β-Lactam Selectivity of Multidrug Transporters AcrB and AcrD Resides in the Proximal Binding Pocket*

Received for publication, January 8, 2014, and in revised form, February 19, 2014 Published, JBC Papers in Press, February 20, 2014, DOI 10.1074/jbc.M114.547794

Naoki Kobayashi*†‡, Norihisa Tamura*‡, Hendrik W. van Veen∥, Akihito Yamaguchi*‡, and Satoshi Murakami*‡†¶

From the †Department of Cell Membrane Biology, Institute of Scientific and Industrial Research, Osaka University, Ibaraki, Osaka 567-0047, Japan, the ‡Faculty of Pharmaceutical Science, Teikyo Heisei University, Nakano, Nakano-ku, Tokyo 164-8530, Japan, the §Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565-0871, Japan, the ¶Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1PD, United Kingdom, and the *Department of Life Science, Tokyo Institute of Technology, Nagatsuta-cho, Midori-ku, Yokohama 226-8501, Japan.

Background: Bacterial multidrug transporters AcrB and AcrD export β-lactam antibiotics.

Results: Charged residues in the proximal binding pocket play a crucial role in β-lactam selectivity.

Conclusion: Proximal pocket acts as a substrate selection filter at the site of entry into the substrate translocation pathway.

Significance: Our understanding of molecular mechanisms of antibiotic recognition is essential for overcoming drug resistance.

β-Lactams are mainstream antibiotics that are indicated for the prophylaxis and treatment of bacterial infections. The AcrA-AcrD-TolC multidrug efflux system confers much stronger resistance on Escherichia coli to clinically relevant anionic β-lactam antibiotics than the homologous AcrA-AcrB-TolC system. Using an extensive combination of chimeric analysis and site-directed mutagenesis, we searched for residues that determine the difference in β-lactam specificity between AcrB and AcrD. We identified three crucial residues at the “proximal” (or access) substrate binding pocket. The simultaneous replacement of these residues in AcrB by those in AcrD (Q569R, I626R, and E673G) transferred the β-lactam specificity of AcrD to AcrB. Our findings indicate for the first time that the difference in β-lactam specificity between AcrB and AcrD relates to interactions of the antibiotic with residues in the proximal binding pocket.

The emergence of multidrug resistance among microbial pathogens affects our ability to treat infectious diseases during chemotherapeutic treatments (1). One important mechanism underlying multidrug resistance is based on active efflux of structurally unrelated drugs from the cell (2). Among bacterial multidrug efflux systems, members of the resistance-nodulation-cell division (RND) family of secondary-active transporters are expressed in Gram-negative bacteria (3). AcrB is a constitutively expressed RND-type transporter in Escherichia coli that determines the intrinsic drug resistance of this organism to a wide range of anionic, cationic, zwitterionic, and neutral antibiotics and toxic compounds. AcrB acts in conjunction with a periplasmic membrane-fusion protein AcrA (3, 4), and an outer membrane porin TolC to form the functional tripartite exporter (5). The tripartite structure enables proton-motive force-dependent export of substrates directly from the cytoplasm and periplasm to the extracellular environment.

AcrD from E. coli is a functional and structural homolog of AcrB that also cooperates with AcrA (5) and TolC (6). However, the AcrAD-TolC export system shows an interesting difference in substrate specificity compared with the AcrAB-TolC system. Although AcrAB-TolC exhibits the broadest substrate specificity among drug efflux systems in E. coli, this efflux pump confers no or weak resistance on cells against anionic β-lactams, such as aztreonam, carbencillin, and sulbencillin (Fig. 1) (6). On the other hand, the AcrAD-TolC system recognizes a more limited range of substrates compared with AcrAB-TolC, but confers high resistance to the anionic β-lactams, and no or weak resistance against typical AcrB substrates, such as novobiocin, erythromycin, acriflavine, crystal violet, ethidium bromide, rhodamine 6G, tetraphenylphosphonium, and benzalkonium (6, 7). For β-lactam antibiotics, it is well known that this resistance is a multifactorial parameter that is dependent on relative drug toxicity and on the kinetics of three processes, active drug efflux, drug entry across the outer membrane, and binding of the antibiotic to its periplasmic target (8, 9). Interestingly, when AcrB and AcrD are expressed separately in the same recipient AcrA and TolC-containing E. coli KAM3 strain, AcrAD-TolC-containing cells show enhanced resistance against specific anionic β-lactam antibiotics compared with AcrAB-TolC-containing cells (6). This difference in β-lactam resistance is therefore most likely dictated by the non-identical AcrB and AcrD proteins (6).

Elkins and Nikaido (10) exchanged the two large periplasmic loops of AcrB and AcrD in E. coli with each other and found that the substrate recognition site is located in these loops. Sim-
are exported by a three-step functionally rotating mechanism. The structures indicate that drugs also include structures of AcrB bound to its substrates, different conformation (16–18). The studies by Murakami et al. (16) showed binding of minocycline or doxorubicin to this site at higher resolution. Both groups suggested that the transport of substrates is a stepwise process of initial substrate binding in the proximal site followed by substrate movement to the distal site during the functional rotation. Smaller substrates might be able to bind to the distal site directly. Together these two substrate-binding sites enable the broad substrate recognition by AcrAB-TolC.

