

Drug-Induced Conformational Changes in Multidrug Efflux Transporter AcrB from *Haemophilus influenzae*[∇]

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In gram-negative bacteria, transporters belonging to the resistance-nodulation-cell division (RND) superfamily of proteins are responsible for intrinsic multidrug resistance. *Haemophilus influenzae*, a gram-negative pathogen causing respiratory diseases in humans and animals, constitutively produces the multidrug efflux transporter AcrB (AcrB_{HI}). Similar to other RND transporters AcrB_{HI} associates with AcrA_{HI}, the periplasmic membrane fusion protein, and the outer membrane channel TolC_{HI}. Here, we report that AcrB_{HI} confers multidrug resistance when expressed in *Escherichia coli* and requires for its activity the *E. coli* TolC (TolC_{EC}) protein. To investigate the intracellular dynamics of AcrB_{HI}, single cysteine mutations were constructed in AcrB_{HI} in positions previously identified as important for substrate recognition. The accessibility of these strategically positioned cysteines to the hydrophilic thiol-reactive fluorophore fluorescein-5-maleimide (FM) was studied *in vivo* in the presence of various substrates of AcrB_{HI} and in the presence or absence of AcrA_{HI} and TolC_{EC}. We report that the reactivity of specific cysteines with FM is affected by the presence of some but not all substrates. Our results suggest that substrates induce conformational changes in AcrB_{HI}.

Multidrug efflux transporters belonging to the resistance-nodulation-cell division (RND) superfamily of proteins are broadly represented in gram-negative bacteria. Previous studies established that these transporters play a major role in the intrinsic and induced resistance of gram-negative bacteria to multiple antimicrobial agents including clinically important antibiotics (reviewed in references 7, 11, 18, and 27). Their contribution to antibiotic resistance in clinical settings made RND-type transporters attractive targets of a search for specific and broad-spectrum inhibitors that could be used to increase the efficacy of antibiotics (8).

RND-type multidrug transporters from various species share a high degree of sequence and, correspondingly, structure conservation. Structural and functional studies showed that RND-type transporters exist as trimers, which are organized into two large domains: the transmembrane hydrophobic domain comprised by 36 transmembrane α -helices (TMS) with each protomer contributing 12 TMSs and the large periplasmic domain exposed to aqueous environment of the periplasm (17). This impressive structure, however, is not sufficient to provide multidrug resistance. RND transporters associate with two accessory proteins. The periplasmic membrane fusion proteins (MFPs) are believed to mediate the interaction between an RND transporter and an outer membrane channel belonging to the outer membrane factor family of proteins (18, 27). Together, these interacting three components span the inner and outer membranes and the periplasm of gram-negative bacteria. Mutations in any of the three proteins completely abolish intrinsic multidrug resistance of gram-negative bacteria.

In the three-component complexes, RND transporters are responsible for drug recognition. Crystallization studies of AcrB from *Escherichia coli* (AcrB_{EC}) revealed several possible drug-interacting sites (16, 25, 26). In the first report, several unrelated drugs were detected in the central cavity formed by three protomers of AcrB_{EC} on the interface between periplasm and the inner membrane (26). Two other sites are located in the periplasmic domains: one is in a prominent cleft on the surface of the periplasmic domain (25); another is deep inside this domain (16). These periplasmic drug binding sites were largely delineated by mutagenesis studies of RND-type transporters from various gram-negative bacteria even before the crystal structures became available (5, 10, 12, 23). Consistent with a high degree of sequence conservation, positions affecting substrate specificity are conserved among various RND transporters. Furthermore, recombinant RND-type multidrug transporters from various gram-negative bacteria are functional when expressed in *E. coli* (23).

Haemophilus influenzae is a gram-negative pathogen causing respiratory diseases in humans and animals. Genetic and sequence studies showed that *H. influenzae* constitutively produces a homolog of AcrB_{EC} (19). Similar to other RND transporters, *H. influenzae* AcrB (AcrB_{HI}) associates with AcrA_{HI}, the periplasmic MFP, and the outer membrane channel TolC_{HI} (24). Inactivation of any of the three proteins AcrA_{HI}, AcrB_{HI}, or TolC_{HI} increased the susceptibility of *H. influenzae* to 16 antimicrobial compounds. In all cases, the susceptibility profiles of AcrA_{HI}, AcrB_{HI}, or TolC_{HI} null mutants were identical, suggesting that these proteins are components of a single pump. Mutations in other RND transporters identified by sequences searches of *H. influenzae* genome did not affect drug susceptibility (24). Therefore, AcrB_{HI}-TolC_{HI} is a major, constitutively expressed multidrug efflux complex of *H. influenzae* and a potent target for the development of specific inhibitors of this transporter.

