MexY-Promoted Aminoglycoside Resistance in *Pseudomonas aeruginosa*: Involvement of a Putative Proximal Binding Pocket in Aminoglycoside Recognition

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**ABSTRACT** The resistance-nodulation-division (RND) family multidrug efflux system MexXY–OprM is a major determinant of aminoglycoside resistance, although the details of aminoglycoside recognition and export by MexY, the substrate-binding RND component of this efflux system, have not been elucidated. To identify regions/residues of MexY important for aminoglycoside resistance, plasmid-borne mexY was mutagenized and mutations that impaired MexY-promoted aminoglycoside (streptomycin) resistance were identified in a ΔmexY strain of *P. aeruginosa*. Sixty-one streptomycin-sensitive mexY mutants were recovered; among these, 7 unique mutations that yielded wild-type levels of MexY expression were identified. These mutations compromised resistance to additional aminoglycosides and to other antimicrobials and occurred in both the transmembrane and periplasmic regions of the protein. Mapping of the mutated residues onto a 3-dimensional structure of MexY modeled on *Escherichia coli* AcrB revealed that these tended to occur in regions implicated in general pump operation (transmembrane domain) and MexY trimer assembly (docking domain) and, thus, did not provide insights into aminoglycoside recognition. A region corresponding to a proximal binding pocket connected to a periplasm-linked cleft, part of a drug export pathway of AcrB, was identified in MexY and proposed to play a role in aminoglycoside recognition. To test this, selected residues (K79, D133, and Y613) within this pocket were mutagenized and the impact on aminoglycoside resistance was assessed. Mutations of D133 and Y613 compromised aminoglycoside resistance, while, surprisingly, the K79 mutation enhanced aminoglycoside resistance, confirming a role for this putative proximal binding pocket in aminoglycoside recognition and export.

**IMPORTANCE** Bacterial RND pumps do not typically accommodate highly hydrophilic agents such as aminoglycosides, and it is unclear how those, such as MexY, which accommodate these unique substrates, do so. The results presented here indicate that aminoglycosides are likely not captured and exported by this RND pump component in a unique manner but rather utilize a previously defined export pathway that involves a proximal drug-binding pocket that is also implicated in the export of nonaminoglycosides. The observation, too, that a mutation in this pocket enhances MexY-mediated aminoglycoside resistance (K79A), an indication that it is not optimally designed to accommodate these agents, lends further support to earlier proposals that antimicrobials are not the intended pump substrates.

*Pseudomonas aeruginosa* is a Gram-negative opportunistic pathogen that is frequently associated with life-threatening nosocomial infections in immunocompromised individuals, especially patients with cystic fibrosis (CF) (1). Intrinsically resistant to many clinically used antimicrobial agents and readily developing resistance during antimicrobial therapy (2), pseudomonal infections are often recalcitrant and difficult to manage (3). Among the major contributors to this intrinsic and acquired antimicrobial resistance are several multidrug efflux systems of the resistance-nodulation-division (RND) family (4). One of these, MexXY-OprM, is particularly noteworthy owing to its role in resistance to aminoglycosides (5, 6), which are uncommon RND pump substrates (4, 7), particularly in clinical *P. aeruginosa* isolates recovered from CF patients (8–11). MexXY proteins are encoded by a two-gene operon (mexY, oprM), while OprM is encoded by the third gene of another multidrug efflux operon, *mexAB-oprM* (12). The *mexXY* operon is inducible by many of its antimicrobial substrates, specifically those, including the aminoglycosides, that target and disrupt the ribosome (13, 14).

