An Elegant Means of Self-protection in Gram-negative Bacteria by Recognizing and Extruding Xenobiotics from the Periplasmic Space* [S]

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Infection of Pseudomonas aeruginosa in cystic fibrosis patients is a major cause of mortality. This organism shows wide ranging antibiotic resistance that is largely attributable to the expression of xenobiotic efflux pump(s). Here, we show a novel mechanism by which the resistance-nodulation-division-type xenobiotic transporter expels potential hazards and protects the interior of the cells. The xenobiotic transporters MexB and MexY preferentially export β-lactam and aminoglycoside antibiotics, respectively. When two large extramembrane loops of MexY were replaced by the corresponding loops of MexB, the hybrid protein exhibited β-lactam selectivity (MexB-type), but failed to recognize aminoglycosides. As the transmembrane segment of MexB was replaced with a corresponding transmembrane segment of MexY, one-by-one for all 12 segments, all the hybrid proteins showed MexB-type antibiotic selectivity. These results clearly demonstrated that the resistance-nodulation-division-type efflux pump in P. aeruginosa selects and transports substrates via the domains that largely protrude over the cytoplasmic membrane. The transmembrane segments were unlikely to have been involved in substrate selectivity. These observations led us to propose a novel mechanism by which the xenobiotic transporters in Gram-negative bacteria select and expel substrates from the periplasmic space before potential hazards penetrate into the cytoplasmic membrane.

Pseudomonas aeruginosa shows intrinsic and mutational resistance to a broad spectrum of antibiotics and this resistance is mainly attributable to a synergy of expression of the xenobiotic efflux transporters and a tight outer membrane barrier (1–4). Multiantibiotic resistance in this organism is a major cause of mortality of hospital patients whose immune activity was lowered by some other factors. P. aeruginosa encodes several RND1 family efflux pumps including MexAB-OprM and MexXY-OprM (3). These transporters consist of the inner membrane spanning the proton-antibiotic antiporter (MexB or MexY), the membrane fusion proteins that are assumed to connect the inner and outer membranes (MexA or MexX) and the outer membrane-associated lipoprotein OprM common to both transporters (3). The inner membrane spanning subunit (RND transporter) has been found to traverse the cytoplasmic membrane 12 times leaving amino and carboxyl termini in the cytoplasm and the TMSSs connected by extra membrane loops (5–7). Although most loops are short, the loops connecting TMS-1 and TMS-2 (loop-1/2) and TMS-7 and TMS-8 (loop-7/8) were found to consist of over 30 amino acid residues largely protruding toward the periplasmic space. Of five charged amino acid residues in the TMSSs of MexB, three in TMS-4 and TMS-10 were shown to be essential for the transporter function probably forming a proton pathway (8). MexB and MexY preferentially export β-lactams and aminoglycosides, respectively, although both transporters export agents such as chloramphenicol, fluoroquinolones, and tetracycline as well (9). The amino acid sequences of these transporters appeared to be about 47% identical (see Supplemental Fig. 1).

Most active transporter proteins recognize the specific stereochemical structure of substrates, while many xenobiotic efflux pumps export structurally dissimilar molecules. Analysis of mutant transporters that altered the substrate selectivity suggested that the mutations were mainly located in TMS. We assessed the question for the substrate recognition site in the RND-type xenobiotic efflux pump by means of domain swapping experiments, and the results let us to propose a novel mechanism of bacterial self-protection against xenobiotics.

Experimental Procedures

Bacterial Strains, Plasmids, and Primers—Bacterial strains and plasmids used are listed in Table 1. Primers are listed in Supplemental Fig. 2.

Construction of Plasmids, Deletion of Chromosomal mexY or mexXY by Gene Replacement, and Other Methods—These are provided in Supplementary Figs. 3 and 4.