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TABLE 2. Antimicrobial susceptibility of the *E. coli* ECM1694 (Δ acrAB) strain carrying plasmid pRAB with AcrAB_{HI} and its variants

Plasmid	MIC (μ g/ml) ^a						
	SDS	NOV	EtB	ERY	CHL	TET	NOR
pSE380 (vector)	40	4	3.125	4	0.4	0.39	0.04
pRAB (AcrAB _{HI})	>10,240	256	200	256	3.2	1.56	0.16
pRAB (C932S)	>10,240	64	200	64	1.6	0.78	0.08
pRAB (C932S E42C)	>10,240	32	50	32	1.6	0.78	0.08
pRAB (C932S T96C)	>10,240	32	100	32	1.6	0.39	0.08
pRAB (C932S A288C)	>10,240	32	100	64	1.6	0.39	0.08
pRAB (C932S L324C)	>10,240	32	100	16	0.8	0.39	0.08
pRAB (C932S L396C)	>10,240	32	100	32	1.6	0.195	0.04
pRAB (C932S I397C)	>10,240	32	100	32	1.6	0.39	0.08
pRAB (C932S G401C)	>10,240	32	100	32	1.6	0.39	0.08
pRAB (C932S I601C)	>10,240	32	100	32	1.6	0.39	0.08
pRAB (C932S Q658C)	>10,240	32	50	8	0.4	0.195	0.08

^a Data are representative of three independent experiments. SDS, sodium dodecyl sulfate; NOV, novobiocin; EtB, ethidium bromide; ERY, erythromycin; CHL, chloramphenicol; TET, tetracycline; NOR, norfloxacin.

the protocol previously described for the MdfA transporter (1). Briefly, prior to the addition of FM, cells (120 optical density units at 600 nm in 1 ml of PBS) were preincubated with substrates (erythromycin, novobiocin, cloxacillin, ethidium bromide, Triton X-100, or inhibitor MC-207,110) in final concentrations of 5 mM (1). After incubation with FM for 30 min at room temperature, the reactions were stopped by the addition of dithiothreitol to a final concentration of 10 mM. Cells were pelleted in a microcentrifuge and resuspended in 2 ml of buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 0.1 mg/ml lysozyme. Cells were broken by sonication, the unbroken cells were removed by centrifugation at $3,220 \times g$ for 15 min, and the supernatant was further centrifuged at $250,000 \times g$ for 60 min. The pellet was resuspended in 0.5 ml of binding buffer containing 50 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 5 mM imidazole (buffer A); then an equal volume of 10% Triton X-100 in buffer A was slowly added to solubilize membrane proteins. After overnight incubation at 4°C, insoluble material was removed by centrifugation at $250,000 \times g$ for 30 min. Supernatants were loaded onto 0.1-ml His-bind resin (Novagen) mini-columns charged with Cu²⁺ and equilibrated with buffer A containing 0.2% Triton X-100 (buffer B). The column was washed with buffer B, and then bound proteins were eluted with a step gradient of imidazole (5 mM, 50 mM, and 500 mM) in buffer B. The majority of AcrB_{HI} was eluted with 500 mM imidazole. Purified AcrB_{HI} and its variants were separated by 8% SDS-PAGE. A Storm 840 phosphor and fluorescent screen imaging system (Amersham Pharmacia-Molecular Dynamics) was used to assess fluorescein labeling of cysteine mutants. Next, gels were stained with Coomassie brilliant blue (CBB) to visualize protein bands and determine amounts of proteins. Fluorescence intensity and protein amount were analyzed using the ImageQuant TL program (Amersham Pharmacia). For each protein band, the fluorescence intensity was normalized on the protein amount determined from the same gel after staining with CBB.

RESULTS

AcrAB_{HI} is expressed in *E. coli* and functional. AcrA_{HI} and AcrB_{HI} were expressed from pRAB plasmid under the IPTG-inducible *P*_{trc} promoter. When pRAB was transformed into the *E. coli* strain ECM1694 (Δ acrAB::kan), cells gained high levels of resistance to multiple antibiotics (Table 2). The increase in MICs was observed even in the absence of IPTG, indicating that both AcrA_{HI} and AcrB_{HI} are expressed as a result of the promoter leakage. Western immunoblotting confirmed that AcrB_{HI} is expressed in *E. coli* and that the addition of IPTG induced severalfold overproduction of this protein (data not shown). The activity of AcrAB_{HI} was completely abolished in the ECM2112 (Δ acrAB Δ tolC) strain. Thus, similar to other recombinant RND transporters produced in *E. coli*, the function of AcrAB_{HI} in *E. coli* required the outer membrane channel TolC_{EC}.

Although disruption of AcrA_{HI} and AcrB_{HI} in *H. influenzae*

led to only two- to eightfold decreases in MICs of such antimicrobials as erythromycin, novobiocin, ethidium bromide, and SDS (19), in *E. coli* the expression of AcrAB_{HI} resulted in a 16- to 128-fold increase in MICs of the same drugs (Table 2). In addition, AcrAB_{HI} conferred elevated resistance to tetracycline, chloramphenicol, and norfloxacin, the antibiotics previously reported to be unaffected by the activity of this transporter in *H. influenzae* cells. We estimate that the uninduced level of AcrAB_{HI} expression in *E. coli* is lower or comparable to that of the native AcrAB_{EC} (data not shown). Thus, increased amounts of AcrAB_{HI} cannot explain the observed changes in MICs and substrate specificity. These data support the previously stated idea (19) that the apparent difference in the substrate specificities of AcrAB in *E. coli* and *H. influenzae* could be attributed to the differences in the size of general porins in the outer membrane of these bacteria.

