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Molecular Construction of a Multidrug Exporter System, AcrAB: Molecular Interaction between AcrA and AcrB, and Cleavage of the N-Terminal Signal Sequence of AcrA[1]

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The AcrAB system of *Escherichia coli* is an intrinsic efflux protein with a broad substrate specificity. AcrA was thought to be localized in the periplasmic space, and to be linked to AcrB and TolC. The AcrAB-TolC system directly exports diverse substrates from the cell interior to the medium. In this study, we have determined the cellular localization of AcrA. By using the osmotic shock method, sucrose density gradient centrifugation, urea washing and Western blotting analysis, we reveal that AcrA is a peripheral inner membrane protein. A mutant plasmid encoding both the AcrA-TetBCt fusion protein and the AcrB-His fusion protein was constructed. Membrane vesicles prepared from cells expressing these fusion proteins were solubilized and AcrB-His was immunoprecipitated with an anti-polyhistidine antibody. After SDS-PAGE, Western blotting was performed with anti-TetBCt antiserum, resulting in the appearance of a 40 KDa band, indicating that AcrA co-precipitated with AcrB. Next we performed site-directed chemical labeling of Cys-introduced mutants of AcrA with [^{14}C]*N*-ethylmaleimide. As judged from the labeling pattern and the molecular mass shift, the N-terminus of AcrA was removed and the mature protein is on the periplasmic surface. On the other hand, C25A mutants retained the N-terminal signal sequence on the cytoplasmic side of the membrane. We conclude that AcrA exists as a complex with AcrB on the periplasmic surface of the inner membrane after removal of the signal sequence.

Key words: AcrAB, drug efflux, multidrug resistance, site-directed mutagenesis, TolC.

The AcrAB proteins of *Escherichia coli* are intrinsic drug efflux proteins thought to be linked to an outer membrane protein, TolC, to form a multicomponent-type drug efflux system with a broad substrate specificity that appears to catalyze efflux at the expense of proton motive force (*1–3*). The AcrAB-TolC system is comprised of three subunits, *i.e.* the inner membrane protein AcrB belongs to the RND (resistance-nodulation-cell division) transporter superfamily (*4*, *5*), the outer membrane protein TolC (*6*), and AcrA. AcrA belongs to a membrane fusion protein family (*7*) and is thought to be localized in the periplasmic space, and to link with AcrB and TolC for direct efflux of the many substrates from the cell interior to the outer medium

Ma *et al.* cloned the *acrAB* gene, and demonstrated that the regions from Pro49 to Gln196, Val371, and Lys374 of AcrA are located in the periplasmic space by means of the alkaline phosphatase fusion method (*1*). They also deduced that the first 24 amino acids of AcrA comprise a prokaryotic lipoprotein signal peptide from the sequence features. The transcription of *AcrAB* is increased under many stress conditions (*8*). The transcription level is also elevated by decanoate, and the AcrAB system transports chenodeoxycholate and taurocholate (*9*). This indicates that one major physiological function of AcrAB is to protect *E. coli* against high concentrations of bile salts in the intestine. To clarify the molecular construction of AcrA, Zgurskaya, and Nikaido constructed a non-lipidated form of AcrA, and determined its hydrodynamic properties by means of analytical ultracentrifugation and dynamic light scattering techniques. The results indicated that AcrA exists in solution as a highly asymmetric monomeric molecule (*10*).

In our laboratory, we established a new method for determining the local milieus of sulfhydryl groups of cysteine residues based on the reactivity of SH modifying reagents, especially for the bacterial tetracycline efflux protein [TetB] (*11–13*). This method is very useful for investigating the surroundings of targeted amino acids replaced by Cys in detail. In this study, we investigate the features of the signal sequence of N-terminal amino acids of AcrA by this method. We also investigate the cellular localization of AcrA, and the possibility of an interaction between AcrA and AcrB.

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Abbreviations: TetB, Tn10-encoded metal-tetracycline/H$^+$ antiporter TetA(B); TetBCt, TetB C-terminal oligopeptide including 14 amino acid residues.

