of pT7-7, an T7-expression plasmid vector, in the cases of YfcF, YibF, and SspA, into the *Nde*I/*Eco*RI site in the cases of YliJ and YghU, and into the *Nde*I/*Hin*dIII site in the cases of YfcG and YncG. The inserted sequences were confirmed by sequencing using a DNA sequencer 3700 (Applied Biosystems).

Site-Directed Mutagenesis—Primers used for site-directed mutagenesis were as follows: 5′-GAT GCC CAC TTT TTC GGC CCT TAT GTG TTA-3′ (forward) and 5′-TAA CAC ATA AGG GCC GAA AAA GTG GGC ATC-3′ (reverse) for Gly mutation of Ser16 of YfcF; 5′-CAC GCT TTG GGC AGA TGC CC-3′ (forward) and 5′-GGG CAT CTG CCC AAA GCG TG-3′ (reverse) for the Ala mutation of Ser16 (S16G) of YfcF; 5′-CAC GCT TTG GGC AGA TGC CC-3′ (forward) and 5′-GGG CAT CTG CCC AAA GCG TG-3′ (reverse) for the S10A mutation of YfcF; 5′-TTT CGC CCC GGC ACC CAA TG-3′ (forward) and 5′-CAT TGG GTG CCG GGG CGA AA-3′ (reverse) for the T9A mutation of YfcG; 5′-TTC GCC CCG ACA CCC GCT GGT CAC AAA ATT-3′ (forward) and 5′-AAT TTT GTG ACC AGC GGG TGT CGG GGC GAA-3′ (reverse) of the N11A mutation of YfcG; and 5′-ACA CCC AAT GGT CAC GCA ATT ACG CTG TTT-3′ (forward) and 5′-AAA CAG CGT AAT TGC GTG ACC ATT GGG TGT-3′ (reverse) for the K14A mutation of YfcG. For example, the S16G mutation of YfcF was performed as follows. The 5′ part of the *yfcF* gene was amplified from the expression plasmid for YfcF by PCR using the YfcF-N and S16G-reverse primers. The template DNA was degraded by the treatment of the PCR solution with *Dpn*I. The 3′ part of the *yfcF* gene was amplified from the expression plasmid by PCR using the S16G-forward and YfcF-C primers. The amplified product was separated on agarose gel electrophoresis. The 5′ and 3′ products were purified with the GENECLEAN® Kit (Qbiogene, Inc.), mixed, and then used as a template for PCR with a pair of primers, YfcF-N and YfcF-C. The amplified DNA was inserted into the expression vector as described above.

Cell-Free Expression—Cell-free production of the GST-like gene products was performed using the expression plasmids described above and according to Kigawa *et al.* (*30*).

Overexpression, Purification, and Assay of GST Activity—The *E. coli* BL21(DE3) cells transformed with an expression plasmid were cultured in the LB medium containing 50 μg/ml ampicillin at 37°C. When the turbidity at 600 nm of the culture reached 0.4–0.6, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM and the culture was continued for additional 16 h. The cells were harvested by centrifugation, resuspended

into 20 mM Tris/HCl (pH 8.0) containing 1 mM EDTA, and then sonicated (22 s, 7 times) on ice. The suspension was centrifuged at 10,000 × *g* and 4°C for 40 min. The supernatant was dialyzed against a buffer containing 20 mM Tris/HCl (pH 8.0) and 1.0 mM EDTA, and then applied to a DE-52 column (22 × 65 mm). After the column had been washed with the same buffer, proteins were eluted with a linear gradient of 0–0.5 M NaCl. Fractions including the overexpressed protein were pooled and the buffer was exchanged with 10 mM potassium phosphate (pH 7.0) by ultrafiltration using an Amicon Ultra15 MW10000 (Millipore). The resulting solution was applied to a hydroxyapatite column (Seikagaku Co., Japan). After the column had been washed with the same buffer, proteins were eluted with a linear gradient of 10–100 mM potassium phosphate (pH 7.0). Fractions containing the overexpressed protein were pooled, and the buffer was exchanged with 20 mM Tris/HCl (pH 8.0) containing 1.0 mM EDTA and 50 mM NaCl. The resulting solution was applied to a Sephacryl S-200 (Amersham Biosystems) column (20 × 900 mm), and the proteins were eluted with the same buffer. Aliquots from the fractions showing absorbance at 280 nm were subjected to SDS-PAGE, and the fractions giving a single band of the overexpressed protein were pooled. The solution was concentrated by ultrafiltration using an Amicon Ultra15 MW 10000. Specific activities of the enzyme toward various substrates were determined as described (*31*, *32*).