In 2011 and 2012, two groups discovered a second substrate-binding site in AcrB in the access protomer (20, 21), in addition to the earlier known substrate-binding site in the binding protomer (16). Both groups suggested that the transport of substrates is a stepwise process of initial substrate binding in the proximal site followed by substrate movement to the distal site during the functional rotation. Smaller substrates might be able to bind to the distal site directly. Together these two substrate-binding sites enable the broad substrate recognition by AcrAB-TolC.

As the Phe cluster in the distal binding pocket of AcrB is partially conserved in AcrD (Phe178, Phe610, and Phe628 of AcrB correspond to Trp178, Phe609, and Phe627, respectively, in AcrD), the difference in substrate recognition between AcrB and AcrD cannot be adequately explained if only interactions between the substrate and aromatic residues are taken into account. We hypothesize that the proximal binding site and other regions in the predicted substrate translocation pathway between the vestibule and the distal substrate binding pocket might participate in substrate recognition. To investigate this hypothesis, we replaced the non-conserved regions in this pathway of crystallized AcrB with the corresponding regions of AcrD (Figs. 2-4). In subsequent site-directed mutagenesis studies we succeeded in generating gain-of-function mutant AcrB proteins which, similar to AcrD but unlike wild-type AcrB, show relatively high resistance to anionic \( \beta \)-lactams. Thus, residues in these RND transporters were identified that are important for the resistance to anionic \( \beta \)-lactams.

**EXPERIMENTAL PROCEDURES**

*Bacterial Strains and Plasmids—* E. coli strains JM109 (22), C7236 (23), and TG1 (24) were used for DNA manipulation, site-directed mutagenesis, and investigation of the phenotypes of the mutants, respectively. Strain KAM3 is an acrB gene-deletion derivative of E. coli TG1 (25), and the acrD gene in KAM3 was deleted to construct KAM3ΔacrD (26). The low copy vectors pTH18cr and pTH19cr (27) were kind gifts from the staff at the cloning vector collection at the National Institute of Genetics (Japan), and were used to express AcrAB and AcrD in which conformational changes in three substrate binding pockets affect accessibility and binding affinity for substrates. In one of the protomers (access protomer), a pocket opens in the periplasmic domain allowing substrate entry. In the second protomer (extrusion protomer), the pocket compresses thus ejecting pre-bound substrate through the top funnel, toward the TolC channel. In the third protomer (binding protomer), the substrate is bound in the pocket by Phe residues that play a role in substrate binding in a multisite binding fashion. Thus, the crystal structures showed the entire route through AcrB for transported substrates, from the vestibule via the substrate binding pocket to the top funnel (19).

In 2002, Murakami et al. determined the first crystal structure of AcrB (14). The crystal structure showed that AcrB exists as a trimer and that each AcrB protomer is composed of three domains: the transmembrane domain, the porter domain (pore domain), and the TolC docking domain (14). Each AcrB protomer has a vestibule open to the outer surface of the plasma membrane from where substrates can potentially be taken up from the periplasm or the outer leaflet of the plasma membrane. These substrates are then extruded to TolC via the top funnel (15). In 2006 and 2007, a number of groups reported asymmetric structures of AcrB in which each protomer has a different conformation (16–18). The studies by Murakami et al. (16) also included structures of AcrB bound to its substrates, minocycline or doxorubicin. The structures indicate that drugs are exported by a three-step functionally rotating mechanism.
KAM3ΔacrD, respectively. Plasmid pACBHLR, a derivative of pUC118 that carries the acrR, acrA, and His-tagged Cys-less acrB genes was described earlier (28). Plasmids pTrcHacrD (7) (a derivative of pTrc99A that carries the His-tagged acrD gene) and pHSGacrD (6) (a derivative of pHSG399 that contains acrD gene) were prepared previously. For use as templates for site-directed mutagenesis, the DNA fragment encoding the N-terminal half of cysteine-less AcrB was subcloned into pUC118 and named pACBRN (28).

Construction of Plasmids Carrying acrRAB and acrD Genes—For correct sequence alignment of AcrB and AcrD, the acrB gene of pACBHLR encoding Cys-less AcrB was first changed to its native form by re-introducing native Cys residues. In addition, an ApaI site was introduced at the 3' end of the acrA gene of pACBHLR, to construct pACBHR2. Then, pTHacrAB-His was constructed by replacement of the Sse8387I-SacI fragment of pTH18cr with the Sse8387I-SacI fragment of pACBHR2 (Fig. 5). For construction of an AcrD-expressing plasmid the HpaI-SalI fragment of pACBHR2 was replaced with the HpaI-SalI fragment of pTrcHacrD, yielding pACBDHR2. Subsequently, the HpaI-SalI fragment of pHSGacrD was replaced with the HpaI-SalI fragment of pACBDHR2 to construct pHSGacrD-His. The HindIII-SalI fragment of pTH19cr was then replaced with the HindIII-SalI fragment of pHSGacrD-His, giving pTHacrD-His (Fig. 5).

