

Effect of Drug Transporter Genes on Cysteine Export and Overproduction in *Escherichia coli*

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L-Cysteine is an important amino acid in terms of its industrial applications. We previously found a marked production of L-cysteine from glucose in recombinant *Escherichia coli* cells expressing an altered *cysE* gene encoding feedback inhibition-insensitive serine acetyltransferase. Also, a lower level of cysteine desulfhydrase (CD) activity, which is involved in L-cysteine degradation, increased L-cysteine productivity in *E. coli*. The use of an L-cysteine efflux system could be promising for breeding L-cysteine overproducers. In addition to YdeD and YfiK, which have been reported previously as L-cysteine exporter proteins in *E. coli*, we analyzed the effects of 33 putative drug transporter genes in *E. coli* on L-cysteine export and overproduction. Overexpression of the *acrD*, *acrEF*, *bcr*, *cusA*, *emrAB*, *emrKY*, *yhjYZ*, and *yojIH* genes reversed the growth inhibition of *tnaA* (the major CD gene)-disrupted *E. coli* cells by L-cysteine. We also found that overexpression of these eight genes reduces intracellular L-cysteine levels after cultivation in the presence of L-cysteine. Amino acid transport assays showed that Bcr overexpression conferring bicyclomycin and tetracycline resistance specifically promotes L-cysteine export driven by energy derived from the proton gradient. When a *tnaA*-disrupted *E. coli* strain expressing the altered *cysE* gene was transformed with a plasmid carrying the *bcr* gene, the transformant exhibited more L-cysteine production than cells carrying the vector only. A reporter gene assay suggested that the *bcr* gene is constitutively expressed at a substantial level. These results indicate that the multidrug transporter Bcr in the major facilitator family is involved in L-cysteine export and overproduction in genetically engineered *E. coli* cells.

L-Cysteine is an important amino acid in terms of its applications in the pharmaceutical, food, and cosmetic industries. However, due to feedback inhibition by L-cysteine of serine acetyltransferase (SAT), which catalyzes the formation of O-acetyl-L-serine (OAS) from acetyl-coenzyme A and L-serine (19, 32), microorganisms do not excrete L-cysteine. To obtain L-cysteine producers, we previously constructed *Escherichia coli cysE* genes that encode altered SATs. These genes were made genetically less sensitive to feedback inhibition by L-cysteine through site-directed or random mutagenesis (22, 34). We found a marked production of L-cysteine plus L-cystine from glucose in recombinant *E. coli* cells expressing the altered *cysE* genes (22, 34). Furthermore, the expression of two cDNAs encoding feedback inhibition-insensitive SATs of *Arabidopsis thaliana* significantly increased productivity (35).

In the same investigation (22), we discovered that proteins with cysteine desulfhydrase (CD) activity were crucial for L-cysteine degradation in *E. coli* and also that CD activity was closely related to the L-cysteine productivity of *E. coli*. CD is known to catalyze the degradation of L-cysteine to pyruvate, ammonia, and sulfide. In *E. coli*, cystathionine- β -lyase, which mainly catalyzes the conversion of cystathionine to homocysteine, pyruvate, and ammonia (10), and tryptophanase, which primarily degrades L-tryptophan to indole, pyruvate, and am-

monia (23), have been shown to exhibit CD activity in vitro and in vivo (10, 24). In addition to the above two enzymes, we recently identified three additional proteins with CD activity as OAS sulfhydrylase A, OAS sulfhydrylase B, and the MalY protein (2). Gene disruption of each protein was significantly effective for overproduction of L-cysteine and L-cystine.

Because high levels of intracellular L-cysteine have been reported to be inhibitory or even toxic (8, 15–17, 26, 33), further improvements in L-cysteine production are expected to use the amino acid efflux system. Recently, export systems for L-cysteine in *E. coli* were identified. The *ydeD* and *yfiK* genes were discovered to augment L-cysteine production when overexpressed (6, 12). *E. coli* cells overexpressing YdeD, which belongs to the PecM family of transporters, excreted considerable amounts of OAS and L-cysteine (6). Overexpression of YfiK, an RthB family member, led to the drastic and parallel secretion of OAS and L-cysteine into the medium (12). L-Cysteine is also exported from the *E. coli* cytoplasm to the periplasm by CydDC, which is an ATP-binding cassette-type transporter required for cytochrome assembly (5, 27). CydDC overexpression conferred resistance to high extracellular L-cysteine concentrations. In *E. coli*, there are 37 open reading frames (ORFs) assumed to be drug transporter genes on the basis of sequence similarities, although the transport abilities of most of them have not yet been established. Five families of drug extrusion translocases have currently been identified based on sequence similarity (4, 28). These are the MF (major facilitator) family, SMR (small multidrug resistance) family, RND (resistance nodulation cell division) family, ABC (ATP-

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binding cassette) family, and MATE (multidrug and toxic compound extrusion) family. Nishino and Yamaguchi (25) cloned all 37 putative drug transporter genes in *E. coli* and investigated their drug resistance phenotypes.

Our objectives in this study were (i) to identify an L-cysteine exporter from 33 putative drug transporter genes in *E. coli*, (ii) to analyze the function of the transporter in L-cysteine export *in vivo* and *in vitro*, and (iii) to test L-cysteine production by *E. coli* cells overexpressing the transporter gene. As a result, we report here that the MF-type transporter Bcr is involved in the export and overproduction of L-cysteine in *E. coli* cells.

MATERIALS AND METHODS

Strains and plasmids. The *E. coli* strains used in this study were JM39 (F⁺ *cysE51* *fr-8*) (9) and JM39Δ*tnaA*, which is a *tnaA* disruptant derived from JM39 (1). Strain DH5α [F⁻ λ⁻ φ80*dlacZ*Δ*M15* Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17*(r_K⁻ m_K⁺) *supE44 thi-1 gyrA96*] was used to subclone the *E. coli* gene.

The 33 multicopy plasmids carrying putative drug transporter ORFs were constructed as reported previously (25). The ORFs were amplified with native promoters and peripheral genes by PCR using primers containing the restriction enzyme site that exists in the multicloning sites of the pUC118 and pUC119 vectors containing the bacterial ampicillin resistance gene (Takara Bio, Ohtsu, Japan). All drug transporter ORFs were arranged to be in the same orientation as the *lac* promoter of pUC vectors, while some of the ORFs were in the reverse orientation. Plasmid pACYC184 (supplied by S. Yasuda), containing the bacterial chloramphenicol resistance gene, was used to clone the altered *cysE* gene that encodes the Met256Ile mutant SAT. Plasmid pNN387 (supplied by R. W. Davis), containing the bacterial chloramphenicol resistance gene, was used for reporter gene assay of the *bcr* gene.