Measurement of Effects of Hydrogen Peroxide on Growth of E. coli—The wild-type or the knockout bacteria were pre-cultured in LB medium with shaking at 37°C overnight. An aliquot of 50 μl from the culture was inoculated into 5.0 ml of fresh LB medium containing 0–3.0 mM hydrogen peroxide. Culture was performed with shaking at 182 rpm and 37°C. Turbidity at 600 nm was measured every hour.

Plasmid Rescue of Knockout Bacteria—The coding regions of the *gst*, *yfcF*, and *yfcG* genes were amplified by PCR using the following pairs of primers: 5′-GCC GGA ATT CAT GAA ATT GTT CTA CAA ACC-3′ (forward) and 5′-GGC CAA GCT TTT ACT TTA AGC CTT CCG CTG-3′ (reverse) for *gst*; 5′-GGC CGA ATT CAT GAG TAA ACC CGC TAT CAC-3′ (forward) and 5′-GCC GAA GCT TTC AGC CAG ATT GCT TCG CCG-3′ (reverse) for *yfcF*; 5′-GCC GGA ATT CAT GAT CGA TCT CTA TTT CGC-3′ (forward) and 5′-GGC CAA GCT TCT ATT AAC TAT CCG AAC GC-3′ (reverse) for *yfcG*. The amplified products were digested with *Eco*RI and *Hin*dIII and inserted into the *Eco*RI/*Hin*dIII site of an expression vector pKK223-3. The JW1627, JW2298, and JW2299 bacteria were transformed with the resulted expression plasmid for *gst*, *yfcF*, and *yfcG*, respectively. The transformed cells were cultured in the LB medium containing 50 μg/ml ampicillin.

RESULTS

YfcF and YfcG Exhibit GST and GSH-Dependent Peroxidase Activities—In addition to *gst* (*20*), *E. coli* has seven genes encoding proteins homologous to known cytosolic GSTs; *yfcF*, *yfcG*, *yibF*, *yliJ*, *yncG*, *yghU*, and *sspA*. To examine whether the gene products have GST activity, they were expressed in a cell-free system (*30*) and their GSH-conjugating activities toward CDNB were assayed.

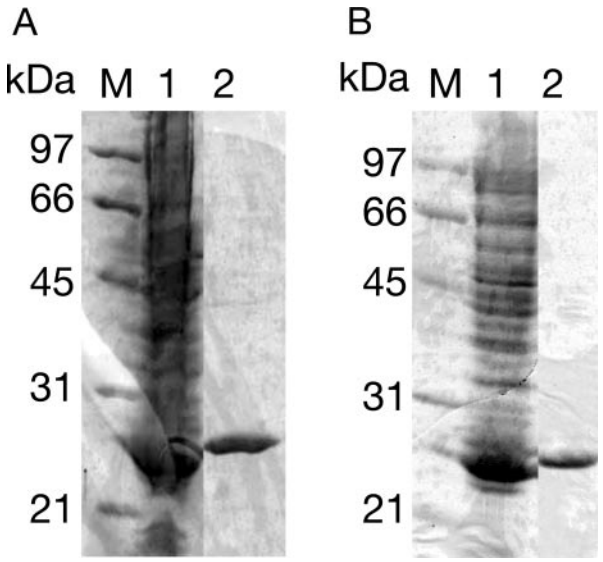

Fig. 1. **SDS-PAGE of the overexpressed YfcF (panel A) and YfcG (panel B).** YfcF and YfcG were over-expressed in *E. coli* BL21(DE3) under the control of T7 promoter. Each protein was purified from the soluble fraction of the lysed bacterial cells. Lane M, molecular size markers. Lane 1, whole cell extract. Lane 2, after purification through DE-52, hydroxyapatite, and Sephacryl S-200 column chromatography.

Significant activity was detected in the solution expressing the YfcF or YfcG protein but not in those expressing the other GST-like proteins (data not shown). To investigate the enzymatic properties, the YfcF and YfcG proteins were over-expressed in *E. coli* under the control of the T7 promoter and were purified to give a single band on SDS-PAGE (Fig. 1).

