JOURNAL OF BACTERIOLOGY, Sept. 2005, p. 5861–5867 Vol. 187, No. 17
0021-9193/05/$08.00+0 doi:10.1128/JB.187.17.5861–5867.2005

The *yliA*, *-B*, *-C*, and *-D* Genes of *Escherichia coli* K-12 Encode a Novel Glutathione Importer with an ATP-Binding Cassette

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Received 28 April 2005/Accepted 30 May 2005

Glutathione protects cells and organisms from oxygen species and peroxides and is indispensable for aerobically living organisms. Moreover, it acts against xenobiotics and drugs by the formation and excretion of glutathione *S* conjugates. In this study, we show that the *yliA*, *-B*, *-C*, and *-D* genes of *Escherichia coli* K-12 encode a glutathione transporter with the ATP-binding cassette. The transporter imports extracellular glutathione into the cytoplasm in an ATP-dependent manner. This transporter, along with γ-glutamyltranspeptidase, has an important role in *E. coli* growth with glutathione as a sole sulfur source.

Glutathione (γ-glutamylcysteinylglycine; GSH) plays a leading role in the protection of cells and organisms by reducing oxygen species and peroxides and acts against xenobiotics and drugs by the formation and excretion of glutathione *S* conjugates (16). Because of its protective effect on organs, glutathione has been dispensed to patients with hepatic diseases in Japan. Glutathione and its derivatives are moved in and out of cells by transporters of several types. The glutathione importers Na^+-dependent transporter (10) and H^+ symporter (9) are known, and the only glutathione transporters with the ATP-binding cassette identified so far are glutathione *S* conjugate exporters (4). As for microbial glutathione transporter, *Saccharomyces cerevisiae* YCF1 of the vacuolar membrane is known as a glutathione *S*-conjugate transporter (14), which also transports reduced glutathione into vacuoles (20), and yeast HGT1 of the plasma membrane is known as a membrane-potential-dependent glutathione importer across the plasma membrane (3). However, there has been no report on the identification of bacterial glutathione transporter.

It has been shown that *Escherichia coli* cells grown in aerobic conditions contain a large amount of glutathione (7). *E. coli* synthesizes glutathione by the sequential actions of γ-glutamylcysteine synthetase (the product of *gshA*) and glutathione synthetase (the product of *gshB*) as in other organisms (1). It excretes glutathione into the culture medium during the exponential phase and the concentration of glutathione in the culture medium reaches maximum in the early stationary phase (18, 26), but thereafter, it is hydrolyzed by γ-glutamyltranspeptidase (GGT) in the periplasm to liberate glutamic acid and cysteinylglycine (22, 26). Cysteinylglycine is taken up into the cytoplasm and then cleaved into cysteine and glycine by aminopeptidases A, B, and N and dipeptidase D to be utilized as a source of cysteine and glycine (25). We have suggested that this is the salvage pathway of cysteine for *E. coli* (23). It was also shown by other researchers in a mammalian cell line (8) and in yeast (15) that GGT catalyzes the initial step of the cleavage of extracellular glutathione for use as a source of cysteine and nitrogen. However, even in the case of a GGT-deficient strain of *E. coli*, the concentration of glutathione in the culture medium decreased gradually after prolonged incubation. This finding prompted us to search for a glutathione transporter which had never been reported in bacteria.

The *ybiK* gene was reported as a member of the *cysB* regulon and it was suggested to encode a protein involved in glutathione transport or metabolism (19), but its mechanism is still unclear. GenBank suggests that four genes, *yliA*, *-B*, *-C*, and *-D*, located downstream of *ybiK*, are transcribed with *ybiK* (Fig. 1). EcoCys (11) suggests that YliA, -B, -C, and -D are uncharacterized members of the ATP-binding cassette superfamily transporters. It suggests that *yliA* and *-B* encode the ATP-binding component and periplasmic binding protein, respectively, and that *yliC* and *-D* encode the plasma membrane components. From the above information, we speculated that YliA, -B, -C, and -D might encode the glutathione transporter.

In this report, we present evidence indicating that *yliA*, *-B*, *-C*, and *-D* indeed encode a novel type of glutathione transporter.

MATERIALS AND METHODS

Bacterial strains, phage, plasmids, and oligonucleotides. The bacterial strains, phage, plasmids, and oligonucleotides used in this study are listed in Table 1. Luria-Bertani (LB) broth (Miller) (17) purchased from Becton Dickinson was used regularly. M9 glucose medium (17) was used as a minimal medium. $MgCl_2$ was used instead of $MgSO_4$ when the effect of Δ*yliAB* mutation on the cell growth was measured. Ampicillin, tetracycline, and kanamycin were added at 100, 10, and 30 μg/ml, respectively, when appropriate. The bacteria were grown at 37°C with reciprocal shaking at 100 rpm.

