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# Membrane Topology of a Multidrug Efflux Transporter, AcrB, in *Escherichia coli*[1]

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**AcrA/B in *Escherichia coli* is a multicomponent system responsible for intrinsic resistance to a wide range of toxic compounds, and probably cooperates with the outer membrane protein TolC. In this study, *acr*AB genes were cloned from the *E. coli* W3104 chromosome. To determine the topology of the inner membrane component AcrB, we employed a chemical labeling approach to analyse mutants of AcrB in which a single cysteine residue had been introduced. The cysteine-free AcrB mutant, in which the two intrinsic Cys residues were replaced by Ala, retained full drug resistance. We constructed 33 cysteine mutants in which a single cysteine was introduced into each putative hydrophilic loop region of the cysteine-free AcrB. The binding of [$^{14}$C]*N*-ethylmaleimide (NEM) to the Cys residue and the competition of NEM binding with the binding of a membrane-impermeant maleimide, 4-acetamide-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS), in intact cells were investigated. The results revealed that the N- and C-terminals are localized on the cytoplasmic surface of the membrane and the two large loops are localized on the periplasmic surface of the membrane. The results supported the 12-membrane–spanning structure of AcrB. Three of the four short periplasmic loop regions were covered by the two large periplasmic loop domains and were not exposed to the water phase until one of the two large periplasmic loops was removed.**

**Key words: AcrB, drug exporter, multidrug resistance, site-directed mutagenesis, topology.**

Lesions in the *acr*AB loci are responsible for increasing susceptibility of *Escherichia coli* to various structurally-unrelated compounds (*1*). The *acr*A and *acr*B genes of *E. coli* were first cloned by Ma *et al.* (*1*). AcrA is a peripheral membrane lipoprotein comprising 397 amino acid residues including a 24–amino-acid signal sequence (*1, 2*), and belongs to the MFP (membrane fusion protein) family (*3*). As shown by a PhoA fusion experiment (*1*) and a chemical labeling experiment (*2*), a significant portion of AcrA is localized in the periplasm. On the other hand, AcrB is an integral cytoplasmic membrane protein comprising 1,049 amino acid residues (*1*), and belongs to the RND (the resistance/ nodulation/cell division) family (*4*). AcrAB is a drug efflux pump system that plays a major role in the multiple-antibiotic-resistance (Mar) phenotype of *E. coli* (*5*), and also mediates active efflux of bile salts (*6*). The acrAB genes are induced by stress conditions (*7*). AcrAB homologs have also been identified as efflux pumps in *Haemophilus influenzae* (*8*) and *Salmonella typhimurium* (*9*). In *Pseudomonas aeruginosa*, there are three intrinsic multidrug efflux systems, MexAB (*10*), MexCD, and MexEF (*11, 12*), which exhibit homology with AcrAB. These multicomponent efflux transporters export drugs across both the cytoplasmic and outer membranes in combination with outer membrane counterparts such as OprM, OprJ, and OprN, which are encoded by the same operons in the *P. aeruginosa* chromosome as MexAB, MexCD, and MexEF, respectively. An outer membrane counterpart of AcrAB is estimated to be TolC and seems to be required for the AcrAB efflux system (*13*), while the TolC mutant phenotype is highly pleiotropic. Since OprM can contribute to the antibiotic resistance of *P. aeruginosa* independent of MexAB (*14*) and overexpressed OprJ is able to compensate for the role of OprM in the MexAB-OprM system (*15*), the interaction between outer membrane components and MexAB or AcrAB may not be strict. Recently, the high resolution crystalline structure of TolC was reported (*16*). The "channel-tunnel" structure of TolC that spans both the outer membrane and periplasmic space is favorable for bypassing the periplasm.

AcrB is a proton-motive force-dependent transporter located in the inner membrane (*17*) and plays a key role in the active drug efflux out of the cell. In this study, we employed a site-directed chemical modification method (*18*) for determination of the membrane topology of AcrB. Recently, two groups reported the 12 transmembrane structure of MexB (*19*) and MexD (*20*), which are homologues of AcrB in *P. aeruginosa*. In their studies, the fusion protein method was used. However, as we previously pointed out (*18*), it is

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not clear whether or not the topology demonstrated with the fusion protein method really reflects the normal structure, because the method inevitably involves truncated protein fragments for fusion with a reporter enzyme. On the other hand, the chemical labeling method has the advantage that the whole protein is used for topology determination. This method is based on the reactivity of cysteine residues introduced by site-directed mutagenesis with membrane-permeant or -impermeant maleimides in intact cells. In this study, we first replaced the two intrinsic cysteine residues of AcrB with Ala. Single-cysteine mutants were constructed on the basis of this wholly-active cysteine-free AcrB and then subjected to a chemical labeling experiment.