The purified YfcF protein was confirmed to exhibit significant GSH-conjugating activity toward CDNB (Table 1). However, the specific activity toward 1 mM CDNB in the presence of 1 mM GSH was as low as 1% of that of GST B1-1, and was inhibited by *S*-hexyl glutathione with the IC_{50} of 0.26 mM. The activity depended specifically on GSH because no activity was detected when γ-glutamylcysteine was used in place of GSH. In addition, YfcF showed GSH-dependent peroxidase activity toward cumene hydroperoxide (Table 1). The kinetic parameters of YfcF in GSH-CDNB conjugation determined at pH 6.5 and 30°C were as follows: k_{cat}, 0.11 s^{-1}; K_m^{GSH}, 0.24 mM; and K_m^{CDNB}, 1.1 mM. The K_m values were not so different from those of GST B1-1, but the k_{cat} value was only about 1% of that of GST B1-1 (*33*). The purified YfcG protein exhibited GSH-conjugation activity toward CDNB and GSH-dependent peroxidase activity toward cumene hydroperoxide also (Table 1), although the activities were so small that the kinetic parameters could not be determined. *E. coli* GST B1-1 is characteristic in exhibiting catalytic activity even at acidic pH as low as 4.5 (*33*). The activities of YfcF and YfcG, however, decreased as pH was lowered from neutral to acidic (data not shown) like many GSTs (*32*).

Ser16 Is Essential for Enzyme Activity of YfcF—The YfcF protein consists of 214 amino acid residues and has primary-structural identity of about 20% with GSTs of the Theta and Zeta classes, and has lower identity with those

Table 1. **Substrate specificity of YfcF and YfcG (at 30°C).**

Substrate (mM)	GSH (mM)	pH	Specific activity (μmol min^{-1} mg^{-1})		
			GST B1-1[a]	YfcF	YfcG
CDNB (1.0)[b]	1.0	6.5	10.1	0.12	0.04
1,2-Dichloro-4-nitrobenzene (1.0)[b]	5.0	7.5	nd	nd	nd
Ethacrynic acid (0.2)[b]	0.25	6.5	0.02	nd	nd
Cumene hydroperoxide (1.2)[c]	1.0	7.0	0.12	0.03	0.05
t-Butyl hydroperoxide (1.2)[c]	1.0	7.0	nd	nd	nd
Hydrogenperoxide (0.15)[c]	1.0	7.0	nd	nd	nd

[a]Nishida *et al*. (*20*). [b]GSH-conjugating activities assayed as described (*31*). [c]GSH-dependent peroxidase activity assayed as described (*32*). nd, not detected.

Fig. 2. **Sequence alignment of the N-terminal regions of YfcF (A) and YfcG (B) with their homologues.** A: Accesion numbers are as follows: *E. coli* YfcF, P77544; *S. typhimurium*, NP_804373; *Y. frederiksenii*, ZP_00830732; *Burkholderia* sp.383, YP_373103; *P. fluorescens*, YP_259509; *A. dehalogenans*, ZP_00401031; *C. violaceum*, NP_902694; Human GSTT1, P30711; Human GSTZ1, O43708. B: Accesion numbers are as follows: *E. coli* YfcG, P77526; *S. typhimurium*, NP_461291; *E. carotovora*, YP_051134; *A. vinelandii*, ZP_00418706; *P. fluorescens*, YP_258398; *M. magnetotactic*, ZP_00052753; *X. campestris*, YP_245111; *B. parapertussis*, NP_885111; *S. cerevisiae* Ure2, P23202; *S. pombe* Gst2, O59827; *S. pombe* Gst1, Q9Y7Q2. The secondary structures of human GSTZ1 (*40*) and *S. cerevisiae* Ure2 (*41*) are indicated with "e" for β-strand and and "h" for α-helix. The catalytic Ser residues of the Theta and Zeta GSTs (*34*, *42*) in the panel A and the Asn residue spatially located near the GSH thiol in Ure2 (*43*) are indicated with "#."

