

Chimeric Analysis of the Multicomponent Multidrug Efflux Transporters from Gram-Negative Bacteria

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Received 20 June 2002/Accepted 5 September 2002

Many multidrug transporters from gram-negative bacteria belong to the resistance-nodulation-cell division (RND) superfamily of transporters. RND-type multidrug transporters have an extremely broad substrate specificity and protect bacterial cells from the actions of antibiotics on both sides of the cytoplasmic membrane. They usually function as three-component assemblies spanning the outer and cytoplasmic membranes and the periplasmic space of gram-negative bacteria. The structural determinants of RND transporters responsible for multidrug recognition and complex assembly remain unknown. We constructed chimeric RND transporters composed of N-terminal residues of AcrB and C-terminal residues of MexB, the major RND-type transporters from *Escherichia coli* and *Pseudomonas aeruginosa*, respectively. The assembly of complexes and multidrug efflux activities of chimeric transporters were determined by coexpression of hybrid genes either with AcrA, the periplasmic component of the AcrAB transporter from *E. coli*, or with MexA and OprM, the accessory proteins of the MexAB-OprM pump from *P. aeruginosa*. We found that the specificity of interaction with the corresponding periplasmic component is encoded in the T60-V612 region of transporters. Our results also suggest that the large periplasmic loops of RND-type transporters are involved in multidrug recognition and efflux.

In *Escherichia coli* and *Pseudomonas aeruginosa* the major, constitutively expressed multidrug transporters are AcrB and MexB, respectively, which belong to the resistance-nodulation-cell division (RND) superfamily (22). Several features distinguish RND-type transporters from other multidrug pumps (MDRs). First, these transporters have a very unusual membrane topology. The predicted structure consists of 12 transmembrane α -helices (TMS) with two large hydrophilic extra-cytoplasmic domains between TMS 1 and 2 and TMS 7 and 8 (6). These unusually large hydrophilic domains (ED1 and ED2) make the membrane topology of RND transporters look very similar to that of receptors rather than transporters. Indeed, the RND transporters share some degree of homology with the cholesterol-sensing domain of HMG-CoA reductase. This finding suggested that these proteins evolved as machinery that senses, or senses and then extrudes, relatively apolar molecules found within the lipid bilayer of membranes (22). Second, unlike the vast majority of MDRs which mostly pump out hydrophobic cations, the RND-type transporters from gram-negative bacteria also interact with neutral, zwitterionic, and anionic compounds (10, 16). Furthermore, some RND transporters provide cell protection on both the cytosolic and the periplasmic sides of the inner membrane. In particular, AcrB and its homologs efficiently expel some β -lactam antibiotics whose target is located in the periplasmic space rather than in cytoplasm (9, 13). In addition, some transported drugs have been shown not to traverse the cytoplasmic membrane

(13). These observations led to the suggestion that (i) these transporters have multiple drug-binding sites to capture drugs from different locations within the cell. Alternatively, (ii) the topology of these transporters is such that both hydrophilic and lipophilic compounds can gain access to drug-binding sites (15, 27). Presently, the structure, number, and location of the multidrug-binding sites in RND transporters remain unknown.

The amino acid sequences of AcrB and MexB are 70% identical. However, despite the high degree of structural and functional similarity, these transporters appear to possess structure variations responsible for several unique features. AcrB and MexB provide a different degree of drug resistance in *E. coli* and *P. aeruginosa* cells, respectively, and also vary in substrate specificities (8, 10). In addition, these transporters interact in a highly specific manner with at least two additional proteins: the periplasmic membrane fusion proteins (MFPs) (AcrA and MexA) and the outer membrane channels (OMFs) (TolC and OprM). These accessory proteins together with a unique RND transporter, AcrB or MexB, form complexes that span both the inner and the outer membranes of gram-negative bacteria and provide an efflux of drugs across two-membrane envelopes directly into the external medium.

In *P. aeruginosa* all three components of multidrug pumps are usually expressed from a single operon, for example, *mexAB-oprM* or *mexCD-oprJ*, providing the coordinated expression of each component. Genetic analysis has indicated that the periplasmic MFPs are specific for each RND transporter and cannot be interchanged between homologous pumps (23). This result implied that, despite the high degree of homology, RND transporters possess structure variations responsible for the specificity of interaction with MFPs. In contrast, a single OMF can support the function of several homologous pumps. The OprM channel encoded in the *mexAB-oprM* operon can also function together with MexCD (23) or

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
<i>P. aeruginosa</i> strain PAO1	Wild type	
<i>E. coli</i> strains DH5 α AG100AX	<i>supE44</i> Δ <i>lacU169</i> <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i> Δ <i>acrAB::kan</i> Δ <i>acrEF::spe</i>	General cloning 12
Plasmids		
pUC18	<i>E. coli</i> cloning vector, Amp ^r	
pUC151A	pUC18 vector carrying the <i>acrAB</i> genes	10
pBP	pUC18 vector expressing <i>acrB</i> under native promoter	This study
pOM	pUC18 vector expressing the <i>oprM</i> gene under P _{lac} promoter	This study
pMAO	pOM derivative carrying the <i>mexA</i> gene	This study
pMABO	pMAO derivative carrying the <i>mexB</i> gene	This study
pPV20	<i>mexAB-oprM</i> genes in the pAK1900 shuttle cloning vector	16

MexXY (1). The versatility of OMFs is also supported by the fact that the enterobacterial MDR operons do not contain genes for these proteins. In contrast, a single protein, multi-functional channel TolC, encoded elsewhere on the chromosome is capable of supporting the transport needs of several MDRs (4) and also the export of such proteins as hemolysin and colicin V (2, 7). Interestingly TolC also supports the activity of the *Pseudomonas* multidrug transporters MexD and MexY when they are expressed in *E. coli* cells in the absence of their native OMFs (11). Based on these and other data, it was proposed that MFPs play a major role in the formation of tripartite complexes and that the inner membrane transporters (IM transporters) do not interact directly with OMFs (21).