## EXPERIMENTAL PROCEDURES

*Materials*—*N*-Ethyl-1-[$^{14}$C]maleimide (1.5 GBq/mmol) was purchased from Perkin Elmer Life Science Products. 4-Acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS) was purchased from Molecular Probes. Monoclonal anti-polyhistidine antibodies were obtained from Sigma. All other materials were of reagent grade and obtained from commercial sources.

*Bacterial Strains*—*E. coli* strains W3104 (*21*), CJ236 (*22*), and TG1 (*23*) were used for preparation of chromosomal DNA, and single-stranded DNA and as a host for transformation after mutagenesis, respectively. *E. coli* N43 (F$^-$, K12 *lac*, *ala*, *mal*, *xyl*, *mtl*, *gal*, *rps*L, *acr*A1) (*1*) was obtained from the National Institute of Genetics (Mishima) for resistance measurements.

*Cloning of the acrAB Locus and Construction of Plasmids*—The full length of the *acr* determinant was cloned from the *E. coli* W3104 chromosome by means of a PCR method using synthetic oligonucleotides, 5′-CAAGTTGCGCCTGCAGGTCC-3′ and 5′-ACGGACACCTTTCGGGATGG-3′, as forward and reverse primers, respectively (Fig. 1). The resulting 5,951-bp fragment was digested with *Sse8387I* and *Aor51HI* restriction enzymes, followed by insertion into pUC118 (purchased from Takara) at the *Pst*I–*Sma*I site. The resulting plasmid, named pAC8, contains *acrR*, *acrA*, and *acrB*, and their promoter region. To detect the AcrB protein, a linker encoding four histidine residues was introduced at the 3′ end of the *acr*B gene by oligonucleotide-directed insertion mutagenesis. The resulting plasmid was named pACBH. Since the intrinsic C-terminal sequence of AcrB is His-His, the resulting His-tagged AcrB has $(His)_6$ at its C-terminus. The two cysteine residues originally contained in AcrB, Cys493 and Cys887, were replaced by alanine by means of site-directed mutagenesis according to Kunkel (*22*) using pACBH as a template and mutagenic primers, 5′-CTCCAGCCCTTGCGGCCACCATGC-3′ and 5′-GTGTTCCTGGCCCTGGCGGCGC-3′, respec-

