

TolC-Dependent Exclusion of Porphyrins in *Escherichia coli*[∇]

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We found that *Escherichia coli tolC* mutants showed increased sensitivity to 5-aminolevulinic acid (ALA), a precursor of porphyrins. The *tolC* mutant cells grown in the presence of ALA showed a reddish brown color under visible light and a strong red fluorescence under near-UV irradiation. Fluorescence spectrometry and high-performance liquid chromatography analysis showed that the *tolC* mutant cells grown in the presence of ALA accumulated a large amount of coproporphyrin(ogen) intracellularly. In contrast, the wild-type cells produced coproporphyrin extracellularly. The *tolC* mutant cells grown in the presence of ALA, which were capable of surviving in the dark, were killed by near-UV irradiation, suggesting that the intracellular coproporphyrin(ogen) renders these cells photosensitive. These results suggest that the TolC-dependent efflux system is involved in the exclusion of porphyrin(ogen)s in *E. coli*.

The antibiotic alaremycin was discovered during the process of screening for inhibitors of bacterial chromosome partitioning using the anucleate cell blue assay in our laboratory (3). The anucleate cell blue assay is designed for detecting anucleate cells resulting from the inhibition of *Escherichia coli* chromosome partitioning. Alaremycin is structurally related to a precursor of porphyrins, namely 5-aminolevulinic acid (ALA), and therefore has been named alaremycin (for ALA-related antibiotic produced by *Streptomyces* sp.). In the course of studying its mechanism of action, we found that *tolC* mutants showed increased sensitivity to alaremycin (data not shown).

The *E. coli* outer membrane channel-tunnel protein TolC is involved in the exclusion of harmful substances such as antibiotics, dyes, organic solvents, and detergents (1, 20, 23, 33, 34). The crystal structure of the TolC protein recently has been determined (17). The TolC protein is composed of a transmembrane domain and a periplasmic domain and forms a homotrimer. The periplasmic barrel structure of TolC is connected to drug efflux pump proteins such as AcrB and AcrE, which are located on the inner membrane. Clamp proteins such as AcrA and ArcF link TolC and pump proteins in the periplasmic space (37, 39). Pump proteins seem to transport toxic cytoplasmic or periplasmic substances into the extracellular space across the outer membrane via the TolC channel.

The TolC-dependent efflux system is not limited to merely being a participant in the exclusion of exogenous harmful substances; it also is involved in the export of intracellular metabolites such as indole (16) and siderophores (5). Although indole, a tryptophan metabolite, is a common compound in *E. coli* cells, high concentrations of indole are harmful to the *E. coli* cells. The *E. coli* cells must, therefore, defend themselves from the toxic action of indole. It has been reported that the TolC-AcrEF efflux pump functions as an indole exporter (16). The TolC-dependent efflux system also is involved in the export of a catecholate siderophore, i.e., enterobactin. It also has

been reported that enterobactin excretion is a two-step process involving the major facilitator EntS and the TolC-dependent efflux system (5).

ALA, which is structurally related to alaremycin, is a heme precursor. In *E. coli*, ALA is formed from glutamyl-tRNA by the action of glutamyl-tRNA reductase, which is encoded by *hemA*, and glutamate-1-semialdehyde aminotransferase, which is encoded by *hemL* (2, 13). ALA is an essential precursor for heme biosynthesis; however, increased concentrations of it induce oxidative stress in the presence of Fe²⁺ ions (4, 11, 28). The second step of heme biosynthesis is catalyzed by porphobilinogen synthase, which is encoded by *hemB* (18). HemB condenses two molecules of ALA to yield porphobilinogen. Further, four molecules of porphobilinogen are condensed to form uroporphyrinogen III by the action of porphobilinogen deaminase, which is encoded by *hemC*, and uroporphyrinogen III synthase, which is encoded by *hemD* (29, 35). Uroporphyrinogen III then is converted to protoporphyrin IX by successive decarboxylation and oxidation by HemE, HemF or HemN, and HemG (9, 14, 24, 26, 30, 40, 41). Finally, ferrochelatase, encoded by *hemH*, converts protoporphyrin IX to heme by inserting Fe²⁺ into protoporphyrin IX (12).