Construction of AcrB/AcrD Chimeras and Mutant AcrB Proteins—To introduce AcrD fragments and residues into the C-terminal half of AcrB, pACBHR2. The replacement of several regions (81–96, 121–144, 291–326, and 822–858) of AcrB was performed by PCR-based mutagenesis (29). In this method a megaprimer was generated in which the acrD gene was amplified with two conventionally designed oligonucleotide primers. Each individual primer was elongated at its 5' end, with additional nucleotides corresponding to the DNA sequence found upstream and downstream, respectively, from the replaced region within the acrD gene. Next, the amplified PCR fragment, i.e. megaprimer, was annealed to the methylated template plasmid and each
strand was elongated. Plasmids pACBRN and pACBHRC provided templates in the replacement of the regions in the N-terminal half of AcrB (81–96, 121–144, and 291–326) and the C-terminal half of AcrB (822–858, respectively. After elongation, the newly created DNA carrying the acrD sequence was unmethylated. Upon DpnI digestion, the methylated template DNA was destroyed. Thus, upon transformation, only the mutated plasmid DNA was recovered from bacteria.

The replacement of the regions (37–48, 175–178, 569–575, 613–617, 626–668, 672–673, and 676–687) and the residues (569, 626, 630, 662, and 673) of AcrB were performed by the method of Kunkel et al. (23) or by the use of a

FIGURE 3. Replaced regions in AcrB. A, side view of the ribbon representation. For a clear view, one protomer is removed. Square area is enlarged in panel C. B, top view of the porter domains from the periplasm. Colored sequences indicate the regions replaced for constructing the chimera designated as “N/H9252–Co/H9252.” The replaced regions are colored as shown in Fig. 2B. Ball-and-stick representation indicates the Phe cluster. The minocycline molecule bound in the distal binding pocket is shown in CPK (Corey-Pauling-Koltun) representation with the position of the C, N, and O atoms indicated by yellow, blue, and red balls, respectively. C, stereo view of the regions in AcrB important for recognition of negatively charged β-lactams. Square region in panel A is expanded and rotated 90 degrees around the 3-fold axis at the center of the trimer for a better view. Substrate translocation pathway (in yellow) including proximal and distal substrate binding pockets, and direction of substrate transport (dashed arrow) are indicated. Green-colored β-sheet sequences indicate Cβ1, -3, -4, and LP regions that determine the difference in substrate specificity between AcrB and AcrD. The important residues (Gln569, Ile626, and Glu673) for recognition of negatively charged β-lactams are shown in CPK representation, and are present in the Cβ1, -3, -4, and LP regions. The space colored in yellow is the solvent-accessible inner cavity of the AcrB molecule, and was created with the program POCASA (42). To put the size of the substrate translocation pathway in AcrB in perspective, a stick model of the β-lactam antibiotic carbenicillin is included at the same scale. The main figure was generated using PyMOL (www.pymol.org/).
QuikChange site-directed mutagenesis kit (Stratagene) with oligonucleotides encoding the regions and residues of AcrD. Mutations were first detected by restriction enzyme analysis, and then verified by DNA sequencing. After verification of the mutations, adequate restriction fragments of the subcloning vectors, pACBRN and pACBHRC, were introduced into the expression vector, pTHacrAB-His, by means of corresponding fragment exchange.

**Expression of AcrB/AcrD Chimeras and AcrB Mutants**—E. coli KAM3ΔacrD was used for expression of the AcrB/AcrD chimeras and AcrB mutants. All proteins have a six-histidine tag at the carboxyl-terminal. Membrane fraction was prepared by ultracentrifugation as described previously (28) and subjected to Western blotting with a mouse anti-polyhistidine antibody (Sigma).

**Drug Resistance Determination**—The minimum inhibitory concentrations (MICs) of drugs and toxic compounds were determined as the concentrations that greatly prevented bacterial growth on YT-agar (0.8% tryptone, 0.5% yeast extract, and 0.5% NaCl) plates with sequential 2-fold dilutions, as described previously (7). Relative MIC values in Fig. 6 were calculated from the MIC values in Table 1 using the following equation.

$$\text{Relative MIC} = \log_2 \left( \frac{\text{MIC cells expressing chimeras or AcrB mutants}}{\text{MIC KAM3ΔacrD control cells}} \right)$$

(Eq. 1)

Negative values for drug susceptible mutants were not plotted in Fig. 6.

**RESULTS**

**Construction and Expression of AcrB/AcrD Chimeras**—To test which sections of the substrate translocation pathway determine the difference in the substrate specificity between AcrB and AcrD, we replaced the 12 regions facing the predicted substrate translocation pathway of AcrB, between vestibule and distal substrate binding pocket, with the corresponding regions of AcrD, except for the regions almost composed of identical residues between AcrB and AcrD (Figs. 2 and 3). Expression of these chimeric proteins in the cytoplasmic membrane of E. coli was confirmed by Western blotting using an anti-histidine tag antibody (Fig. 4A).