Single cysteine mutants of AcrB_{HI} are functional. To investigate the intracellular dynamics of AcrB_{HI}, we introduced unique cysteine residues into the putative substrate binding sites of AcrB_{HI} (AcrB_{HI}-Cys). Previously, Mao et al. (10) identified several spontaneous mutations in *P. aeruginosa* MexD that altered the substrate specificity of this transporter. MexD and AcrB_{HI} share 29% identity and 53% homology with each other. We reasoned that the overall topology of substrate binding sites is conserved in RND transporters. Using sequence alignment and structure analysis, we identified the amino acid residues located in the sites of AcrB_{HI} similar to the sites of MexD (Table 1). Prior to mutagenesis, a single cysteine residue, C932, of the native AcrB_{HI} was replaced with serine [AcrB_{HI}(C932S)]. Western immunoblotting analysis showed that AcrB_{HI}(C932S) is expressed to the level comparable to that of the wild-type AcrB_{HI} (data not shown). As judged by MIC measurements, this cysteineless AcrB_{HI} was functional although MICs were two- to fourfold lower for all tested substrates (Table 2).

We next introduced nine unique cysteine residues into AcrB_{HI}(C932S) (Fig. 1C and Table 1). Three mutations L396C, I397C, and G401C are located in the TMS4, which is proposed to be the major route for the proton translocation (17). The E42C substitution is exposed to the central cavity of AcrB_{HI}, the site of substrate binding to AcrB_{EC} identified in

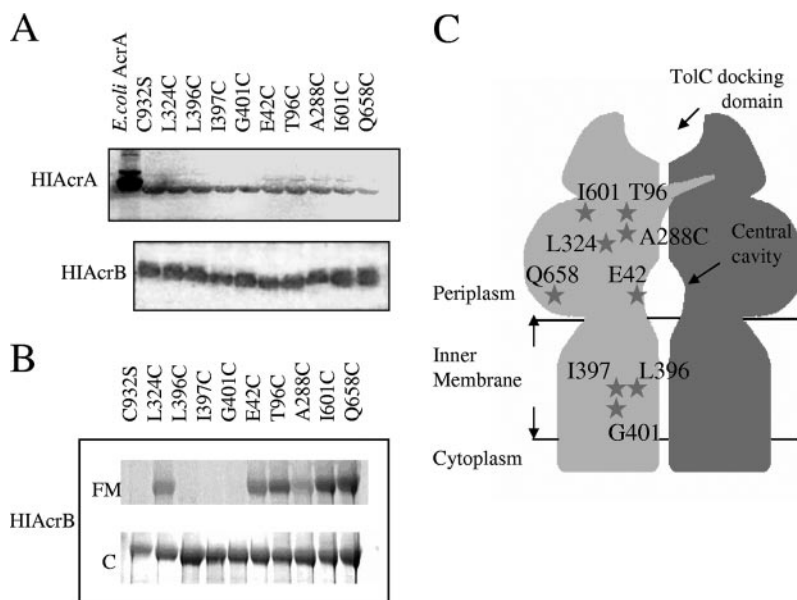


FIG. 1. Expression and accessibility to FM of AcrB_{HI} and its variants. (A) Western immunoblotting of membrane fractions (15 μ g of total protein per lane) isolated from cells carrying pRAB plasmids with the indicated AcrB_{HI} mutants. AcrA_{HI} was visualized using rabbit anti-AcrA antibody, and AcrB_{HI} was visualized with a nickel-activated derivative of horseradish peroxidase. (B) Accessibility of AcrB_{HI}-Cys to FM. Whole cells containing the indicated AcrB_{HI} variants were incubated with 0.3 mM FM for 30 min at 37°C (see Materials and Methods). Purified AcrB_{HI} variants were separated by 8% SDS-PAGE and visualized by fluorescence using a Storm Imager (FM). After fluorescence scanning protein bands were visualized by CBB (C) staining. (C) Schematic representation of AcrB_{HI} and positions of introduced cysteine residues based on the structure of AcrB_{EC} (17). HI-AcrA, AcrA_{HI}; HI-AcrB, AcrB_{HI}.

one of the structural studies (26). Substitutions T96C, A288C, and L324C are located in the major depression of the periplasmic domain, which was recently implicated in substrate binding (16). Finally, I601C and Q658C are located in the external cleft of the periplasmic domain and in proximity to the putative interaction site with the periplasmic AcrA_{HI} (17, 25). All AcrB_{HI}-Cys mutants were expressed at about the same level and retained their functionality (Fig. 1A and Table 2). However, AcrB_{HI}(Q658C) provided only partial levels of resistance to erythromycin and ethidium bromide and lost activity against chloramphenicol (Table 2). The AcrB_{HI} carrying E42C and L324C mutations was only partially active against ethidium bromide and erythromycin, respectively. All Cys mutations with the exception of E42C failed to confer resistance against tetracycline and displayed only a twofold increase in resistance against norfloxacin.

Cysteines located in the periplasmic domain of AcrB_{HI} but not in the TMS4 are accessible for labeling with the hydrophilic thiol-reactive probe FM. Our experimental approach is based on two assumptions: (i) since many substrates of AcrB_{HI} are bulky molecules, a cysteine residue located in the substrate binding site should be accessible to a relatively large thiol-reactive probe; (ii) binding of substrate is expected to modulate the reactivity of a cysteine residue located in proximity to substrate binding sites. Previously, the substrate protection of cysteines from thiol-reactive probes was successfully used to delineate substrate binding sites in various transporters (4, 15).