In *E. coli*, heme is used as a cofactor by cytochromes in the electron transfer system (36) and by a hydrogen peroxide-scavenging enzyme named catalase (19). *E. coli* cannot utilize exogenous heme or porphyrins, and no heme/porphyrin uptake systems have been identified thus far. Therefore, heme is synthesized de novo by the above-mentioned heme biosynthesis pathway. It is reported that heme is incorporated into apo cytochromes in the periplasmic space probably by the action of the cytochrome *c* maturation system CcmABCDEFGH (31). However, the manner in which porphyrin molecules are transported from the cytoplasm to the periplasmic space across the inner membrane remains unknown (7).

During our study of the mechanism of action of alaremycin, we found that *E. coli tolC* mutants showed increased sensitivity to exogenous ALA. *tolC* mutant cells grown in the presence of ALA exhibited a strong red fluorescence under near-UV irradiation. Moreover, *tolC* mutant cells grown in the presence of ALA accumulated a large amount of porphyrin(ogen)s intra-

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cellularly, in contrast to the wild-type cells, which produced porphyrins extracellularly. These results suggest that the TolC-dependent efflux system is involved in porphyrin(ogen) exclusion in *E. coli*.

MATERIALS AND METHODS

Strains, plasmid, and growth medium. The *E. coli* K-12 strain JA300 (*F⁻ thr leuB6 tpnC1117 thi rpsL20 hsdS*), its *tolC* derivative JA300T (JA300 but with *tolC*), and the *tolC* plasmid pMX, carrying the wild-type *tolC* gene on the low-copy-number vector pMW119 (Nippon Gene Co. Ltd., Tokyo, Japan), were used in this study (1). P1 phage-mediated transduction was used to construct *fur::Tn5* derivatives of JA300 and JA300T by using BN904 (*F⁻ gltA6 fur::Tn5 galK30 pyrD36 relA1 rpsL129 thi-1 supE44 λ⁻*) as the donor. The cells were grown in P medium containing 1% polypepton, 0.5% NaCl, and 0.1% glucose (pH 7.2).

Determination of the MIC. The MIC of ALA was determined on P agar plates using conventional dilution methods. A total of 10^4 cells were spotted on a test agar plate, followed by incubation at 30°C for 24 h in the dark. The lowest concentration of ALA that completely inhibited colony formation was determined as the MIC.

Photosensitivity of the cells grown in the presence of ALA. Each bacterial strain was grown at 30°C in P medium in the presence or absence of 10 µg/ml of ALA for 24 h. The cells were harvested by centrifugation, quickly washed with saline, and suspended in the same solution. The cells were suspended in a glass plate by being stirred and then were exposed to 366-nm near-UV light for 5 to 15 min. A 15-W UV lamp with a 366-nm filter placed at a distance of 5 cm from the plate was used for this purpose. After dilution, the irradiated cells were spread on P plates. The plates were incubated at 30°C for 24 h in the dark, and the number of viable cells was counted.

Extraction and measurement of intracellular ALA. ALA was extracted using the method of Kanjo et al. (15). Briefly, each bacterial strain was grown at 30°C in P medium until an optical density at 660 nm of 0.9 was attained. Cells were harvested from 90 ml of the cultures by centrifugation at $5,800 \times g$ for 10 min and then were suspended in 3 ml of fresh P medium. ALA then was added to the cell suspension at a final concentration of 100 or 300 µg/ml. The cells were incubated at 30°C for the indicated time periods. Further, 1 ml of the cell suspension was centrifuged and quickly washed twice with ice-cold water, and then the cells were suspended in 200 µl of 10% trichloroacetic acid, followed by the addition of 200 µl of a 1 M sodium acetate solution and 40 µl of methyl acetoacetate to the cell suspension. The mixture was boiled for 10 min at 100°C. After the mixture had cooled down to room temperature, 600 µl of ethyl acetate was added to the mixture. After centrifugation at $15,300 \times g$ for 1 min at 4°C, the organic phase was used for the chromogenic reaction. Ehrlich's reagent (200 µl) was added to 400 µl of the organic phase, and the A_{553} of the aqueous phase was measured after 10 min.