In this study, we have taken advantage of functional differences between AcrB and MexB to search for the region(s) involved in drug transport and/or interaction with accessory proteins. Our approach is based on the construction of chimeric AcrB/MexB proteins and assaying their activity in the complexes with AcrA-TolC or MexA-OprM proteins.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Luria-Bertani medium (10 g of Bacto-tryptone, 10 g of Bacto yeast extract, and 5 g of NaCl per liter) was used to grow all bacterial cultures. Ampicillin (100 μ g/ml), kanamycin (35 μ g/ml), and spectinomycin (50 μ g/ml) were used for selection. All plasmids (Table 1; Fig. 1) were constructed by common cloning techniques (18) and propagated in the DH5 α strain of *E. coli*. Nucleotides of the *acrAB* and *mexAB-oprM* loci are numbered according to the methods of Ma et al. (9) and Poole et al. (16).

To construct pBP, pUC151A was first digested with *BlnI* and *XhoI* restriction enzymes, treated with T4 DNA polymerase, and self-ligated to produce pUC151XB. Next, a 3,554-bp *SacI-NsiI* fragment of pUC151XB was ligated into a *SacI-PstI*-treated pUC18 vector. Expression plasmids for OprM (pOM) and MexA-OprM (pMAO) were generated by PCR amplification and subcloning into *XbaI-HindIII* sites and *SacI-XbaI* sites of pUC18 and pOM, respectively. The pMABO plasmid was generated by cloning the PCR-amplified *mexB* gene in the *XbaI* and *SpeI* sites of pMAO.

Construction of expression plasmids for AcrB/MexB hybrid proteins. To construct expression plasmids for AcrB/MexB hybrid proteins, we designed PCR primers complementary to the *acrB* and *mexB* regions indicated in Fig. 1 (the sequences of primers are available upon request). Primers contained added restriction sites, if required. For amplification of *mexB* regions, the chromosomal *P. aeruginosa* DNA or pPV20 was used as a template. For amplification of *acrB* regions, pUC151A or pBP was used as a template.

All plasmids for the expression of chimeric genes downstream of *acrA* are derivatives of pUC151A. Plasmid pAcrAB377 was constructed by replacement of

a *PpuMI-NsiI* fragment of pUC151A with a PCR-amplified 2,019-bp fragment of *mexB*. To construct pAcrAB518, pAcrAB569, and pAcrAB612, the corresponding chimeric genes were assembled by PCR. In the expression plasmids, the chimeric genes replaced the *DraIII-NsiI* fragment of pUC151A. pAcrAB589 and pAcrAB849 were constructed by replacement of the *PpuMI-HindIII* fragment of pUC151A with *acrB589/mexB* and *acrB849/mexB* chimeric genes. *acrB589/mexB* and *acrB849/mexB* were assembled by cloning of corresponding *mexB* fragments into *HincII-PstI* sites of pUC18 to produce pBSpe and pBMfe, respectively, and subsequent subcloning of *acrB* fragments into *XmaI-SpeI*-treated pBSpe and *XmaI-MfeI*-treated pBMfe.

All plasmids for the expression of chimeric AcrB/MexB genes in a single operon with MexA and OprM are derivatives of pMAO plasmid. The chimeric *acrB612/mexB* and *acrB849/mexB* genes were PCR amplified using pAcrAB612 and pAcrAB849 as templates. The obtained DNA fragments were subcloned into a *XbaI-SpeI*-treated pMAO plasmid to yield pMexAB612 and pMexAB849. Expression plasmid pMexAB377 was constructed by the replacement of the *PpuMI-SpeI* fragment of pMexAB849 with the *PpuMI-SpeI* fragment of pMABO. To construct pMexAB60 and pMexAB182, the corresponding DNA fragments of the *acrB* gene were PCR amplified using pBA plasmid as a template with forward primer containing an *XbaI* site and the reverse primer containing sequences complementary to both *acrB* and *mexB*. The PCR products were then used as forward primers to amplify the *mexB* gene, with pPV20 as a template. After treatment with *XbaI* and *SpeI*, the obtained 3,150-bp DNA fragments were subcloned into the pMAO plasmid to yield pMexAB182 and pMexAB60.

When PCR was used in the construction of a plasmid, the final structure was verified by DNA sequencing. PCRs were performed with a Peltier PTC-200 thermal cycler (MJ Research) with Biolase DNA polymerase (BioLine). Restriction endonucleases, alkaline phosphatase, and T₄ DNA ligase were obtained from New England BioLabs Inc. or Gibco BRL.

MIC determinations. For the determination of MICs of various antimicrobial agents, exponentially growing cultures (optical density at 600 nm of 1.0) were inoculated at a density of 10⁴ cells per ml into Luria-Bertani medium in the presence of twofold-increasing concentrations of the drug under investigation. Cell growth was determined after overnight incubation at 37°C.

Cross-linking, preparation of membrane fractions, and immunoblotting analysis. Membrane fractions of the dithiobis(succinimidylpropionate) (DSP)-treated and nontreated AG100AX *E. coli* cells expressing different hybrids or wild-type proteins were prepared as described previously (26). Protein concentrations were determined using the Bio-Rad protein assay with bovine serum albumin as a standard. Equal protein amounts of membrane fractions containing different hybrids were loaded onto sodium dodecyl sulfate (SDS)-polyacrylamide gels. Membrane fractions containing wild-type AcrB and AcrB849/MexB were loaded onto gels in concentrations 5 to 10 times lower than other samples because of the higher reactivity of anti-AcrB antibody with these two proteins. SDS-polyacrylamide gel electrophoresis was performed by standard methods through 5% stacking and 8 or 10% separating gels. For immunoblotting, proteins were transferred electrophoretically to polyvinylidene difluoride membrane (Millipore) in 3-(cyclohexylamino)-1-propanesulfonic acid-NaOH (10 mM; pH 11) and methanol (10%). Protein visualization was performed according to standard protocols with anti-AcrA (24), anti-AcrB (25), and anti-OprM (a kind gift of K.