Strain and plasmid construction. P1 transduction, DNA manipulation, and transformation were performed by the standard methods (17, 21). Gene disruption was performed according to the method of Datsenko and Wanner (5). Briefly, a Δ*yliAB* strain was constructed as follows. The DNA fragment of Kohara clone (12) #208 containing the *yliA* gene region was cloned onto pUC18 to obtain pSH1517. The FRT-Kan^r-FRT fragment was amplified by PCR using oligonucleotides pKD13-1 and pKD13-4 as primers and pKD13 as a template with ExTaq DNA polymerase (Takara, Kyoto, Japan). The fragment was blunt ended with a blunting kit (Takara, Kyoto, Japan) and ligated with the 3.1-kb NruI and SacII (blunt-ended) fragment of pSH1517, which deleted most of the *yliA*

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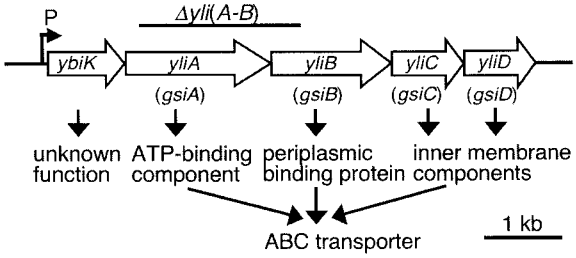

FIG. 1. The structure of the *ybiK-yliABCD* operon. The order, size, and products of the genes and the location of the promoter suggested by GenBank and EcoCys were diagrammed. The region of DNA deleted in our Δ*yliAB* mutation is shown by the bar above the genes. Gene names in parentheses are the new names proposed, after glutathione importer.

gene and the N-terminal region of *yliB* (Fig. 1). The obtained plasmid was cleaved with EcoRI and HindIII, and the 1.8-kb fragment was used to transform strain TK251 by electroporation. Kanamycin-resistant transformant SI26 was obtained. The Δ*yliAB*::Kan^r strain was transduced into the strains with appropriate backgrounds by the P1*vir* phage. The transductants were transformed with pCP20 at 30°C to eliminate the Kan^r determinant. Then, pCP20 was cured by incubating the strains at 37°C. The deletion made on the genome was confirmed by colony PCR using oligonucleotides yliA-1 and yliA-2.

Δ*gshA*::Kan^r and Δ*gshA* were constructed similarly. The DNA fragment containing the *gshA* gene region was amplified by PCR using oligonucleotides −286 and +1746 as primers and genomic DNA of *E. coli* as a template. The fragment was cleaved with PvuI and PstI and ligated with pBR322 cleaved with the same restriction endonucleases to obtain pFK68. The blunt-ended FRT-Kan^r-FRT fragment obtained above was ligated with the 5.4-kb BglII (blunt-ended) and BssHII (blunt-ended) fragment of pFK68. The obtained plasmid was cleaved with AseI and ScaI, and the 2.6-kb fragment was used to transform strain TK251 by electroporation. Kanamycin-resistant transformant SI95 was obtained. Δ*gshA*::Kan^r was moved into the strain SI35, and Kan^r determinant was eliminated as described above. The deletion made on the genome was confirmed by colony PCR using oligonucleotides gshA-1 and gshA-2.

The plasmids with the *yliA*, *-B*, or *-C* gene deleted from pSH1596 were constructed as follows. The NcoI site was inserted 3 bp after the stop codon of *yliD* of pSI152 to obtain pSH1569 with yliD-E NcoI and yliD-E NcoI-comp as mutagenic primers by the QuikChange method (Stratagene) except KOD plus DNA polymerase (Toyobo, Osaka, Japan) was used. The DNA fragment between the AatII and NcoI sites, located upstream of *ybiK*, was deleted from pSH1569, and pSH1584 was made. The DNA fragment containing the coding region of the *lacZ* gene was amplified by PCR using oligonucleotides LacZ-fusion-up and LacZ-fusion-down and pMC1871 as a template. The fragment was cleaved with NcoI and HindIII and ligated with pSH1584 cleaved with the same restriction endonucleases to obtain pSH1596. Then, *yliA* gene was looped out from pSH1596 using oligonucleotides delyliA and delyliA-comp as mutagenic primers by the QuikChange method. The correctness of the DNA sequence of the mutated plasmid (pSH1597) was confirmed. DH5α was transformed with pSH1597, and the colonies of the transformant formed on the LB plate supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were blue, as well as the DH5α transformed with pSH1596. This indicates that the Δ*yliA* deletion does not interfere with the transcription of the downstream genes of the operon, as intended. The *yliB* and *-C* genes were deleted from pSH1596 similarly. This Δ*yliA* deletion extends from the fifth base from the stop codon of *ybiK* to the base just before the stop codon of *yliA*. The Δ*yliB* deletion extends from the base just after the initiation codon of *yliB* to the initiation codon of *yliC*. The Δ*yliC* deletion extends from the initiation codon of *yliC* to the base just before the initiation codon of *yliD*.