EXPERIMENTAL PROCEDURES

Materials—*N*-[Ethyl-1-^{14}C]maleimide (1.5 GBq/mmol) was purchased from DuPont–New England Nuclear. The oligonucleotides used for site-directed mutagenesis were synthesized by Sawady Technologies (Tokyo). Monoclonal anti-polyhistidine antibody HIS-1 and anti-penicillinase-[Enterobacter cloacae] antibody were purchased from SIGMA (Saint Louis, MO) and ROCKLAND (Gilbertsville, PA), respectively. Rabbit anti-TetBCt antiserum was prepared in our laboratory using a synthetic oligopeptide corresponding to the C-terminal 14 amino acids of TetB (*14*). All other materials were of reagent grade and obtained from commercial sources.

Bacterial Strains and Plasmids—*E. coli* CJ236 was used for oligonucleotide-directed mutagenesis by the Kunkel method (*15*). *E. coli* TG1 (*16*) was used for transformation after mutagenesis. *E. coli* N43 (F$^-$ K-12 *lac, ara, mal, xyl, mtl, gal, rpsL, acrA1*) (*1*) was used to determine the minimum inhibitory concentrations of ethidium bromide and novobiocin, and for the biochemical experiments in this study. N43 is a strain hypersensitive to many drugs because the IS2 element is inserted within the second codon of *acrA*. The *acrAB* gene was cloned from the *E. coli* W3104 strain (*17*) by PCR and then ligated into pUC118. The resultant plasmid named pAC8 was used as a wild-type *acrAB* plasmid. After introducing an *Nru*I site at the 3′-terminus of *acrA*, a TetBCt-tagged *acrA* gene was constructed by ligation of the DNA fragment encoding the TetB C-terminal 14–amino acids to the *Nru*I site of *acrA*, resulting in a plasmid named pACATC (Fig. 1). The $(His)_6$-tagged *acrB* gene was constructed by oligonucleotide-directed site-specific mutagenesis, resulting in plasmid pACABH. A Cys-less mutant *acrA* gene (pACACL) was constructed using the site-directed oligonucleotides and the pAC8 plasmid as a template. Cys-introduced mutants were constructed by the mutagenic oligonucleotide and pACATC or pACACL as a template. pACATCBH, which encodes TetBCt-tagged AcrA and $(His)_6$-tagged AcrB, was constructed by recombining a DNA fragment of pACABH encoding $(His)_6$-tagged AcrB with the corresponding fragment pACATC (Fig. 1).

Fractionation by the Osmotic Shock Method, Sucrose Density Gradient Centrifugation, and 5 M Urea Wash—The osmotic shock experiment was performed according to the method of Neu *et al.* (*18*). After harvesting an overnight culture of *E. coli*, the cells were washed with phosphate-buffered saline, resuspended in 30 mM Tris-HCl buffer (pH 7.3) containing 10 mM EDTA and 20% sucrose, and then shaken gently for 10 min at 24°C. After centrifugation (10,000 ×*g* for 10 min), the cells were quickly resuspended in cold water and then shaken gently for 10 min in an ice water bath. Then the shocked cells and the supernatant (shock fluid) were separated by centrifugation (25,000 ×*g* for 40 min). The resultant cells were resuspended in 50 mM potassium phosphate buffer (pH 7.0), and disrupted by brief sonication. After the removal of the unbroken cells, total cell membranes were separated by ultracentrifugation (200,000 ×*g* for 30 min). Then the total membranes were subjected to sucrose density gradient centrifugation [30–45% (w/w) sucrose, 200,000 ×*g* for 12 h] in order to separate the inner and outer membranes. The inner and outer membrane fractions were resuspended in the same potassium phosphate buffer. The outer membrane fraction was washed twice with 2% sarkosyl to remove inner membrane contamination. The inner membranes were then treated with 5 M urea at 4°C for 1 h. After ultracentrifugation, peripheral membrane proteins were collected in the supernatant.

Labeling of AcrA Proteins with [^{14}C]N-Ethylmaleimide (NEM) and Prevention by 4-Acetamido-4-maleimidylstilbene-2,2′-disulfonic Acid (AMS)—The [^{14}C]NEM binding experiments was performed according to the method of Kimura *et al.* (*11, 12*). Briefly, intact cells were incubated with or without 5 mM AMS for 30 min at 30°C, then labeled with 0.5 mM [^{14}C]NEM for 5 min. The reaction was stopped by dilution with excess NEM. The cells were disrupted by brief sonication and the membrane fractions were collected by ultracentrifugation. The sonicated membranes (1 mg membrane protein) were solubilized in 1% Triton X-100 and 0.1% SDS. Then the AcrA-TetBCt proteins were immunoprecipitated using anti-TetBCt antiserum. After SDS polyacrylamide electrophoresis, the radioactive bands were visualized with a BAS-1000 Bio-Imaging Analyser (Fuji Film, Tokyo).