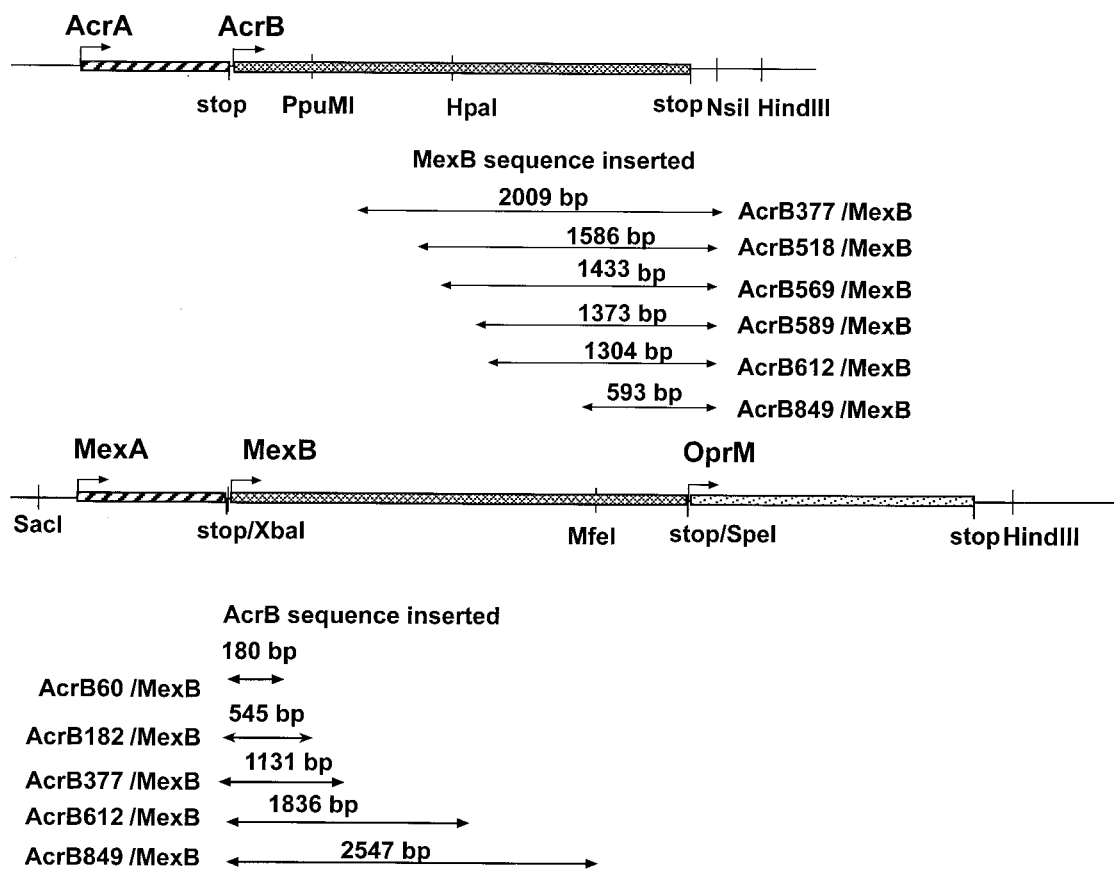


FIG. 1. Construction of chimeric AcrB/MexB transporters. Physical maps of *acrAB* and *mexAB-oprM* operons are shown. Arrows indicate the DNA fragments corresponding to *acrB* or *mexB* present in chimeric constructs. Numbers in the names of constructs correspond to the N-terminal amino acid residues of AcrB constituting the chimeric transporters. Only restriction sites relevant to cloning are shown.

Poole) antibodies and alkaline phosphatase-conjugated anti-rabbit antibodies (Sigma).

RESULTS

AcrB and MexB demonstrate distinct substrate specificities in *E. coli*. Previous studies demonstrated that the MexAB-OprM multidrug efflux complex from *P. aeruginosa* is functional when expressed in *E. coli* cells (19). However, the pattern of resistance to different antimicrobials is less broad than that attributed to MexAB-OprM in *P. aeruginosa*. The substrate specificities of AcrAB-TolC and MexAB-OprM appear to be very similar in *E. coli*, consistent with the high degree of homology between corresponding components of these complexes (AcrA and MexA, 57.7% identity; AcrB and MexB, 69.8% identity).

The high degree of homology between the inner membrane components enabled us to detect the expression of MexA and MexB proteins by immunoblotting analysis with polyclonal antibody raised against AcrA and AcrB proteins. In agreement with previously reported data (19), we found that the MexAB-OprM complex is expressed in *E. coli* cells (Fig. 2) and is functionally active, as judged by the restoration of the drug-resistant phenotype of the AG100AX strain, which is deficient in the AcrAB and AcrEF pumps (Table 2).

Although both AcrAB and MexAB-OprM conferred resis-

tance to a broad spectrum of antimicrobials, the levels of resistance provided by these transporters differed substantially for a number of compounds (Table 2). In particular, *E. coli* cells expressing MexAB-OprM were capable of surviving in higher concentrations of cinoxacin (CIN; 8-fold difference in MICs compared to AcrAB-expressing cells). On the other hand, MexAB-OprM-containing cells were less resistant than AcrAB-expressing cells to oleandomycin (OLE; 16-fold difference), erythromycin (ERY; 8-fold difference), and ethidium bromide (EtB; 4-fold difference). It is noteworthy that the MexAB-OprM complex provides higher levels of resistance to the latter drugs when expressed in the *P. aeruginosa* envelope, presumably because of the difference in the permeability properties of the outer membranes of *E. coli* and *P. aeruginosa* (8).