To investigate whether the constructed cysteine residues of AcrB_{HI} are accessible from the aqueous environment of the periplasm, whole *E. coli* cells expressing various AcrB_{HI}-Cys mutants were treated with the hydrophilic, thiol-reactive flu-

orophore FM. The reaction was terminated by the addition of dithiothreitol, and AcrB_{HI}-Cys variants were purified from membrane fractions and analyzed as described in Materials and Methods.

Figure 1B shows that no fluorescent signal was detected in the cysteineless AcrB_{HI}(C932S) and mutants with Cys residues L396C, I397C, and G401C located in TMS4. However, Cys residues located in the periplasmic loops were accessible to FM, albeit to different degrees. As expected, I601C and Q658C exposed to the outer cleft of the periplasmic domain of AcrB_{HI} were the most readily reacting with FM. The A288C mutation was the least accessible to FM (~10 to 30% of Q658C reactivity). In the crystal structure of the homologous AcrB_{EC}, this A288C residue is located deep in the periplasmic drug binding pocket. The E42C, T96C, and L324C residues were partially reactive, with ~30 to 50% of accessibility compared to Q658C. In the AcrB_{EC} structure, E42C is accessible from the central cavity formed by three AcrB_{HI} protomers, and T96C and L324C lie in the periplasmic domain (16).

All cysteines are less accessible to FM in TolC_{EC}- and AcrA_{HI}-deficient *E. coli*. For its function in *E. coli* AcrAB_{HI} requires the outer membrane channel TolC_{EC}. Therefore, we next investigated the accessibility of AcrB_{HI}-Cys mutants to FM in the *E. coli* strain lacking the chromosomal TolC_{EC}. Consistent with previous observations (6), the high levels of AcrAB_{HI} expression were toxic to the *tolC*_{EC} strain. The levels of AcrAB_{HI} mutants varied significantly, and expression was often completely lost after storage of the transformed strains at -80°C. Therefore, for these experiments we used only freshly transformed cells. Surprisingly, although the overall pattern of

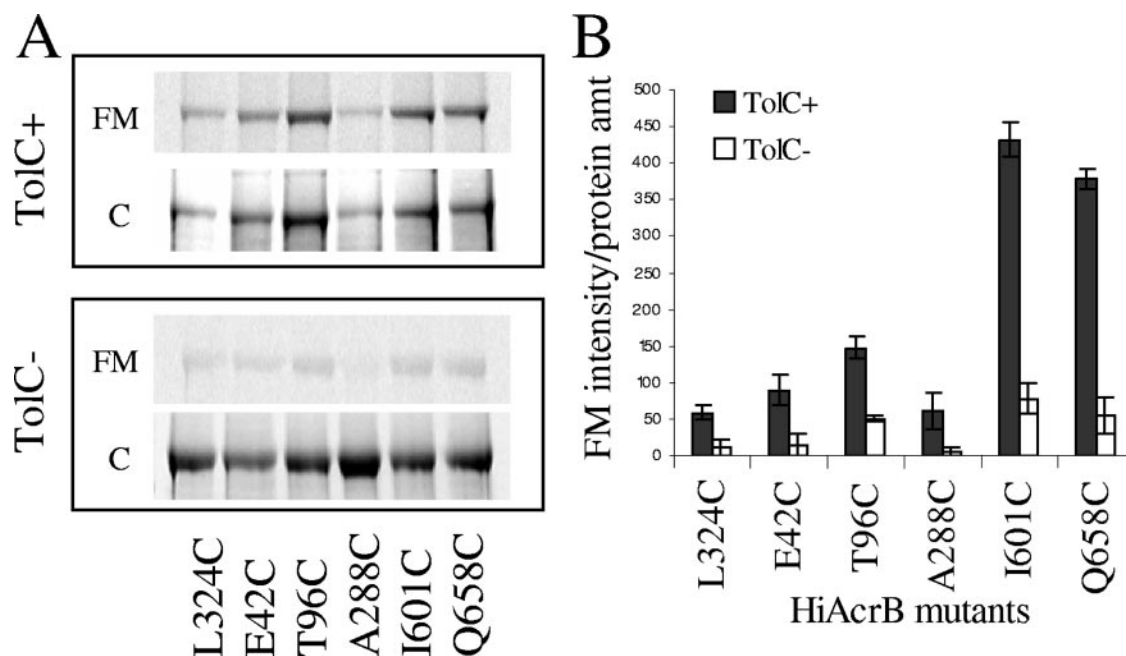


FIG. 2. FM accessibility of Cys residues in the periplasmic loops of AcrB_{HI}-Cys is reduced in the absence of TolC_{EC}. (A) AcrB_{HI} variants were produced in *E. coli* strains AG100AX (Δ acrAB Δ acrEF) and ECM2112 (Δ acrAB Δ tolC) and analyzed for FM accessibility as described in the legend of Fig. 1 and in Materials and Methods. (B) Fluorescence intensities of AcrB_{HI}-Cys variants (counts) shown in panel A are normalized based on the amount (amt) of AcrB_{HI} protein present (counts) as determined by CBB staining of SDS-PAGE gels. Error bars are standard deviations ($n = 3$). HiAcrB, AcrB_{HI}.

