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Glutathione Transporter of *Escherichia coli*: Aiming at the Direct Production of Glutathione

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Research aims

Like other organisms, *E. coli* synthesizes glutathione by γ-glutamylcysteine synthetase and glutathione synthetase[1]. *E. coli* excretes glutathione into the culture medium during the exponential phase and glutathione concentration in the culture medium reaches maximum in the early stationary phase[5,10], but thereafter it is hydrolyzed by γ-glutamyltranspeptidase (GGT) in the periplasm to liberate glutamic acid and cysteinylglycine[7,10]. Glutamic acid and cysteinylglycine are taken up into the cytoplasm to be utilized as source of amino acids[9]. Even in the case of GGT-deficient strain of *E. coli*, however, glutathione concentration 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[6], but its mechanism was unclear. GenBank suggests that four genes, *yliA*, *B*, *C* and *D*, locate downstream of *ybiK*, are transcribed with *ybiK* (Figure 1). EcoCys[3] suggests that YliA, B, C and D constitute an uncharacterized member of the ATP-binding cassette superfamily transporters. It suggests that *yliA* and *B* encode an ATP-binding component and a periplasmic binding protein, respectively, and that *yliC* and *D* encode plasma membrane components. From the above information, we speculated that YliA, B, C and D might encode a glutathione transporter.

Methods

Measurement of glutathione. Reduced glutathione was measured with an HPLC (model LC-9A; Shimadzu) equipped with a Shim-pack Amino-Na column and a fluorescence detector (model RF-535), with *o*-phthalaldehyde as the detection reagent[8]. Reduced and oxidized glutathione could be measured separately by this method. Total glutathione was measured with glutathione reductase by the method of Fahey *et al*.[2]

Transport assay. Transport assay was performed as described previously[4] using [^{35}S] glutathione (final 2 nM; 35.4 Tbq/mmol) except M9 glucose medium was used instead of M63 medium. When the effects of verapamil and carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) were determined, cells were preincubated in the presence of these

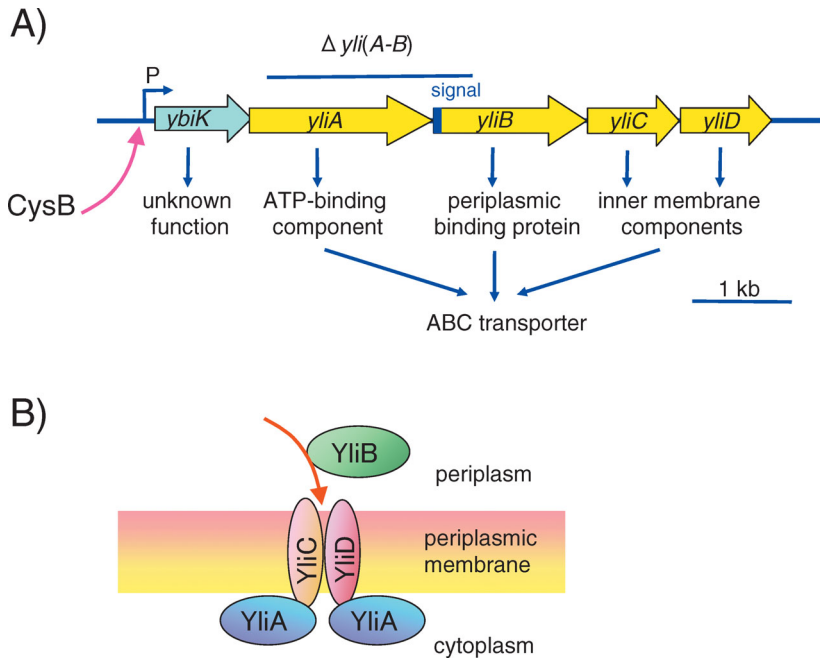

Fig. 1. The structure of *ybiK-yliABCD* operon. (A) The order, size and products of the genes, and the location of the promoter suggested by GenBank and EcoCys were diagrammed. The region of DNA deleted in our *Δyli*(*A-B*) mutation is shown by the bar above the genes. (B) The predicted structure of YliABCD transporter.

chemicals for 30 min at 37°C prior to the addition of labeled glutathione.