Media and cultivation. Luria-Bertani (LB) complete medium (30) and M9 minimal medium (30) were used for general cultivations. The *E. coli* recombinant strains were grown in LB or M9 medium containing 0.4% glucose (wt/vol). If necessary, ampicillin (50 μg/ml) and/or chloramphenicol (100 μg/ml) was added. For solid media, 2% agar was added. Growth was monitored by measurement of the optical density at 610 nm (OD₆₁₀).

C1 medium with sodium thiosulfate (C1+TS) was used for the production of L-cysteine and L-cystine (35). The pH was adjusted to 7.0 using KOH. For the production of amino acids, a loopful of cells cultured for 24 h on LB solid medium containing ampicillin and chloramphenicol at 30°C was inoculated into 20 ml of C1+TS in a 500-ml flask and cultured at 30°C for 72 h on a reciprocal shaker maintained at 120 strokes per min (35). Growth was measured by the absorbance (OD₅₆₂) of the culture broth after appropriate dilution with 0.1 N HCl.

Construction of plasmids. The enzymes used for DNA manipulations were obtained from Takara Bio. The *yde* gene involved in efflux of L-cysteine and OAS (6) was cloned into pUC118. A DNA fragment of the *yde* gene containing the putative promoter and terminator regions was prepared by PCR from genomic DNA from strain JM39, using oligonucleotide primers 5'-TTT GGG AAG CTT CTT ATC CGC CAG GAT ACC AAA ACC-3' and 5'-GTT TGG GGA TCC CCA GTT GTT ACT TCT TGT GCC AAT G-3' (underlining indicates the positions of HindIII and BamHI sites, respectively). The unique amplified band of 1.3 kb was digested with HindIII and BamHI and then ligated to the large fragment of pUC118 digested with HindIII and BamHI to construct pUCydeD. The nucleotide sequence of the *ydeD* gene was confirmed by DNA sequencing.

To coexpress the Met256Ile mutant SAT with the L-cysteine exporter, the 1.2-kb BamHI-SalI fragment from plasmid pCEM256I (22) was ligated to the large fragment of pACYC184 digested with BamHI and SalI to construct pACYC-M256I.

We also constructed a plasmid carrying only the *bcr* gene as follows. The plasmid pUCbcr was digested with Eco8I and BstPI to recover the 5.1-kb fragment. This fragment carried the incomplete *rsuA* gene, with a truncation of the sequence encoding the carboxyl terminus from amino acid residues 70 to 232, and the entire *bcr* gene with its native promoter. Both ends of the fragment were blunted by T4 DNA polymerase and self-ligated to construct plasmid pUCbcr-rsuA.

Intracellular and extracellular contents of L-cysteine. After cultivation in 2 ml of LB medium containing ampicillin at 37°C for 12 h to stationary phase, culture samples were diluted with LB medium to an OD₆₁₀ of 2.0. The cells from 1 ml of diluted suspension were harvested, washed twice with distilled water, and suspended in 0.1 ml of distilled water. The cells were incubated in a water bath,

and intracellular amino acids were extracted by boiling for 10 min. After centrifugation (1 min at 15,000 × g), the supernatant was used as intracellular samples. The amount of L-cysteine was determined by the method of Gaitonde, as described previously (13). One hundred microliters of sample which had been treated with Gaitonde reagent (250 mg ninhydrin dissolved in a mixture of 4 ml of HCl and 16 ml of acetic acid) was heated in a boiling water bath for 5 min and then cooled rapidly on ice before dilution to 400 μl using 100% ethanol. After 30 min at room temperature, the reaction products were measured at 560 nm.

Culture supernatants were used as extracellular samples. The concentrations of L-cysteine and L-cystine were determined by a microbioassay with *Pediococcus acidilactici* IFO 3076, as described by Tsunoda et al. (36). Since L-cysteine in the culture fluid was easily oxidized to L-cystine, which was slightly soluble in water, the culture fluids were assayed after L-cystine was dissolved with 0.5 N HCl.

Amino acid transport assays. *E. coli* cells were cultured in 2 ml of LB medium containing ampicillin to the stationary growth phase. Culture samples were diluted with LB to an OD₆₁₀ of 1.0. The cells from 1 ml of diluted suspension were harvested, washed with 100 mM Tris-HCl (pH 7.3) containing 100 mM NaCl and 0.5 mM MgCl₂, and suspended in 1 ml of M9 medium containing 0.4% glucose. Amino acid uptake assays were performed by incorporation of L-[³⁵S]cysteine (1,075 Ci/mmol; 1 Ci = 37 GBq), L-[¹⁴C]leucine (20 mCi/mmol), L-[¹⁴C]valine (55 mCi/mmol), L-[¹⁴C]proline (246 mCi/mmol), L-[¹⁴C]serine (50 mCi/mmol), L-[¹⁴C]arginine (20 mCi/mmol), L-[¹⁴C]glutamic acid (55 mCi/mmol), and L-[¹⁴C]methionine (55 mCi/mmol) (New England Nuclear, Wilmington, Del.). One milliliter of cell suspension was preincubated for 5 min at 30°C, and the reactions were initiated at 30°C by the addition of nonlabeled and labeled amino acids to final concentrations of 200 μM and 1.0 μCi/ml, respectively. At intervals, 100-μl aliquots of the cell suspension were rapidly removed, collected on nitrocellulose filters (0.45-μm pore size) (Advantec, Tokyo, Japan), and washed twice with 10 ml of 100 mM Tris-HCl (pH 7.3) containing 100 mM NaCl and 0.5 mM MgCl₂ under vacuum within 1 min to avoid significant losses of amino acids from the cells. Amino acid export assays were performed as follows. The above reaction mixtures containing nonlabeled and labeled amino acids were incubated for 60 min at 30°C, washed with 100 mM Tris-HCl (pH 7.3) containing 100 mM NaCl and 0.5 mM MgCl₂, and suspended in 1 ml of M9 medium containing 0.4% glucose. After incubation at 30°C, 100-μl aliquots of the cell suspension were removed, collected on nitrocellulose filters, and washed twice with 10 mM morpholineethanesulfonic acid-Tris (pH 7.0). The filters were dried, and intracellular labeled amino acids were determined by liquid scintillation counting. Amino acid uptake activities were calculated in nmol per min per mg dry weight. The export rate was also determined based on the remaining labeled amino acid content in the cells. *E. coli* cells were de-energized with carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (200 μM [final concentration]) to collapse the proton gradient.