The AcrAB pump needs, for its efficient action, the presence of the multifunctional outer membrane channel TolC (4). Although TolC- and AcrAB-deficient phenotypes are very similar, they are not identical, presumably because TolC provides the route for drugs across the outer membrane for other multidrug transporters of *E. coli* (20). Despite the high homology between AcrAB and MexAB, the TolC protein does not seem to interact with and support the function of MexAB. When we introduced *mexAB* into *E. coli* cells in the absence of the OprM channel, the cells remained highly susceptible to different drugs, demonstrating that all three components, MexA, MexB,

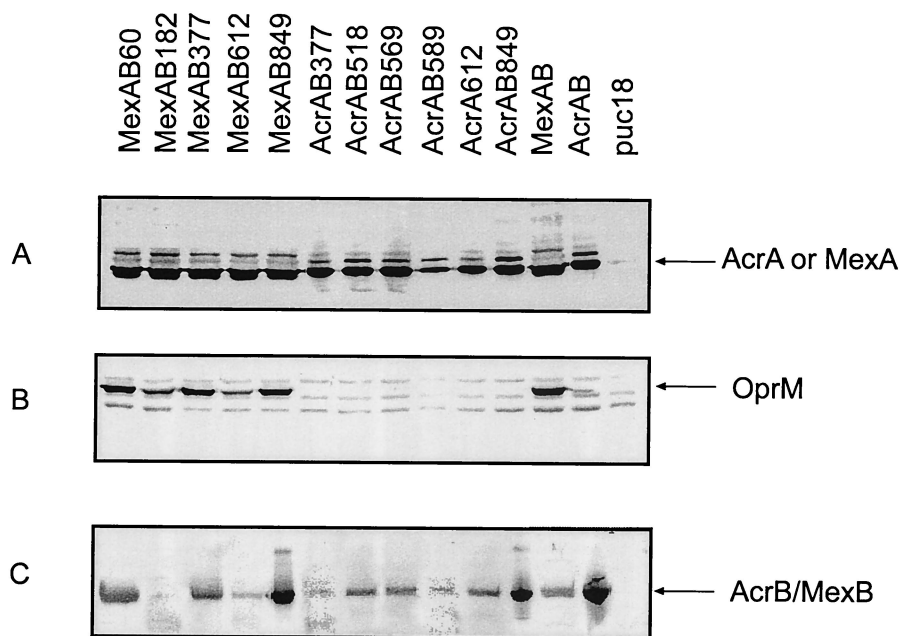


FIG. 2. Expression of wild-type and chimeric AcrB/MexB in *E. coli*. Cells expressing wild-type AcrB and MexB as well as chimeric AcrB/MexB transporters in operons with either AcrA (AcrAB) or MexA/OprM (MexAB) were harvested and analyzed by Western blotting with anti-AcrA (A), anti-OprM (B), or anti-AcrB (C) antibodies. The expression of AcrA, MexA, and OprM was detected in whole cells (A and B). AcrB, MexB, and chimeric AcrB/MexB proteins were visualized in membrane fractions of corresponding cells (C). Arrows indicate the positions of expressed proteins.

and OprM, must be present to protect *E. coli* from multiple drugs (data not shown). Consistent with this result is the finding that the OprM protein, when produced in TolC-deficient cells, does not rescue the drug-sensitive phenotype of these cells. Thus, all three components of the *Pseudomonas* efflux transporter MexAB-OprM must be present in *E. coli* cells to provide protection against multiple drugs.

Construction and expression of chimeric AcrB/MexB molecules. Comparative sequence analyses have indicated that the N- and C-terminal halves of RND proteins share sequence

similarities, suggesting that they may have evolved via tandem gene duplication events (17). Using deletion analysis we found that neither N- nor C-terminal halves of the AcrB transporter can act autonomously (data not shown). This result suggested that both halves of multidrug transporters are required for multidrug transport or interaction with accessory proteins.

To identify regions of the RND transporters responsible for the interactions with accessory proteins and/or for the transport activities, we generated a series of hybrid AcrB/MexB molecules (Fig. 1 and 3). The hybrid *acrB/mexB* genes were

TABLE 2. Antimicrobial susceptibility of *E. coli* cells expressing wild-type and chimeric AcrAB and MexAB-OprM efflux pumps

Construct	MIC ($\mu\text{g/ml}$) ^c														
	SDS	CA	NOV	EtB	OLE	ERY	PUR	LIN	CIN	NAL	CF	PRO	TET	CTET	NOR
pUC18	40	2,325	4	3.125	4	4	2	32	2	1	0.8	6.25	0.32	0.04	0.01
AcrAB	>10,240	>9,300	256	800	4,096	256	256	1,024	2	8	12.8	25	2.56	0.4	0.16
MexAB-OprM	10,240	>9,300	256	100	256	32	64	512	16	16	12.8	25	2.56	0.8	0.08
AcrAB849^a	>10,240	>9,300	256	200	512	256	256	256	8	8	6.4	25	2.56	0.4	0.08
MexAB849 ^b	40	2,325	2	6.25	4	4	2	16	2	1	0.8	6.25	0.16	0.01	0.01
AcrAB612	>10,240	>9,300	256	200	256	64	32	256	1	1	1.6	6.25	0.64	0.1	0.04
MexAB612	40	2,325	2	3.125	2	4	2	16	2	1	0.8	6.25	0.32	0.02	0.005
AcrAB589	40	2,325	4	6.25	2	4	1	32	2	1	0.4	6.25	0.32	0.01	0.01
AcrAB569	40	2,325	4	6.25	2	4	2	32	2	1	0.8	6.25	0.32	0.01	0.02
AcrAB518	40	2,325	4	6.25	2	4	2	32	2	1	0.8	6.25	0.32	0.01	0.02
AcrAB377	40	2,325	2	3.125	2	2	2	32	2	1	0.4	6.25	0.16	0.01	0.02
MexAB377	40	4,650	2	3.125	4	4	2	16	2	1	0.8	6.25	0.16	0.02	0.01
MexAB182	40	2,325	2	3.125	4	4	2	16	2	1	0.8	3.125	0.32	0.04	0.04
MexAB60	10,240	>9,300	64	6.25	64	8	32	32	8	2	0.8	6.25	0.64	0.1	0.08

^a The numbers correspond to AcrB N-terminal amino acid residues present in hybrid transporters. Functional constructs and corresponding MIC values are shown in bold.