Results

Swapping of Extramembrane Loops—Taking the advantage of sequence similarity, clear substrate selectivity, and the shared outer membrane subunit between MexB and MexY, we carried out domain-swapping experiments. We took first the MexY transporter, deleted loop-1/2 and replaced it with loop-1/2 of MexB. Similarly, loop-7/8 of MexY was replaced with that of MexB. The wild-type MexB conferred resistance against β-lactam antibiotics (aztreonam), nalidixic acid, and chloramphenicol to an MIC level of 0.13, 50, and 50 μg ml⁻¹, respectively, but not against gentamicin (aminoglycoside) (0.1 μg ml⁻¹) (Fig. 1a). The MexY transporter conferred resistance against gentamicin, nalidixic acid, and chloramphenicol to an MIC level of 0.78, 12.5, and 6.25 μg ml⁻¹, respectively, but not against β-lactam (0.2 μg ml⁻¹) (Fig. 1b). If loop-1/2 or loop-7/8 alone was responsible for substrate selectivity, one would expect

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pect that cells expressing such a hybrid protein would exhibit antibiotic susceptibility similar to that in cells producing the MexAB-OprM efflux pump. Alternatively, if the TMSs were solely responsible for selecting antibiotics, the hybrid protein might exhibit MexY-type antibiotic selectivity. Cells expressing the hybrid protein with loop-1/2 or loop-7/8 of MexB and the rest of the domains from MexY appeared totally non-functional (Fig. 1a and b). The results suggested that MexY-TMSs alone could not select the substrate or that the transporter might require both loop-1/2 and loop-7/8 from the same source. It is also possible that the construction of the loop-TMS joint site could not select the substrate or that the transporter might not be able to select substrates or that the transporter might not be able to select substrates.

Next, we replaced both loop-1/2 and loop-7/8 of MexY with the corresponding loops derived from MexB. Cells expressing a mosaic protein showed the aztreonam MIC of 3.13 \( \mu \text{g mL}^{-1} \), which was identical to the MIC in cells expressing the wild type MexB (Fig. 1c). The cells exhibited the gentamicin MIC that was identical to that of MexY-negative cells, which was 0.1 \( \mu \text{g mL}^{-1} \) (Fig. 1c). One may argue that substrate selectivity by the periplasmic domains may be limited to very hydrophilic substrates, such as \( \beta \)-lactams and aminoglycosides. However, this might not be the case, because selectivity of this MexB-MexY mosaic protein toward more hydrophobic substrates was also changed from the MexY-type to the MexB-type whose naldixic acid and chloramphenicol MICs were both 50 \( \mu \text{g mL}^{-1} \) (Fig. 1c). These results clearly indicated that the RND xenobiotic transporters in _P. aeruginosa_ select substrates by means of two large periplasmic domains.

Since the above domain swapping experiment was successful in the presence of MexA, the question of whether or not two large periplasmic domains cooperate with membrane fusion protein in the xenobiotic export can be investigated. We have carried out such an experiment by expressing the above-mentioned MexB(loop-1/2+loop-7/8/MexY(TMS)s) hybrid protein in the host producing MexX, but lacking MexA, MexB, and MexY. As seen in Fig. 1b, this mosaic protein failed to transport any substrate so far tested, and it became evident that loop-1/2 and loop-7/8 were unable to cooperate with MexX, but did cooperate with MexA. Therefore, it is highly likely that two periplasmic domains interact with the partner membrane fusion protein.

To ascertain whether or not lack of antibiotic transport activity in the constructs carrying either one of two large periplasmic domains is due to low level protein expression, we tested the presence of histidine-tagged MexB or MexY in cells harboring the respective plasmids. As seen in Fig. 1, all the cells harboring the plasmid encoding mexBHis, mexYHis, mexB(loop-1/2+loop-7/8/MexY(TMS)s)His showed comparable expression of proteins reactive with anti-hexahistidine antibody. These results clearly indicated that the undetectable function of MexB(loop-1/2+loop-7/8/MexY(TMS)s)His was not attributable to low level expression of the hybrid protein.