**Alteration of Drug Resistance of Cells Expressing AcrB/AcrD Chimeras**—First, we simultaneously replaced all 12 regions, Nβ1-Co8 of AcrB (Fig. 2), with the corresponding regions of AcrD, and investigated the substrate specificity by determining MICs for a variety of antibiotics, antiseptics, detergents, and...
that the simultaneous replacement of Nβ1, Nβ3, Nβ4, Nβ6, and Nβ8 impairs the functionality of the chimera. On the other hand, Cβ1-Cα8, in which only regions in the carboxyl-terminal half of AcrB were replaced with those of AcdD, showed higher resistance against carbenicillin and sulbenicillin, and lower resistance against many other compounds than AcrB (Table 1). This pattern of resistance was close to that of AcdD itself, suggesting that the Cβ1-Cα8 regions include crucial sections that determine the difference in substrate specificity between AcrB and AcdD. Then, a new set of chimeras was prepared in which regions between Cβ1 and Cα8 were combined (Table 1, Fig. 2).

A combination of four regions, Cβ1, -3, -4, and LP, gave a resistance profile close to that of AcdD (Figs. 2 and 6, Table 1), that is, high resistance against AcrD-specific drugs (the anionic β-lactams; aztreonam, carbenicillin, and sulbenicillin) and low or no resistance against AcrB-specific drugs (such as erythromycin, acriflavine, ethidium bromide, and benzalkonium). This mutant was equally well expressed as wild-type AcdD and AcrB (Fig. 4).

Role of Charged Residues in Substrate Specificity—As described above, AcrD exports the negatively charged β-lactams more efficiently than AcrB. This fact encouraged us to focus on the charged residues located in the Cβ1, -3, -4, and LP regions. There are five residues that vary in charge between AcrB and AcdD in Cβ1, -3, -4, and LP: Gln569, Ile626, Ser630, Met662, and Glu673 in AcrB correspond to Arg568, Arg625, Arg629, Arg661, and Gly672 in AcdD, respectively (Fig. 2A). We constructed mutant AcrB proteins in which these residues were replaced by the corresponding residues of AcdD (Q569R, I626R, S630R, M662R, and E673G). All mutants were expressed in the plasma membrane of E. coli (Fig. 4B). As indicated in Table 1, the simultaneous replacement of these five residues resulted in greatly increased MIC values for negatively charged β-lactams (aztreonam, carbenicillin, and sulbenicillin). Surprisingly, further replacement studies including all possible combinations of these residues demonstrated that simultaneous replacement of three residues (Q569R, I626R, and E673G) was sufficient for high resistance against the negatively charged β-lactams and cefamandole (FAM) than wild-type AcrB. The C7 side chain of cefamandole is similar to the C6 side chain of carbenicillin and sulbenicillin (Fig. 1).

Among the single replacements of Q569R, I626R, and E673G in AcrB, only the I626R substitution significantly increased resistance to negatively charged β-lactams (Table 1), suggesting
| Strain and plasmid | KAM3acrD | pTHacrAB-His (native AcrAB) | AcrB/AcrD chimera | AcrB mutant |
|---------------------|-----------|----------------------------|-------------------|-------------|
|                     | MIC (μg/ml)* | MIC (μg/ml)* | MIC (μg/ml)* |MIC (μg/ml)* |
| ATM                 | 1         | 0.5                  | 1                | 0.5         |
| CAR                 | 4         | 4                   | 2                | 2           |
| SBPC                | 204       | 1024                | 256              | 8           |
| OXA                 | 8         | 128                 | 32               | 8           |
| MIPC                | 8         | 128                 | 32               | 8           |
| NAF                 | 8         | 128                 | 32               | 8           |
| FAM                 | 8         | 128                 | 32               | 8           |
| NOV                 | 8         | 128                 | 32               | 8           |
| ERY                 | 8         | 128                 | 32               | 8           |
| TET                 | 8         | 128                 | 32               | 8           |
| MIN                 | 8         | 128                 | 32               | 8           |
| NAL                 | 8         | 128                 | 32               | 8           |
| NOR                 | 8         | 128                 | 32               | 8           |
| ENX                 | 8         | 128                 | 32               | 8           |
| TMP                 | 8         | 128                 | 32               | 8           |
| TRI                 | 8         | 128                 | 32               | 8           |
| ACR                 | 1         | 1                   | 1                | 1           |
| CRV                 | 1         | 1                   | 1                | 1           |
| ETR                 | 1         | 1                   | 1                | 1           |
| B6G                 | 1         | 1                   | 1                | 1           |
| TPP                 | 1         | 1                   | 1                | 1           |
| BZK                 | 1         | 1                   | 1                | 1           |
| SDS                 | 1         | 1                   | 1                | 1           |
| DOC                 | 1         | 1                   | 1                | 1           |

*Relative MICs were calculated as ratios between the MICs for KAM3acrD and KAM3acrD carrying the plasmid. Abbreviations for drugs: ATM, aztreonam; CAR, carbenicillin; SBPC, sulbenicillin; OXA, oxacillin; MIPC, cloxacinil; NAF, nafillin; FAM, cefamandole; NOV, novobiocin; ERY, erythromycin; TET, tetracycline; MIN, minocycline; NAL, nalidixic acid; NOR, norfloxacinn; ENX, enoxacin; TMP, trimethoprim; TRI, triclosan; ACR, acriflavine; CRV, crystal violet; ETR, ethidium bromide; B6G, rhodamine 6G; TPP, tetrathenylphosphonium; BZK, benzalkonium; DOC, deoxycholate.