Extraction and fractionation of porphyrins. Total porphyrins were extracted using the method of Cox and Charles (8). Each bacterial strain was grown at 30°C in P medium until an optical density at 660 nm of 0.9 was attained, and then ALA was added to the broth at a final concentration of 10 µg/ml. The cell culture (20 ml) was centrifuged at $5,800 \times g$ for 5 min to separate the cells from the medium. The cells were suspended in 20 ml of ice-cold water. Ethyl acetate-glacial acetic acid (3:1, vol/vol; 10 ml) was added to the medium and the cell suspension. The mixtures then were centrifuged at $5,800 \times g$ for 10 min, and then the organic layer was recovered. The ethyl acetate extracts were washed with 1 ml of ice-cold water. Porphyrins were extracted from the ethyl acetate fractions using 3 ml of 3 M hydrochloric acid solution twice. The fluorescence spectra of the resultant hydrochloric acid solutions were collected using an F-2000 fluorescence spectrophotometer (Hitachi Co. Ltd., Tokyo, Japan) with an excitation wavelength of 405 nm. The fluorescence intensity at 596 nm was determined as an index of the total porphyrin content.

The porphyrin composition was analyzed according to the method of Nitzan et al. (25) with a minor modification. Hydrochloric acid solutions containing total porphyrins were separated using a high-performance liquid chromatography (HPLC) system equipped with a photodiode array detector (Waters Co., Chicago, IL) and an octadecyl silica gel column (ODS-100V; 5-µm particle size; 150 by 4.6 mm; Tosoh Co. Ltd., Tokyo, Japan). Porphyrins were eluted for 20 min at a flow rate of 1 ml/min with a linear gradient from 100% solvent A (10% acetonitrile in 1 M ammonium acetate; pH 5.1) to 100% solvent B (methanol-acetic acid, 10:1 [vol/vol]), followed by elution again with 100% solvent B.

Isolation of the periplasmic cell fraction. The cells that were grown in the presence of 10 µg/ml ALA were suspended in an ice-cold solution of 0.5 M sucrose, 0.1 M Tris-HCl, and 1 mM Na-EDTA (pH 8.0). After a 10-min incu-

bation on an ice bath, lysozyme was added to the solution at a final concentration of 16 µg/ml, and immediately thereafter an equal volume of ice-cold water was added. The suspension was again incubated on an ice bath for 5 min, and then MgSO₄ was added at a final concentration of 18 mM. The suspension was centrifuged at $5,800 \times g$ for 10 min. The clear supernatant was considered to contain the periplasmic cell fraction. The degree of contamination of this fraction with cytoplasmic material was estimated by comparing the malate dehydrogenase activity in this fraction to that in the total cell lysate. Malate dehydrogenase activity was assayed by determining the initial rate of NAD⁺ reduction at 25°C. The reaction mixture contained 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 0.5 mM NAD⁺, the cytoplasmic cell fraction, and 40 mM L-malate. The reaction was initiated by the addition of L-malate, and the increase in the levels at A_{340} was monitored using a spectrophotometer (U-2800; Hitachi).

RESULTS

ALA sensitivity of *tolC* mutants. During the study of the mechanism of action of alaremycin, we found that the *tolC* mutants showed increased sensitivity to alaremycin (data not shown). We presumed that the TolC-dependent efflux system was involved in alaremycin efflux. Since the structure of alaremycin is similar to that of ALA, we also examined the ALA sensitivity of the *tolC* mutants. As expected, the *tolC* mutants showed increased sensitivity to ALA. The MIC of ALA was 1.25 mg/ml and 2.5 mg/ml for the *tolC* mutant strain JA300T and the wild-type strain JA300, respectively. The ALA sensitivity of the *tolC* mutant was restored to that of the wild-type strain by the complementation of the former with a plasmid carrying the *tolC* gene.