Measurement of glutathione. Reduced glutathione was measured using standard compounds (Sigma-Aldrich) with a high-pressure liquid chromatography (HPLC) instrument (model LC-9A; Shimadzu, Kyoto, Japan) equipped with a Shim-pack Amino-Na column and a fluorescence detector (model RF-535; Shimadzu), with *o*-phthalaldehyde as the detection reagent (24). Reduced and oxidized glutathione could be measured separately by this method. Total glutathione was measured with glutathione reductase (Sigma-Aldrich) by the method of Fahey et al. (6).

Transport assay. Transport assay was performed as described previously (13) using [^{35}S]glutathione (final concentration, 2 nM; 35.4 Tbq/mmol; PerkinElmer) except M9 glucose medium was used instead of M63 medium. When the effects of verapamil (Nacalai Tesque, Kyoto Japan) and carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) (Nacalai Tesque, Kyoto Japan) were determined, cells were preincubated in the presence of these chemicals for 30 min at 37°C prior to the addition of labeled glutathione.

RESULTS

Effect of Δ*ggt* and Δ*yliAB* on the concentration of extracellular glutathione. Since the wild-type *E. coli* accumulates only several μM glutathione at a maximum in the medium and it is difficult to measure such a low concentration of glutathione, the strains were transformed with a plasmid, pSH1391, which contains the *gshA* and *gshB* genes on pBR322, to overproduce glutathione-synthesizing enzymes. There was little difference in the growth among the strains used. The effects of Δ*ggt* and Δ*yliAB* on the concentration of extracellular glutathione were compared (Fig. 2). The concentration decreased after reaching the maximum during the early stationary phase when either *ggt* or *yliAB* was normal. That Δ*ggt yliA*$^+$ *yliB*$^+$ strain accumulates much more glutathione in the medium than the *ggt*$^+$ Δ*yliAB* strain indicates that GGT is more effective in reducing the concentration of extracellular glutathione than the YliABCD transporter. On the other hand, the extracellular glutathione of the Δ*ggt* Δ*yliAB* strain gradually increased even during the stationary phase. When the Δ*ggt* Δ*yliAB* strain was complemented with pACYC177 containing the *ybiK*$^+$-*yliA*$^+$ *yliB*$^+$ *yliC*$^+$ *yliD*$^+$ operon, the extracellular GSH was dramatically decreased.

Transport assay of the YliABCD transporter. Transport assay was performed using [^{35}S]glutathione and GGT-deficient derivatives (Fig. 3). The Δ*yliAB* strain transported practically no glutathione, while its *yliA*$^+$ *yliB*$^+$ derivative obviously transported glutathione. Moreover, Δ*yliAB* strain transformed with pACYC177 containing *ybiK*$^+$-*yliA*$^+$ *yliB*$^+$ *yliC*$^+$ *yliD*$^+$ complemented the GSH transport phenotype. However, the same strain transformed with pACYC177 containing *ybiK*$^+$-*yliA*$^+$ *yliB*$^+$ *yliC*$^+$ did not complement the phenotype (Fig. 3A).

The plasmids containing *ybiK*$^+$-*yliA*$^+$ *yliB*$^+$ *yliC*$^+$ *yliD*$^+$ but with *yliA*, *-B*, or *-C* deleted were also constructed. The Δ*yliAB* strain was transformed with these plasmids, and a transport assay was performed. Transport of glutathione was not observed when the operon deleted either *yliA*, *-B*, or *-C* (Fig. 3B).

Effect of Δ*yliAB* mutation on the intracellular concentration of glutathione. Glutathione-synthesis-deficient (Δ*gshA*) derivatives of the above strains were grown overnight in the minimal medium supplemented with or without 1 mM reduced glutathione. The cells were then opened by ultrasonication, and the amount of glutathione accumulated inside the cells was measured by HPLC (Fig. 4). All the glutathione found was in reduced form, and no oxidized form was observed. The amount of total glutathione measured with glutathione reductase agreed well with that of reduced glutathione found by HPLC (data not shown). When these four strains were grown in the minimal medium without glutathione, no detectable glutathione was found inside these strains. Although Δ*yliAB* mutation decreased the accumulation of glutathione inside the cells, nonnegligible accumulation of glutathione was observed even in the strain with Δ*yliAB* Δ*ggt* Δ*gshA* (strain SI100). The

TABLE 1. Bacterial strains, phage, plasmids, and oligonucleotides used in this study