of the Beta and other classes. Genes encoding a protein that is >50% identical to YfcF, however, are found in some bacterial genomes (Fig. 2A), although none of the gene products has been characterized so far. YfcF and the bacterial homologues are thought to constitute a novel class of GST. Many cytosolic GSTs are known to lower pK_a of the thiol group of GSH by allowing the hydroxy group of a Tyr or Ser residue to interact with it (*5*, *34*). The catalytic Tyr or Ser residue is located on the loop between the first β-strand and the first α-helix of the GST fold. Sequence alignment of the N-termini indicates that Ser16 of YfcF is the only residue conserved in this region, and suggests that Ser16 corresponds to the catalytic Ser residue of the Theta and Zeta class GSTs (Fig. 2A). To examine whether or not Ser16 is important for catalytic activity, it was replaced with Gly by site-directed mutagenesis. The specific activity toward CDNB of the S16G mutant was less than 2% of that of the wild-type protein (Fig. 3). Since the CD spectra were indistinguishable between the wild-type and the S16G mutant proteins as shown in Fig. 4A, it is thought that the Gly mutation of Ser16 reduced the catalytic activity without altering the secondary structure. Ser16 was also replaced with Ala, but the mutant protein expressed was insoluble and could not be assayed. On the other hand, Ser10 was demonstrated to be nonessential for the catalytic activity because its Ala mutation did not decrease the enzyme activity (Fig. 3). Thus, Ser16 is essential for the catalytic activity of YfcF and is probably the counterpart of the catalytic Ser residue of the class Theta and Zeta GSTs.

Asn11 Is Essential for Enzyme Activity of YfcG—The YfcG protein consists of 215 amino acid residues. Genes encoding a protein >50% identical to YfcG are found in several bacterial genomes, although none of the gene products has been characterized. YfcG is about 30% identical to the GST-like domain of fungal Ure2 protein, a negative regulator of enzymes of nitrogen metabolism (*35*) with prion property (*36*). It has been reported that Ure2 lacks typical GST activity but has glutathione peroxidase activity (*37*). On the other hand, the YfcG homologues in *Schizosaccharomyces pombe*, Gst2 and Gst1 (24–31% in identity), are reported to have GST activity toward

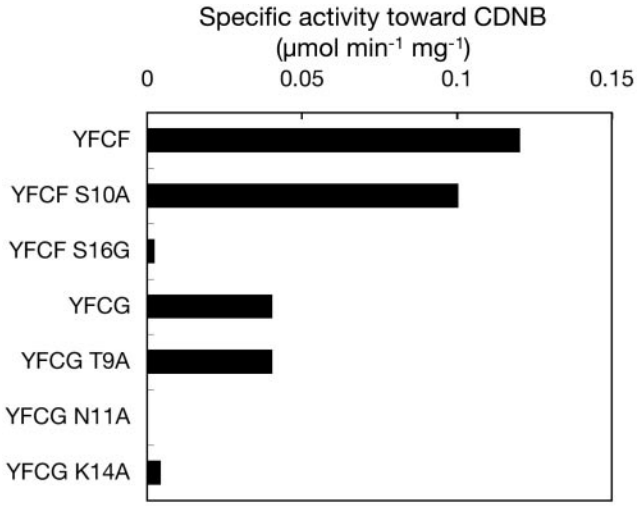

Fig. 3. **Effects of site-directed mutagenesis on enzyme activities of YFCF and YFCG.** Specific activity toward CDNB in the presence of 1 mM GSH and 1 mM CDNB at pH 6.5 and 30°C was determined.