The tripartite MexXY-OprM pump is comprised of a periplasmic membrane fusion protein (MexX), the inner-membrane (IM) drug/H+ antiporter (MexY; the RND component), and the outer-membrane (OM) channel (OprM) (15, 16). Crystal structures of the related AcrB protein from *Escherichia coli* indicate that RND components exist as asymmetric homotrimers with individual monomers, in a concerted fashion, adopting one of three conformational states that represent different steps of the drug export process—access, binding, and extrusion (also known as loose, tight, and open, respectively) (17, 18). Individual monomers are proposed to cycle sequentially through these conformational states, as sub-
strates first enter the pump (access state), bind (binding state), and are then extruded into the OM channel-forming constituent through an opening at the top of the RND component (extrusion state) in what has been termed a functionally rotating drug transport mechanism (17). RND monomers are comprised of a transmembrane (TM) region of 12 α-helices (TM1 to TM12) responsible for coupling the TM proton flux to pump operation, mutations in the MexY-equivalent proximal binding pocket impact aminoglycoside resistance, highlighting the probable involvement of the previously defined export pathway for aminoglycosides (Table 1), while the cloned wild-type (WT) mexY gene on plasmid pCL10 restored resistance (Table 1). As such, it is not clear that the heterogeneous-identified export pathways, while likely present in MexY, are used by aminoglycosides, which are much more hydrophilic than the typical RND/AcrB substrates (23). To identify regions/residues of MexY involved in the MexY-mediated aminoglycoside resistance and so provide insights into aminoglycoside accommodation by this RND pump, mexY mutations that compromise assembly and general pump operation, mutations in the MexY-equivalent proximal binding pocket impact aminoglycoside resistance, highlighting the probable involvement of the previously defined export pathway for other agents in aminoglycoside capture and extrusion by MexY.

### RESULTS AND DISCUSSION

**Isolation of MexY mutants exhibiting reduced aminoglycoside resistance.** To identify mutations in mexY that compromised MexY activity with respect to aminoglycoside resistance, a MexY-bearing prRK415 derivative, plasmid pCL10, was mutagenized and introduced into a *P. aeruginosa* PAO1 *ΔmexY* derivative, strain K3315. Elimination of the mexY gene in K3315 conferred susceptibility to known MexXY-OprM antimicrobial substrates, including aminoglycosides (Table 1), while the cloned wild-type (WT) mexY gene on plasmid pCL10 restored resistance (Table 1). As such, pCL10 derivatives carrying mexY mutations that compromise aminoglycoside resistance could be selected by their failure to restore MexY<sub>WT</sub> levels of aminoglycoside resistance. Thus, hydroxylamine-mutated pCL10-bearing K3315 (2,600 colonies) 

| Strain | Plasmid | MexY mutation | MIC (µg/ml)<sup>a</sup> | STR | PAR | NEO | AMI | ERY | SPC | CAM | NOR<sup>b</sup> | CEF<sup>d</sup> |
|--------|---------|---------------|-----------------|-----|-----|-----|-----|-----|-----|-----|-----|-------|-------|
| K767   | None    | WT            | 32              | 256 | 32  | 0.5 | 512 | 512 | 256 | 64  | ND<sup>c</sup> | ND    |
| K3315  | None    | None          | 2               | 8   | 4   | 0.5 | 64  | 64  | 32  | 0.5 | 1     |       |
| K3315  | pRK415  | None          | 2               | 16  | 4   | 0.5 | 64  | 64  | 32  | 0.5 | 1     |       |
| K3315  | pCL10   | WT            | 8               | 64  | 8   | 1   | 512 | 512 | 256 | 64  | ND    | ND    |
| K3315  | pCL14   | G216D         | 2               | 16  | ND  | ND  | 64  | 64  | 32  | 0.5 | 1     |       |
| K3315  | pCL13   | R184H         | 4               | 32  | ND  | ND  | 256 | 256 | 32  | ND  | ND    | ND    |
| K3315  | pCL11   | S16F          | 4               | 32  | ND  | ND  | 256 | 256 | 32  | ND  | ND    | ND    |
| K3315  | pCL17   | A960T         | 4               | 16  | ND  | ND  | 256 | 256 | 32  | ND  | ND    | ND    |
| K3315  | pCL15   | V339M         | 4               | 16  | 8<sup>e</sup> | 1  | 512 | 256 | 32  | 2   | 1     |       |
| K3315  | pCL12   | R166C         | 4               | 32  | ND  | ND  | 256 | 512 | 32  | 2   | 4     |       |
| K3315  | pCL16   | P562S         | 8               | 32  | ND  | ND  | 256 | 512 | 32  | ND  | ND    | ND    |
| K3315  | pCL19   | D133A         | 4               | 16  | 4   | 0.5 | 256 | 1024 | 32 | ND  | ND    | ND    |
| K3315  | pCL20   | D133S         | 4               | 16  | 4   | 0.5 | 256 | 1024 | 32 | ND  | ND    | ND    |
| K3315  | pCL18   | K79A          | 8               | 128 | 8<sup>e</sup> | 1  | 512 | 1024 | 32 | ND  | ND    | ND    |
| K3315  | pCL21   | Y613A         | 4               | 16  | 4   | 0.5 | 512 | 256 | 32  | 2   | 2     |       |