Component	Description or sequence	Source or reference
Strains		
JJP150	F$^-$ ϕ*ybiK-lacZ* Δ*ybiK*::Kan^r *cysA nupC*::Tn*10 araD139* Δ(*argF-lac*)*U169 rpsL150 deoC1 relA1 thiA ptsF25 ftb5301*	D. P. Clark (19)
MG1655	F$^-$ prototrophic	C. A. Gross
SH639	F$^-$ Δ*ggt-2*	Spontaneous Tet^s mutant of SH703 selected on fusaric acid plate; laboratory stock
SH702	F$^-$ *zhg*::Tn*10*	26
SH703	F$^-$ Δ*ggt-2 zhg*::Tn*10*	26
SH1504	F$^-$ Δ*ggt-2 cysA nupC*::Tn*10*	SH639 × P1(JJP150); this work
SH1525	SH1504 but Δ*yliAB*::Kan^r	SH1504 × P1(SI26); this work
SH1527	MG1655 but *cysA nupC*::Tn*10*	MG1655 × P1(JJP150); this work
SH1535	SH1527 but Δ*yliAB*::Kan^r	SH1527 × P1(SI26); this work
SH1552	pSI152/SI35	This work
SH1554	pSI151/SI35	This work
SH1555	pSI152/SI37	This work
SH1617	pSH1596/SI35	This work
SH1618	pSH1597/SI35	This work
SH1619	pSH1599/SI35	This work
SH1620	pSH1616/SI35	This work
SI26	TK251 but Δ*yliAB*::Kan^r	This work
SI28	SH703 but Δ*yliAB*::Kan^r	SH703 × P1(SI26); this work
SI35	SH703 but Δ*yliAB*	SI28 was transformed with pCP20 and grown at 37°C; this work
SI37	pSH1391/SI35	This work
SI49	pSH1391/SH703	This work
SI95	TK251 but Δ*gshA*::Kan^r	This work
SI96	SI35 but Δ*gshA*::Kan^r	SI35 × P1(SI95); this work
SI97	SI35 but Δ*gshA*	SI96 was transformed with pCP20 and grown at 37°C; this work
SI99	pSI80/SI97	This work
SI100	pSI83/SI97	This work
SI101	SH702 but Δ*yliAB*::Kan^r	SH702 × P1(SI26); this work
SI102	SH702 but Δ*yliAB*	SI101 was transformed with pCP20 and grown at 37°C; this work
SI103	pSH1391/SI102	This work
SI104	pSH1391/SH702	This work
SI105	SH703 but Δ*gshA*::Kan^r	SH703 × P1(SI95); this work
SI106	SH703 but Δ*gshA*	SI105 was transformed with pCP20 and grown at 37°C; this work
SI109	pSI83/SI106	This work
SI153	pSI151/SI97	This work
SI154	pSI152/SI97	This work
TK251	pKD46/MG1655	This work
Phage Kohara #208		Y. Kohara (12)
Plasmids		
pCP20	pSC101 replicon (ts) *bla*$^+$ *cat*$^+$ Flp (λRp) *cI857*	CGSC[a] (5)
pFK68	ColEI replicon *rop*$^+$ Tet^r Amp^s *gshA*$^+$; 2.1-kb DNA fragment containing *gshA* was ligated with pBR322 cleaved with PvuI (blunt ended) and PstI (blunt ended)	This work
pKD13	*oriR*γ *bla*$^+$ FRT-Kan^r-FRT	CGSC (5)
pKD46	*oriR101* replicon *repA101*^{ts} *araC*$^+$ *gam*$^+$-*bet*$^+$-*exo*$^+$ (*araBp*) *bla*$^+$	CGSC (5)
pMC1871	ColEI replicon *rop*$^+$ Tet^r *'lacZ*	Pharmacia
pSH1391	ColEI replicon *rop*$^+$ Tet^s Amp^s Kan^r *gshA*$^+$ *gshB*$^+$	Laboratory stock
pSH1517	ColEI replicon Amp^r *yliA*$^+$; 2.3-kb CspI fragment (blunt ended) of Kohara #208-λDNA was ligated with pUC18 cleaved with HincII	This work
pSH1569	pSI152 but the NcoI site was inserted 3 bp after the stop codon of *yliD*	This work
pSH1584	pSH1569 but deleted DNA between the AatII (blunt-ended) site and NcoI site which locate upstream of *ybiK*	This work
pSH1596	p15A replicon *bla*$^+$ Kan^s *ybiK*$^+$-*yliA*$^+$ *yliB*$^+$ *yliC*$^+$ *yliD*$^+$-*lacZ*$^+$; *lacZ* fragment amplified by P CR using oligonucleotides LacZ-fusion-up and LacZ-fusion-down, and pMC1871 as a template was ligated with pSH1584 cleaved with NcoI and HindIII	This work
pSH1597	p15A replicon *bla*$^+$ Kan^s *ybiK*$^+$-*yliB*$^+$ *yliC*$^+$ *yliD*$^+$-*lacZ*$^+$; DNA region corresponds to the coding region of *yliA* looped out from pSH1596 using oligonucleotides delyliA and delyliA-comp	This work