**Q569R, I626R, S630R, M662R, and E673G are located in C1, C3, C3, C4, and LP of AcrB, respectively.**
that I626R (corresponding to Arg$^{625}$ in AcrD) is most important for recognition of the negatively charged β-lactams in AcrB. I626R is located in the proximity of the distal substrate binding pocket containing Phe residues that were shown to interact with drugs by hydrophobic or aromatic-aromatic interactions (16). Single mutations at Q569R and E673G in AcrB confer no or somewhat decreased resistance against the negatively charged β-lactams, but these substitutions increased the resistance against these β-lactams when combined with mutations at other positions (Table 1). These results suggest that the introduction of Arg at position 569 and removal of Glu at position 673 in AcrB (corresponding to Arg$^{568}$ and Gly$^{672}$ in AcrD) contribute to its recognition of the negatively charged β-lactams.

AcrD confers no or very low resistance against positively charged compounds including ethidium$^+$, rhodamine-6G, and the tetracyphenylphosphonium ion (TPP$^+$), whereas the transport of these substrates by AcrB is significant (Table 1). Consistent with this difference, we found that the replacement of AcrB side chains of positions 569, 626, and 673 by those in AcrD decreased, but did not completely abolish, the AcrB-mediated resistance in cells to the positively charged compounds (Fig. 6, Table 1).

Taken together, via the systematic replacement of regions and residues in AcrB by corresponding regions and residues in AcrD (Fig. 3C), we identified three residues in AcrB (Gln$^{569}$, Ile$^{626}$, and Glu$^{673}$), which when replaced by those in AcrD (Arg$^{568}$, Arg$^{625}$, and Gly$^{672}$, respectively) shift the substrate specificity of AcrB to the specificity of AcrD.

**DISCUSSION**

In the co-crystal structures of AcrB and substrates, each of the three protomers of the AcrB-substrate complex has a different conformation. Only one protomer of the complex has a substrate bound in the Phe-rich distal binding pocket (Fig. 3). The crystal structures of AcrB also revealed that each protomer forms a channel between vestibule and the distal binding pocket. This channel was predicted to be a substrate-translocation pathway. However, it is not clear whether substrate specificities of AcrB and its homologs are determined by the Phe-rich distal binding pocket only, or also by regions in the substrate-translocation pathway leading to the distal pocket. AcrD is an AcrB homolog that transports negatively charged β-lactams more effectively than AcrB. In our chimera approach, we show that substitution of regions Cβ1, -3, -4, and LP in AcrB by those in AcrD transfers the ligand specificity of AcrD to AcrB (Table 1, Figs. 3C and 6). These regions are mainly located in the space between the β-sheets in the uptake channel near the proximal binding pocket (Fig. 3C). In the distal substrate binding pocket of AcrB, six Phe residues are presented. It was suggested that utilization of different combinations of Phe residues provide a basis for the broad substrate recognition in the distal binding pocket. Indeed, when these Phe residues were substituted by Ala, several mutants changed their substrate specificity (30). However, these aromatic residues were not altered in the AcrB chimera containing Cβ1, -3, -4, and LP elements from AcrD, suggesting that the aromatic residues in the distal substrate binding pocket are not determinants of the specificity for anionic β-lactams. Instead, we found that simultaneous replacement of Q569R, I626R, and E673G in the Cβ1, Cβ3, and LP regions of AcrB, respectively, by the corresponding residues in AcrD (Arg, Arg, and Gly, respectively) is critical for recognition by AcrB of negatively charged β-lactam substrates of AcrD; aztreonam, carbenicillin, and sulbenicillin. Cβ4 is less critical for recognition of negatively charged β-lactams. However, substitution of Cβ4 reduces the recognition of AcrB-specific substrates by both wild-type AcrB and Cβ1, -3, and LP (Table 1, Figs. 3C and 6). Hence, selectivity for these β-lactams might already be determined by residues in the uptake channel, after which aromatic residues in the binding pockets of AcrB and AcrD would contribute to the binding of the preselected substrates (Fig. 3C and 7). In addition, this region might be used as the binding site for comparably large substrates, like rifampicin and erythromycin (see below).

In previous work (31–33), the importance of the charged residues in substrate recognition was demonstrated for multidrug transcription regulator proteins BmrR and QacR. Glu$^{134}$ of BmrR has an electrostatic interaction with a positively charged TPP$^+$ ion in the BmrR-TPP$^+$ crystal structure (31), which is disrupted in an E134A substitution mutant (31). Among the three residues in AcrD that determine recognition of negatively charged β-lactams, the Arg at position 626 is most important, which is most likely due to its position close to the distal binding pocket.