AcrB_{HI}-Cys accessibility to FM remained the same, the reactivity of all mutants was substantially lower in the *tolC*_{EC} mutant compared to the wild type (Fig. 2). This result suggested that either the lack of TolC_{EC} dramatically changed the

AcrB_{HI} conformation or, more likely, that the outer membrane of the *tolC*_{EC} mutant is less permeable to FM.

The growing body of evidence suggests that MFPs could be directly involved in transport reactions by interacting with sub-

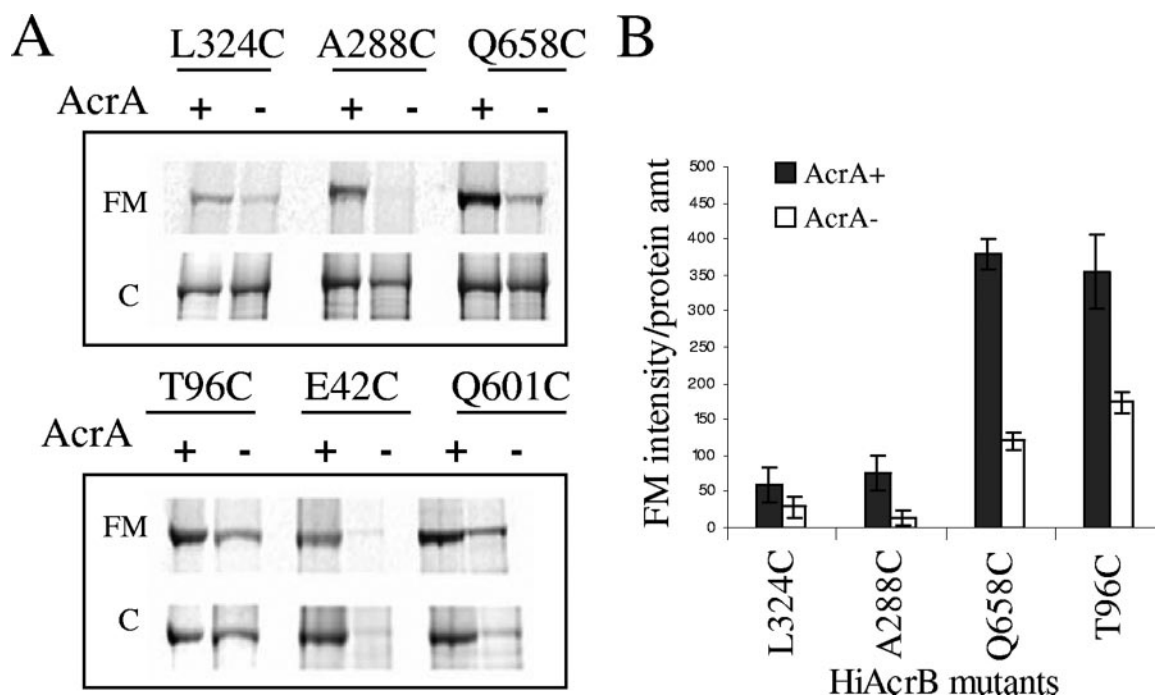


FIG. 3. The lack of AcrA_{HI} negatively affects the expression and FM accessibility of AcrB_{HI}-Cys. (A) AcrB_{HI}-Cys variants were produced in *E. coli* AG100AX (Δ acrAB Δ acrEF) in the presence and absence of AcrA_{HI} and analyzed for FM accessibility as described in the legend of Fig. 1 and in Materials and Methods. (B) Fluorescence intensities of AcrB_{HI}-Cys variants shown in panel A are normalized as described in the legend of Fig. 2. Error bars are standard deviations ($n = 3$). HiAcrB, AcrB_{HI}; amt, amount.

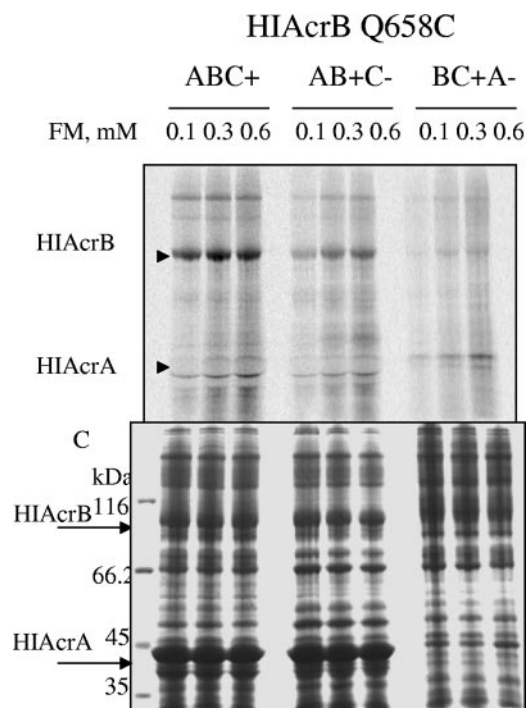


FIG. 4. Cells lacking either TolC_{EC} or AcrA_{HI} are less permeable for FM. Cells expressing AcrB_{HI}(Q658C) in the presence (+) or absence (-) of TolC_{EC} and AcrA_{HI} (indicated by B, C, and A, respectively, above the lanes) were treated with increasing concentrations of FM for 30 min at room temperature. Total membrane fractions were isolated, and proteins were separated by 12% SDS-PAGE followed by fluorescence imaging (top panel) and then CBB staining (bottom panel; C). Positions of AcrB_{HI} and AcrA_{HI} are indicated by arrows. Total fluorescence intensity of all proteins bands, not only AcrB_{HI}, decreased in the absence of either TolC_{EC} or AcrA_{HI}. HI-AcrA, AcrA_{HI}; HI-AcrB, AcrB_{HI}.