Similar intracellular accumulation of ALA by wild-type and *tolC* mutant cells. We suspected that the TolC-dependent efflux system was involved in ALA efflux and that the *tolC* mutants had accumulated a larger amount of ALA intracellularly than the wild-type cells. As a precursor of heme biosynthesis, ALA is an essential compound for *E. coli* cell growth. An increase in intracellular ALA concentration was, however, shown to be harmful to the *E. coli* cells, because ALA induces oxidative stress in the presence of ferric ions (4, 11, 28). Thus, the effect of the *fur* mutation, which causes the increased uptake of iron ions, on the ALA sensitivity of the *E. coli* cells was examined. Even in the presence of 1 mM FeCl₃, the introduction of the *fur::Tn5* mutation resulted in only a twofold increase in the ALA sensitivity in both the wild-type and the *tolC* mutant strains. This suggested that the intracellular ALA concentration was not markedly increased even if cells were exposed to high concentrations of ALA. We therefore examined the intracellular ALA concentrations in the wild-type and *tolC* mutant cells after they had been exposed to high concentrations of ALA. As shown in Fig. 1, the intracellular ALA concentration increased slightly in the first 30 min and reached a plateau thereafter when the cells were exposed to 100 or 300 µg/ml of ALA. No significant difference in the intracellular ALA concentration was observed between the wild-type and *tolC* mutant cells, suggesting that ALA was quickly excluded by the TolC-independent efflux system or that the incorporated ALA was rapidly metabolized to porphobilinogen by porphobilinogen synthase, which is encoded by the *hemB* gene, and to other downstream metabolites, i.e., porphyrin(ogen)s.

***tolC* mutant cells showed red fluorescence under near-UV irradiation.** During the ALA accumulation experiments, we noticed that the *tolC* mutant cells grown in the presence of 10 µg/ml of ALA, a concentration at which the growth of the *tolC*

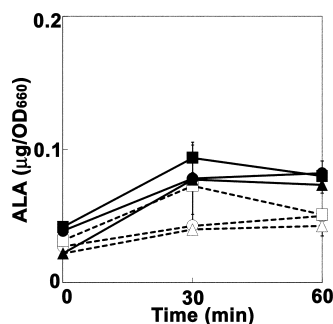


FIG. 1. Accumulation of ALA in the wild-type and *tolC* mutant cells exposed to exogenous ALA. The cells were exposed to 100 (open symbols) or 300 (closed symbols) $\mu\text{g/ml}$ ALA at 30°C . After the indicated time periods, ALA was extracted from the cells and was quantified as described in Materials and Methods. Circles, JA300 (wild-type strain); triangles, JA300T (containing *tolC*); squares, JA300T/pMX. The means (symbols) and standard deviations (error bars) are shown ($n = 3$). OD_{660} , optical density at 660 nm.

mutants was not inhibited, showed a reddish brown color under visible light (Fig. 2, upper). Since heme and porphyrin(ogen)s, which are synthesized from ALA, are pigments that yield a red fluorescence, we suspected that the *tolC* mutant cells accumulated porphyrin(ogen)s intracellularly. As expected, the *tolC* mutant cells showed a strong red fluorescence under near-UV irradiation of 366 nm (Fig. 2, lower), which was a characteristic of porphyrin(ogen)s. In contrast, the wild-type cells showed a bluish white color under near-UV irradiation of 366 nm. This suggested that the *tolC* mutant cells accumulated a large amount of porphyrin(ogen)s intracellularly, while the wild-type cells did not.