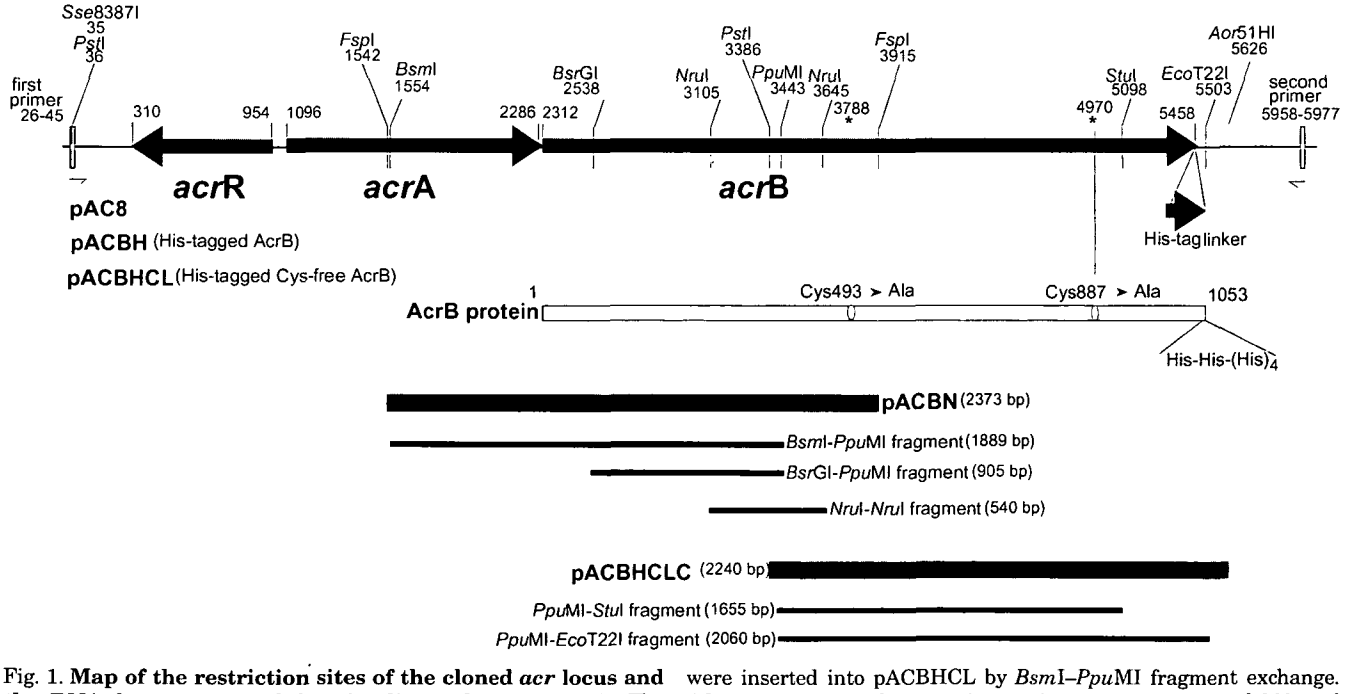


Fig. 1. **Map of the restriction sites of the cloned *acr* locus and the DNA fragments used for site-directed mutagenesis.** The 5,951-bp acr locus of *E. coli* chromosome DNA was amplified by PCR using forward and reverse primers, then digested with *Sse8387I* and *Aor51HI*. The resulting fragment was cloned into pUC118 (pAC8). Then, a $(His)_4$ linker was inserted at the 3′ end of the *acrB* gene (pACBH). Cys493 and Cys887 of AcrB were replaced with alanine by site-directed mutagenesis of pACBH to obtain Cys-free AcrB (pACBHCL). The N- and C-terminal halves of His-tagged Cys-free AcrB were separately subcloned (pACBN and pACBHCLC). Single-cysteine-introducing mutagenesis at positions 3, 39, 133, 258, 319, 336, 340, 359, 364, 365, 367, 369, and 421 was performed in pACBN. The mutations at amino acid positions 3, 39, 133, 258, 336, 365, and 367 were inserted into pACBHCL by *Bsm*I–*Ppu*MI fragment exchange. After mutagenesis, the mutations at 319, 340, 359, 364, and 369, and that at 421 were inserted into pACBHCL by *Bsr*GI–*Ppu*MI and *Nru*I–*Nru*I fragment exchange, respectively. Mutagenesis at amino acid positions 388, 390, 462, 465, 469, 501, 576, 715, 836, 863, 892, 894, 921, 923, 926, 949, 963, 997, 1002, 1034, and 1043 was performed in pACBHCLC. The mutations at 388, 390, 462, 465, 469, 501, 576, 715, 836, 863, 892, 894, 921, 923, and 1002, and those at 926, 949, 997, 1034, and 1043 were inserted into pACBHCL through exchange of the *Ppu*MI-*Stu*I and *Ppu*MI-*Eco*T22I fragments, respectively. Bold arrows indicate the ORFs encoding AcrR, AcrA, and AcrB. The gray bar indicates the AcrB protein. Asterisks indicate the positions of cysteine-free mutations.

tively. Both primers contain silent mismatches to generate a new restriction site for *BglI* in addition to the mismatches causing amino acid replacements. The resulting plasmid was named pACBHCL.

*Construction of Plasmids Encoding Single-Cysteine Mutant AcrB*—For site-directed mutagenesis, the DNA fragments encoding the N- and C-terminal halves of AcrB were separately subcloned into pUC118 (Fig. 1). pACBN, which encodes the N-terminal 536 amino acid residues of AcrB and the C-terminal 248 residues of AcrA, was contructed by subcloning the 2,373-bp *FspI–FspI* fragment of pAC8 into the *SmaI* site of pUC118. pACBHCLC, which encodes the C-terminal 689 amino acid residues of cysteine-free AcrB plus 4 histidines, was constructed by removing the *PstI–PstI* fragment from pACBHCL. The mutagenesis for constructing the N3C, A39C, S133C, S258C, S319C, S336C, V340C, L359C, A364C, T365C, I367C, T369C, and A421C mutants was performed with pACBN as a template. The mutagenesis for the F388C, I390C, S462C, A465C, Q469C, V501C, V576C, S715C, S836C, S863C, Y892C, S894C, L921C, N923C, Y926C, A949C, A963C, S997C, A1002C, S1034C, and S1043C mutants was performed with pACBHLC as a template. The mutagenesis was performed with mutagenic oligonucleotides containing mismatches for the desired amino acid replacements with silent mismatches producing new restriction enzyme sites. Mutations were first detected by restriction enzyme analysis, then verified by DNA sequencing. After verification of the mutations, adequate restriction fragments of pACBN and pACBHCLC containing the mutations were introduced into pACBHCL by means of corresponding fragment exchange.