Reporter gene assay. A 400-bp fragment containing the 5' upstream region of the *bcr* gene was amplified by PCR with plasmid pUCbcr (25) and oligonucleotide primers 5'-CGC GCG GCC GCG GTG CTG ATG ACT GAT GAT-3' and 5'-CGC AAG CTT AAC GGG CTC CTG AAA GTC ATT-3' (underlining indicates the positions of NotI and HindIII sites, respectively). The PCR products were digested with NotI and HindIII and inserted into the NotI-HindIII site proximal to the coding region of *lacZ* in pNN387 to generate pNNbcr-P. Plasmid pNNbcr-P was used to transform strain JM39Δ*tnaA*. After incubation of the resultant transformant in LB medium containing chloramphenicol at 37°C to the exponential growth phase, tetracycline (1 μg/ml) or 10 mM L-cysteine was added. At intervals (0 min to 180 min), the cells were harvested and lysed. β-Galactosidase activity was then measured at 30°C by means of the hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside to produce *o*-nitrophenol and galactose. β-Galactosidase activity (in Miller units) was calculated using the following formula: (OD₄₂₀ of *o*-nitrophenol × 1,000/min/ml)/OD₆₀₀ of the culture (21).

Nucleotide sequence accession numbers. The GenBank accession numbers for the *bcr* and *ydeD* genes are X63703 and D90796, respectively.

RESULTS

Overexpression of eight drug transporter genes reverses growth inhibition of *tnaA*-disrupted *E. coli* cells by L-cysteine. Although the mechanisms are not yet understood, it has previously been observed that the growth of *E. coli* cells is inhibited by excess L-cysteine (15). It was demonstrated that proteins with CD activity are involved in L-cysteine degradation in *E. coli* cells (1, 2, 22). Therefore, *E. coli* cells with a lower level of CD activity would be much more sensitive to L-cysteine than

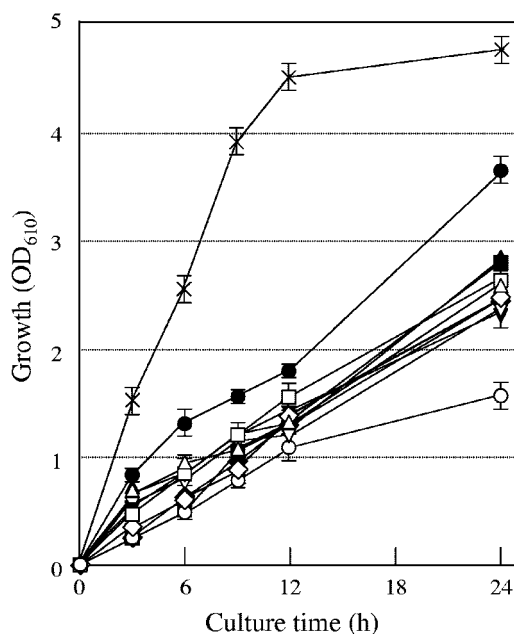


FIG. 1. Growth (OD_{610}) of *E. coli* strains in LB medium plus 30 mM L-cysteine at 37°C. Cells of strain JM39 with pUC118 (x) and of JM39 Δ *tnaA* with pUC118 (o), pUCydeD carrying *ydeD* (Δ), pUCbcr carrying *bcr* (\square), pUCemrAB carrying *emrAB* (∇), pUCemrKY carrying *emrKY* (\diamond), pUCcusA carrying *cusA* (\bullet), pUCacrD carrying *acrD* (\blacktriangle), pUCacrEF carrying *acrEF* (\blacksquare), pUCybjYZ carrying *ybjYZ* (\blacktriangledown), or pUCyojIH carrying *yojIH* (\blacklozenge) were grown in LB medium at 37°C. At stationary phase, cells were diluted 1:100 in 5 ml of LB medium plus 30 mM L-cysteine. Values indicate means and standard deviations of results from three independent experiments.

wild-type cells. Among the five CD proteins identified in *E. coli*, tryptophanase, encoded by the *tnaA* gene, contributes primarily to L-cysteine degradation (2). As shown in Fig. 1, the growth of *tnaA*-disrupted JM39 Δ *tnaA* was significantly inhibited relative to that of wild-type JM39 when the cells were cultured in LB medium plus 30 mM L-cysteine at 37°C. Both strains showed the same level of growth when cultured in LB medium (data not shown).

Because high levels of L-cysteine are toxic to the cell, one might expect that the active efflux of a toxic metabolite such as L-cysteine is mediated by a family of transmembrane proteins referred to as drug transporters. We therefore transformed JM39 Δ *tnaA* cells with plasmids carrying one of 33 ORFs assumed to be drug transporter genes in *E. coli* and examined the effects of their overexpression on the growth of *E. coli* cells in the presence of L-cysteine (Fig. 1). Of these 33 genes, the *acrD*, *acrEF*, *bcr*, *cusA*, *emrAB*, *emrKY*, *ybjYZ*, and *yojIH* genes significantly reversed the growth inhibition of JM39 Δ *tnaA* cells by L-cysteine after cultivation for 24 h. We also found that the overexpression of YdeD, which is involved in the efflux of L-cysteine and OAS (6), improves the growth of JM39 Δ *tnaA* cells in the presence of L-cysteine.

In contrast, overexpression of either the *acrAB*, *emrD*, *emrE*, *fsr*, *mdfA*, *mdlAB*, *sugE*, *yajR*, *yceE*, *yceL*, *yddA*, *ydeA*, *ydeF*, *ydgFE*, *ydhC*, *ydhE*, *ydiM*, *yebQ*, *yegMNOB*, *yhiH*, *yhiUV*, *yidY*, *yieO*, *yjiO*, or *ynfM* gene had no influence on the growth of JM39 Δ *tnaA* cells in the presence of L-cysteine (data not

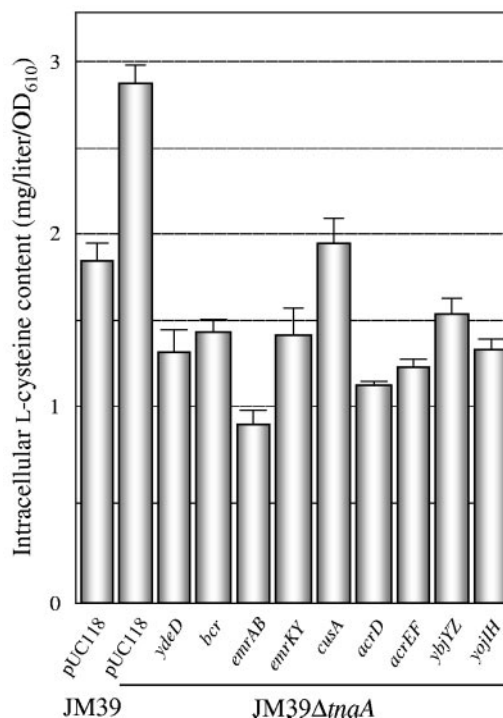


FIG. 2. Intracellular L-cysteine contents of *E. coli* strains with plasmids carrying drug transporter genes. Cells were grown in LB medium plus 30 mM L-cysteine at 37°C for 12 h, and the intracellular L-cysteine content was measured. Values indicate means and standard deviations of results from three independent experiments.

shown). Accordingly, the eight putative drug transporter genes (the *acrD*, *acrEF*, *bcr*, *cusA*, *emrAB*, *emrKY*, *ybjYZ*, and *yojIH* genes) were chosen as likely candidates for an L-cysteine exporter.