Results

Effect of Δggt and Δyli(A-B) mutations on the concentration of extracellular glutathione. Since the wild-type *E. coli* accumulates only several μM glutathione at maximum in the medium and it is difficult to measure such a low concentration of glutathione, the strains were transformed with plasmid pSH1391, which contains *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 *Δggt* and *Δyli*(*A-B*) mutations on the concentration of extracellular glutathione were compared (Figure 2). The glutathione concentration decreased after reaching maximum during the early stationary phase when either *ggt* or *yliAB* was normal. On the other hand, the extracellular glutathione of the *Δggt Δyli*(*A-B*) strain gradually increased even during the stationary phase. When the *Δggt Δyli*(*A-B*) strain was complemented with pACYC177 containing *ybiK*$^+$-*yliA*$^+$*B*$^+$*C*$^+$*D*$^+$ operon, the extracellular glutathione was dramatically decreased.

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

Effect of Δyli(A-B) mutation on the intracellular concentration of glutathione. Glutathione synthesis-deficient (*ΔgshA*) derivatives of the above strains were grown overnight in the minimal medium supplemented with and without 1 mM reduced glutathione. The cells were then lysed by ultrasonication and the amount of glutathione accumulated inside the cells was measured by HPLC (Figure 4). All the glutathione found was 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 the *Δyli*(*A-B*) mutation decreased the accumulation of glutathione inside the cells, non-negligible accumulation of glutathione was observed even in the strain with *Δyli*(*A-B*) *Δggt ΔgshA* (strain SI100). The *Δyli*(*A-B*) mutation was complemented with pACYC177 containing *ybiK*$^+$-*yliA*$^+$*B*$^+$*C*$^+$*D*$^+$ (strain SI154), but not with pACYC177 containing *ybiK*$^+$-*yliA*$^+$*B*$^+$*C*$^+$ (strain SI153).

Transport of glutathione by YliABCD transporter depends on ATPase activity. Effect of ATPase inhibitor verapamil on glutathione transport by YliABCD transporter was determined. Transport of glutathione by YliABCD transporter was strongly inhibited in the presence of 10 mM verapamil (Figure 5). Membrane potential inhibitor, CCCP,

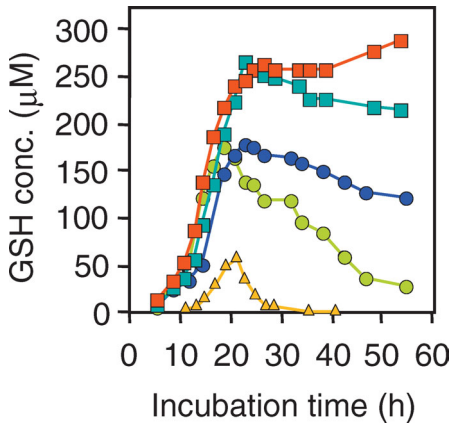

Fig. 2. Glutathione concentration of the culture media. Strains SI37 (*Δggt Δyli*(*A-B*); red square), SI49 (*Δggt*; blue square), SH1555 (pACYC177::*ybiK*$^+$-*yliA*$^+$*B*$^+$*C*$^+$*D*$^+$/*Δggt Δyli*(*A-B*); yellow triangle), SI103 (*Δyli*(*A-B*); blue circle), and SI104 (*ggt*$^+$ *yliA*$^+$*B*$^+$; green circle) were grown in 100 ml minimal medium. At the time indicated, 2-ml of culture was subtracted. OD_{610} 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.