Continued on following page

TABLE 1—*Continued*

Component	Description or sequence	Source or reference
pSH1599	p15A replicon *bla*$^+$ Kan^s *ybiK*$^+$-*yliA*$^+$ *C*$^+$ *D*$^+$-*lacZ*$^+$; DNA region corresponds to the coding region of *yliB* looped out from pSH1596 using oligonucleotides delyliB and delyliB-comp	This work
pSH1616	p15A replicon *bla*$^+$ Kan^s *ybiK*$^+$-*yliA*$^+$ *yliB*$^+$ *yliD*$^+$-*lacZ*$^+$; DNA region corresponds to the coding region of *yliC* looped out from pSH1596 using oligonucleotides delyliC and delyliC-comp	This work
pSI80	p15A replicon *bla*$^+$ Kan^s *ybiK*$^+$-*yliA*$^+$ *yliB*$^+$ *yliC*$^+$ *yliD*$^+$ *yliE*$^+$ *yliF*$^+$ *yliG*$^+$; 13.5-kb AatI (blunt-ended) fragment of Kohara #208-λDNA was ligated with pACYC177 cleaved with BamHI (blunt ended) and XhoI (blunt ended)	This work
pSI83	p15A replicon *bla*$^+$ Δ*kan*; pACYC177 was cleaved with BamHI (blunt ended) and XhoI (blunt ended) and self-ligated	This work
pSI151	p15A replicon *bla*$^+$ Kan^s *ybiK*$^+$-*yliA*$^+$ *yliB*$^+$ *yliC*$^+$; pSI80 was cleaved with EcoRI (blunt ended) and HindIII (blunt ended) and self-ligated	This work
pSI152	p15A replicon *bla*$^+$ Kan^s *ybiK*$^+$-*yliA*$^+$ *yliB*$^+$ *yliC*$^+$ *yliD*$^+$; pSI80 was cleaved with SalI (blunt ended) and HindIII (blunt ended) and self-ligated	This work
Oligonucleotides		
pKD13-1	GTGTAGGCTGGAGCTGCTTC	
pKD13-4	ATTCCGGGGATCCGTCGACC	
yliA-1	AGTCTGCAACGCGGTGAGA	
yliA-2	GCTGAGAACGGCTGTTTGAGG	
yliD-E NcoI	GGATCCGAAAATTAAAGGATAGTTACCATGGAATATTGCTTG AAAGGGTAATCACC	
yliD-E NcoI-comp	GGTGATTACCCTTTCAAGCAATATTCCATGGTAACTATCCTTT AATTTTCGGATCC	
LacZ-fusion-up	CCGCCGGCGCCATGGTCGTTTTACAACGTCGTGACTGGG[b]	
LacZ-fusion-down	GGGAAGCTTATTATTAATGATGATGATGATGATGTTTTTGACA CCAGACCAACTGGTAATGG	
delyliA	GCCACACAGTGATGAATAACATTCAGGCGGAG	
delyliA-comp	CTCCGCCTGAATGTTATTCATCACTGTGTGGC	
delyliB	CAGGCGGAGAATAAAATGCTTAATTACGTTATCAAACG	
delyliB-comp	CGTTTGATAACGTAATTAAGCATTTTATTCTCCGCCTG	
delyliC	CAACGCAGGGAGTGGAATGCGACTATTTAACTGG	
delyliC-comp	CCAGTTAAATAGTCGCATTCCACTCCCTGCGTTG	
−286	GGGCGATCGCCATTGCGTAAAACATCGCGC	
+1746	GGGAACTGCAGGCGCTTCCATC	
gshA-1	ATTGTGAAGATAGTTTACTGACTAGA	
gshA-2	GGTAAAGCGTTACGCTATG	

[a] CGSC, *E. coli* Genetic Stock Center.
[b] Underlining shows the NcoI site at the initiation codon.

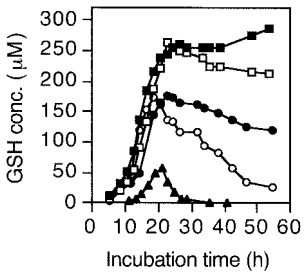

FIG. 2. Glutathione concentration of the culture media. Strains SI37 (Δ*ggt* Δ*yliAB*) (filled square), SI49 (Δ*ggt*) (open square), SH1555 (pACYC177::*ybiK*$^+$-*yliA*$^+$ *yliB*$^+$ *yliC*$^+$ *yliD*$^+$/Δ*ggt* Δ*yliAB*) (filled triangle), SI103 (Δ*yliAB*) (filled circle), and SI104 (*ggt*$^+$ *yliA*$^+$ *yliB*$^+$) (open circle) were grown in 100 ml minimal medium. At the times indicated, 2 ml of culture was subtracted. An optical density at 610 nm was measured using 1 ml of the 2-ml culture. Another 1 ml was centrifuged, and the concentration of glutathione of the culture fluid was measured with glutathione reductase.