strates or communicating conformational changes taking place in the inner membrane transporter to the outer membrane channel (2, 3, 22). To investigate if AcrA_{HI} affects the reactivity of cysteines in AcrB_{HI}-Cys, DNA sequence encoding AcrA_{HI} was deleted from the plasmids carrying AcrB_{HI} with E42C, T96C, A288C, L324C, I601C, and Q658C substitutions. The AcrA_{HI}-less constructs were then studied for the ability to confer a multidrug resistance phenotype in AG100AX cells, expression of AcrB_{HI}, and accessibility to FM.

No multidrug resistance phenotype was conferred by AcrB_{HI} variants in the absence of AcrA_{HI}, demonstrating that none of the endogenous AG100AX proteins can complement the AcrA_{HI} function (data not shown).

The lack of AcrA_{HI} affected accessibility as well as expression of AcrB_{HI}-Cys variants. Although the expression of all AcrB_{HI} variants was somewhat lower in the absence of AcrA_{HI}, E42C and I601C variants were expressed at the lowest levels (Fig. 3). Surprisingly, similar to the TolC_{EC}-deficient strain, the accessibility of all AcrB_{HI}-Cys variants was reduced in the absence of AcrA_{HI} (Fig. 3). The increase in Cys accessibility of AcrB_{HI} would be expected if any of these residues were in a direct contact with AcrA_{HI}.

Both TolC_{EC}⁻ and AcrA_{HI}⁻ strains are highly susceptible to various compounds. Possibly the major response of *E. coli* to

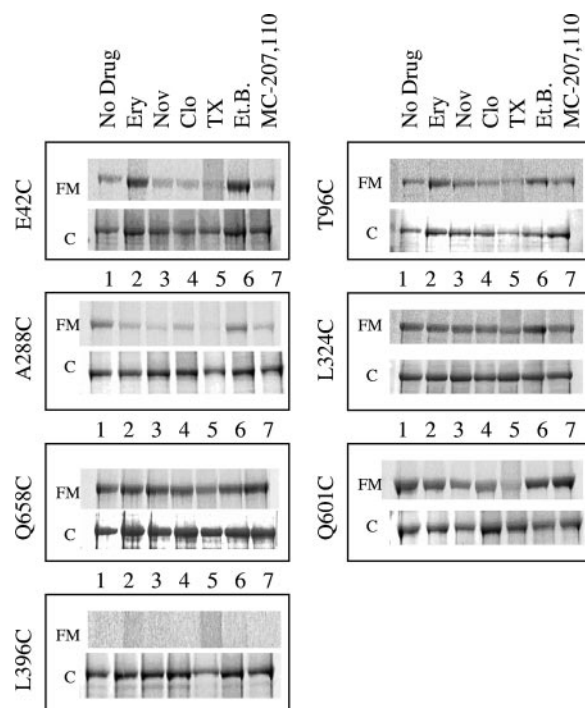


FIG. 5. Substrates modulate the accessibility of AcrB_{HI}-Cys to FM. Whole cells containing the indicated AcrB_{HI} variants were pretreated for 30 min at 37°C with PBS buffer alone (No drug) or containing a 5 mM concentration of the following compounds: erythromycin (Ery), novobiocin (Nov), cloxacillin (Clo), Triton X-100 (TX), ethidium bromide (Et.B.), or inhibitor MC-207,110. After this pretreatment cells were labeled with FM and analyzed as described in the legend of Fig. 1. For each variant, the top panel shows fluorescence intensity (FM) and the bottom panel shows CBB (C) staining.

the lack of active efflux is to decrease the permeability of the outer membrane. To test whether FM permeability is the major reason for decreased labeling of cysteines in mutants lacking TolC_{EC} or AcrA_{HI}, cells expressing AcrB_{HI}(Q658C) were treated with increasing concentrations of FM; total membrane fractions were then isolated and analyzed by SDS-PAGE (Fig. 4). Even at the lowest concentration of FM (0.1 mM) Q658C was readily labeled with FM in cells expressing all three proteins. The fluorescence intensity slightly increased at 0.3 mM FM and remained constant at 0.6 mM FM. Similarly, the extent of labeling of Q658C increased with increasing concentrations of FM in TolC_{EC}⁻ and AcrA_{HI}⁻ strains but was substantially lower than that in the presence of the functional transporter. In addition to AcrB_{HI}(Q658C), a number of other membrane proteins were labeled with FM. Overall accessibility profiles of total membrane fractions correlated with that of AcrB_{HI}(Q658C), indicating that the labeling of all proteins, not only AcrB_{HI}, decreased in the absence of TolC_{EC} or AcrA_{HI}. We conclude that the lack of either TolC_{EC} or AcrA_{HI} negatively affects accessibility of all proteins to FM, presumably due to the change in outer membrane permeability.