Some residues important for substrate specificity of RND-type multidrug transporters that were identified in previous studies, co-localize in the predicted substrate translocation pathway identified in this investigation (Fig. 3) (13, 34). In their work on the AcrB-homolog MexD from *P. aeruginosa*, Mao et al. (13) reported spontaneous mutations Q34K, E89K, A292V, P328L, F608S, and N673K, which increased individually the MexD-dependent resistance against aztreonam and carbenicillin. These residues correspond to Gln$^{34}$, Gln$^{89}$, Gly$^{290}$, Pro$^{326}$, Phe$^{610}$, and Thr$^{676}$ in AcrB. The residues Gln$^{34}$ and Thr$^{676}$ that were replaced by Lys in the study by Mao et al. (13) are located in the proximal binding pocket and the vicinity of the uptake channel, and are close to Gln$^{569}$, Ile$^{626}$, and Glu$^{673}$ residues identified in our study (Fig. 3C). However, the positively charged amino acids at positions 34, 89, and 676 in MexD mutants were not observed in wild-type MexD, or in related transporters MexB and AcrD, which do export aztreonam and carbenicillin. Furthermore, Gln$^{34}$ is conserved in AcrB homologs with different substrate specificities (13). Therefore, residues at positions 34, 89, and 676 might not directly contribute to the recognition of charged substrates in native RND transporters.

Recently, Nakashima et al. (20) reported the crystal structures of AcrB bound to the high-molecular mass drugs, rifampicin and erythromycin. These drugs bind to the proximal binding pocket in the access monomer of AcrB. The structures suggest that the high-molecular mass drugs initially bind to the proximal pocket specifically, and are then forced into the distal pocket. In this study, we show that the side chains in AcrB at positions 569 and 673 in the proximal pocket are important for selection of the negatively charged β-lactams. Glu$^{673}$ is located in the entrance of the uptake channel of AcrB that leads to the
The distal substrate-binding chamber (Fig. 7), where this residue might interfere with the entry of the negatively charged β-lactams (Fig. 7). Replacement of Glu673 by Gly (as present in AcrD) overcomes this interference. Because Gln569 is also located in this entrance in AcrB, substitution of Gln569 by Arg (as in AcrD) would provide a positive charge around the entrance of the channel and support entry of anionic β-lactam antibiotics. Indeed, for another small substrate, minocycline, binding was shown in the distal binding pocket rather than the proximal pocket (16), and replacement of residues in the distal pocket was found to affect AcrAB-TolC-mediated minocycline resistance. A difference in the utilization of the two substrate binding pockets by different substrates would expand the diversity of substrates that can be transported by this type of tripartite pump.

Our data on the importance of I626R for the recognition of anionic β-lactams by AcrB might show analogy to observations for the human ATP-binding cassette multidrug transporter ABCG2 (also referred to as the breast cancer resistance pro-
tein), which is capable of transporting chemotherapeutic drugs from cancer cells (35, 36). Residue 482 in the intracellular loop of TM3 is known to affect substrate specificity. Wild-type ABCG2, with an Arg at position 482, mediates efficient export of anionic methotrexate, but not of cationic rhodamine 123 and doxorubicin. In contrast, ABCG2 with a Gly or Thr at position 482 mediates export of rhodamine 123 and doxorubicin, but not methotrexate (37–39). The recent observations on the determining role of residue 482 in drug transport by ABCG2 but not in drug binding (40, 41) might point to the presence of residue 482 in a substrate selection filter that controls access to ligand binding surfaces. However, in the interpretation of our findings for AcrB it is also possible that β-lactam antibiotics bind to the proximal binding pocket in a similar fashion as larger substrates, such as erythromycin and rifampicin. This would make the transport reaction by AcrB a two-step process with sequential binding to proximal and distal binding pockets for all substrates.

β-Lactams are mainstream antibiotics that are indicated for the prophylaxis and treatment of infections by bacterial pathogens. We identified regions in AcrB and AcrD that determine the difference in β-lactam specificity between these homologous multidrug transporters. In particular, when AcrD and AcrB are compared, side chains at three positions play an important role in the difference in recognition of negatively charged β-lactams. Upon the introduction of AcrD residues Arg<sup>568</sup>, Arg<sup>625</sup>, and Gly<sup>672</sup> at corresponding positions in AcrB, the ability to interact with these β-lactams is transferred from AcrD to AcrB. In view of the location of these positions in current crystal structures of AcrB, our results suggest for the first time that the difference in β-lactam selectivity between AcrB and AcrD resides in the proximal binding pocket.

Acknowledgments—E. coli KAM3 was kindly provided by Tomofusa Tsuchiya and Yuji Morita (Okayama University, Okayama, Japan).