Accumulation of porphyrin(ogen)s in *tolC* mutant cells. We extracted intracellular porphyrin(ogen)s from the wild-type and *tolC* mutant cells grown in the presence of $10 \mu\text{g/ml}$ ALA. Porphyrinogens, if present, would have been oxidized during the extraction procedures; therefore, the extracted samples should have contained porphyrins (10, 27). The total porphyrin concentrations were determined by fluorescence spectrometry with an excitation wavelength of 405 nm. As shown in Fig. 3, the *tolC* mutant cells accumulated a large amount of porphy-

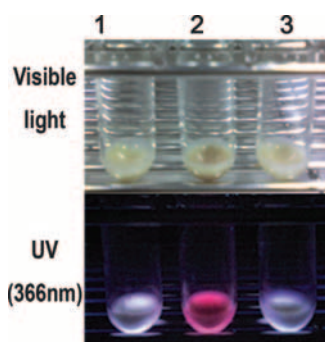


FIG. 2. Red fluorescent pigments accumulated in the *tolC* mutant cells grown in the presence of ALA. Cells grown at 30°C for 24 h in the presence of $10 \mu\text{g/ml}$ ALA were harvested by centrifugation and were photographed under visible light (upper) or near-UV irradiation of 366 nm (lower). Lanes: 1, JA300 (wild-type strain); 2, JA300T (containing *tolC*); 3, JA300T/pMX.

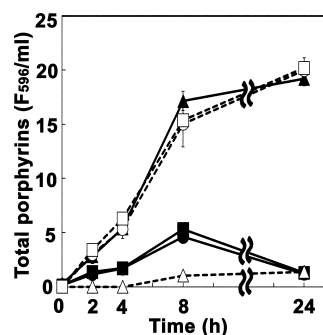


FIG. 3. Intracellular and extracellular porphyrin(ogen)s produced by the wild-type and *tolC* mutant cells grown in the presence of ALA. The cells were grown at 30°C in the presence of $10 \mu\text{g/ml}$ ALA for the indicated time periods. The cells were separated from the growth medium by centrifugation. Porphyrin(ogen)s were extracted from the cells as well as from the medium and were quantified as described in Materials and Methods. Circles, JA300 (wild-type strain); triangles, JA300T (containing *tolC*); squares, JA300T/pMX; open symbols, medium fraction; closed symbols, cell fraction. The means (symbols) and standard deviations (error bars) are shown ($n = 3$). F_{596} , fluorescence intensity at 596 nm with an excitation wavelength of 405 nm.

rin(ogen)s intracellularly. On the other hand, the wild-type cells accumulated a smaller amount of porphyrins. We speculated that the TolC-dependent efflux system is involved in porphyrin(ogen) efflux. If our speculation was accurate, the wild-type cells would excrete porphyrin(ogen)s into the medium. As expected, the wild-type cells released a large amount of porphyrins in the medium, an amount that was almost comparable to the intracellular porphyrin(ogen) levels in the *tolC* mutant cells. The total amounts of porphyrin(ogen)s produced by the wild-type and *tolC* mutant cells were almost identical. We also examined if heme synthesis was stimulated in the *tolC* mutant cells grown in the presence of ALA. However, notable heme accumulation was not observed even in the presence of the *fur* mutation, which caused an increase in the iron uptake (data not shown).

ALA is metabolized to protoporphyrinogen IX via uroporphyrinogen III and coproporphyrinogen III. Uroporphyrinogen III, coproporphyrinogen III, and protoporphyrinogen IX are converted to uroporphyrin III, coproporphyrin III, and protoporphyrin IX, respectively, by auto- or enzymatic oxidation. Finally, ferrochelatase, which is encoded by *hemH*, converts protoporphyrin IX to heme by inserting Fe^{2+} into protoporphyrin IX. Porphyrins produced by the wild-type and *tolC* mutant strains grown in the presence of ALA were analyzed by fluorescence spectrometry. The fluorescence spectra of the total porphyrin fractions extracted from the wild-type medium and that extracted from the *tolC* mutant cells matched well with that of coproporphyrin (Fig. 4). This was confirmed by HPLC analysis. As shown in Fig. 5, the wild-type and *tolC* mutant strains grown in the presence of ALA produced mainly coproporphyrin. Coproporphyrin accounted for 94% of the total porphyrins extracted from the wild-type medium and 97% of the total porphyrins extracted from the *tolC* mutant cells. Since it is considered that porphyrinogens are oxidized to porphyrins during export or extraction procedures, it is possible that the *tolC* mutant cells accumulated coproporphyrinogen intracellularly. On the other hand, the extracellularly accumu-