RESULTS

Construction of Tagged and Mutant Genes, and Verifying the Expression and Activity—To begin this study, we constructed the series of AcrA mutants listed in Table I. The expression of these proteins was verified by Western blotting using anti-TetBCt antiserum (*18*). The TetBCt-tagged AcrA protein was detected at about the 40 kDa position (data not shown). The minimum inhibitory concentration (MIC) values for ethidium bromide and novobiocin are shown in Table I. *E. coli* N43 cells showed hypersensitivity to these compounds since this strain has defects in the *acrA* locus. The plasmid pAC8, which encodes the wild-type AcrA and AcrB, conferred a high level of resistance to ethidium bromide (1,024 μg/ml) and novobiocin (256 μg/ml). When a TetBCt tag was introduced to AcrA, the resistance was

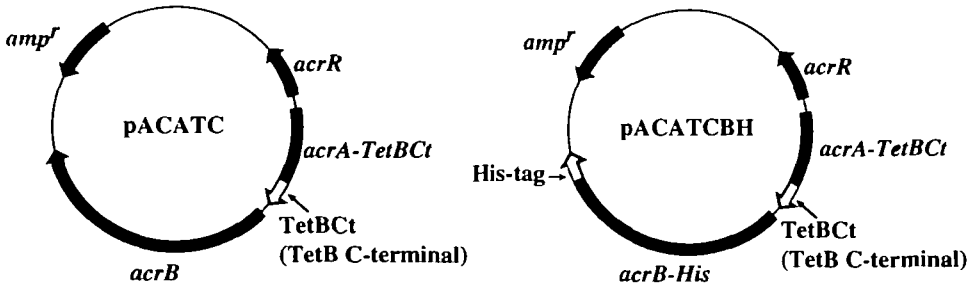

Fig. 1. **Structures of the plasmids pACATC and pACATCBH.** The *acrA* and *acrB* genes were cloned with the *acrR* gene from the genome of *E. coli* W3104 by PCR and then ligated into pUC118. TetBCt and $(His)_6$ tags were fused at the C-terminals of *acrA* and *acrB* genes, respectively.

decreased but remained significant. All other Cys-less and Cys-introduced mutants also showed reduced but significant resistance, indicating that the mutations did not cause serious conformational changes.

Cellular Localization of AcrA Detected by the Osmotic Shock Method, Sucrose Density Gradient Centrifugation, and Membrane Washing with 5 M Urea—E. coli N43 cells were co-transformed with pACATCBH, which encodes AcrA-TetBCt, AcrB-His, and β-lactamase, and pLGT2, which encodes TetB. β-lactamase and TetB were used as periplasmic and inner membrane protein controls, respectively. These proteins were detected by Western blotting using anti-TetBCt antiserum (AcrA-TetBCt and TetB), monoclonal anti-polyhistidine antibody (AcrB-His), and anti-penicillinase antibody (β-lactamase). All were expressed simultaneously in *E. coli* N43 (Fig. 2A, lane 1). First, the periplasmic proteins were separated by the osmotic shock procedure described in "EXPERIMENTAL PROCEDURES." As shown in Fig. 2A (lane 2), β-lactamase was observed mainly in the osmotic shock fluid, whereas AcrA-TetBCt and AcrB-His were not observed in the fluid at all, but were present in the shocked cell precipitates (Fig. 2A, lane 3) along with TetB. The resulting cells were disrupted with a French press, and the total membrane fraction was separated by ultracentrifugation. AcrA-TetBCt, AcrB-His, and TetB were not observed in the supernatant (Fig. 2A, lane 4) but were detected in the total membrane fraction (Fig. 2A, lane 5). Then, the inner (IM) and outer membranes (OM) were separated by sucrose density gradient ultracentrifugation. AcrA-TetBCt, AcrB-His, and TetB were all detected in the IM fraction (Fig. 2A, lane 7) and not in the OM fraction (Fig. 2A, lane 6), indicating that AcrA and AcrB are inner membrane-bound proteins.