REFERENCES
1. Livermore, D. M. (2002) Multiple mechanisms of antimicrobial resistance in <i>Pseudomonas aeruginosa</i>: our worst nightmare? <i> Clin. Infect. Dis.</i> 34, 634–640
2. Nikaido, H. (1998) Antibiotic resistance caused by Gram-negative multidrug efflux pumps. <i>Clin. Infect. Dis.</i> 27, S32–41
3. Sulavik, M. C., Houseweart, C., Cramer, C., Jiwani, N., Murgolo, N., Greene, J., DiDomincio, B., Shaw, K. J., Miller, G. H., Hare, R., and Shinmer, G. (2001) Antibiotic susceptibility profiles of <i>Escherichia coli</i> strains lacking multidrug efflux pump genes. <i>Antimicrob. Agents Chemother.</i> 45, 1126–1136
4. Dinh, T., Paulsen, I. T., and Saier, M. H., Jr. (1994) A family of extracytoplasmic proteins that allow transport of large molecules across the outer membranes of Gram-negative bacteria. <i>J. Bacteriol.</i> 176, 3825–3831
5. Paulsen, I. T., Park, J. H., Choi, P. S., and Saier, M. H., Jr. (1997) A family of gram-negative bacterial outer membrane factors that function in the export of proteins, carbohydrates, drugs and heavy metals from Gram-negative bacteria. <i>FEMS Microbiol. Lett.</i> 156, 1–8
6. Nishino, K., Yamada, J., Hirakawa, H., Hirata, T., and Yamaguchi, A. (2003) Roles of TolC-dependent multidrug transporters of <i>Escherichia coli</i> in resistance to β-lactams. <i>Antimicrob. Agents Chemother.</i> 47, 3030–3033
7. Nishino, K., and Yamaguchi, A. (2001) Analysis of a complete library of putative drug transporter genes in <i>Escherichia coli</i>. <i>J. Bacteriol.</i> 183, 5803–5812
8. Lim, S. P., and Nikaido, H. (2010) Kinetic parameters of efflux of pencillins by the multidrug efflux transporter AcrAB-TolC of <i>Escherichia coli</i>. <i>Antimicrob. Agents Chemother.</i> 54, 1800–1806
9. Nagano, K., and Nikaido, H. (2009) Kinetic behavior of the major multidrug efflux pump AcrB of <i>Escherichia coli</i>. <i>Proc. Natl. Acad. Sci. U.S.A.</i> 106, 5854–5858
10. Elkins, C. A., and Nikaido, H. (2002) Substrate specificity of the RND-type multidrug efflux pumps AcrB and AcrD of <i>Escherichia coli</i> is determined predominantly by two large periplasmic loops. <i>J. Bacteriol.</i> 184, 6490–6498
11. Eda, S., Maseda, H., and Nakae, T. (2003) An elegant means of self-protection in Gram-negative bacteria by recognizing and extruding xenobiotics from the periplasmic space. <i>J. Biol. Chem.</i> 278, 2085–2088
12. Tikhonova, E. B., Wang, Q., and Zgurskaya, H. I. (2002) Chimeric analysis of the multicomponent multidrug efflux transporters from Gram-negative bacteria. <i>J. Bacteriol.</i> 184, 6499–6507
13. Mao, W., Warren, M. S., Black, D. S., Satou, T., Murata, T., Nishino, T., Gotoh, N., and Lomovskaya, O. (2002) On the mechanism of substrate specificity by resistance nodulation division (RND)-type multidrug resistance pumps: the large periplasmic loops of MexD from <i>Pseudomonas aeruginosa</i> are involved in substrate recognition. <i>Mol. Microbiol.</i> 46, 889–901
14. Murakami, S., Nakashima, R., Yamashita, E., and Yamaguchi, A. (2002) Crystal structure of bacterial multidrug efflux transporter AcrB. <i>Nature</i> 419, 587–593
15. Murakami, S., and Yamaguchi, A. (2003) Multidrug-exporting secondary transporters. <i>Curr. Opin. Struct. Biol.</i> 13, 443–452
16. Murakami, S., Nakashima, R., Yamashita, E., Matsumoto, T., and Yamaguchi, A. (2006) Crystal structures of a multidrug transporter reveal a functionally rotating mechanism. <i>Nature</i> 443, 173–179
17. Seeger, M. A., Schiefer, A., Eicher, T., Verrey, F., Diederichs, K., and Poss, K. M. (2006) Structural asymmetry of AcrB trimer suggests a peristaltic pump mechanism. <i>Science</i> 313, 1295–1298
18. Sennhauser, G., Amstutz, P., Bränd, C., Storchenger, O., and Grütter, M. G. (2007) Drug export pathway of multidrug exporter AcrB revealed by DARPin inhibitors. <i>PLoS Biol.</i> 5, e7
19. Murakami, S. (2008) Multidrug efflux transporter, AcrB—the pumping mechanism. <i>Curr. Opin. Struct. Biol.</i> 18, 459–465
20. Nakashima, R., Sakurai, K., Yamaski, S., Nishino, K., and Yamaguchi, A. (2011) Structures of the multidrug export AcrB reveal a proximal multisite drug-binding pocket. <i>Nature</i> 480, 565–569
21. Eicher, T., Cha, H. I., Seeger, M. A., Brandstätter, L., Del-Delik, J., Bohnert, J. A., Kern, W. V., Verrey, F., Grütter, M. G., Diederichs, K., and Poss, K. M. (2012) Transport of drugs by the multidrug transporter AcrB involves an access and a deep binding pocket that are separated by a switch-loop. <i>Proc. Natl. Acad. Sci. U.S.A.</i> 109, 5687–5692
22. Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. <i>Gene</i> 33, 103–119
23. Kunkel, T. A. (1985) Rapid and efficient site-specific mutagenesis without phenotypic selection. <i>Proc. Natl. Acad. Sci. U.S.A.</i> 82, 488–492
24. Taylor, J. W., Ott, J., and Eckstein, F. (1985) The rapid generation of oligonucleotide-directed mutations at high frequency using phosphorothioate-modified DNA. <i>Nucleic Acids Res.</i> 13, 8765–8785
25. Morita, Y., Kodama, K., Shiota, S., Mine, T., Kataoka, A., Mizushima, T., and Tsujiya, T. (1998) NovM, a putative multidrug efflux protein, of <i>Vibrio parahaemolyticus</i> and its homolog in <i>Escherichia coli</i>. Antimicrob. Agents Chemother. 42, 1778–1782
26. Hirakawa, H., Nishino, K., Hirata, T., and Yamaguchi, A. (2003) Comprehensive studies of drug resistance mediated by overexpression of response regulators of two-component signal transduction systems in <i>Escherichia coli</i>. <i>J. Bacteriol.</i> 185, 1851–1856
27. Hashimoto-Gotoh, T., Yamaguchi, M., Yasojima, K., Tsuimura, A., Wakahayashi, Y., and Watanabe, Y. (2000) A set of temperature sensitive-replication/-segregation and temperature resistant plasmid vectors with different copy numbers and in an isogenic background (chloramphenicol, kanamycin, lacZ, repA, par, polA). <i>Gene</i> 241, 185–191
28. Murakami, S., Tamura, N., Saito, A., Hirata, T., and Yamaguchi, A. (2004)
Extramembrane central pore of multidrug exporter AcrB in *Escherichia coli* plays an important role in drug transport. *J. Biol. Chem.* 279, 3743–3748