^b All constructs expressing chimeric pumps in a single operon with the *mexA* gene also contain the *oprM* gene.

^c Abbreviations: SDS, sodium dodecyl sulfate; CA, cholic acid; NOV, novobiocin; LIN, lincomycin; EtB, ethidium bromide; ERY, erythromycin; OLE, oleandomycin; NOR, norfloxacin; PUR, puromycin; NAL, nalidixic acid; CF, chloramphenicol; CIN, cinoxacin; PRO, proflavine; TET, tetracycline; CTET, chlortetracycline.

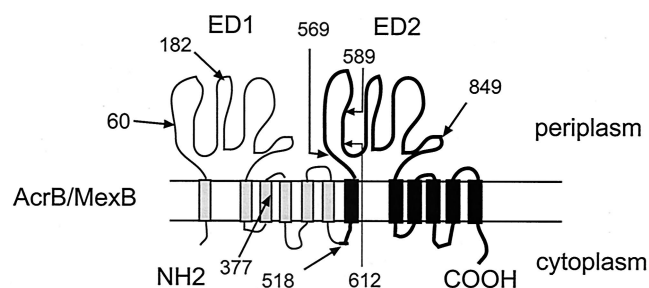


FIG. 3. Membrane topology of AcrB and MexB. These transporters were proposed to consist of 12 TMSs with two large extracytoplasmic domains (ED1 and ED2). The N- and C-terminal halves of AcrB and MexB share sequence similarities. Arrows with numbers show the sites of fusions between AcrB and MexB amino acid sequences and the number of N-terminal amino acid residues from AcrB in chimeric AcrB/MexB transporters.

cloned either immediately downstream of the *acrA* gene to assay their functionality with this protein (AcrA-AcrB/MexB) or between the *mexA* and *oprM* genes, in a manner to preserve the chromosomal arrangement of these genes in a single operon (MexA-AcrB/MexB-OprM) (Fig. 1). In these arrangements, the role of N-terminal residues in the function and assembly of multicomponent drug transporters can be evaluated by assessment of the functional activity of the AcrB/MexB hybrids in combination with AcrA and TolC, whereas the role of the C-terminal residues can be assessed in combination with MexA and OprM proteins. The native *acrA* promoter has provided the efficient expression of the *acrA-acrB/mexB* operon, but the expression of the *mexA-acrB/mexB-oprM* operon was dependent on the *P_{lac}* promoter of the pUC18 vector.

All constructs were transformed into *E. coli* cells deficient in AcrAB and AcrEF multidrug transporters, and their expression was assayed by immunoblotting (Fig. 2). As judged by the Western blotting of whole-cell extracts prepared from *E. coli*

expressing either AcrA-AcrB/MexB or MexA-AcrB/MexB-OprM, all constructs produced similar amounts of MexA and AcrA (Fig. 2A). However, the levels of expression of chimeric AcrB/MexB proteins were affected by the position of junctions between AcrB and MexB (Fig. 2C). The amounts of AcrB849/MexB were comparable to that of the wild-type AcrB protein and higher than the amount of wild-type MexB, regardless of whether this hybrid was coexpressed with AcrA or with MexA/OprM proteins. All other hybrids, with the exception of AcrB589/MexB and AcrB182/MexB, were produced at levels comparable to that of the wild-type MexB. When the link between the AcrB and MexB portions was located either in the N-terminal region of ED1 or in the similar position in ED2, the hybrids were unstable, independently of the periplasmic proteins. In particular, AcrB589/MexB and AcrB182/MexB were present in membranes at low levels. The accessory proteins seem to affect the expression of the AcrB377/MexB hybrid. This protein was produced in larger amounts when expressed in combination with the MexA and OprM proteins.

The specificity of interaction with accessory proteins is encoded within the T60-V612 region of AcrB and MexB transporters. Previous chemical cross-linking studies have shown that the periplasmic lipoprotein AcrA interacts specifically with the inner-membrane-associated AcrB transporter and that this complex is assembled even in the absence of the outer membrane component TolC (26). We used the same approach to determine which hybrid AcrB/MexB molecules can form complexes with AcrA or MexA proteins. To stabilize protein complexes, intact *E. coli* cells expressing different constructs were treated with cross-linker DSP (26). As previously reported, the AcrA and AcrB proteins form a stable complex in the inner membrane of *E. coli* (Fig. 4). Under the same conditions, MexA and MexB form complexes with molecular masses similar to those of AcrAB. In our assay we did not detect high-molecular-weight species that could indicate the presence of the tripartite MexA-MexB-OprM complex.