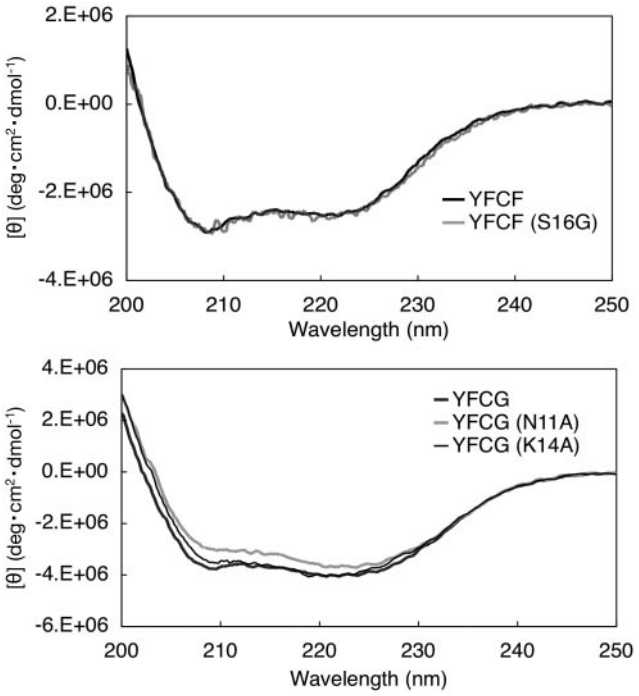

Fig. 4. **CD spectra.** Panel A, the spectra of wild-type YfcF and its S16A mutant. Panel B, the spectra of wild-type YfcG and its N11A and K14A mutants. The CD spectra were measured with a Jasco J-720 spectropolarimeter at 30°C using the water-jacketed quartz cell with a light path of 1 mm. The proteins were solved in 20 mM Tris/HCl (pH 8.0) containing 1.0 mM EDTA at the concentrations as follows: YfcF, 3.0 μM; YfcF(S16A), 4.0 μM; YfcG, 3.2 μM; YfcG(N11A), 2.8 μM; and YfcG(K14A), 3.2 μM.

CDNB and to play a role in response to oxidative stress also (*38*). In primary-structural comparison of the N-terminal region of YfcG with those of its homologues, only Asn11 is a completely conserved polar residue located on or around the putative loop region between the first β-strand and the first α-helix (Fig. 2B). When Asn11 was replaced with Ala by site-directed mutagenesis, no significant activity toward CDNB was detected. The Ala mutation of Thr9, however, which is conserved among the bacterial homologues but not in the fungal homologues, did not alter the activity. The CD spectrum of the N11A mutant was almost equivalent with that of the wild-type protein (Fig. 4B). Therefore, Asn11 is thought to be important for the catalytic mechanism rather than maintaining the folding structure. The Asn residue is conserved in all of the YfcG homologues as a P*N*X*X*K motif (Fig. 2B). The Ala mutation of Lys14 reduced the enzyme activity to ~10% level of the wild-type enzyme without altering the CD spectrum (Fig. 4B), but did not abolish the activity completely (Fig. 3). Therefore, Asn11 seems to be essential for the catalytic mechanism although Lys14 is important partly. YfcG and its bacterial homologues are thought to constitute another novel class of GST.

GST B1-1, YfcF, and YfcG Are Important for Defense against Oxidative Stress—Thus, *E. coli* was found to have GSTs of at least three distinct types including GST B1-1. Their biological roles, however, are unknown. Since all of them showed GSH-dependent peroxidase activity toward cumene hydroperoxide (Table 1), we examined whether or not the deletions of each gene increase the susceptibility to hydrogen peroxide in the bacterial growth. In the presence of 2–3 mM hydrogen peroxide, the growth rate of the wild-type bacteria (BW25113) was lowered to some extent (Fig. 5A). This growth inhibition was more remarkable in the cases of the mutant bacteria in which one of the three GST genes was deleted (Fig. 5, B, C, and D).

To confirm the importance of the GSTs for resistibility to hydrogen peroxide, the wild-type or mutant protein was expressed in each deletion mutant. When the bacteria transformed with the expression plasmid vector, pKK223-3, without any inserted DNA were cultured in the presence of 50 μg/ml ampicillin, they were more susceptible to hydrogen peroxide than each parent strain. By transformation of JW1627 (Δ*gst*), JW2298 (Δ*yfcF*), and JW2299 (Δ*yfcG*) with the expression plasmids for the respective deleted GSTs, the resistibility to hydrogen peroxide was increased in all the cases (Fig. 6). Neither of the transformations, JW2298 (Δ*yfcF*) with YfcF(S16G) and JW2299 (Δ*yfcG*) with YfcG(N11A), however, increased the resistibility. On the other hand, transformation of JW2299 (Δ*yfcG*) with YfcG(K14A), whose gene product retains ~10% enzyme activity, increased the resistibility to hydrogen peroxide. Thus, the enzyme activities of GST B1-1, YfcF, and YfcG are thought to participate in defense against growth inhibition caused by hydrogen peroxide.