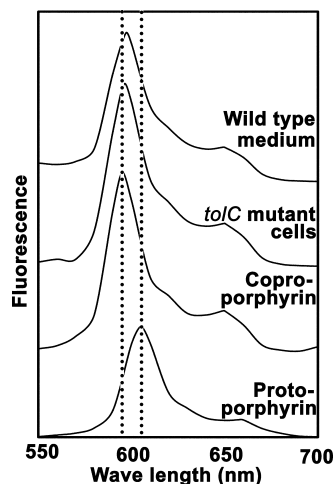


FIG. 4. Fluorescence spectra of porphyrins produced by the wild-type and *tolC* mutant cells. The fluorescence spectra of the total porphyrin fractions extracted from the wild-type medium and the *tolC* mutant cells at 24 h, as shown in Fig. 3, were collected at an excitation wavelength of 405 nm. The spectra of authentic coproporphyrin and protoporphyrin also are shown.

lated molecules in the wild-type medium might have been coproporphyrin itself.

It has been reported that enterobactin excretion is a two-step process involving the major facilitator EntS and the TolC-dependent efflux system (5). It also is possible that porphyrin(ogen) exclusion also is a two-step process, i.e., porphyrin(ogen) are transported to the periplasm by a TolC-independent mechanism and then transported across the outer membrane by the TolC-dependent efflux system. We therefore examined the intracellular localization of accumulated porphyrin(ogen)s in the cells grown in the presence of ALA. It was revealed that a substantial proportion of porphyrins ($97.4\% \pm 0.44\%$ in the *tolC* mutant cells and $67.5\% \pm 0.45\%$ in the wild-type cells) was recovered from the periplasmic cell fraction. The degree of contamination of the periplasmic cell fraction with cytoplasm was 11.3 and 21.3% for the *tolC* mutant and wild-type cells, respectively; this was determined using malate dehydrogenase as a marker enzyme for the cytoplasm. This finding indicates that TolC is involved in the transport of porphyrins from the periplasm into the medium. Since the periplasmic environment is more oxidative than that of

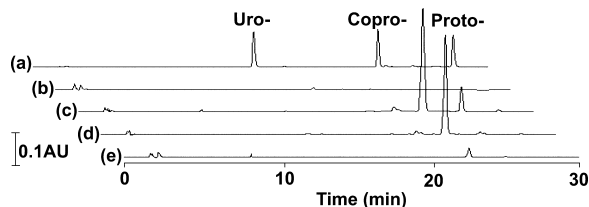


FIG. 5. Composition of porphyrins. The total porphyrin fractions at 24 h, as shown in Fig. 3, were separated by HPLC as described in Materials and Methods. (a) Authentic samples. Uro-, uroporphyrin; Copro-, coproporphyrin; Proto-, protoporphyrin. (b) Wild-type cells. (c) Wild-type medium. (d) *tolC* mutant cells. (e) *tolC* mutant medium. The vertical scale shown on the left represents 0.1 absorbance unit (AU) at 393 nm.

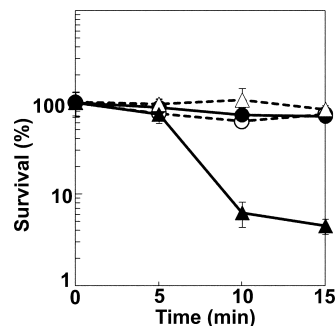


FIG. 6. Photosensitivity of the *tolC* mutant strain grown in the presence of ALA. Cells grown at 30°C for 24 h in the presence (closed symbols) or absence (open symbols) of 10 µg/ml ALA were exposed to near-UV light at 366 nm, and the number of viable cells was counted as described in Materials and Methods. Circles, JA300 (wild-type strain); triangles, JA300T (containing *tolC*). The means (symbols) and standard deviations (error bars) are shown ($n = 3$).

the cytoplasm, the *tolC* mutant cells might accumulate coproporphyrin rather than coproporphyrinogen.