Δ*yliAB* mutation was complemented with pACYC177 containing *ybiK*$^+$-*yliA*$^+$ *yliB*$^+$ *yliC*$^+$ *yliD*$^+$ (strain SI154), but not with pACYC177 containing *ybiK*$^+$-*yliA*$^+$ *yliB*$^+$ *yliC*$^+$ (strain SI153).

Transport of glutathione by the YliABCD transporter depends on ATPase activity. Effect of the ATPase inhibitor verapamil on glutathione transport by the YliABCD transporter was determined. Transport of glutathione by the YliABCD transporter was strongly inhibited in the presence of 10 mM verapamil (Fig. 5). Membrane potential inhibitor, CCCP, had no effect at 100 μM (data not shown).

Effect of the Δ*yliAB* mutation on cell growth. The ability of the YliABCD transporter to utilize glutathione as a sole sulfur source was also investigated. The *cysA* gene encodes sulfate permease, and a *cysA* mutant cannot grow with SO_4^{2-} in the medium and it is a cysteine auxotroph. The *cysA* Δ*ggt* (sulfate transport and GGT-deficient) strain grew weakly on minimal medium with 0.3 mM glutathione as a sole sulfur source (Fig. 6b, row 4), while almost no growth was observed for its Δ*yliAB* derivative on the same plate (Fig. 6b, row 3). The doubling times of the *cysA*, *cysA* Δ*yliAB*, *cysA* Δ*ggt*, and *cysA* Δ*yliAB* Δ*ggt*

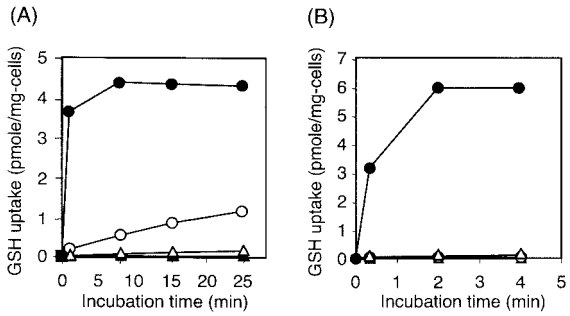

FIG. 3. Glutathione uptake in a transporter assay. (A) Strains SI35 (Δ*ggt* Δ*yliAB*) (filled triangles), SH703 (Δ*ggt*) (open circles), SH1552 (pACYC177::*ybiK*$^+$-*yliA*$^+$ *yliB*$^+$ *yliC*$^+$ *yliD*$^+$/Δ*ggt* Δ*yliAB*) (filled circles), and SH1554 (pACYC177::*ybiK*$^+$-*yliA*$^+$ *yliB*$^+$ *yliC*$^+$/Δ*ggt* Δ*yliAB*) (open triangles). (B) Strains SH1617 (pACYC177::*ybiK*$^+$-*yliA*$^+$ *yliB*$^+$ *yliC*$^+$ *yliD*$^+$-*lacZ*$^+$/Δ*ggt* Δ*yliAB*) (filled circles), SH1618 (pACYC177::*ybiK*$^+$-*yliB*$^+$ *yliC*$^+$ *yliD*$^+$-*lacZ*$^+$/Δ*ggt* Δ*yliAB*) (open circles), SH1619 (pACYC177::*ybiK*$^+$-*yliA*$^+$ *yliC*$^+$ *yliD*$^+$-*lacZ*$^+$/Δ*ggt* Δ*yliAB*) (filled triangles), and SH1620 (pACYC177::*ybiK*$^+$-*yliA*$^+$ *yliB*$^+$ *yliD*$^+$-*lacZ*$^+$/Δ*ggt* Δ*yliAB*) (open triangles).

strains in minimal medium supplemented with 0.3 mM glutathione at 37°C were 1.7, 2.1, 3.2, and 12 h, respectively.

DISCUSSION

Our previous study of glutathione metabolism in *E. coli* indicated that catabolism of extracellular glutathione is initiated by the cleavage of its γ-glutamyl linkage by GGT (22, 26). However, the concentration of extracellular glutathione decreased gradually after prolonged incubation even when the *ggt* gene was deleted (Fig. 2, SI49) and the existence of a glutathione importer was suspected. As described in the introduction, we predicted that YliABCD is a glutathione importer. When both Δ*ggt* and Δ*yliAB* were deleted, the concentration of the extracellular glutathione did not decrease up to 55 h (Fig. 2, SI37). These results indicate that GGT and the YliABCD transporter are the two determinants that decrease the extracellular glutathione. Since *yliA* and *-B* are located upstream of *yliC* and *-D* and the promoter of the operon before *ybiK* (Fig. 1), the complementation test was performed using the plasmid containing whole operon.