Overexpression of eight drug transporter genes reduces intracellular L-cysteine levels in *tnaA*-disrupted *E. coli* cells. We assumed that transformants overexpressing L-cysteine exporter candidates excreted considerable amounts of L-cysteine into the medium and had decreased intracellular L-cysteine levels. *E. coli* cells were cultivated in LB medium plus 30 mM L-

TABLE 1. Drug transporter genes in *E. coli* being considered as L-cysteine exporter candidates

Drug transporter family	Gene	Known function (reference)
MF	<i>bcr</i>	Resistance to bicyclomycin, tetracycline, fosfomycin, sulfathiazole, etc. (3, 25, 37)
	<i>emrAB</i>	Resistance to deoxycholate, CCCP, etc. (20, 25)
RND	<i>emrKY</i>	Resistance to deoxycholate (25)
	<i>cusA</i>	Efflux of copper ion (14)
	<i>acrD</i>	Resistance to deoxycholate, SDS, novobiocin, etc. (25, 29)
	<i>acrEF</i>	Resistance to acriflavine, ethidium bromide, rhodamine 6G, deoxycholate, etc. (18, 25)
ABC	<i>ybjYZ</i>	Resistance to erythromycin (25)
	<i>yojIH</i>	Putative transporter

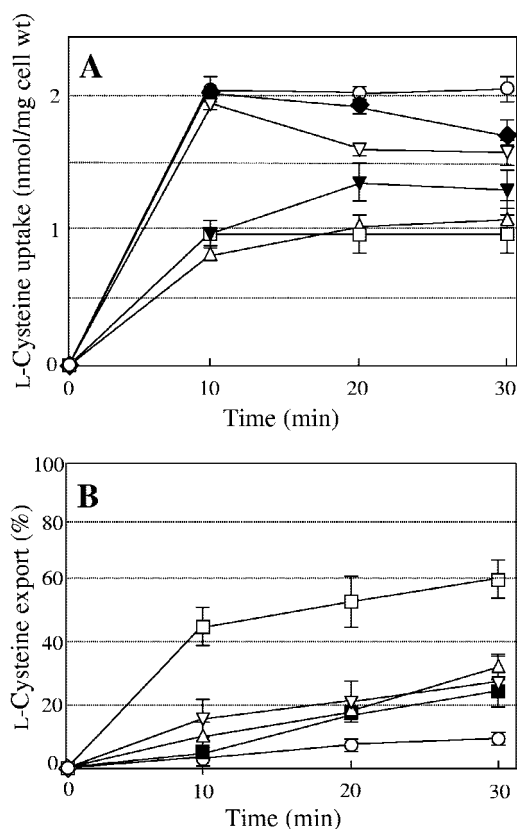


FIG. 3. Time courses of L-cysteine uptake (A) and export (B) by cells of strain JM39 Δ *tnaA* with pUC118 (○), pUCydeD carrying *ydeD* (△), pUCbcr carrying *bcr* (□), pUCemrAB carrying *emrAB* (▽), pUCacrEF carrying *acrEF* (■), pUCybjYZ carrying *ybjYZ* (▼), or pUCyoiH carrying *yoiH* (◆). Values indicate means and standard deviations of results from three independent experiments.

cysteine at 37°C for 12 h, and the intracellular L-cysteine levels were examined (Fig. 2). No L-cysteine was detected in any of the strains when they were cultivated in LB medium (data not shown). The intracellular L-cysteine level in JM39 Δ *tnaA* carrying vector only was approximately 1.5-fold higher than that in JM39, probably due to lower CD activity. After cultivation in the presence of L-cysteine for 12 h, the intracellular L-cysteine contents of eight transformants were significantly reduced, to 30 to 70% of that of JM39 Δ *tnaA* harboring the vector. We also observed that YdeD overexpression resulted in a 50% decrease in the L-cysteine level. These results suggest that these eight genes might be involved in L-cysteine export into the medium. Table 1 lists the eight drug transporter genes being considered as L-cysteine exporter candidates.

Bcr overexpression promotes L-cysteine export in *tnaA*-disrupted *E. coli* cells. To determine whether any genes were involved in L-cysteine export, we tested JM39 Δ *tnaA* cells overexpressing eight genes and YdeD for the uptake and export of radiolabeled L-cysteine. As shown in Fig. 3, the cells overexpressing YdeD showed a prominent decrease in the uptake and an increase in the export of L-cysteine relative to the cells carrying the vector only. Among the eight drug transporter genes tested, five genes had somewhat negative effects on uptake and positive effects on export, with those effects being

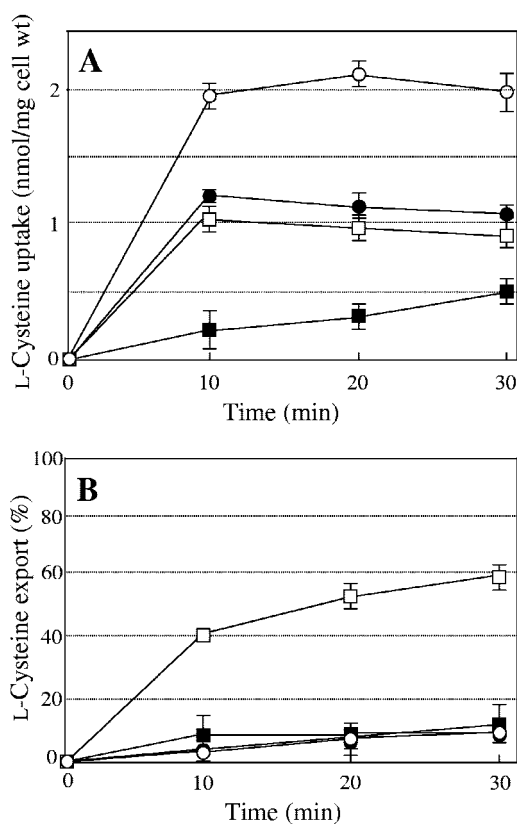


FIG. 4. Time courses of L-cysteine uptake (A) and export (B) in the absence (open symbols) or presence (filled symbols) of CCCP by cells of strain JM39 Δ *tnaA* with pUC118 (circles) or pUCbcr carrying *bcr* (squares). Values indicate means and standard deviations of results from three independent experiments.

greater in the case of the *bcr* gene. Bcr, from the MF family, and YbjYZ, from the ABC family, significantly blocked L-cysteine uptake, with levels ranging from 40% to 50% of that observed in the cells carrying the vector only (Fig. 3A). EmrAB and YoiH also reduced L-cysteine uptake after 10 min and 20 min, respectively. Bcr and EmrAB, from the MF family, and AcrEF, from the RND family, significantly accelerated L-cysteine export compared with the cells carrying the vector only (Fig. 3B). In particular, the Bcr protein highly exported L-cysteine, removing up to about 60% of the initial amount in the cells. However, the transport activities of other ORFs (AcrD, CusA, and EmrKY) were virtually unchanged from that in the cells harboring the vector only (data not shown). These results indicated that the Bcr protein would be an effective exporter of L-cysteine in *E. coli*, in addition to YdeD.