*Labeling of Mutant AcrB Proteins with [$^{14}$C]N-Ethylmaleimide (NEM) and Its Prevention by 4-Acetoamido-4′-maleimidylstilbene-2,2′-disulfonic Acid (AMS)*—Competitive chemical labeling of membrane proteins was performed as previously reported (*18*). Briefly, *E. coli* TG1 cells harboring each mutant plasmid were grown in 2 × YT broth to the mid-logarithmic phase, then 25 μg/ml novobiocin was added as an inducer, and cultivation was continued for 2 h. The cells were then harvested and washed with 50 mM MOPS-KOH buffer (pH 7.0) containing 0.1 M KCl, followed by suspension in the same buffer and adjustment of the absorbance at 530 nm to 85. Five microliters of 100 mM AMS or the same volume of distilled water was added to 100 μl of the cell suspension, followed by incubation for 30 min at 30°C. Subsequently, 0.1 mM (final) [$^{14}$C]NEM was added to the reaction mixture, followed by incubation for 5 min at 30°C. The mixture was diluted with 900 μl of the same buffer containing 5 mM unlabeled NEM. Immediately after the dilution, the cells were collected and washed once with the same buffer, then disrupted by brief sonication. After removal of unbroken cells, the membrane fraction was collected by ultracentrifugation with a Beckman Ultracentrifuge Optima TL. The resultant precipitate was solubilized with 1% Triton X-100 and 0.1% SDS. The AcrB protein was immunopreciptated with anti-polyhistidine antibodies and Pansorbin *Staphylococcus aureus* cells (Calbiochem), and the precipitate was subjected to SDS–polyacrylamide gel electrophoresis, followed by Coomassie Brilliant Blue staining. The resulting gel was soaked in Amplify (Amersham), dried and exposed to an imaging plate for visualization with a BAS-1000 Bio-Imaging Analyzer (Fuji Film, Tokyo).

In the case of labeling using sonicated membrane vesicles, membranes were prepared from *E. coli* TG1cells harboring mutant plasmids by brief sonication. These membranes (0.5 mg of membrane protein) were used in place of intact cells as described above.

## RESULTS

*Cloning of the acrAB Locus and Drug Resistance*—The *acr* locus encoding AcrR, AcrA, and AcrB was amplified by PCR from *E. coli* chromosome DNA, then cloned in pUC118 as described under "EXPERIMENTAL PROCEDURES." The entire sequence of the *acrB* gene in the resulting plasmid, pAC8, was determined using an ABI PRISM 310 DNA sequencer. As a result, three mismatches (G2404→A, C3813→T, and T4186→C) were found in comparion with the sequence reported by Ma *et al.* (*1*). Among them, the C3813→T mismatch causes an amino acid change from Ala501 to Val, while the other two mismatches are silent. Direct sequencing of the chromosomal *acrB* gene revealed that these mismatches were due to the misreading by Taq DNA polymerase. However, the mismatches did not alter the drug resistance profile of the AcrB protein, because the site-directed back-mutant V501A showed the same resistance profile as pAC8 (data not shown). When *E. coli* N43 cells, in which the *acrA* gene in the chromosome was interrupted with a 1,372-bp insertion sequence (*1*) and, as a result, the strain exhibited hypersensitivity to various structurally unrelated compounds, were transformed with pAC8, the resistance of the cells was restored to the level of wild-type cells (Table I).

*Construction and Resistance Pattern of Single Cysteine AcrB Mutants*—AcrB is an intrisic cytoplasmic membrane protein composed of 1,049 amino acid residues, while AcrA is a peripheral membrane protein comprising 397 amino acid residues. According to the hydropathy profile, AcrB is predicted to contain 12 transmembrane segments with two large hydrophilic loop regions between TM1 and TM2, and TM7 and TM8 (Fig. 2). To determine the membrane topology of AcrB by site-directed chemical labeling, we first constructed cysteine-free polyhistidine-tagged AcrB. AcrB contains two intrinsic cysteine residues, C493 and C887. The codons encoding these two cysteine residues were replaced with alanines by site-directed mutagenesis. The resulting plasmid, pACBHCL, encoding the cysteine-free polyhistidine-tagged AcrB exhibited drug resistance comparable to the wild type when novobiocin (Table II) and other toxic

TABLE I. **Drug resistance levels of *E. coli* TG1, N43 (acr⁻), and N43 cells harboring plasmid pAC8.** The resistance was measured by the agar dilution method and expressed as the minimum inhibitory concentration (MIC).

| Drug | MIC (μg/ml) | | | |
|---|---|---|---|---|
| | TG1 | N43 (acr⁻) | N43/pUC118 (vector control) | N43/pAc8 |
| Ethidium bromide | 512 | 16 | ND* | 1,024 |
| SDS | >16,384 | 32 | ND* | >16,384 |
| Tetracycline | ND* | 0.25 | ND* | 0.25 |
| Rifampin | 16 | 8 | 8 | 16 |
| Acriflavin | 512 | 16 | 8 | 256 |
| Novobiocin | 128 | 4 | 4 | 256 |
| Erythromycin | 32 | 4 | 1 | 64 |

*ND, not determined.

compounds were tested (data not shown), indicating that this AcrB mutant retained the original topology and that none of these cysteines are essential for the function. On the basis of pACBHCL, we constructed 33 single-cysteine AcrB mutants focusing on the putative hydrophilic loop regions and the N- and C-terminals (Fig. 2). The levels of resistance of these mutants against novobiocin were measured (Table II). As shown in Table II, 24 of the 33 mutants exhibited comparable resistance levels to the Cys-free AcrB. The V340C, A364C, and T365C mutants showed reduced but significant resistance. However, 6 other mutants, S319C, L359C, I367C, V576C, S836C, and L921C, retained almost no resistance. With respect to these non-resistant mutants, we planned that the active mutant(s) involved the same loop regions.