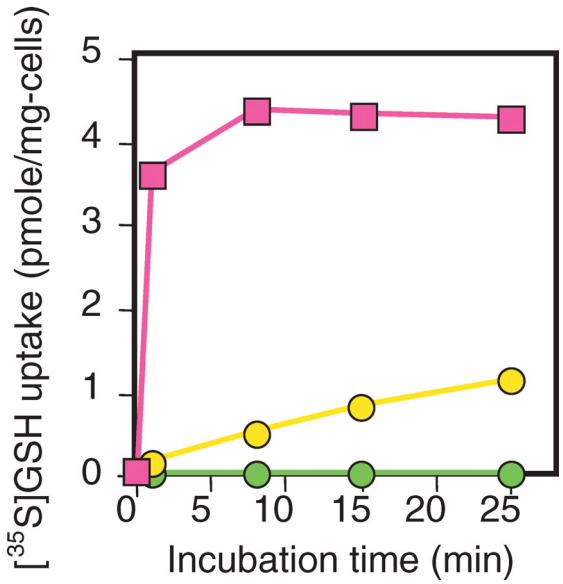

Fig. 3. Glutathione uptake in a transporter assay. Strain SI35 (*Δggt Δyli*(*A-B*); green circles), SH703 (*Δggt*; yellow circles), SH1552 (pACYC177::*ybiK*$^+$-*yliA*$^+$*B*$^+$*C*$^+$*D*$^+$/*Δggt Δyli*(*A-B*); pink squares).

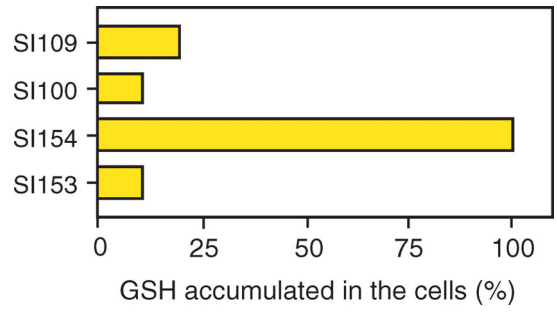

Fig. 4. Accumulation of glutathione in the cells grown in minimal medium supplemented with 1 mM glutathione. Strains SI100 (pACYC177/*ΔgshA Δggt Δyli*(*A-B*)), SI109 (pACYC177/*ΔgshA Δggt*), SI153 (pACYC177::*ybiK*$^+$-*yliA*$^+$*B*$^+$*C*$^+$/*ΔgshA Δggt Δyli*(*A-B*)), SI154 (pACYC177::*ybiK*$^+$-*yliA*$^+$*B*$^+$*C*$^+$*D*$^+$/*ΔgshA Δggt Δyli*(*A-B*)) 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 strain SI154. Seventy four nmole of glutathione per mg-cells were found in strain SI154.

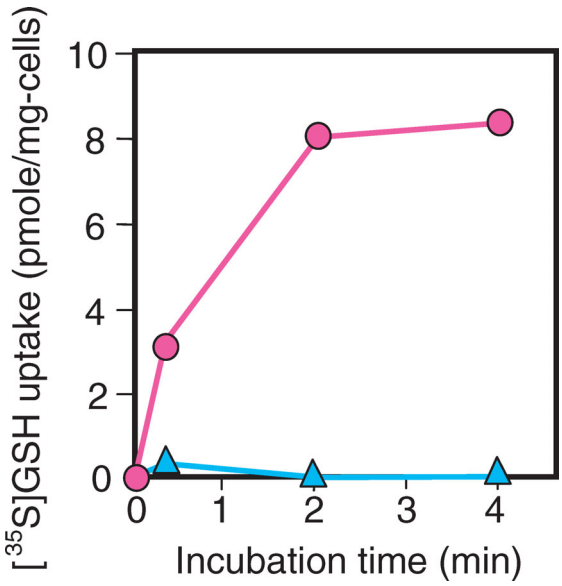

Fig. 5. Effect of verapamil on glutathione uptake. Glutathione uptake of strain SH1552 was measured in the absence of verapamil (pink circles), and in the presence of 10 mM verapamil (blue triangles).

had no effect at 100 μM (data not shown).