The Bcr sequence showed that it belongs to the MF family, a large family of integral membrane proteins with 12 transmembrane domains (11). Most members of the MF family are dependent on the proton motive force for substrate transport. To determine whether Bcr requires the proton motive force to transport L-cysteine, we tested whether the effect of Bcr could be prevented by the protonophore CCCP (Fig. 4). The incubation of JM39 Δ *tnaA* cells overexpressing Bcr with CCCP significantly diminished the uptake (Fig. 4A) and export of L-cysteine, such that the rate was indistinguishable from that

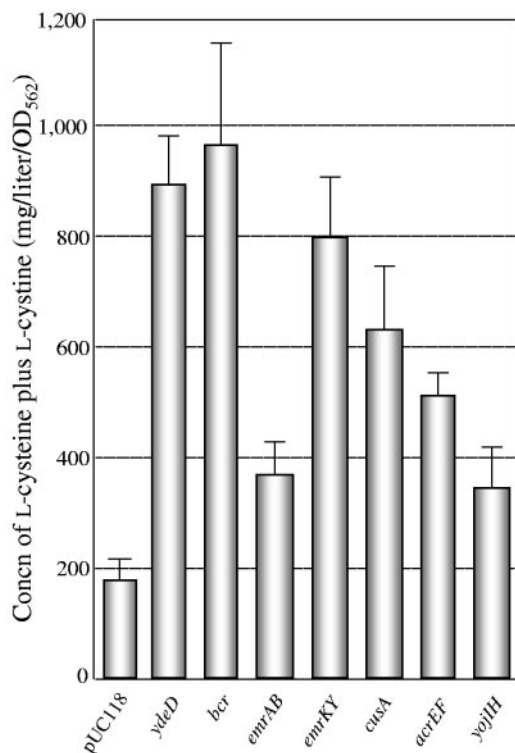


FIG. 5. Production of L-cysteine plus L-cystine by *E. coli* strain JM39 Δ *tnaA* with pACYC-M256I carrying the altered *cysE* gene and plasmids carrying drug transporter genes after 48 h of cultivation. Detailed procedures for culture are described in the text. The concentration of L-cysteine plus L-cystine was determined by a microbiobassay. Values indicate means and standard deviations of results from three independent experiments.

observed with JM39 Δ *tnaA* cells carrying pUC118 (Fig. 4B). Interestingly, the addition of CCCP also resulted in a 50% decrease in L-cysteine uptake in the cells carrying the vector only, suggesting that there is another protein(s) involved in L-cysteine uptake. These results confirm that the Bcr transporter derives energy for L-cysteine export from the proton gradient.

To further examine the substrate specificity of Bcr for amino acids, we performed various amino acid transport assays, including assays with hydrophilic residues (L-proline and L-serine), hydrophobic residues (L-leucine and L-valine), a basic residue (L-arginine), an acidic residue (L-glutamic acid), and a sulfur-containing residue (L-methionine). However, Bcr overexpression had no discernible effect on the uptake and export of these amino acids (data not shown). These results suggest that L-cysteine may be the only amino acid exported by Bcr.

Bcr overexpression contributes to L-cysteine production in *tnaA*-disrupted *E. coli* cells that express an altered *cysE* gene. In order to confirm whether the overexpression of drug transporter genes was involved in L-cysteine production in *E. coli* cells, each plasmid or the vector pUC119 was introduced into JM39 Δ *tnaA* harboring pACYC-M256I, which carries an altered *cysE* gene encoding feedback-insensitive Met256Ile mutant SAT. The transformed cells were grown in C1+TS medium, and the medium was analyzed for its L-cysteine and

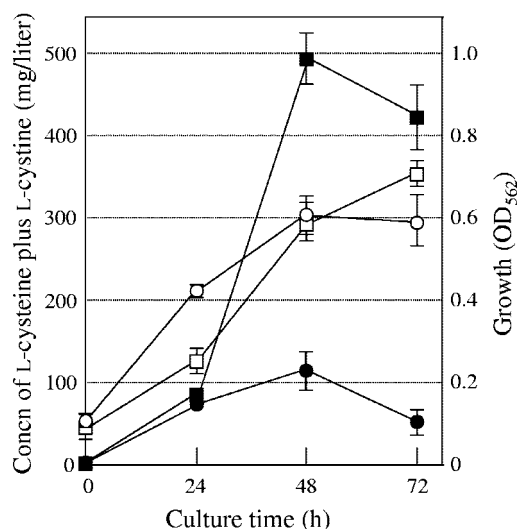


FIG. 6. Time courses of growth (OD₅₆₂) (open symbols) and L-cysteine plus L-cystine production (filled symbols) by cells of strain JM39 Δ *tnaA* with pACYC-M256I carrying the altered *cysE* gene and pUC118 (circles) or pUCbcr carrying *bcr* (squares). Detailed procedures for culture are described in the text. Values indicate means and standard deviations of results from three independent experiments.

L-cysteine contents. As shown in Fig. 5, the cells overexpressing Bcr, EmrAB, EmrKY, CusA, AcrEF, and YojIH after 48 h of cultivation showed approximately two- to five-fold increases in L-cysteine production. It is noteworthy that when the *bcr* gene was overexpressed, the L-cysteine level was approximately fivefold higher than that of the cells harboring the vector only. This finding is in agreement with the results of the in vitro transport assay (Fig. 3). A significant effect on L-cysteine production was also observed in *ydeD*-overexpressing cells. However, AcrD and YbjYZ did not cause increases in L-cysteine production (data not shown).

Figure 6 shows the time courses of growth and L-cysteine production by cells of strain JM39 Δ *tnaA* harboring pACYC-M256I and pUC118 or pUCbcr (carrying *bcr*). Both types of transformed cells showed the same level of growth after 72 h of cultivation, although the growth of cells overexpressing Bcr was inhibited after 24 h of cultivation. The amounts of increasing L-cysteine produced by the cells overexpressing Bcr were approximately five- to sixfold higher than those observed in the cells carrying the vector only after 48 h and 72 h of cultivation. These results clearly indicate that Bcr plays important roles in L-cysteine export in *E. coli* cells and that Bcr overexpression is effective in L-cysteine production by *E. coli* cells. However, the decrease in L-cysteine production commenced after 48 h of cultivation, probably because of the remaining CD enzymes.