Finally, the inner membranes were washed with 5 M urea to remove peripheral membrane proteins. As shown in Fig. 2B, 74% of AcrA-TetBCt was released in the supernatant fraction (lane 1) and only 26% remained in the precipitate (lane 2). On the other hand, the AcrB-His and TetB proteins were obtained mainly in the precipitate. When the inner membranes were washed with potassium phosphate buffer, only 30% of the AcrA-TetBCt proteins were released in the supernatant (lane 3), while 70% were precipitated (lane 4). These results indicate that AcrA is a peripheral inner membrane protein, while AcrB, like TetB, is an integral membrane protein.

Co-Precipitation of the AcrA and AcrB Proteins—In order to investigate the interaction between AcrA and AcrB, sonicated membranes prepared from *E. coli* N43 cells carrying pACATCBH and pLGT2 were solubilized, and the AcrB-His proteins were immunoprecipitated using an anti-polyhistidine antibody. The AcrA-TetBCt proteins were detected in the precipitates by Western blotting using an anti-TetBCt antibody. As controls, sonicated membranes were also prepared from cells carrying pACATC, which encodes AcrA-TetBCt and non-tagged AcrB, and cells carrying pACABH, which encodes non-tagged AcrA and AcrB-His. All these cells also carried plasmid pLGT2. In Fig. 3, odd-numbered

TABLE I. **Ethidium bromide and novobiocin resistance levels of *E. coli* N43* cells harboring the mutant plasmids.**

Plasmid	Encoded Acr proteins	MIC of ethidium bromide (μg/ml)	MIC of novobiocin (μg/ml)
No plasmid		16	2
pAC8	AcrA, AcrB	1,024	256
pACATC	AcrA-TetBCt, AcrB	512	32
pACACL	Cys-less AcrA, AcrB	1,024	128
pACAN4C	N4CAcrA-TetBCt, AcrB	512	32
pACAT60C	T60CAcrA-TetBCt, AcrB	512	32
pACACLTC	Cys-less AcrA-TetBCt, AcrB	512	32
CL/N4C	Cys-less N4CAcrA-TetBCt, AcrB	512	32
CL/T60C	Cys-less T60CAcrA-TetBCt, AcrB	512	16
pACATCBH	AcrA-TetBCt, AcrB-His	256	32

The resistance levels are indicated as the minimum inhibitory concentrations (MIC) determined by the agar dilution method. *N43 strain: The chromosomal *acrA* gene was disrupted by insertion mutation (*acrA*::IS2).