*Expression and Reactivity of the AcrB Mutants with [$^{14}$C]-NEM in Sonicated Membrane Vesicles*—Sonicated membranes prepared from *E. coli* TG1 cells harboring the mutant plasmids were incubated with 0.1 mM [$^{14}$C]NEM for 5 min at 30°C. After solubilization of the membranes, AcrB proteins were precipitated with anti-polyhistidine antibodies. The CBB-stained AcrB bands on SDS-PAGE gels are shown in Fig. 3 (upper panel). The level of expression of S836C mutant, which exhibits no resistance, was significantly lower than those of the other mutants, but expression was clearly detectable. The levels of expression of all the other mutants were normal, except for the N3C and Y926C mutants, of which the expression was slightly reduced. Therefore, the decreased resistance of some mutants was not due to the decrease in their expression.

Binding of [$^{14}$C]NEM to the mutant AcrB proteins is shown in Fig. 3 (lower panel). Although all of these 33 mutations were introduced into hydrophilic loop regions in the original putative topology based on the hydropathy profile (not shown), 14 mutants, S336C, V340C, T365C, I367C, T369C, F388C, I390C, S863C, S894C, L921C, Y926C,

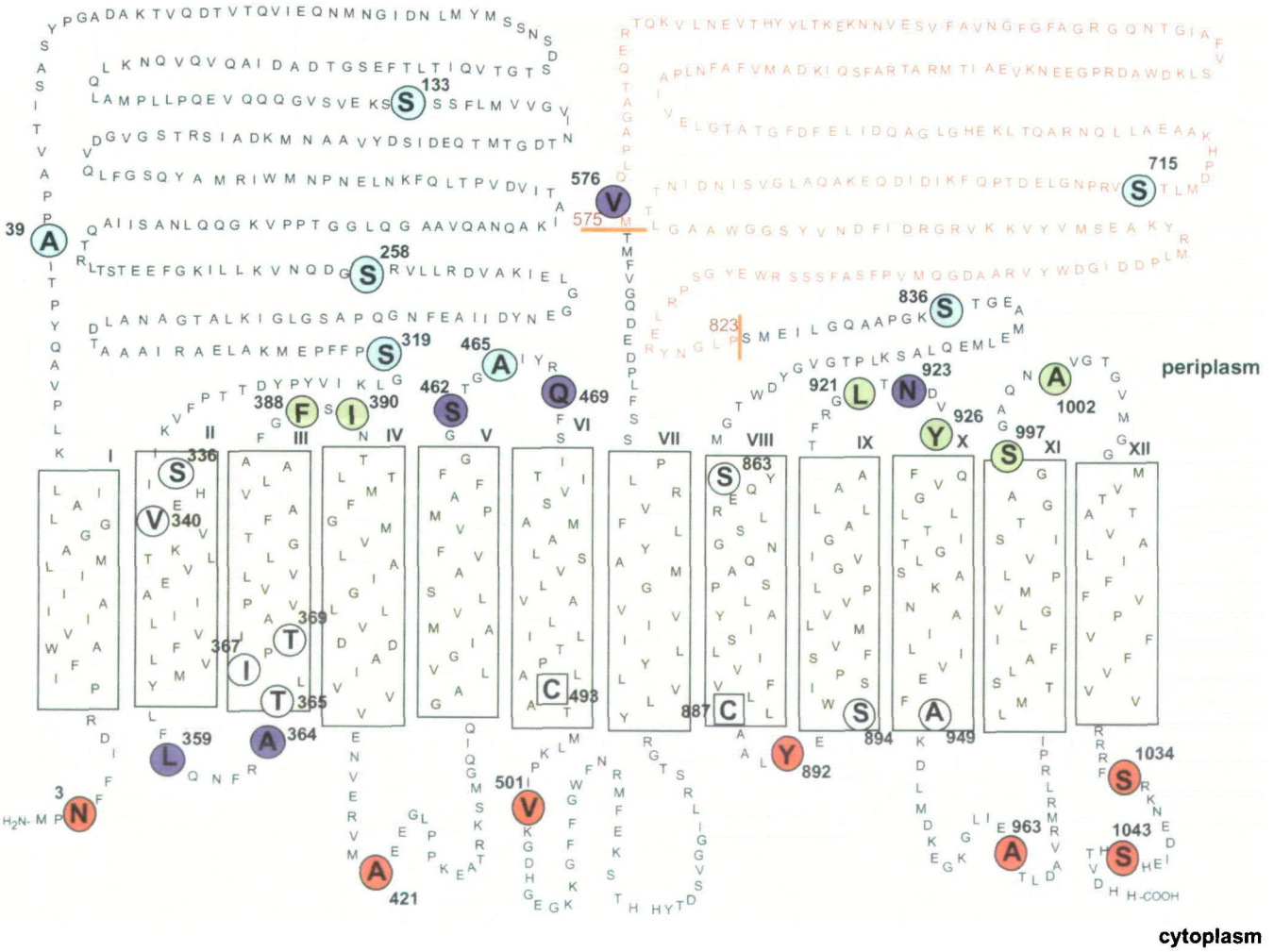


Fig. 2. **Membrane topology of AcrB determined in this study and the locations of the introduced cysteines.** The model was first constructed on the basis of hydropathy analysis, then modified on the basis of the results of this study. Two Cys residues, Cys493 and Cys887, which were replaced by alanine are depicted in large bold letters in open squares. The residues replaced by a cysteine residue are depicted in large bold letters enclosed in circles with numbers indicating the positions. The results of labeling with [$^{14}$C]NEM and blocking by AMS are indicated: blue circles, NEM labeling was completely or largely blocked by AMS; red circles, labeling was not affected by AMS; purple circles, labeling was observed in sonicated membranes but not in intact cells; green circles, labeling was observed only when one of the two large periplasmic domains was removed; open circles, labeling was observed in neither intact cells nor sonicated membranes. The region in orange letters is the region from Met575 to Pro823 missing from the truncated mutant.