Photosensitivity of *tolC* mutants grown in the presence of ALA. It is known that porphyrin(ogen)s function as photosensitizers. Previous studies reported that the *hemH* mutant strain, which accumulated protoporphyrin(ogen) due to a defect in ferrochelatase, showed photosensitivity (21, 22, 45). We therefore examined the photosensitivity of the *tolC* mutant strain, which accumulated coproporphyrin(ogen). The wild-type and *tolC* mutant cells were grown in the presence of 10 µg/ml ALA for 24 h in the dark. The cells then were exposed to near-UV light at 366 nm, following which the number of viable cells was counted. As shown in Fig. 6, the *tolC* mutant cells grown in the presence of ALA exhibited decreased survival following the near-UV irradiation compared to the survival of wild-type cells grown under identical conditions. The *tolC* mutant cells grown in the absence of ALA did not show such photosensitivity. These results showed that coproporphyrin(ogen) synthesized from exogenous ALA functions as a photosensitizer in the *tolC* mutant cells.

DISCUSSION

There are many genes in the *E. coli* chromosomal DNA that encode proteins associated with drug efflux. In particular, the outer membrane channel-tunnel protein, TolC, plays an important role in drug efflux. The TolC protein effluxes many harmful compounds in partnership with several inner membrane pump proteins, such as AcrAB and AcrEF. The TolC-dependent efflux system mostly exports periplasmic compounds to the extracellular space across the outer membrane (17, 20, 23).

The TolC-dependent efflux system also plays a role in the efflux of endogenous compounds; for example, the TolC and AcrEF pump is involved in indole efflux. Although indole, a tryptophan metabolite, is a common compound in the *E. coli* cells, high concentrations of indole are harmful to *E. coli* cells (16). TolC is involved in the export of useful compounds as well. Enterobactin, involved in the acquisition of extracellular Fe^{3+} , is transported by TolC (5).

E. coli can utilize both de novo-synthesized and exogenous

ALA. ALA is synthesized from glutamyl-tRNA by the action of glutamyl-tRNA reductase, which is encoded by *hemA*, and glutamate-1-semialdehyde aminotransferase, which is encoded by *hemL* (2, 13). On the other hand, extracellular ALA is incorporated into the cells via the dipeptide transporter system (43). Although ALA is a heme precursor, increased intracellular ALA concentrations cause oxidative stress in the presence of Fe²⁺ ions (4, 11, 28). Since ALA-induced reactive oxygen acts on the guanine base of DNA, ALA can be a mutagenic compound. Therefore, intracellular ALA concentrations are strictly regulated (42, 44). In this study, we examined the intracellular ALA concentrations in the wild-type and *tolC* mutant cells grown in the presence of ALA; however, no significant differences in the intracellular ALA concentration were noted between the wild-type and *tolC* mutant cells.

In the 1970s, Doss and Philippe-Dormston demonstrated that exogenous ALA stimulates porphyrin biosynthesis in several bacteria, including *E. coli* (10, 27). They also showed that the synthesized porphyrin(ogen)s then are exported into the medium. ALA is metabolized to porphobilinogen by porphobilinogen synthase, which is encoded by the *hemB* gene (18). Further, porphobilinogen is metabolized to heme via uroporphyrinogen III, coproporphyrinogen III, and protoporphyrinogen IX (26). As shown in Fig. 2, the *tolC* mutant cells grown in the presence of ALA showed a reddish brown color under visible light and a strong red fluorescence under near-UV irradiation. We presumed that the *tolC* mutant cells accumulated porphyrin(ogen)s intracellularly, because porphyrin(ogen)s are pigments that yield a red fluorescence. Hence, we measured the amount of accumulated porphyrin(ogen)s in the *E. coli* cells grown in the presence of ALA. As expected, the *tolC* mutant had accumulated a large amount of porphyrin(ogen)s intracellularly, while the wild-type cells had not. In contrast, the wild-type *E. coli* cells grown in the presence of ALA excreted porphyrins in the medium. A large amount of porphyrins was detected in the wild-type culture medium but not in the *tolC* mutant culture medium. These results suggest that the ALA incorporated into the cells was metabolized to porphyrin(ogen)s, and the excess porphyrin(ogen)s were exported by the TolC-dependent efflux system.