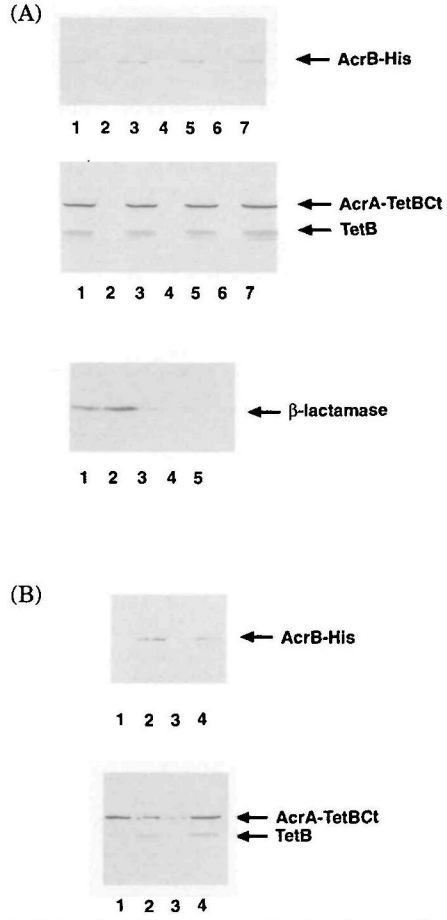

Fig. 2. **Cellular localization of the AcrA protein.** (A) Fractionation of *E. coli* N43 cells carrying pACATCBH and pLGT2, which express AcrA-TetBCt, AcrB-His, TetB, and β-lactamase, by the osmotic shock method, sucrose density gradient centrifugation as described under "EXPERIMENTAL PROCEDURES." AcrA-TetBCt and TetB were detected by Western blotting using anti-TetBCt antiserum (middle panel). β-Lactamase (lower panel) and AcrB-His (upper panel) were detected using anti-penicillinase antibody and anti-polyhistidine antibody, respectively. Lane 1, whole cells; lane 2, osmotic shock fluid; lane 3, shocked cells; lane 4, supernatant of shocked cell lysate; lane 5, total cell membrane; lane 6, outer membrane; lane 7, inner membrane. (B) 5 M urea or phosphate-buffer washing of the inner membrane. Lanes 1, supernatant of urea-washed inner membrane; lanes 2, precipitate of urea washed inner membranes; lanes 3, supernatant of phosphate buffer-washed inner membrane; lanes 4, precipitate of phosphate buffer–washed inner membrane.

lanes represent the immunoprecipitates and even-numbered lanes represent the total membrane protein fractions. As shown in Fig. 3 (lane 3), a 40 KDa AcrA-TetBCt band was clearly detected in the immunoprecipitate, indicating that the AcrA-TetBCt proteins co-precipitated with the AcrB-His proteins. Since a TetB protein band was not observed in the immunoprecipitate (lane 3) while TetB proteins were certainly present in the total membrane fraction (lane 4), it is clear that the co-precipitation is due to the specific AcrA-AcrB interaction. In lane 1, no AcrA-TetBCt band was detected because non-tagged AcrB proteins were not precipitated by the anti-polyhistidine antibody. Similarly, no band was visualized in lane 5 because no TetBCt-tagged protein was present in the precipitate. These results clearly indicate that the AcrA-TetBCt proteins immunoprecipitate specifically with AcrB-His.

[^{14}C]NEM Binding of the Cys Mutants and Its Prevention by a Membrane-Impermeable Maleimide, AMS—AcrA has only one Cys residue (Cys25), which does not react with [^{14}C]*N*-ethylmaleimide (Fig. 4). We constructed five AcrA mutants, that is, N4C (Asn 4→Cys), T60C (Thr 60→Cys), and CL (Cys 25→Ala), N4C/CL, and T60C/CL, and tagged the C-terminals of these mutants with a TetBCt epitope. In order to determine the localization of the introduced cysteine residues, we investigated the binding of a membrane-permeable SH reagent, [^{14}C]NEM, with these Cys mutants, and the blocking of the NEM binding by a membrane-impermeable maleimide, AMS, in intact cells as described in "EXPERIMENTAL PROCEDURES." As shown in Fig. 4 (upper panel), the molecular masses of the CL mutants were about 3 kDa greater than that of the AcrA-TetBCt. 3 kDa corresponds approximately to the molecular mass of an oligopeptide comprising 24 amino acids of the N-terminal of AcrA, indicating that in the wild-type AcrA, the N-terminal 24 amino acids are removed as a signal sequence. Cys25 is located at the putative cleavage site of a signal peptidase and the Cys25→Ala mutation probably prevents the cleavage by the signal peptidase. The amounts of the Cys-free mutants (CL/T60C, CL, and CL/N4C) and the T60C mutant were less than those of the wild-type (AcrA-TetBCt) and the N4C mutant (Fig. 4, upper panel), probably due to the expression levels of these mutants. With respect to the [^{14}C]NEM binding (Fig. 4, lower panel), the N4C mutant showed no reactivity with [^{14}C]NEM regardless of the presence of AMS, supporting the assumption that the N-terminus of AcrA is removed. On the other hand, the CL/N4C mutant showed high reactivity with [^{14}C]NEM and the binding of NEM was not inhibited by AMS, indicating that position 4 is located inside the cytoplasmic membrane. In contrast, the binding of [^{14}C]NEM to the T60C mutant was completely inhibited by AMS, indicating that position 60 is outside the cytoplasmic membrane.