A949C, S997C, and A1002C, exhibited no [$^{14}$C]NEM binding. Thus, these residues seem to be embedded in the hydrophobic interior. Since the other 19 mutants showed various degrees of [$^{14}$C]NEM binding, these residues are exposed to the aqueous phase as expected. Considering the intervals between the NEM-reactive mutations and the presence of hydrophobic segments, the polypeptide chain seems to cross the membrane between Asn3 and Ala39 (35AA), Ser319 and Leu359 (39AA), Ala364 and Ala421 (56AA), Ala421 and Ala465 (43AA), Gln469 and Ala501 (31AA), Ala501 and Val576 (74AA), Ser836 and Tyr892 (55AA), Tyr892 and Asn923 (30AA), Asn923 and Ala963 (39AA), and Ala963 and Ser1034 (70AA) (Fig. 3). For the polypeptide chain to cross the membrane once as an α-helical form, a length of about 20 amino acids is required. Out of the 10 putative transmembrane regions listed above, the segments between Ala364 and Ala421 (56AA), and Ser1034 and Ala963 (70AA) contain highly hydrophobic segments composed of 41 and 56 amino acid residues, respectively. Therefore, the polypeptide chain may cross the membrane twice, in each of these regions. On the other hand, since another two long segments between Ala501 and Val576 (74AA), and Ser836 and Tyr892 (55AA) contain long hydrophilic segments and the hydrophobic segments comprise less than or around 30 residues, the polypeptide chain may cross the membrane once in these regions.

TABLE II. **Novobiocin resistance levels of *E. coli* N43 (acr$^-$) cells carrying a derivative of pACBHCL encoding single-cysteine mutants of AcrB.** The resistance was measured by the agar dilution method and expressed as the minimum inhibitory concentration (MIC).

| Mutant | MIC (μg/ml) | Mutant | MIC (μg/ml) |
|---|---|---|---|
| N43 (host) | 4 | A465C | 256 |
| pAC8 | 256 | Q469C | 128 |
| pACBHCL | 128 | V501C | 128 |
| N3C | 256 | V576C | 2 |
| A39C | 128 | S715C | 128 |
| S133C | 128 | S836C | <1 |
| S258C | 128 | S863C | 128 |
| S319C | 8 | Y892C | 128 |
| S336C | 128 | S894C | 128 |
| V340C | 64 | L921C | <1 |
| L359C | 4 | N923C | 128 |
| A364C | 32 | Y926C | 128 |
| T365C | 16 | A949C | 128 |
| I367C | <1 | A963C | 128 |
| T369C | 128 | S997C | 128 |
| F388C | 128 | A1002C | 256 |
| I390C | 128 | S1034C | 128 |
| A421C | 128 | S1043C | 128 |
| S462C | 128 | | |