DISCUSSION

E. coli has eight genes that code for proteins homologous to known cytosolic GSTs. Among the gene products, only GST B1-1 has been known to have GSH-dependent enzyme activities, *i.e.*, GST and GSH-dependent peroxidase activities (*20*). In this study, YfcF and YfcG as well as GST B1-1 were found to exhibit both the activities (Table I). Although the GST activity toward CDNB detected on YfcF and YfcG were only about 1% of that of GST B1-1, their GSH-dependent peroxidase activities toward cumene hydroperoxide were as high as 25–40% of that of GST

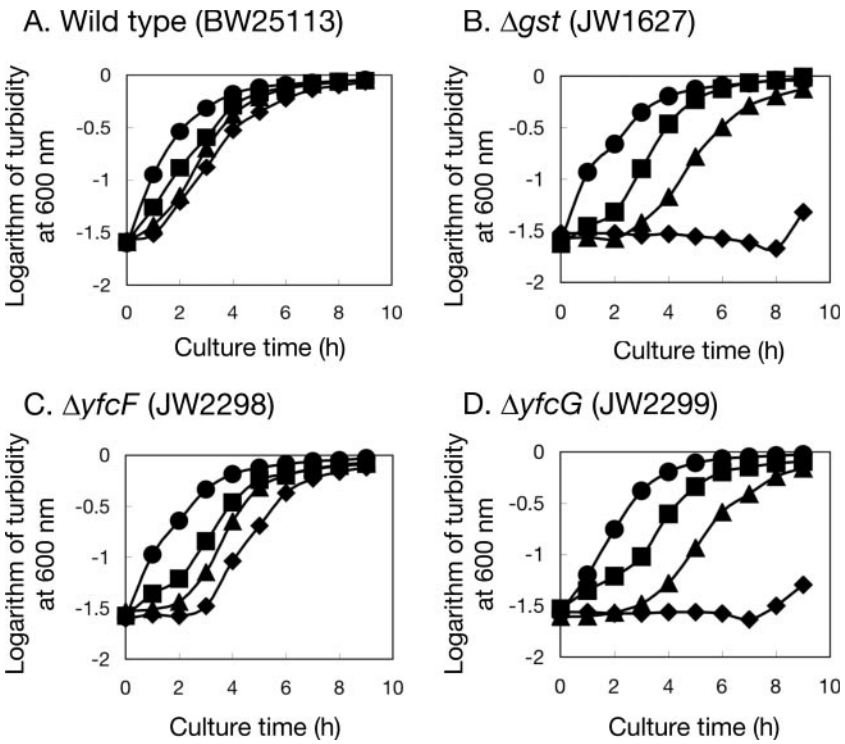

Fig. 5. **Effects of hydrogen peroxide on growth.** The parent strain and mutant strains were cultured in LB medium with shaking at 37°C. Culture was started with inoculating a 50 μl aliquot from the preculture into a fresh 5 ml LB medium containing 0–3.0 mM hydrogen peroxide. Logarithm of the turbidity at 600 nm, plotted on the ordinate, was measured every hour, plotted on the abscissa. A typical result is shown for each strain. Each plot was the average of duplicate samples. Panel A, BW25113, wild-type; B, JW1627, Δ*gst*; C, JW2298, Δ*yfcF*; D, JW2299, Δ*yfcG*. Closed circle, without hydrogen peroxide; closed square, 2.0 mM hydrogen peroxide; closed triangle, 2.5 mM hydrogen peroxide; and closed diamond, 3.0 mM hydrogen peroxide.

B1-1 (Table 1). Proteins >50% identical to YfcF or YfcG are encoded in some bacterial genomes (Fig. 2), but they have limited homology with the GSTs of the Beta and other classes already characterized. Therefore, each of YfcF and YfcG is thought to constitute a new GST class with its bacterial homologues, and this is the first time that the proteins of these groups are experimentally shown to have enzyme activity.