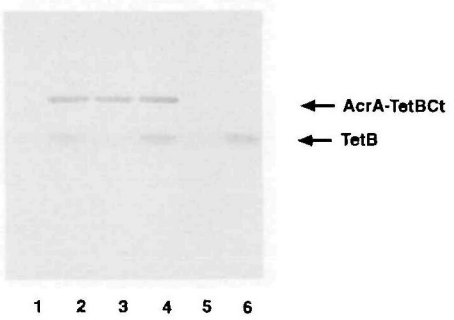

Fig. 3. **Co-immunoprecipitation of AcrA and AcrB.** Sonicated membranes were solubilized in 1% Triton X-100 and 0.1% SDS and the AcrB-His proteins were precipitated with anti-polyhistidine antibody. Then AcrA-TetBCt proteins were detected in the precipitates by Western blotting using anti-TetBCt antibody. As a control, sonicated membranes before solubilization were also subjected to Western blotting. Lanes 1, 3, and 5 represent the immunoprecipitates and lanes 2, 4, and 6 represent the sonicated membranes. Sonicated membranes were prepared from *E. coli* N43 cells carrying pACATC/pLGT2 (lanes 1 and 2), pACATCBH/pLGT2 (lanes 3 and 4), or pACABH/pLGT2 (lanes 5 and 6).

DISCUSSION

In this study, we show that (i) AcrA interacts physically with AcrB, and (ii) the N-terminus of AcrA is cleaved upon maturation as a signal sequence and that Cys25 is essential for this cleavage. The N-terminus of C25A-AcrA traverses the cytoplasmic membrane. In Fig. 5, we summarize the results of this study.

We showed that AcrA is a peripheral inner membrane protein and that the mature part of the protein is located on the outside surface of the membrane. The co-immunoprecipitation experiment indicates that AcrA exists as a complex with AcrB. Zgurskaya and Nikaido used the fluorescence energy transfer technique to show that AcrA associates with the lipid bilayer surface (*5*). They also reported that Cys25 in AcrA is modified by a lipid moiety, but that this lipid modification is not functionally essential (*10*).

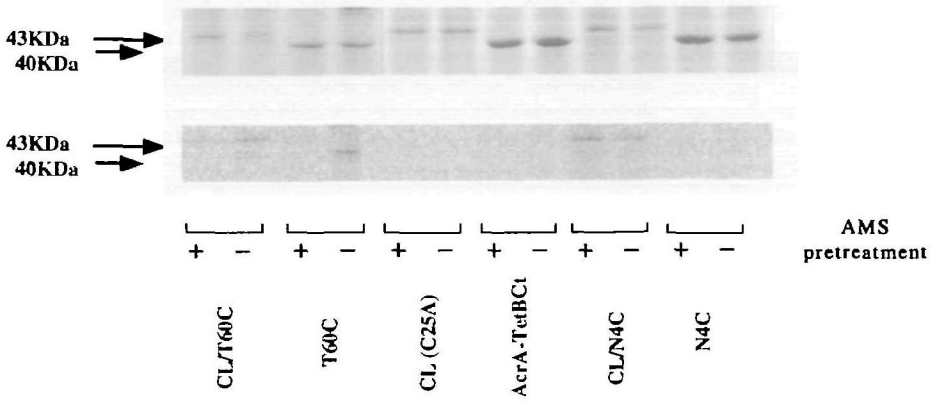

Fig. 4. **Binding of [^{14}C]*N*-ethylmaleimide to cysteine-introduced mutant AcrA proteins and its prevention by membrane-impermeable maleimide, AMS, in intact cells.** The experiments were performed as described in "EXPERIMENTAL PROCEDURES." Upper panel, Coomassie Brilliant Blue staining of the SDS–polyacrylamide gel; lower panel, radioactive bands visualized with a BAS-1000 Bioimaging Analyzer.