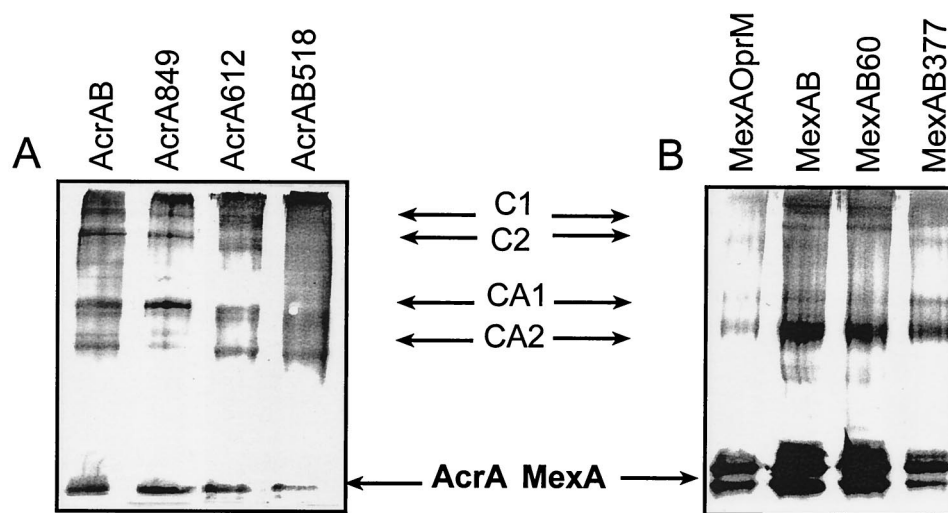


FIG. 4. Western blotting analysis of AcrA (A) and MexA (B) in *E. coli* cells cross-linked with DSP. Positions of oligomeric AcrA and MexA are indicated as CA1 and CA2. Higher-molecular-weight complexes C1 and C2 presumably contain the periplasmic and inner membrane components of AcrAB-TolC (A) and MexAB-OprM (B) complexes, respectively. The membrane fraction of cells expressing MexA and OprM proteins without MexB (panel B, lane1) is shown as a negative control.

The high-molecular-weight complexes reacting with both anti-AcrA (Fig. 4) and anti-AcrB (data not shown) antibodies were detected in the membrane fractions of *E. coli* cells expressing AcrA-AcrB612/MexB, AcrA-AcrB849/MexB, and MexA-AcrB60/MexB-OprM hybrid transporters. This result is consistent with the finding that these AcrB/MexB hybrid proteins are functionally active and that the interaction between the periplasmic component and the IM transporter is required for the efflux of drugs (Table 2 and see below). The interactions between AcrB612/MexB, AcrB849/MexB, and AcrA appear to be very specific, as evidenced by the inability of AcrB612/MexB or AcrB849/MexB to associate with MexA and OprM (data not shown) and the lack of functional activity of these hybrids (Table 2). Thus, the N-terminal 612 amino acid residues of AcrB contain the structural determinants for specific association with AcrA.

Of five hybrids expressed with MexA and OprM, only AcrB60/MexB restored the drug-resistant phenotype to *E. coli* cells, suggesting that only the first 60 N-terminal amino acid residues of MexB can be substituted by corresponding regions of the AcrB transporter. Taken together, these results suggested that the specificity of interaction with the periplasmic MFPs is encoded in the T60-V612 region of the IM transporter.

We could not detect reproducibly the complexes between the periplasmic proteins and hybrid pumps for other constructs. The expression of AcrB182/MexB and AcrB589/MexB hybrids was substantially lower than that of the wild-type AcrB or MexB proteins. One explanation for the absence of detectable complexes is the low sensitivity of the *in vivo* chemical cross-linking technique. Also, the low amount of hybrid proteins in the membrane fractions could be an indication that these proteins are not properly folded within the membrane and, as a result, are vulnerable to the action of proteases.

Drug specificity of chimeric AcrB and MexB efflux transporters. Cells expressing AcrA-AcrB612/MexB, AcrA-AcrB849/MexB, and MexA-AcrB60/MexB-OprM displayed increased resistance to a number of tested compounds compared with cells transformed with the vector alone (Table 2). However, the transport activity of these hybrid pumps differed substantially from the wild-type AcrB and MexB transporters. Since the complex formation appears to be unaffected in AcrA-AcrB612/MexB, AcrA-AcrB849/MexB, and MexA-AcrB60/MexB-OprM (see above and Fig. 4), the observed differences in substrate specificities or efficiencies of efflux among these hybrids arise most probably from the structural variations in the IM transporters that affect the multidrug recognition or multidrug transport mechanisms.

All three functional chimeras recognized and expelled the detergents SDS and cholic acid (CA) and novobiocin (NOV) with efficiencies comparable to that of the wild-type AcrAB or MexAB pumps (Table 2). This result shows that the 60 amino acid residues of the N terminus and the C-terminal amino acid residues of AcrB and MexB are interchangeable with respect to efflux of these substrates.

The wild-type AcrB and MexB transporters differ from each other in the specificity to several compounds (Table 2). Surprisingly, the chimeric AcrA-AcrB849/MexB pump, which contains only the last 200 C-terminal residues from MexB, showed the substrate specificities characteristic of both wild-type trans-

porters. *E. coli* cells expressing this pump were indistinguishable from those with the wild-type AcrB transporter in susceptibility to the majority of tested compounds (Table 2). On the other hand, the specificity of the AcrA-AcrB849/MexB pump to EtB, lincomycin (LIN), and CIN was similar to that of the wild-type MexB transporter. This result suggested that the structure variations responsible for differences in transport of the latter compounds reside in the C-terminal 200 amino acid residues of the AcrB and MexB transporters. For other substrates, these residues are not sufficient to convert the specificity of the AcrB849/MexB hybrid to a MexB-like one.

The AcrA-AcrB612/MexB pump contains an additional 235 C-terminal amino acid residues from MexB. All these residues are located entirely in the extracytoplasmic domain ED2. This chimeric transporter showed, distinct from other constructs, substrate specificity. AcrB612/MexB transported LIN, EtB, ERY, OLE, and puromycin (PUR) with the efficiency of the wild-type MexB. This result suggested that the region V612-S/K849 of RND transporters might contribute to the specificity toward these drugs. The AcrB612/MexB hybrid, however, lost the ability to expel other substrates, including CIN, nalidixic acid (NAL), or proflavine (PRO), which are efficiently expelled by the wild-type pumps and AcrB849/MexB hybrid. Perhaps the binding of these compounds occurs in different site of transporter. The presence of the V612-S/K849 region from MexB could disrupt this site in the AcrB transporter.