The reactivity of thiol groups in E42C, T96C, and A288C is modulated by AcrB_{HI} substrates. All AcrB_{HI}-Cys variants are competent in multidrug recognition, as demonstrated by complementation of multidrug-susceptible phenotypes of

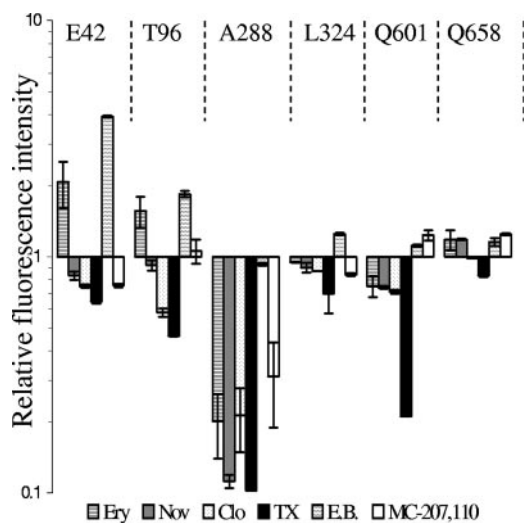


FIG. 6. Substrates modulate the accessibility of AcrB_{HI}-Cys to FM. Fluorescence intensities of AcrB_{HI}-Cys variants shown in Fig. 5 were normalized as described in the legend of Fig. 2 and expressed as a F_1/F_0 ratio, where F_1 is the normalized fluorescence intensity of AcrB_{HI} pretreated with drug and F_0 is the normalized fluorescence intensity of AcrB_{HI} pretreated with buffer alone (No drug). Error bars are standard deviations ($n = 3$). Ery, erythromycin; Nov, novobiocin; Clo, cloxacillin; TX, Triton X-100; EB, ethidium bromide.

acrAB-deficient *E. coli* (Table 2). The only exception is the Q658C variant, which was found to be partially deficient in efflux of erythromycin and chloramphenicol. To test whether substrates affect the accessibility of Cys in AcrB_{HI}, whole cells of AcrB_{HI}-Cys variants were preincubated with selected substrates: the antibiotics erythromycin, novobiocin, and cloxacillin; the detergent Triton X-100; the dye ethidium bromide; and the broad-spectrum inhibitor of RND-type transporters MC-207,110. Previously, MC-207,110 was shown to be a substrate of these transporters also (9). Next, cells were exposed to FM, and AcrB_{HI}-Cys was analyzed as described above.

As seen in Fig. 5 and 6, Triton X-100 reduced labeling of cysteines in all positions, albeit to different degrees. It is unlikely that the effect of Triton X-100 is specific to AcrB_{HI} since AG100AX (Δ *acrAB* Δ *acrEF*) is resistant to this detergent, and the overproduction of AcrB_{HI} did not affect susceptibility to Triton X-100 (data not shown). Perhaps this detergent interferes with either cysteine or FM reactivity. The accessibility of cysteine residues in the four positions I601C, Q658C, L324C, and L396C was not affected by pretreatment of cells with various compounds. The L396C residue located in the TMS4 remained inaccessible to FM, suggesting that binding of a substrate does not lead to opening of the transmembrane domain. In the presence of all tested substrates, except Triton X-100, the I601C, Q658C, and L324C residues were labeled to the same degree, suggesting that these three residues are not directly involved in binding of substrates or located in the structurally rigid regions of AcrB_{HI}.

The three mutations E42C, T96C, and A288C displayed variable accessibility to FM depending on specific substrates (Fig. 5 and 6). The E42C and T96C substitutions showed similar responses. The preincubation with ethidium bromide and erythromycin visibly increased the accessibility of E42C and

T96C to FM, whereas other substrates slightly inhibited labeling of these residues. The protection against labeling with FM is expected if these two residues are directly involved in binding of substrates. This result suggests that these two residues are located in the structurally flexible regions of AcrB_{HI} and that substrates promote conformational changes in AcrB_{HI}.

In addition to E42C and T96C, the accessibility of A288C was also affected by substrates. However, all tested substrates with the exception of ethidium bromide protected A288C from FM. This result suggests that substrates restrict access of FM to the A288C residue. In the crystal structure of AcrB_{EC}, the homologous G290 residue is located in the large binding pocket within ~ 8 Å distance from the bound substrate (16). Thus, the protection of the A288C residue could arise either from direct substrate interference or from the conformational changes in the protein induced by substrates.

DISCUSSION

In this report, we present evidence that AcrB_{HI} is a dynamic protein undergoing conformational changes in the presence of substrates. We introduced single cysteine substitutions based on the sequence alignment and structure modeling of AcrB_{HI} and MexD from *P. aeruginosa* (Table 1). Previously Mao et al. (10) showed that mutations in these positions enable MexD transporter to pump out the normally nontransported antibiotic carbenicillin. In addition, these mutations also had a substantial impact, both positive and negative, on the MexD-mediated transport of numerous other substrates, suggesting that these residues play an important role in substrate recognition and/or transport. In the high-resolution structure of AcrB_{EC}, the homologous Cys substitutions could be tentatively assigned to three distinct regions. These three regions map to the three putative substrate binding regions identified in the recent structural studies: the central cavity (E42C) (26), the large pocket in the periplasmic domain (T96C, A288C, and L324C) (16), and the external cavity of the periplasmic domain (Q658C and I601C) (25). In addition, three single cysteines were introduced into TMS4 of AcrB_{HI} in the vicinity of the proposed proton channel (17).