### Table I

| Strains or plasmids | Description | Source or Ref. |
|---------------------|-------------|----------------|
| _P. aeruginosa_     |             |                |
| PAO4290             | _lev-10 argF10 aph-9004; FP-_- | (20) |
| TNP071              | _ΔmexB_; derivative of PAO4290 | (20) |
| TNP073              | _ΔmexAB_; derivative of PAO4290 | (20) |
| TNP088              | _ΔmexXY_ of TNP071 | This study |
| TNP089              | _ΔmexY_ of TNP073 | This study |
| E. coli             | _E. coli_ strain for transformation |                |
| DH5α               | Laboratory stock | (21) |
| S17-1               | Mobilizer strain |                |
| Plasmids            |             |                |
| pK19mobSacB        | Broad-host-range mobilizable vector with sacB, Kmr | (22) |
| pKMS-X/Y           | pK19mobSacB derivative carrying the _mexXY_ genes and its franking regions | This study |
| pKMS-XY            | pK19mobSacB derivative used for the deletion of the _mexXY_ genes | This study |
| pKMS-XXY           | pK19mobSacB derivative used for the deletion of the _mexXY_ genes | This study |
| pUCP20             | Broad-host-range vector; Amp’ | (23) |
| pUCP-mexB          | pUCP20 derivative carrying _mexB_ gene | This study |
| pUCP-mexY          | pUCP20 derivative carrying _mexY_ gene | This study |
| pUCP-B<sub>His</sub> | pUCP20 derivative carrying histidine-tagged-mexB gene | This study |
| pUCP-Y<sub>His</sub> | pUCP20 derivative carrying histidine-tagged-mexY gene | This study |
| pYLI1/2E<sub>His</sub> | pUCP20 derivative carrying loop1/2-substituted mexX gene | This study |
| pYLI7/8E<sub>His</sub> | pUCP20 derivative carrying loop7/8-substituted mexY gene | This study |
| pTM1-1/2 + 7/8B<sub>His</sub> | pUCP20 derivative carrying loop1/2 and 7/8-substituted mexX gene | This study |
| pTM1-1/2Y          | pUCP20 derivative carrying TMS1-substituted mexB gene | This study |
| pTM1-2Y            | pUCP20 derivative carrying TMS2-substituted mexB gene | This study |
| pTM1-3Y            | pUCP20 derivative carrying TMS3-substituted mexB gene | This study |
| pTM1-4Y            | pUCP20 derivative carrying TMS4-substituted mexB gene | This study |
| pTM1-5Y            | pUCP20 derivative carrying TMS5-substituted mexB gene | This study |
| pTM1-6Y            | pUCP20 derivative carrying TMS6-substituted mexB gene | This study |
| pTM1-7Y            | pUCP20 derivative carrying TMS7-substituted mexB gene | This study |
| pTM1-8Y            | pUCP20 derivative carrying TMS8-substituted mexB gene | This study |
| pTM1-9Y            | pUCP20 derivative carrying TMS9-substituted mexB gene | This study |
| pTM1-10Y           | pUCP20 derivative carrying TMS10-substituted mexB gene | This study |
| pTM1-11Y           | pUCP20 derivative carrying TMS11-substituted mexB gene | This study |
| pTM1-12Y           | pUCP20 derivative carrying TMS12-substituted mexB gene | This study |

**Swapping of Transmembrane Segments**—Although the above experiment demonstrated that loop-1/2 and loop-7/8 were responsible for substrate recognition, this does not rule out the possibility that these loops select substrates by coupling with TMS(s). If the TMS of MexB participates in substrate recognition, replacement of the TMS of MexB with that of MexY must result in hybrid protein dysfunction in the transport of the MexB substrate. Thus, we replaced the TMS of the MexB transporter with that of MexY such that TMS-1 of MexB was replaced by TMS-1 of MexY and so on. The results revealed that all MexY-TMSs were fully cooperative with loop-1/2 and loop-7/8 of MexB in the transporter function and showed that aztreonam transport activity was comparable with that of intact MexB. There were considerable variations in the activity of the hybrid transporter whose proteins having TMS-5, -6, and -7.
showed low transport activity, yet all the proteins exhibited MexB-type substrate selectivity (Fig. 2). The hybrid proteins were unable to transport the MexY substrate, gentamicin. Therefore, it became evident that the TMS of MexB was not directly involved in the selection of either β-lactam or aminoglycoside antibiotics. Substrate selectivity of these hybrid proteins for other antibiotics such as nalidixic acid and chloramphenicol appeared to be the MexB-type without exception.