A transport assay was performed using [^{35}S]glutathione and

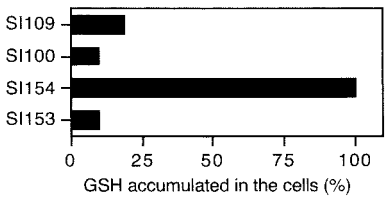

FIG. 4. Accumulation of glutathione in the cells grown in minimal medium supplemented with 1 mM glutathione. Strains SI100 (pACYC177/Δ*gshA* Δ*ggt* Δ*yliAB*), SI109 (pACYC177/Δ*gshA* Δ*ggt*), SI153 (pACYC177::*ybiK*$^+$-*yliA*$^+$ *yliB*$^+$ *yliC*$^+$/Δ*gshA* Δ*ggt* Δ*yliAB*), and SI154 (pACYC177::*ybiK*$^+$-*yliA*$^+$ *yliB*$^+$ *yliC*$^+$ *yliD*$^+$/Δ*gshA* Δ*ggt* Δ*yliAB*) were grown in minimal medium supplemented with 1 mM reduced glutathione for 12 h. The amount of glutathione found in the cells was expressed as relative to that for strain SI154. Seventy-four nanomoles of glutathione per mg of cells was found in strain SI154.

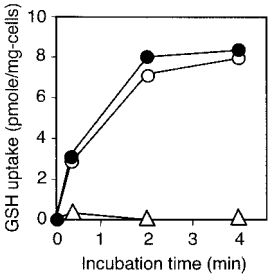

FIG. 5. Effect of verapamil on glutathione uptake. Glutathione uptake of strain SH1552 was measured in the absence of verapamil (filled circles) and in the presence of 1 mM (open circles) and 10 mM verapamil (open triangles).

GGT-deficient derivatives (Fig. 3). This is because GGT cleaves glutathione in the periplasm and cysteinylglycine uptake into the cytoplasm occurs. In fact, a *ggt*$^+$ Δ*yliAB* strain took up a nonnegligible amount of ^{35}S in a transport assay (data not shown). To avoid an overestimation of glutathione uptake by the transporter, GGT-deficient strains were used in this experiment. It was shown that uptake of ^{35}S into the cells depends on the YliABCD transporter in GGT-deficient strains (Fig. 3A). The plasmid with *yliA*$^+$ *yliB*$^+$ *yliC*$^+$ *yliD*$^+$ could complement the Δ*yliAB* mutation, but the plasmid with *yliA*$^+$ *yliB*$^+$ *yliC*$^+$ could not (Fig. 3A). This result strongly suggests that this Δ*yliAB* mutation has a polar effect on the downstream genes. Therefore, as shown in Fig. 3B, only one of the *yliA*, *yliB*, and *yliC* genes was carefully deleted with the intent of not causing a polar effect on the downstream genes. None of the deletions caused a polar effect, confirmed by the expression of the *lacZ* gene inserted just after the *yliD* gene. Our findings indicate that YliA, -B, -C, and -D are the components of the transporter because the transport activity was abolished if any one of them was deleted (Fig. 3A and B). The *ybiK* gene exists in front of *yliABCD*, and the possibility that they constitute an operon has been suggested (Fig. 1). Since the function of YbiK is not clear and its involvement in glutathione transport or metabolism has been suggested (19), there is a possibility that YbiK processes glutathione and then only a part of its mole-

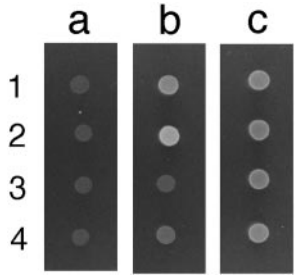

FIG. 6. Growth of *E. coli* strains on a minimal medium plate supplemented with glutathione as the sole sulfur source. Strains were grown overnight in 5 ml LB medium, washed twice, and suspended with 5 ml of 1× M9 buffer. One μl of cell suspension was plotted on plates and grown overnight at 37°C. Strains 1 (SH1535; *cysA* Δ*yliAB*::Kan^r), 2 (SH1527; *cysA*), 3 (SH1525; *cysA* Δ*ggt* Δ*yliAB*::Kan^r), and 4 (SH1504; *cysA* Δ*ggt*) were grown on minimal medium supplemented with 0.05 mM thiamine without any sulfur source (column a) or with 0.3 mM glutathione (column b) or 0.3 mM cysteine (column c) as a sole sulfur source.