*Prevention of [$^{14}$C]NEM Binding by AMS in Intact Cells*—To determine whether the introduced cysteine residues are located inside or outside of the cytoplasmic membrane, we investigated the prevention of [$^{14}$C]NEM binding by a membrane impermeable SH reagent, AMS, in intact cells. *E. coli* TG1 cells expressing mutant AcrB proteins were preincubated in the absence or presence of AMS, then treated with [$^{14}$C]NEM, as described under "EXPERIMENTAL PROCEDURES." In this experiment, only Cys mutants reactive with [$^{14}$C]NEM in sonicated membranes were tested. To our surprise, five mutants, L359C, A364C, S462C, Q469C, V576C, and N923C, exhibited no [$^{14}$C]NEM binding in intact cells even in the absence of AMS (Fig. 4, upper panel). [$^{14}$C]NEM binding to N3C, A421C, V501C, Y892C, S1034C, and S1043C was not affected by AMS preincubation, indicating that these mutations are on the inside (cytoplasmic side) of the membrane. The binding to A963C was very low in the absence of AMS, while it was not affected by AMS. Thus, position 963 also seems to be located on the cytoplasmic side. In contrast, [$^{14}$C]NEM binding to S258C, S319C, A465C, S715C, and S836C was completely prevented by preincubation with AMS, and the binding to A39C and S133C was largely prevented by AMS, indicating that these residues are located on the outside (periplasmic side) of the membrane. Judging from these results, the N- and C-terminals are located on the cytoplasmic side, and loop 4-5, loop 6-7, loop 8-9, and loop 10-11 are also located on the cytoplasmic side. There are 281 amino acid residues between Ala39 and Ser319, while the [$^{14}$C]-NEM binding to the mutants in this area was completely prevented by AMS. Thus the region between TM1 and TM2 seems to form a large periplasmic domain (loop 1-2). Similarly, the region between TM7 and TM8 including Val576, Ser715, and Ser836 also forms a large periplasmic loop region (loop 7-8), because this region is highly hydrophilic and there is no AMS-independent mutation in this region. Loop 5-6 is also located on the periplasmic side. Although the location of loop 2-3 could not be determined from the AMS competition results, this loop region seems to be on

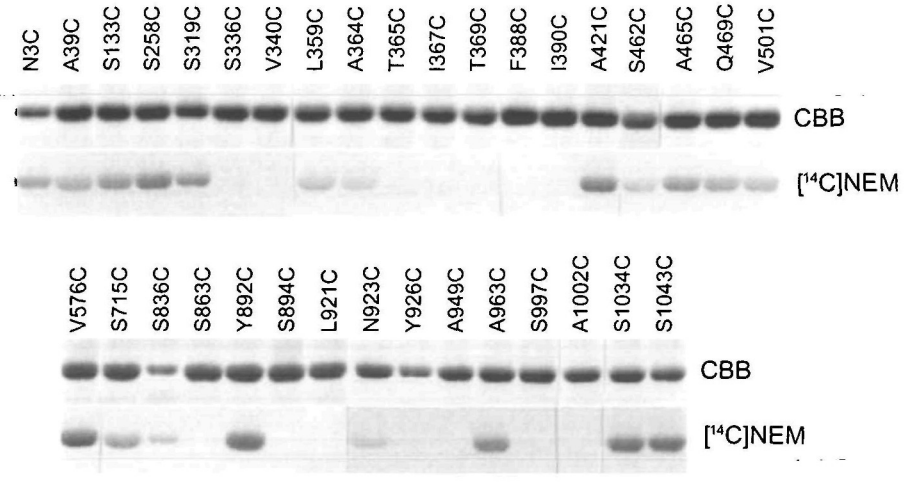


Fig. 3. **[$^{14}$C]NEM binding to the cysteine residues introduced into the predicted loop regions of AcrB in sonicated membranes.** Sonicated membranes from *E. coli* cells carrying mutant AcrB proteins were incubated in the presence of 0.1 mM [$^{14}$C]NEM. After solubilization, the proteins were precipitated with anti-polyhistidine antibodies and Pansorbin, then subjected to SDS-PAGE. Protein bands (upper panel) and radioactive bands (lower panel) were visualized by Coomassie Brilliant Blue staining and autoradiography with a BAS-1000 Bioimaging analyser.

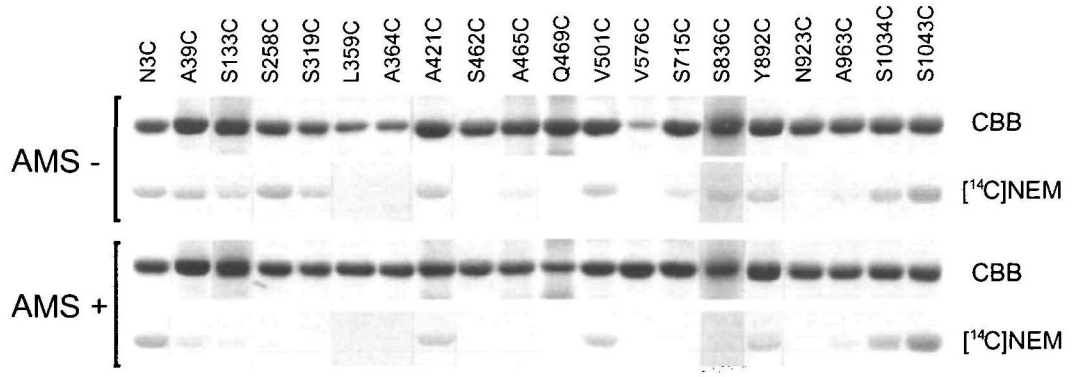


Fig. 4. **Prevention of [$^{14}$C]NEM binding to mutant AcrB proteins by AMS in intact cells.** *E. coli* cells carrying AcrB proteins were incubated in the presence or absence of 5 mM AMS, then incubated with 0.1 mM [$^{14}$C]NEM. Cells were disrupted by sonication and then, after solubilization, the proteins were precipitated with anti-polyhistidine antibodies and Pansorbin. Then the protein bands and radioactive bands were visualized as in Fig. 3.