Effect of Δyli(A-B) mutation on the cell growth. The ability of YliABCD transporter to utilize glutathione as a sole sulfur source was also investigated. The *cysA* gene encodes a sulfate permease and *cysA* mutants cannot grow with SO_4^{2-} in the medium; they are cysteine auxotrophs. The *cysA Δggt* (sulfate transport and GGT deficient) strain grew weakly on minimal medium with 0.3 mM glutathione as a sole sulfur source, while almost no growth was observed for its *Δyli*(*A-B*) derivative on the same plate. The doubling times of *cysA*, *cysA Δyli*(*A-B*), *cysA Δggt*, and *cysA Δyli*(*A-B*) *Δggt* strains in minimal medium supplemented with 0.3 mM glutathione at 37°C were 1.7, 2.1, 3.2, and 12 h, respectively.

Conclusion

All of the results indicate that YliABCD compose 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.

This is the first report not only of bacterial glutathione transporter, but also of a glutathione importer with 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 ATP-binding cassette indicates that there is more diversity in the mechanism of glutathione transport across cell membranes than previously considered.

References

1) P. Apontoweil and W. Berends. 1975. Glutathione biosynthesis in *Escherichia coli* K 12. Properties of the enzymes and regulation. *Biochim. Biophys. Acta* **399**: 1–9.
2) R. C. Fahey, S. Brody, and S. D. Mikolajczyk. 1975. Changes in the glutathione thiol-disulfide status of *Neurospora crassa* conidia during germination and aging. *J. Bacteriol*. **121**: 144–151.
3) P. D. Karp, M. Arnaud, J. Collado-Vides, J. Ingraham, I. T. Paulsen, and M. H. Saier, Jr. 2004. The *E. coli* EcoCyc database: no longer just a metabolic pathway database. *ASM News* **70**: 25–30.
4) T. Koyanagi, T. Katayama, H. Suzuki, and H. Kumagai. 2004. Identification of the LIV-I/LS system as the third phenylalanine transporter in *Escherichia coli* K-12. *J. Bacteriol*. **186**: 343–350.
5) R. A. Owens and P. E. Hartman. 1986. Export of glutathione by some widely used *Salmonella typhimurium* and *Escherichia coli* strains. *J. Bacteriol*. **168**: 109–114.
6) J. Parry and D. P. Clark. 2002. Identification of a CysB-regulated gene involved in glutathione transport in *Escherichia coli*. *FEMS Microbiol. Lett*. **209**: 81–85.
7) H. Suzuki, W. Hashimoto, and H. Kumagai. 1993. *Escherichia coli* K-12 can utilize an exogenous γ-glutamyl peptide as an amino acid source, for which γ-glutamyltranspeptidase is essential. *J. Bacteriol*. **175**: 6038–6040.
8) H. Suzuki, S. Izuka, H. Minami, N. Miyakawa, S. Ishihara, and H. Kumagai. 2003. Use of bacterial γ-glutamyltranspeptidase for enzymatic synthesis of γ-D-glutamyl compounds. *Appl. Environ. Microbiol*. **69**: 6399–6404.
9) H. Suzuki, S. Kamatani, E.-S. Kim, and H. Kumagai. 2001. Aminopeptidases A, B and N, and dipeptidase D are the four cysteinylglycinases of *Escherichia coli* K-12. *J. Bacteriol*. **183**: 1489–1490.
10) H. Suzuki, H. Kumagai, and T. Tochikura. 1987. Isolation, genetic mapping and characterization of *Escherichia coli* K-12 mutants lacking γ-glutamyltranspeptidase. *J. Bacteriol*. **169**: 3926–3931.