Substitutions with cysteines in all selected positions did not lead to notable changes in AcrB_{HI} activity or substrate specificity (Table 2) with the exception of the E42C, L324C, and Q658C mutants, which were partially defective in the transport of at least one substrate from among erythromycin, ethidium bromide, and chloramphenicol. Thus, none of the mutated amino acid residues is critical in multidrug efflux. However, the reduced activities of the E42C, L324C, and Q658C mutants suggest that these residues could contribute to the binding or transport of drugs.

Consistent with structure predictions, cysteine residues placed into the periplasmic domain were accessible for labeling with the large, hydrophilic probe FM, albeit to different degrees. E42C, T96C, A288C, and L324C residues were labeled only partially, indicating that the reactivity of these residues could be limited due to hindrance from surrounding amino acid residues. Alternatively, partial accessibility could indicate the heterogeneity of AcrB_{HI} conformations. Recently, two independent studies suggested that during transport reaction AcrB_{EC} cycles through three different conformations (16, 21).

Interestingly, the access to T96C, A288C, and L324C from the periplasm differs in these three conformations, with the least access in the “tight-binding” conformer. Furthermore, the conformational transitions of AcrB_{EC} also affect the central cavity, the site of E42C. Thus, partial reactivity of these residues could be due to the conformational heterogeneity of AcrB_{HI}.

Located in the external cleft of the periplasmic domain, I601C and Q658C were readily labeled with FM. Interestingly, AcrA_{HI}, which presumably binds to this region of AcrB_{HI}, did not protect these residues from labeling. In contrast, Q658C was visibly less accessible to FM in the absence of AcrA_{HI}. In addition, the accessibilities of T96C, A288C, and L324C also decreased in the absence of AcrA_{HI}. A similar decrease in Cys accessibility was also observed for the TolC-deficient strain of *E. coli*. This decrease in FM labeling was not restricted to AcrB_{HI} (Fig. 4). FM labeling of other membrane proteins was also reduced in TolC_{EC}⁻ and AcrA_{HI}⁻ backgrounds, suggesting that the change in the FM permeability is the major reason for this effect. Previous studies showed that cells lacking TolC_{EC} replace the outer membrane porin OmpF with OmpC, the porin with the much smaller permeability (13). Such substitution could restrict the access of the hydrophilic FM to the periplasm. Similarly, using N-terminal protein sequencing, we found that ECM2112, the TolC_{EC}⁻ strain used in this study, produces OmpC. Interestingly, in the AG100AX strain, which is hypersusceptible to multiple drugs due to the lack of major efflux pumps, OmpF is also replaced by OmpC (E. B. Tikhonova and H. Zgurskaya, unpublished data). Thus, replacement of OmpF with the less permeable OmpC seems to be a common response of *E. coli* to the increased multidrug susceptibility due to the lack of active efflux.

In addition, TolC_{EC} itself could be a route for FM to reach the periplasm and the transporter. Defects in TolC_{EC}, for example, led to hyperresistance to the glycopeptide antibiotic vancomycin, which is too large to pass through the general porins (20). In this case, the lack of AcrA_{HI} could keep TolC_{EC} in the closed conformation, leading to the decrease in FM accessibility.

Low fluorescence signals precluded unequivocal determination of the effect of substrates on AcrB_{HI}-Cys reactivity in the absence of TolC_{EC} or AcrA_{HI} (Fig. 2 and 3; also data not shown). The effect of substrates became apparent when all three components of the operational pump were present. The Cys in A288C was protected from labeling with FM by almost all tested compounds (the only exception is ethidium bromide) including MC-207,110 inhibitor. This result is consistent with the recently reported structure of the ligand-bound AcrB_{EC}, in which the homologous G290 residue is in proximity to bound substrates (16). However, this approach cannot distinguish between hindrance effects caused by substrate binding versus conformational changes. Thus, either one of these factors or both could contribute to protection of the A288C residue from FM.

Two other residues, E42C in the central cavity and T96C located in the periplasmic domain, were also differentially affected by substrates. In contrast to A288C, some substrates (erythromycin and ethidium bromide) significantly enhanced the reactivity of E42C and T96C, suggesting that the conformation of AcrB_{HI} changes in response to substrate binding. A similar substrate-dependent enhancement of labeling with thiol-reactive reagents was also reported for cysteines intro-

duced into the multidrug efflux transporter MdfA (1). Interestingly, the modulation of cysteine accessibility of E42C and T96C depended on the specific substrate. Both erythromycin and ethidium bromide are positively charged compounds, and preincubation with these compounds enhanced labeling with FM. In contrast, negatively charged novobiocin and cloxacillin did not affect the labeling or slightly protected the E42C and T96C residues. This result suggests that various compounds could bind to different surfaces on AcrB_{HI}.

None of the other accessible Cys residues of the AcrB_{HI} mutants changed their reactivity in response to the presence of substrates. In contrast to MexD, all cysteine substitutions were well tolerated by AcrB_{HI} transporter and did not lead to significant changes in MICs. Only L324C and Q658C substitutions partially reduced resistance to several tested compounds (Table 2). Therefore, the lack of effect of substrates in the case of Q658C and L324C could be due to defects in substrate binding. However, it is also possible that these residues, similar to Q601C, do not contribute to substrate binding and located in the conformationally rigid regions of AcrB_{HI}.

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