Protoporphyrin(ogen) is known to function as a photosensitizer. It has been reported that the *E. coli hemH* mutant, which accumulates protoporphyrin(ogen) in its cells, shows photosensitivity (8, 21, 22, 45). In this study, we showed that the *tolC* mutant cells grown in the presence of ALA exhibited decreased survival following the near-UV irradiation, while the wild-type cells survived under the same conditions. Since the *tolC* mutant cells mainly accumulated coproporphyrin(ogen), the intracellular coproporphyrin(ogen) functioned as a photosensitizer like protoporphyrin(ogen). Heme biosynthesis is regulated at the ALA synthesis step through feedback inhibition that is dependent on the heme concentration (42, 44). In the presence of exogenous ALA, however, heme biosynthesis is not repressed, and therefore a large amount of heme precursor porphyrin(ogen)s is produced (32). It also has been reported that protoporphyrinogen synthase, which is encoded by *hemF*, is inhibited by protoporphyrin (6). This is probably the reason why mainly coproporphyrin(ogen) was accumulated in the ALA-treated cells. These findings match well with previous observations (10, 27). The function of the TolC-dependent

efflux system may be to prevent the intracellular accumulation of excess porphyrin(ogen)s when cells are exposed to high concentrations of ALA. This is a new physiological role of the TolC-dependent efflux system in *E. coli*.

The TolC outer membrane channel-tunnel protein functions together with inner membrane efflux pump proteins. Therefore, an inner membrane pump(s) or exporter(s) also should be involved in porphyrin exclusion in combination with TolC. It was shown that a substantial portion of porphyrins that accumulated in the *tolC* mutant cells was recovered from the periplasmic cell fraction. This indicates that porphyrin(ogen) exclusion is a two-step process, as is the case for enterobactin excretion. In this process, porphyrin(ogen)s are transported to the periplasm by a TolC-independent mechanism and then are transported across the outer membrane by the TolC-dependent efflux system. It has been reported that the cytochrome *c* maturation system CcmABCDEFGH may be involved in the incorporation of heme into apo cytochromes in the periplasmic space (31). In this system, porphyrin molecules are to be transported from the cytoplasm to the outer side of the cytoplasmic membrane. However, the manner in which porphyrin molecules are transported from the cytoplasm to the periplasmic space across the inner membrane remains unknown (7). We examined the ALA sensitivity and porphyrin(ogen) accumulation in the *ccm* mutant strains; none of the mutant strains exhibited increased ALA sensitivity or porphyrin(ogen) accumulation (data not shown). We also examined the ALA sensitivity and porphyrin(ogen) accumulation in mutant strains with a known drug efflux pump mutation that is predicted to function along with TolC. Among the strains that we examined, namely, *acrAB*, *acrD*, *acrEF*, *emrAB*, *macAB*, *mdtEF* (*yhiUV*), *mdtABC* (*yegMNO*), and *emrKY* mutant strains, no mutant strains showed increased ALA sensitivity or porphyrin(ogen) accumulation (data not shown). In particular, it has been reported that *Staphylococcus aureus* HrtAB, which belongs to the MacAB family of ABC-type efflux carriers, is a possible heme transporter (38). However, *macAB* mutation showed no effect on porphyrin accumulation in *E. coli*, as mentioned above. An inner membrane pump that remains to be identified may be involved in porphyrin exclusion, or several pump proteins may simultaneously function as porphyrin exporters.

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