YfcF and YfcG are thought to be important at least for defense against oxidative stress for the reasons as follows: (i) the *yfcF*- and *yfcG*-deficient strains were more susceptible to hydrogen peroxide than the parent strain (Fig. 5); (ii) the *yfcF*- and *yfcG*-deficient bacteria were restored to be resistant to hydrogen peroxide by transformation with the expression plasmids for the wild-type YfcF and YfcG proteins, respectively (Fig. 6); and (iii) neither YfcF(S16G) nor YfcG(N11A), both of which had impaired catalytic activity, restored the knockout bacteria by transformation to be resistant to hydrogen peroxide (Figs. 4 and 5). In addition to YfcF and YfcG, GST B1-1 was demonstrated to be important for resistibility to hydrogen peroxide: deletion of the *gst* gene decreased the resistibility, which was restored by transformation with the expression plasmid for GST B1-1. We could not examine whether the restoration of the restibility required the enzyme activity of GST B1-1 because no mutant that abolished or greatly reduced the enzyme activity without altering the folding structure was available. The importance of *E. coli* GST B1-1 for defense against oxidative stress, however, is consistent with the report on *P. mirabilis* GST B1-1 (*24*). Thus, all the three GSTs were suggested to be important for defense against oxidative stress. Some eukaryotic GSTs are known to play a critical role in the system that protects against reactive oxygen species by catalyzing the breakdown of peroxidized lipid and oxidized DNA (*39*). Since all of GST B1-1, YfcF, and YfcG exhibited GSH-dependent peroxidase activity to a model substrate cumene hydroperoxide, their contributions to resistibility to hydrogen peroxide are presumed to depend on their peroxidase activity toward organic peroxides generated during oxidative stress, in analogy with the eukaryotic GSTs.

GST B1-1, YfcF, and YfcG are different from one another not only in the classification based on primary structure but also in the type of catalytic residue. The results with site-directed mutagenesis suggest that Ser16 of YfcF and Asn11 of YfcG are essential for both the enzyme activity (Fig. 3) and the resistibility of the host bacteria to hydrogen peroxide (Fig. 6). The corresponding Ser and Asn residues are conserved among the proteins homologous to YfcF and YfcG, respectively. Ser16 is thought to be the catalytic residue of YfcF and to correspond to the catalytic Ser of the Theta, Zeta, and Delta class GSTs (Fig. 2). On the other hand, Asn11 of YfcG is conserved among the homologues as a PN*XX*K motif, and Ala mutation of Lys14 reduced the enzyme activity also (Fig. 3). Although these facts suggest the importance of the Lys residue in the PN*XX*K motif, the YfcG(K14A) mutant retained ~10% activity of YfcG (Fig. 3) and its expression in the *yfcG*-deficient bacteria could restore the host bacteria resistible to hydrogen peroxide (Fig. 6). Therefore, the Asn residue in the PN*XX*K motif, rather than the Lys residue, is primarily important for catalytic mechanism. Thus, the Ser and Asn residues are thought to be the catalytic residue of YfcF and YfcG, respectively. On the other hand, no residue has been demonstrated to be essential for catalytic mechanism of GST B1-1. None of the side chains of Tyr5 and Ser11 is suggested to interact with the GSH thiol group (*21*), and analysis involving site-directed mutagenesis suggests that none of the side chains of Tyr-5 (*20*), Cys-10 (*33*), and Ser-11 (Inoue *et al.*, unpublished data) is essential for