Functional analysis of the *bcr* gene. The plasmid pUCbcr used to overexpress the *bcr* gene also contains another gene, *rsuA* (formerly designated *yejD*), encoding 16S rRNA pseudouridine synthase (25, 31, 38). As shown in Fig. 7A, actual expression of the Bcr protein was confirmed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of crude cell extracts. Moreover, as reported previously (25), pUCbcr conferred resistance to tetracycline (Fig. 7B). However, one cannot exclude the possibility that the positive effects of Bcr on

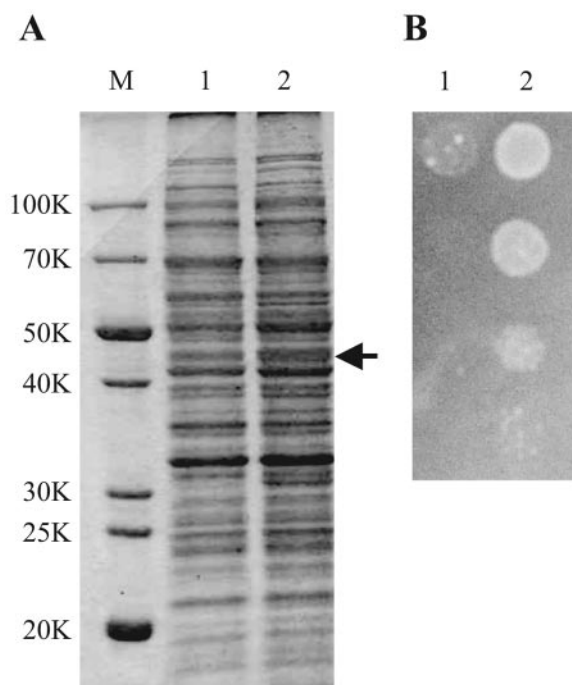


FIG. 7. Overexpression of *bcr* gene in *E. coli*. (A) SDS-polyacrylamide gel electrophoresis of strain JM39 Δ *tnaA* with pUC118 (lane 1) or pUCbcr carrying *bcr* (lane 2). After cultivation in LB medium at 37°C for 12 h, crude extracts were isolated and separated in a 12% SDS-polyacrylamide gel. Each lane was loaded with a sample containing 30 μ g of protein. The gel was stained with Coomassie brilliant blue R-250. Molecular mass standards are shown at the left (lane M). The arrow indicates the position of the Bcr protein (43K). (B) Growth phenotypes on tetracycline-containing medium of strain JM39 Δ *tnaA* with pUC118 (lane 1) and pUCbcr carrying *bcr* (lane 2). After cultivation in LB medium at 37°C for 12 h, serial dilutions (10^{-2} to 10^{-5}) of approximately 10^8 cells (from top to bottom) were spotted onto an LB plate containing 2 μ g of tetracycline/ml. The plate was incubated at 37°C for 12 h.

L-cysteine export and production were due to overexpression of the *rsuA* gene. We therefore constructed plasmid pUCbcr-*rsuA*, carrying only the *bcr* gene, and analyzed the function of the Bcr protein. Strain JM39 Δ *tnaA* with pACYC-M256I and pUCbcr-*rsuA* produced L-cysteine plus L-cystine (410 ± 55 mg/liter) after 48 h of cultivation at the same rate as strain JM39 Δ *tnaA* with pACYC-M256I and pUCbcr (422 ± 125 mg/liter). It was also found that strain JM39 Δ *tnaA* harboring pUCbcr-*rsuA* was resistant to tetracycline (data not shown). These results verify that the Bcr protein alone functions properly and is sufficient to increase L-cysteine production in a CD-disrupted *E. coli* strain that expresses an altered SAT.

The *bcr* gene has been reported to code for resistance to tetracycline (4-fold) and fosfomycin (4-fold), in addition to bicyclomycin (16-fold), in *E. coli* cells (25). We suspected that *bcr* gene expression might be under the control of these drugs or L-cysteine. To test this, we examined the effects of tetracycline and L-cysteine on *bcr* gene expression by a reporter gene assay. We transformed strain JM39 Δ *tnaA* with plasmid pNNbcr-P and the vector (pNN387), and the obtained transformants were cultivated in LB medium for 3 h in the presence

or absence of tetracycline or L-cysteine. When cultured in LB medium only, the cells harboring pNNbcr-P yielded substantial levels of β -galactosidase activity (33 to 39 units). However, the β -galactosidase activities in the cells harboring pNNbcr-P were virtually unchanged after the addition of tetracycline (34 to 39 units) or L-cysteine (31 to 38 units). These results showed that exogenous L-cysteine or tetracycline does not induce the expression of the *bcr* gene.

DISCUSSION

Although there are 37 ORFs in the chromosomal DNA of *E. coli* that can be assumed to be genes encoding drug transporters, none of these had yet been reported to have L-cysteine transport activity. This is the first report of an L-cysteine exporter identified by the overexpression of putative drug transporters. We found that Bcr in the MF family is apparently involved in L-cysteine export and overproduction in *E. coli* cells.

In addition to the *bcr* gene, seven other drug transporter genes, *acrD*, *acrEF*, *cusA*, *emrAB*, *emrKY*, *ybjYZ*, and *yojIH*, were picked up as candidates for the L-cysteine exporter. In the MF-type drug transporter family, EmrAB was identified as a transporter involved in multidrug resistance (20). EmrAB overexpression from a plasmid conferred resistance to a broad range of compounds, including deoxycholate and CCCP (25). EmrKY overexpression also conferred resistance to various drugs, such as doxorubicin, rhodamine 6G, and benzalkonium (25). Based on the intracellular L-cysteine levels in CD-disrupted *E. coli* cells (Fig. 2), EmrAB, AcrD, AcrEF, and YojIH also appeared to be promising L-cysteine exporters. As shown in Fig. 5, however, there is a poor correlation between the intracellular L-cysteine level and L-cysteine production. A transport assay using radiolabeled amino acids also showed that the L-cysteine export capability of the cells overexpressing Bcr was much higher than those observed in the case of other transporters and even of a previously characterized exporter, YdeD.