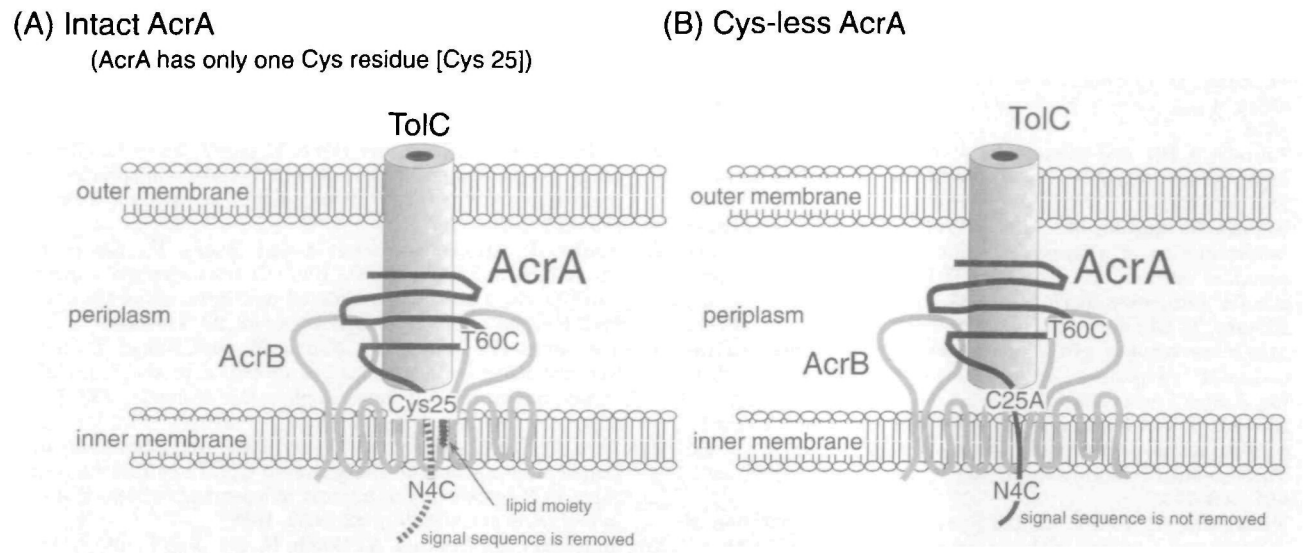

Fig. 5. **Schematic model of the molecular construction of the AcrAB-TolC system in *E. coli*.** A "channel tunnel" crystal structure over 140 Å of TolC was reported by V. Koronakis *et al.* (*27*). The TolC channel may directly dock with the inner membrane channel formed by AcrB. AcrA probably works as a joint of TolC and AcrB.

Although there is a possibility that this lipid modification participates in the interaction between AcrA and the inner membrane, an important factor for the interaction of AcrA with the inner membrane may be the intermolecular interaction between AcrA and AcrB.

We found that there is a difference in molecular mass between the wild-type AcrA and the Cys-less mutant. Using the site-directed chemical labeling technique, we demonstrated that the N-terminus of AcrA is cleaved upon maturation as a signal sequence, and that Cys25 is essential for this cleavage. From the amino acid sequence, it has been proposed that the first 24 amino acids of AcrA might comprise a prokaryotic lipoprotein signal sequence and that Cys25 is the N-terminal amino acid of the mature AcrA (*1*). Zgurskaya and Nikaido found that N-terminal truncated AcrA accumulates in the cytoplasm, and that a fusion protein comprising the OmpA-signal sequence and the N-terminal truncated AcrA [OmpA-AcrA-$(His)_6$] is partially cleaved by the signal peptidase (*10*). Our results indicate that cleavage of the N-terminal region of AcrA requires translocation of the AcrA protein, however, cleavage of the signal sequence is not required for this function because the CL mutant retains significant activity.

The MexAB-OprM systems (*19*) as well as MexCD-OprJ (*20*) and MexEF-OprN (*21*) systems in *Pseudomonas aeruginosa* are homologues of AcrAB-TolC systems in *Escherichia coli*. MexA and MexB contribute to the determination of substrate specificity in this system, as indicated by the results of chimeric experiments involving MexAB-OprM and MexCD-OprJ (*20*). Since OprM-, MexA-, and MexB-deficient mutants are all susceptible to many antibiotics, all of these subunits are essential for the system (*22*, *23*). The outer membrane components of Mex-efflux systems can be interchanged while the inner membrane components are not interchangeable (*24*). Recently, Yoneyama *et al.* (*25*) conducted a vigorous study of the localization of MexA and its interaction with the inner membrane. They found that MexA anchors the inner membrane *via* the fatty acid moiety, however, the mutant MexA protein without fatty acid is fully functional. Their observations are consistent with our findings. The MexA protein seems to be fixed on the periplasmic surface of the inner membrane *via* direct physical interaction with the inner membrane component, MexB, without anchoring the inner membrane. The results presented here provide good insight into understanding the molecular construction of bacterial multicomponent-type drug efflux systems.

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