The N-terminal 60 amino acid residues of AcrB, which are present in AcrB60/MexB, dramatically reduced the spectrum of substrates of the wild-type MexB transporter. Although AcrB60/MexB conferred resistance to PUR, CIN, SDS, CA, and NOV, this hybrid had only partial or no activity against the rest of the tested drugs.

Taken together our results suggest that such substrates as SDS, CA, and NOV differ from others in terms of recognition and transport efficiency by AcrB/MexB transporters. The partial replacements of N-terminal or C-terminal residues in AcrB/MexB transporters did not change the ability of hybrids to expel these compounds from cells. The 200 C-terminal amino acid residues of MexB are sufficient to convert the specificity of AcrB/MexB hybrids to a MexB-like specificity to EtB, OLE, and CIN. In addition, two other regions of AcrB/MexB appear to be essential for transport of various substrates. The N-terminal residues M1-T60 and amino acid residues located in the V612-S/K849 region of ED2 strongly affect the substrate specificities of hybrid transporters.

DISCUSSION

In this study we investigated the structural organization of RND transporters by constructing chimeric proteins containing the regions of two multidrug transporters, AcrB and MexB. These transporters function as the tripartite complexes AcrAB-TolC and MexAB-OprM in *E. coli* and *P. aeruginosa*, respectively, and provide an efficient protection of cells against a multitude of structurally dissimilar antimicrobial agents (8, 10). In agreement with previous studies (19), we found that the functional MexAB-OprM complex can be efficiently expressed in *E. coli* cells, suggesting that no other proteins are required for the functional activity of this transporter. Surprisingly, the outer membrane channel TolC did not complement the func-

tion of OprM, as judged from the drug susceptibility of TolC-containing *E. coli* cells expressing plasmid-borne MexA and MexB proteins. Similarly, OprM when expressed in TolC-deficient *E. coli* cells did not render the cells drug resistant. This finding was somewhat unexpected, since the OprM channel has been shown to be readily interchangeable between *Pseudomonas* MDR pumps and to function efficiently with MexCD and MexXY transporters (1, 5). Furthermore, both MexCD and MexXY proteins can function with the TolC channel when expressed in *E. coli* (11, 19). Thus, despite the interchangeability of TolC and OprM in some transporters, these channels interact with AcrAB and MexAB, respectively, in a highly specific manner.

Besides structural determinants responsible for the specificity of interaction, the RND transporters may possess variations defining differences in substrate specificities. The efficiency and substrate specificities of AcrAB-TolC and MexAB-OprM are difficult to compare when they are expressed in their native environments because of the well-established difference in the permeability properties of the outer membrane of *E. coli* and *P. aeruginosa* (14). However, when both pumps are expressed in *E. coli* their functional differences can be assessed. AcrB and MexB appeared to have very similar substrate specificities; however, they provided different levels of resistance to some compounds (Table 2). In particular, MexAB-OprM provides higher levels of resistance against CIN. On the other hand, AcrAB-TolC is more efficient in pumping out positively charged compounds such as EtB and OLE. Thus, despite the high degree of primary sequence homology between the components, these pumps do not efflux all substrates with equal efficiency.

We have taken advantage of the differences described above between AcrAB-TolC and MexAB-OprM to identify the regions of the RND transporters involved in specific interactions with the other two components of complexes and/or for regions defining substrate specificities. Among 11 hybrid pumps, only AcrA-AcrB612/MexB, AcrA-AcrB849/MexB, and MexA-AcrB60/MexB-OprM were capable of multidrug efflux, demonstrating that the complexes are assembled and that the IM hybrid transporters retained their function. We have verified the complex assembly by using an *in vivo* cross-linking technique (26). Although we cannot detect the tripartite complexes by using this assay, the complexes between the periplasmic components and the IM transporters were found for all functional hybrid pumps (Fig. 4). Thus, the structural determinants defining the specificity of interaction with the periplasmic components of complexes are encoded within the T60-V612 region of the IM transporters. This region spans the most of ED1, TMS 2 through TMS 7, and the 75 amino acid residues of ED2.

Since neither AcrA nor MexA proteins possess transmembrane segments, it is unlikely that TMS 2 through TMS 7 contain structure variations defining the specificity of interaction with MFPs. Thus, amino acid residues of ED1 and ED2 presumably interact with accessory MFPs. Interestingly, the hybrid transporters AcrB589/MexB and AcrB612/MexB differ by only 22 amino acid residues. However, in contrast to AcrB612/MexB, AcrB589/MexB is deficient in the complex assembly. Similarly, AcrB182/MexB, which contains an extra 122 amino acids of AcrB compared to AcrB60/MexB, is a nonfunctional construct. Both these regions are entirely lo-

AcrB <i>E. coli</i> :	590 610
<i>Klebsiella pneumoniae</i> :	VLNEVTHYY LTKEKNN VESVFAV
<i>Enterobacter cloacae</i> :	VLDEMTNYY LTKEKDN VESVFAV
<i>Salmonella enterica</i> :	VLDEMTDY FLTKEKDN VESVFAV
MexB <i>P. aeruginosa</i> :	VVEKVENYY FTEKNN IMSVFS-
	VVDSMREY LLEKES SSVSVFTV

FIG. 5. Alignment of the V590-V612 region of AcrB homologs from bacteria belonging to the γ -*Proteobacteria* division and MexB from *P. aeruginosa*. The conserved residues in AcrB and its close homologs that differ in MexB are in boldface and highlighted.

cated in the moderately conserved regions of ED1 or ED2. Figure 5 shows the alignment of the V590-V612 regions of RND-type multidrug transporters from bacteria belonging to the γ -*Proteobacteria* group. Among 22 amino acid residues of this region, 5 are highly conserved in transporters from enterobacteria and differ in the MexB transporter. The site-specific mutagenesis of these residues is under way to determine their role in drug efflux and complex assembly.