From the RND-type family, three genes, *acrEF*, *acrD*, and *cusA*, were listed as candidates. AcrEF (18) and AcrD (29) have been identified as drug exporters. AcrEF overexpression from a plasmid conferred resistance to acriflavine, ethidium bromide, rhodamine 6G, and deoxycholate (25). Although the *acrD* gene was reported to mediate aminoglycoside resistance (29), this gene conferred resistance to some other compounds (deoxycholate, SDS, novobiocin, etc.) in addition to aminoglycosides (29). *CusA* overexpression did not show any resistance to the 26 representative antimicrobial agents (25), despite a report that the gene product was involved in the efflux of copper (14). In the amino acid transport assay, however, only AcrEF overexpression seemed to somewhat increase L-cysteine export.

Among putative ABC-type drug exporters, only YbjYZ overexpression conferred erythromycin-specific resistance on *E. coli* cells (25). Thus, it appears that the *ybjYZ* gene is a novel macrolide resistance determinant. Judging from the growth phenotype results and intracellular L-cysteine levels in CD-disrupted *E. coli* cells, the overexpression of YbjYZ and YojIH might be involved in L-cysteine efflux. However, an amino acid transport assay showed that only YbjYZ overexpression re-

sulted in a slight decrease in L-cysteine uptake. In conclusion, our results using 33 putative drug transporter genes indicate that the Bcr protein is involved in L-cysteine export and overproduction in *E. coli* cells.

It was previously reported that the *bcr* gene, when present on a high-copy-number plasmid, confers resistance to the dike-topiperazine antibiotic bicyclomycin (3). *E. coli* cells overexpressing Bcr were also resistant to some other drugs, such as tetracycline, fosfomicin (25), and sulfathiazole (37). However, disruption of the *bcr* gene did not increase the susceptibility to L-cysteine and tetracycline (our unpublished results), although the *E. coli* strain lacking this gene resulted in the loss of bicyclomycin resistance (3). This is probably because there are several export systems in *E. coli* for L-cysteine (YdeD and YfiK) (6, 12) and tetracycline (MdfA, AcrAB, etc.) (26). Analysis using radioactive amino acids showed that the Bcr protein has specificity for L-cysteine and does not significantly export any amino acids tested, including L-serine, which has a large side chain volume, and L-valine, which inhibits the growth of *E. coli* cells (7).

It is presumed that Bcr is energized by a proton gradient. When CCCP was added to dissipate the proton motive force, the transport activity for L-cysteine significantly decreased (Fig. 4). This is the first experimental indication that the Bcr protein utilizes the proton motive force for L-cysteine transport. Among 19 putative transporters of the MF family, most possess 12 hydrophobic regions, which may be transmembrane segments, and a conserved GXXXX(R/K)XGR(R/K) motif in the putative cytoplasmic loop between the hydrophobic regions (39). An altered Bcr protein with a high transport activity or substrate specificity for L-cysteine might be useful for further improvements in L-cysteine production. Such engineering of the *bcr* gene is currently under way.

It is not clear which form of L-cysteine is transported across the membrane. It is possible that L-cysteine is exported as free L-cysteine, as L-cystine, or as 2-methyl-2,4-thiazolidinedicarboxylic acid (CP), which is the product of a nonenzymatic condensation reaction of L-cysteine with pyruvate in the cytoplasm or the medium (6). Therefore, one cannot rule out the possibility that YdeD and YfiK do not export free L-cysteine, but L-cysteine bound in CP. We found that both the Bcr and YdeD proteins significantly export free L-cysteine (Fig. 3). To examine the substrate of these L-cysteine exporters in further detail, we will analyze the intracellular and extracellular metabolite profiles of L-cysteine-producing *E. coli* cells overexpressing these genes.

Both *ydeD* and *yfiK* are expressed at very low levels (6, 13). No regulation could be detected for the expression of these two genes, but the phenotype of a *ydeD* insertion mutant and the chromosomal organization of the *yfiK* gene support the possibility that these genes are regulated (6, 13). On the other hand, nothing is yet known about the regulation of expression of the *bcr* gene. The plasmid pUCbcr used here possesses two genes, *bcr* and *yejD* (putative regulator) (25). When the *bcr* gene alone was cloned into the expression vector under the control of the *trc* promoter, the drug resistance pattern of the recombinant plasmid was comparable to that of pUCbcr (25). This and the results of the reporter gene assay suggest that the *bcr* gene is constitutively expressed at a substantial level. Although it is unknown whether YejD belongs to the family of

known regulators, it is unlikely that YejD regulates the expression of the *bcr* gene.

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REFERENCES

- Awano, N., M. Wada, A. Kohdoh, T. Oikawa, H. Takagi, and S. Nakamori. 2003. Effect of cysteine desulfhydrase gene disruption on L-cysteine overproduction in *Escherichia coli*. *Appl. Microbiol. Biotechnol.* **62**:239–243.
- Awano, N., M. Wada, H. Mori, S. Nakamori, and H. Takagi. 2005. Identification and functional analysis of cysteine desulfhydrases in *Escherichia coli*. *Appl. Environ. Microbiol.* **71**:4149–4152.
- Bentley, J., L. S. Hyatt, K. Ainley, J. H. Parish, R. B. Herbert, and G. R. White. 1993. Cloning and sequence analysis of an *Escherichia coli* gene conferring bicyclomycin resistance. *Gene* **127**:117–120.
- Bolhuis, H., H. W. van Veen, B. Poolman, A. J. Driessen, and W. N. Konings. 1997. Mechanisms of multidrug transporters. *FEMS Microbiol. Rev.* **21**:55–84.
- Cook, G. M., and R. K. Poole. 2000. Oxidase and periplasmic cytochrome assembly in *Escherichia coli* K-12: CydDC and CcmAB are not required for haem-membrane association. *Microbiology* **146**:527–536.
- Daßler, T., T. Maier, C. Winterhalter, and A. Böck. 2000. Identification of a major facilitator protein from *Escherichia coli* involved in efflux of metabolites of the cysteine pathway. *Mol. Microbiol.* **36**:1101–1112.
- De Felice, M., C. Squires, M. Levintal, J. Guardiola, A. Lamberti, and M. Iaccarino. 1977. Growth inhibition of *Escherichia coli* K-12 by L-valine: a consequence of a regulatory pattern. *Mol. Gen. Genet.* **156**:1–7.
- Delaney, J. M., D. Ang, and C. Georgopoulos. 1992. Isolation and characterization of the *Escherichia coli* *htrD* gene, whose product is required for growth at high temperatures. *J. Bacteriol.* **174**:1240–1247.
- Denk, D., and A. Böck. 1987. L-Cysteine biosynthesis in *Escherichia coli*: nucleotide sequence and expression of the serine acetyltransferase (*cysE*) gene from the wild-type and cysteine excreting mutant. *J. Gen. Microbiol.* **133**:515–525.
- Dwivedi, C. M., R. C. Ragin, and J. R. Uren. 1982. Cloning, purification, and characterization of β -cystathionase from *Escherichia coli*. *Biochemistry* **21**:3064–3069.
- Eisenberg, D., E. Schwarz, M. Komaromy, and R. Wall. 1984. Analysis of membrane and surface protein sequences with the hydrophobic moment plot. *J. Mol. Biol.* **179**:125–142.
- Franke, I., A. Resch, T. Daßler, T. Maier, and A. Böck. 2003. YfiK from *Escherichia coli* promotes export of O-acetylserine and cysteine. *J. Bacteriol.* **185**:1161–1166.
- Gaitonde, M. K. 1967. A spectrophotometric method for the direct determination of cysteine in the presence of other naturally occurring amino acids. *Biochem. J.* **104**:627–633.
- Grass, G., and C. Rensing. 2001. Genes involved in copper homeostasis in *Escherichia coli*. *J. Bacteriol.* **183**:2145–2147.
- Harris, C. L. 1981. Cysteine and growth inhibition of *Escherichia coli*: threonine deaminase as the target enzyme. *J. Bacteriol.* **145**:1031–1035.
- Harris, C. L., and L. Lui. 1981. Cysteine and growth inhibition of *Escherichia coli*: depression of the *ilvGEDA* operon. *Biochem. Biophys. Res. Commun.* **101**:1145–1151.
- Kari, C., Z. Nagy, P. Kovacs, and F. Hernadi. 1971. Mechanism of the growth inhibitory effect of cysteine on *Escherichia coli*. *J. Gen. Microbiol.* **68**:349–356.
- Kawamura-Sato, K., K. Shibayama, T. Horii, Y. Iimura, Y. Arakawa, and M. Ohta. 1999. Role of multiple efflux pumps in *Escherichia coli* in indole expulsion. *FEMS Microbiol. Lett.* **179**:345–352.
- Kredich, N. M. 1983. Regulation of cysteine biosynthesis in *Escherichia coli* and *Salmonella typhimurium*, p. 115–132. In K. M. Herrmann and R. L. Somerville (ed.), *Amino acids: biosynthesis and genetic regulation*. Addison-Wesley Publishing Company, London, United Kingdom.
- Lomovskaya, O., and K. Lewis. 1992. Emr, an *Escherichia coli* locus for multidrug resistance. *Proc. Natl. Acad. Sci. USA* **89**:8938–8942.
- Miller, J. 1972. *Experiments in molecular genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Nakamori, S., S. Kobayashi, C. Kobayashi, and H. Takagi. 1998. Overproduction of L-cysteine and L-cystine by *Escherichia coli* strains with a