To verify the proper expression of the hybrid proteins, crude membrane fractions were subjected to Western blotting analysis using antibody against MexB (loop-1/2). The results showed that all the constructs expressed the hybrid protein in an amount comparable with intact MexB, except for the protein containing TMS-6 from MexY. However, the substrate selectivity and transport activity of this hybrid protein was comparable with that of the others. Therefore, we concluded that variable transport activity of the hybrid proteins containing TMS from MexY was not attributable to the low level expression.

**DISCUSSION**

Studies on the substrate recognition site of xenobiotic transporters in mammalian and prokaryote cells showed the following results. Mutations, which altered the substrate specificity of P-glycoprotein and multidrug resistant proteins, were scattered in TMSs and cytoplasmic loops. Thus, it has been assumed that these transporters select substrates from either cytoplasmic and exoplasmic leaflets of the membrane, which are mechanisms proposed as the "vacuum cleaner model" and "flippase model," respectively (10–12). Substrate recognition in the xenobiotic transporters of the prokaryote have been investigated in Bmr, LmrP, MfdA, QuacA, and EmrE, revealing that most, if not all, mutations affecting substrate recognition have been identified in the TMS(s) (13–16).

Recently, the x-ray crystallographic structure of AcrB, an *Escherichia coli* homologue of MexB, has been solved showing the presence of three major domains: a cluster of 12 TMSs, a pore-forming domain, and a TolC-docking domain (5). The pore domains formed a central cavity, designated as a vestibule, that connected with three channel openings toward the periplasm, and a funnel-like TolC-docking domain. The authors predicted that these vestibules are likely candidates for the substrate-collecting ducts. Since the amino acid identity of AcrB and MexB appeared to be about 70%, it is likely that the three-dimensional structure of MexB was much like that of AcrB. In fact, the x-ray crystallographic analysis of AcrB confirmed our previous results that TMS-4 and -10 of MexB are close to each other (8).

This raises the question of which part of the RND transporters collects the substrates. It has been proposed that the substrate-collecting site(s) of the RND transporters are located at both the inner membrane and the cytoplasm (2, 17). Since the
results presented in this paper clearly demonstrated that membrane insoluble substrates were selected in the periplasmic domains, it is highly likely that the substrate-collecting ducts in MexB and MexY are located in the periplasmic domain. If this proposal is valid, one can answer the following interesting questions: (i) how do the cells protect the cytoplasmic membrane from membrane deteriorating surfactants, such as sodium dodecyl sulfate and bile salts? (ii) How are lipid-insoluble antibiotics such as β-lactams and aminoglycosides efficiently transported? (iii) How do these low substrate-selective transporters prevent leakage of cytoplasmic materials?

The following model plausibly explains the mechanism by which the RND transporters might collect, recognize, and transport xenobiotics. Substrates that have diffused into the periplasmic space across the outer membrane are sucked up into the periplasmic pore inlets before diffusing into the inner membrane. They are then subjected to filtration by the substrate-recognizing site probably located in the pore domains and subsequently transported to the extracellular milieu (Fig. 3). The xenobiotics may not penetrate the cytoplasm of cells with a tight outer membrane barrier and powerful xenobiotic transporters, which protect the interior of the cells from potential hazards. This is an elegant and efficient means of safeguard. However, this new model is not mutually exclusive of the hypothesis that the transporters collect substrates from the inner membrane. During the course of this manuscript preparation, we learned that MexD mutations with altered substrate selectivity were mapped in the large periplasmic domains (18) and that domain-swapping experiments have been carried out with different combinations of the transporters (19). The results are partially consistent with the present results and, therefore, can be regarded as supporting the new model proposed in this report.

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