The comparison of functional activities of AcrB612/MexB, AcrB849/MexB, and AcrB60/MexB (Table 2) suggested that the recognition of negatively charged compounds, such as the detergents SDS and CA and NOV, is distinct from other compounds. These substrates are expelled by all three hybrids with an efficiency similar to that in the wild-type transporters AcrB and MexB. However, hybrid pumps differed significantly in the efflux of other tested compounds.

The AcrA-AcrB849/MexB chimeric pump displayed a MexB-like specificity to the substrates CIN, EtB, OLE, and LIN (Table 2). In this hybrid, only the C-terminal 200 amino acid residues are from MexB. Thus, the structural variations responsible for differences in substrate specificities of AcrB and MexB to these substrates appear to localize in the C-terminal domains of RND transporters. Alignment of these regions in AcrB and MexB indicated that these domains are highly conserved (data not shown). The only variable regions in this domain, which might be responsible for the observed differences between AcrB and MexB, are the cytoplasmic loop connecting TMS 10 and TMS 11 and the C-terminal cytoplasmic tail.

On the other hand, AcrA-AcrB849/MexB was similar to the wild-type AcrB in recognition of ERY and PUR, suggesting that other regions of AcrB are also involved in interactions with substrates. AcrA-AcrB612/MexB, containing an additional 235 C-terminal residues from MexB, transported these substrates with an efficiency comparable to that of the wild-type MexB transporter. This result suggested that the residues V612-S/K849 located in the extracytoplasmic domain ED2 play an important role in transport of these substrates. The alignment of the V612-S/K849 regions in AcrB and MexB is shown in Fig. 6. These regions appear to differ substantially in the distribution of charged amino acids, with the obviously high density of negative charges in the MexB protein. On the contrary, AcrB contains an extra number of positively charged amino acids. The reverse distribution of charges in AcrB612/MexB and AcrB849/MexB could explain the inability of AcrB612/MexB to pump out quinolones (CIN and NAL) and other substrates (chloramphenicol [CF], PRO, and tetracycline [TET]). The important role of extracytoplasmic domains in the

	10	20	30	40	50	60
MexB	MSKFFIDRPIFAWVIALVIMLAGGLSILSLPQYPAIAPPAAVQVSYPGASAETVQDT					
	:	:	:	:	:	:
AcrB	MPNFFIDRPIFAWVIAIIMLAGGLAILKLPVAQYPTIAPPAVTISASYPGADAKTVQDT					
	10	20	30	40	50	60
MexB	VTGFNFAGRGQSSGMAFIMLKPWEERPGENSVFELAKRAQMHHFFSKDAMVFAFA					
	:	:	:	:	:	:
AcrB	VNGFGFAGRGQNTGIAFVSLKDWADRPGEENKVEAITMRATRAFSSQIKDAMVFAFN					
	620	630	640	650	660	
MexB	PPSVLELGNATGFDLFLQDQAGVGHEVLLQARNKFLMLAAQNP-ALQVRVPRNGMSDEFPQY					
	:	:	:	:	:	:
AcrB	LPAILVELGTATGFDLFLQDQAGVGHEVLLQARNKFLMLAAQNP-ALQVRVPRNGLETFPQF					
	670	680	690	700	710	720
MexB	KLEIDDEKASALGVSLADINSTVSIAWGSSYVNDFIDRGRVKRVYLQGRPDARMNPDDL					
	:	:	:	:	:	:
AcrB	KIDIDQEKALGVSLADINSTVSIAWGSSYVNDFIDRGRVKKVYVMSEAKYRMLPDDIG					
	730	740	750	760	770	780
MexB	KWYVRNDKGEVMPFNAFATGKWEYGSPLERYNGVPAMEILGEPAPGLSSGDAMAAVEEIVK					
	:	:	:	:	:	:
AcrB	DWYVRAADGQVMPFSAFSSSRWEYGSPLERYNGVLSMEILGQAAPGKSTGEAMELMEQLAS					
	790	800	810	820	830	840

FIG. 6. Alignment of AcrB and MexB sequences in functionally important regions. Amino acid residues that differ in these two transporters are highlighted.

drug specificities of RND transporters has also been demonstrated in domain replacement studies of the AcrB and AcrD transporters (3).

It is noteworthy that MexA-AcrB60/MexB-OprM appears to be functionally similar to AcrB612/MexB. This similarity is obvious in the specificities to NAL, PUR, CF, PRO, tetracycline, and chlortetracycline (CTET). The AcrB60/MexB construct contains only 60 N-terminal amino acid residues from AcrB. Since structural variations in the M1-T60 region of AcrB/MexB profoundly affect the specificity toward such a broad spectrum of substrates, this result suggests that all these substrates might interact with the same drug-binding region of AcrB/MexB. Perhaps this drug-binding site comprises amino acid residues from both the N- and C-terminal domains of RND transporters. Indeed, the N-terminal residues of all our constructs originate from the AcrB transporter. The shortest region of AcrB required for the function with AcrA was found to be 612 N-terminal residues (AcrB612/MexB). Still, similar to MexA-AcrB60/MexB-OprM, the AcrA-AcrB612/MexB hybrid pump appears to be defective in transport of several substrates. On the other hand, cells expressing AcrA-AcrB849/MexB were resistant to all tested compounds. Thus, amino acid residues from both the N- and C-terminal domains could contribute to the substrate specificity of RND transporters. However, we cannot exclude the possibility that the defects in transport of particular substrates are caused by partial misfolding of chimeric transporters.

ACKNOWLEDGMENTS

This work was supported by a Scientist Development grant from the American Heart Association to H.I.Z.

This study was begun in the lab of H. Nikaido. We are indebted to Hiroshi Nikaido for his continuing support and critical reading of the manuscript. We thank K. Poole for providing polyclonal anti-OprM antibody.

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