- genetically altered serine acetyltransferase. *Appl. Environ. Microbiol.* **64**:1607–1611.
23. **Newton, W. A., and E. F. Snell.** 1964. Catalytic properties of tryptophanase, a multifunctional pyridoxal phosphate enzyme. *Proc. Natl. Acad. Sci. USA* **51**:382–389.
 24. **Newton, W. A., Y. Morino, and E. F. Snell.** 1965. Properties of crystalline tryptophanase. *J. Biol. Chem.* **240**:1211–1218.
 25. **Nishino, K., and A. Yamaguchi.** 2001. Analysis of a complete library of putative drug transporter genes in *Escherichia coli*. *J. Bacteriol.* **183**:5803–5812.
 26. **Park, S., and J. A. Imlay.** 2003. High levels of intracellular cysteine promote oxidative DNA damage by driving the Fenton reaction. *J. Bacteriol.* **185**:1942–1950.
 27. **Pittman, M. S., H. Corker, G. Wu, M. B. Binet, A. J. G. Moir, and R. K. Poole.** 2002. Cysteine is exported from the *Escherichia coli* cytoplasm by CydDC, an ATP-binding cassette-type transporter required for cytochrome assembly. *J. Biol. Chem.* **277**:49841–49849.
 28. **Putman, M., H. W. van Veen, and W. N. Konings.** 2000. Molecular properties of bacterial multidrug transporters. *Microbiol. Mol. Biol. Rev.* **64**:672–693.
 29. **Rosenberg, E. Y., D. Ma, and H. Nikaido.** 2000. AcrD of *Escherichia coli* is an aminoglycoside efflux pump. *J. Bacteriol.* **182**:1754–1756.
 30. **Sambrook, J., and D. W. Russell.** 2001. *Molecular cloning: a laboratory manual*, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
 31. **Sivaraman, J., V. Sauvé, R. Larocque, E. A. Stura, J. D. Schrag, M. Cygler, and A. Matte.** 2002. Structure of the 16S rRNA pseudouridine synthase RsaA bound to uracil and UMP. *Nat. Struct. Biol.* **9**:353–358.
 32. **Soda, K.** 1987. Microbial sulfur amino acids: an overview. *Methods Enzymol.* **143**:453–459.
 33. **Sorensen, M. A., and S. Pedersen.** 1991. Cysteine, even in low concentrations, induces transient amino acid starvation in *Escherichia coli*. *J. Bacteriol.* **173**:5244–5246.
 34. **Takagi, H., C. Kobayashi, S. Kobayashi, and S. Nakamori.** 1999. PCR random mutagenesis into *Escherichia coli* serine acetyltransferase: isolation of the mutant enzymes that cause overproduction of L-cysteine and L-cystine due to the desensitization to feedback inhibition. *FEBS Lett.* **452**:323–327.
 35. **Takagi, H., N. Awano, S. Kobayashi, C. Kobayashi, M. Noji, K. Saito, and S. Nakamori.** 1999. Overproduction of L-cysteine and L-cystine by expression of genes for feedback inhibition-insensitive serine acetyltransferase from *Arabidopsis thaliana* in *Escherichia coli*. *FEMS Microbiol. Lett.* **179**:453–459.
 36. **Tsunoda, T., S. Eguchi, and K. Narumi.** 1961. On the bioassay of amino acids. II. Determination of arginine, aspartic acid and cysteine. *Amino Acids* **3**:7–13.
 37. **Vedantam, G., G. G. Guay, N. E. Austria, S. Z. Doktor, and B. P. Nichols.** 1998. Characterization of mutations contributing to sulfathiazole resistance in *Escherichia coli*. *Antimicrob. Agents Chemother.* **42**:88–93.
 38. **Wrzesinski, J., A. Bakin, K. Nurse, B. G. Lane, and J. Ofengand.** 1995. Purification, cloning, and properties of the 16S RNA pseudouridine 516 synthase from *Escherichia coli*. *Biochemistry* **34**:8904–8913.
 39. **Yamaguchi, A., T. Kimura, Y. Someya, and T. Sawai.** 1993. Metal-tetracycline/H⁺ antiporter of *Escherichia coli* encoded by transposon Tn10. The structural resemblance and functional difference in the role of the duplicated sequence motif between hydrophobic segments 2 and 3 and segments 8 and 9. *J. Biol. Chem.* **268**:6496–6504.