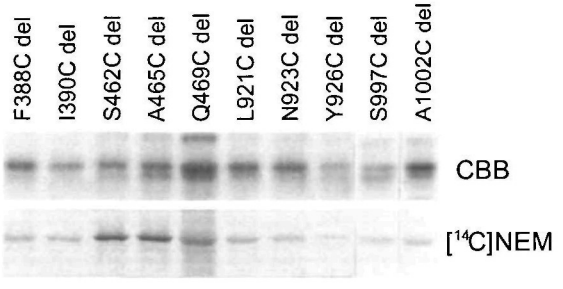


Fig. 5. **[$^{14}$C]NEM binding to the loop 7-8–truncated cysteine mutants of AcrB in sonicated membranes.** The 249 amino acid residues from Met575 to Pro823 were deleted in the single-cysteine mutants. The [$^{14}$C]NEM binding and visualization were performed as in Fig. 3.

the cytoplasmic side of the membrane because it must be opposite loop 1-2 beyond TM2.

*[$^{14}$C]-NEM Binding to Cys Mutants as to Putative Loop 3-4, Loop 5-6, Loop 9-10, and Loop 11-12 in the Loop 7-8 Deletion Mutant*—Cys mutants as to the putative short periplasmic loop regions were generally not reactive with NEM even in membrane vesicles, except for those as to loop 5-6. The two large periplasmic loop domains may prevent NEM molecules from gaining access to these small loops. Therefore, we examined the NEM reactivity of Cys mutants when one of these two domains was removed, the 249 amino acid residues from Thr574 to Pro823 being deleted (Fig. 2). Figure 5 shows the [$^{14}$C]NEM binding to the mutants as to the putative small periplasmic loops in the loop 7-8 truncated mutant. Although the expression of AcrB was greatly reduced by the truncation, [$^{14}$C]NEM clearly bound to all the mutants, indicating that these short loops are covered by the large periplasmic domain and, when this domain is absent, they are exposed to the aqueous phase. These findings also support the prediction that loop 3-4, loop 9-10, and loop 11-12 are located on the periplasmic surface of the membrane.

## DISCUSSION

The results of this study are summarized in Fig. 2. Our results are in good agreement with the model comprising 12 transmembrane segments with two large periplasmic loop domains between TM1 and TM2, and TM7 and TM8. The N- and C-terminals are located on the cytoplasmic side. Three of the four short periplasmic loops are covered by the large periplasmic loop domains.

The topology of AcrB (Fig. 2) is essentially the same as those of MexB (*19*) and MexD (*20*), which were determined by the reporter-enzyme fusion method. In this study, the 12-membrane-spannning structure with two large periplasmic loops was evidently confirmed to be the general topology of RND type bacterial drug efflux transporters by means of a more accurate method involving the whole proteins. In comparison with the prediction based on hydropathy analysis, TM2 and TM3 were shifted in the N-terminal direction by about 5 or 6 residues, that is, Ser336 and Val340 in TM2, and Thr365, Ile367 and Thr369 in TM3 were originally predicted to be located in loop 1-2 and loop 2-3, respectively. Similarly, the boundaries between TM8, TM9 and TM10, and the respective next loops were slightly shifted. However, the experimentally determined transmembrane regions were strikingly coincident with the prediction overall.

Although the roles of the two large periplasmic domains are not yet known, we showed that these domains cover the periplasmic surface of AcrB. Recent determination of the crystalline structure of TolC (*16*) revealed the unique molecular structure of this protein, that is, a long tunnel of over 100 angstroms, which is connected with the outer membrane channel domain, protrudes from the outer membrane into the periplasmic space. This seems enough for direct interaction of TolC with the surface of the inner membrane or AcrB. Considering our results, the large periplasmic domain of AcrB may play some role in the docking with the TolC "chunnel" (*24*). Zgruskaya and Nikaido recently reported that AcrA, which is a membrane fusion protein, exists as a trimer in the AcrAB-TolC complex (*17*). TolC is also a trimer with a structural repeat in each subunit, that is, six repeated units per trimer. It will be interesting to determine whether AcrB is also a trimer, and to investigate the molecular interaction between AcrB and TolC by means of, for example, site-directed cross-linking of mutationally introduced cysteine residues (*25*).

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