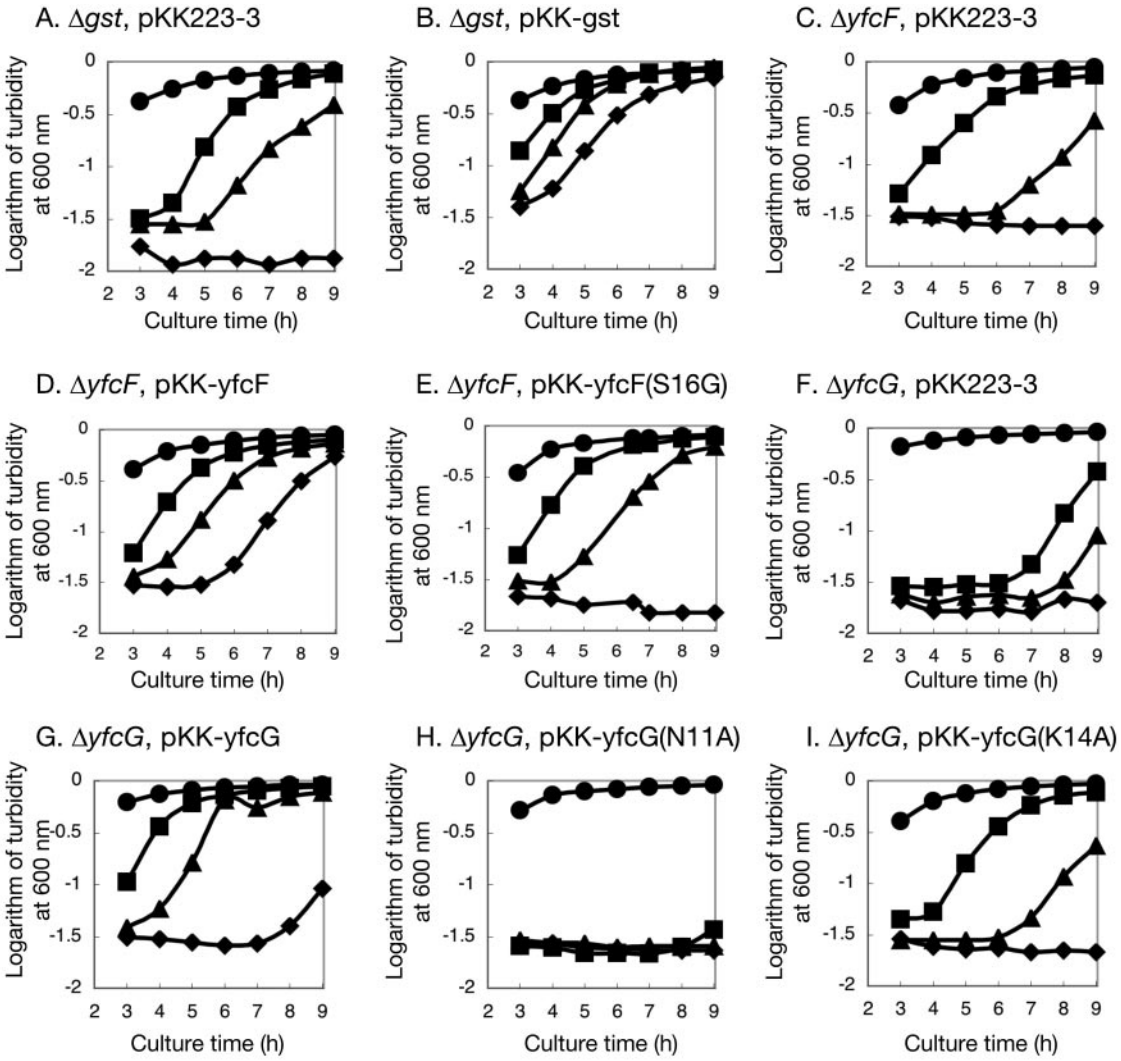

Fig. 6. **Rescue of the knockout bacteria by transformation.** The transformed bacteria were cultured in LB medium containing 50 μg/ml ampicillin with shaking at 37°C. Logarithm of the turbidity at 600 nm, plotted on the ordinate, was measured every hour, plotted on the abscissa. Panel A, JW1627 (Δ*gst*) transformed with a plasmid vector pKK223-3 (mock); B. JW1627 (Δ*gst*) transformed with an expression plasmid for GST B1-1; C, JW2298 (Δ*yfcF*) transformed with pKK223-3 (mock); D, JW2298 (Δ*yfcF*) transformed with an expression plasmid for YfcF; E, JW2298 (Δ*yfcF*) transformed with an expression plasmid for YfcF(S16G); F, JW2299 (Δ*yfcG*) transformed with pKK223-3 (mock); G, JW2299 (Δ*yfcG*) transformed with an expression plasmid for YfcG; H, JW2299 (Δ*yfcG*) transformed with an expression plasmid for YfcG(N11A); I, JW2299 (Δ*yfcG*) transformed with an expression plasmid for YfcG(K14A). The concentrations of hydrogen peroxide were as follows: closed circle, 0 mM; closed square, 2.0 mM; closed triangle, 2.5 mM; and closed diamond, 3.0 mM.

catalytic activity, distinct from YfcF, YfcG, and the other class GSTs.

In conclusion, *E. coli* has three GSTs of distinct classes at least, which have different catalytic residues. In spite of the diversity, they are thought to commonly function in defense against oxidative stress. This seems consistent with the hypothesis that GSTs constitute a very ancient protein superfamily that has evolved from a thioredoxin-like ancestor in response to the development of oxidative stress (*6*).

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