29. Geiser, M., Cébe, R., Drewello, D., and Schmitz, R. (2001) Integration of PCR fragments at any specific site within cloning vectors without the use of restriction enzymes and DNA ligase. *BioTechniques* 31, 88–90, 92

30. Bohnert, J. A., Schuster, S., Seege, M. A., Fähnrich, E., Pos, K. M., and Kern, W. V. (2008) Site-directed mutagenesis reveals putative substrate binding residues in the *Escherichia coli* RND efflux pump AcrB. *J. Bacteriol.* 190, 8225–8229

31. Zheleznova, E. E., Markham, P. N., Neyfakh, A. A., and Brennan, R. G. (1999) Structural basis of multidrug recognition by BmrR, a transcription activator of a multidrug transporter. *Cell* 96, 353–362

32. Schumacher, M. A., Miller, M. C., Grkovic, S., Brown, M. H., Skurray, R. A., and Brennan, R. G. (2001) Structural mechanisms of QacR induction and multidrug recognition. *Science* 294, 2158–2163

33. Vázquez-Laslop, N., Markham, P. N., and Neyfakh, A. A. (1999) Mechanism of ligand recognition by BmrR, the multidrug-responding transcriptional regulator: mutational analysis of the ligand-binding site. *Biochemistry* 38, 16925–16931

34. Middlemiss, J. K., and Poole, K. (2004) Differential impact of MexB mutations on substrate selectivity of the MexAB-OprM multidrug efflux pump of *Pseudomonas aeruginosa*. *J. Bacteriol.* 186, 1258–1269

35. Gutmann, D. A., Ward, A., Urbatsch, I. L., Chang, G., and van Veen, H. W. (2010) Understanding polyspecificity of multidrug ABC transporters: closing in on the gaps in ABCB1. *Trends Biochem. Sci.* 35, 36–42

36. Hardwick, L. J., Velamakanni, S., and van Veen, H. W. (2007) The emerging pharmacotherapeutic significance of the breast cancer resistance protein (ABCG2). *Br. J. Pharmacol.* 151, 163–174

37. Allen, I. D., Jackson, S. C., and Schinkel, A. H. (2002) A mutation hot spot in the Bcrp1 (Abcg2) multidrug transporter in mouse cell lines selected for doxorubicin resistance. *Cancer Res.* 62, 2294–2299

38. Chen, Z. S., Robey, R. W., Belinsky, M. G., Shchaveleva, I., Ren, X. Q., Sugimoto, Y., Ross, D. D., Bates, S. E., and Krug, G. D. (2003) Transport of methotrexate, methotrexate polyglutamates, and 17β-estradiol 17-(β-D-glucuronide) by ABCG2: effects of acquired mutations at R482 on methotrexate transport. *Cancer Res.* 63, 4048–4054

39. Miwa, M., Tsukahara, S., Ishikawa, E., Asada, S., Imai, Y., and Sugimoto, Y. (2003) Single amino acid substitutions in the transmembrane domains of breast cancer resistance protein (BCRP) alter cross resistance patterns in transfectants. *Int. J. Cancer* 107, 757–763

40. Ejendal, K. F., Diop, N. K., Schweiger, L. C., and Hrycyna, C. A. (2006) The nature of amino acid 482 of human ABCG2 affects substrate transport and ATP hydrolysis but not substrate binding. *Protein Sci.* 15, 1597–1607

41. Pozza, A., Perez-Victoria, J. M., Sardo, A., Ahmed-Belkacem, A., and Di Pietro, A. (2006) Purification of breast cancer resistance protein ABCG2 and role of arginine-482. *Cell Mol. Life Sci.* 63, 1912–1922

42. Yu, J., Zhou, Y., Tanaka, I., and Yao, M. (2010) Roll: a new algorithm for the detection of protein pockets and cavities with a rolling probe sphere. *Bioinformatics* 26, 46–52