cule containing ^{35}S is taken up into the cell. To deny this possibility and to show that the whole glutathione molecule is taken up by the YliABCD transporter, the concentration of glutathione accumulated inside glutathione-synthesis-deficient mutants grown in medium supplemented with glutathione was measured (Fig. 4). These results clearly show that the whole glutathione molecule was transported into the cell by the YliABCD transporter. Parry and Clark suggested the involvement of YbiK in glutathione transport or metabolism (19). Since they used strains with the *ybiK*::Kan^r mutation, which has polar effect on *yliA*, *-B*, *-C*, and *-D*, their suggestion of the involvement of YbiK in glutathione transport might have been derived from this polar effect. A study on the role of YbiK is under way, and the results will be reported elsewhere.

Transport of glutathione was inhibited by verapamil, but not by CCCP. This indicates that this transport system depends on ATPase activity and not on membrane potential (Fig. 5).

To determine whether the transport of glutathione by the YliABCD transporter has physiological meaning, the effect of its absence on the growth of *E. coli* by using glutathione as a sole sulfur source was observed. The *cysA* mutant grew on a minimal medium plate supplemented with glutathione as a sole sulfur source (Fig. 6b, row 2). The growth of the *cysA* Δ*yliAB* strain (Fig. 6b, row 1) was obviously weaker than that of the *cysA* strain. The growth of the *cysA* Δ*ggt* strain was severely retarded (Fig. 6b, row 4), and almost no growth was observed for the *cysA* Δ*ggt* Δ*yliAB* strain on the same plate (Fig. 6b, row 3). The growth of cells in column c (minimum medium supplemented with cysteine as a sulfur source (Fig. 6c) was less than that of the *cysA* mutant on minimum medium supplemented with glutathione (Fig. 6b, row 2). We should mention that the addition of this much cysteine inhibits cell growth. The doubling time of these strains in the liquid minimal medium was compared, and the results indicated that both GGT and the YliABCD transporter are important in the growth of *E. coli* with glutathione as a sole sulfur source.

Although no detectable glutathione uptake was observed in the Δ*ggt* Δ*yliAB* strain by transport assay, there was some accumulation of glutathione inside the Δ*ggt* Δ*yliAB* Δ*gshA* strain grown in minimal medium supplemented with glutathione (Fig. 4, strain SI100). In fact, the *cysA* Δ*ggt* Δ*yliAB* strain could grow in minimal medium supplemented with glutathione as a sole sulfur source (Fig. 6b, row 3); however, its doubling time was extremely long. It is possibile that a nonspecific glutathione uptake system besides YliABCD and GGT exists in *E. coli*.

Boos and Lucht (2) reviewed the periplasmic binding-protein-dependent ABC transporters of *E. coli*, and they proposed a consensus sequence of the ATP-binding cassette subunits. The amino acid sequence of YliA conserved many of the consensus residues. YliA has repeats of Walker motif A-linker peptides-Walker motif B (Walker motif A, residues 55 through 63 and 363 through 371; linker peptides, residues 175 through 183 and 470 through 478; Walker motif B, residues 195 through 201 and 490 through 496). According to the MOTIF program (GenomeNet, Japan), YliB has a bacterial extracellular solute-binding protein family 5 signature (residues 76 through 98) and YliC and -D have binding-protein-dependent transport system inner membrane component signatures (residues 197 through 225 and 188 through 216, respectively). Also, the SignalP program (Technical University of Denmark) predicts that the first 26 amino acids of YliB constitute a signal peptide. The SOSUI program (Department of Biotechnology, Tokyo University of Agriculture and Technology) predicted that YliC and -D have six and seven transmembrane helices, respectively. All of these indicate that YliABCD composes an ATP-binding cassette superfamily transporter, YliA and -B being an ATP-binding component and a periplasmic binding protein, respectively, and YliC and -D being plasma membrane components. We propose the name *gsi* for these genes, after glutathione importer. *yliA*, *-B*, *-C*, and *-D* would be renamed *gsiA*, *-B*, *-C*, and *-D*, respectively.

This is the first report not only of bacterial glutathione transporter but also of a glutathione importer with an ATP-binding cassette among all organisms. The homology search suggests that *Escherichia coli* O157:H7, *Shigella flexneri*, *Salmonella enterica* serovar Typhi, and *Salmonella enterica* serovar Typhimurium have homologues. Our finding of a new glutathione importer with an ATP-binding cassette indicates that there is more diversity in the mechanism of glutathione transport across cell membranes than previously considered.

ACKNOWLEDGMENTS

This work was supported by Grant-in-Aid for Scientific Research no. 15580061 to H.S. from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and by a research grant from the Noda Institute for Scientific Research to H.S. T.K., S.I., and A.O. are supported by the 21st Century COE Program of the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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