*<sup>a</sup>The antimicrobial susceptibility of *P. aeruginosa ΔmexY* strain K3315 carrying the indicated plasmids expressing wild-type (WT) MexY or MexY derivatives with the indicated amino acid substitutions is reported. Results for WT strain K767 and plasmid-free K3315 are provided for comparison purposes.

*<sup>b</sup>MIC values lower than that measured for pCL10-carrying K3315 expressing WT MexY are bolded. MIC values higher than that measured for pCL10-carrying K3315 are italicized.

*<sup>c</sup>STR: streptomycin; PAR: paromomycin; NEO: neomycin; AMI: amikacin; ERY: erythromycin; SPC: spectinomycin; CAM: chloramphenicol; NOR: norfloxacin; CEF: cefepime.

*<sup>d</sup>ND MICs were determined in the presence of 8 µg/ml chloramphenicol. The NOR MIC for all strains in the absence of chloramphenicol was 0.5 µg/ml.

*<sup>e</sup>The V393M mutation compromises neomycin resistance.

*<sup>f</sup>At half the NEO MIC (4 µg/ml), MexY<sub>V339M</sub>-expressing K3315 grew reproducibly slower than K3315 expressing MexY<sub>WT</sub> (data not shown), indicating that the V339M mutation compromises neomycin resistance.

*<sup>g</sup>At half the NEO MIC (4 µg/ml), MexY<sub>K79A</sub>-expressing K3315 grew reproducibly faster than K3315 expressing MexY<sub>WT</sub> (data not shown), indicating that the K79A mutation enhances neomycin resistance.
resistance could not be assessed. To evaluate the impact of the MexY<sub>V339M</sub> mutation on resistance to additional antimicrobials, it was necessary to induce mexX expression with the nonsubstrate chloramphenicol. As seen in Table 1, chloramphenicol increased resistance to the quinolone norfloxacin and the β-lactam cepepime in strain K3315 expressing wild-type mexY but not in the same strain harboring a plasmid without an insertion or expressing the inactive MexY<sub>G216D</sub> variant (Table 1; see legend for MICs determined in the absence of chloramphenicol), consistent with chloramphenicol-promoted drug resistance being mediated by MexXY. Chloramphenicol-treated strain K3315 expressing MexY<sub>V339M</sub> showed an increase (relative to the vector-only control) in resistance to norfloxacin but not to cepepime, indicating that this mutation compromised resistance to the β-lactam but not the quinolone. Thus, although the V339M mutation negatively impacts resistance to aminoglycosides, it does not do so selectively.

Noticeably, most of the identified mutations (except S16F and R166C) occur at residues that are conserved among a variety of RND family multidrug transporters, including those that accommodate aminoglycosides (MexY, AxyY, AmrB, AdeB, SmeZ, and AcrD) and those that do not (MexB, MexD, AcrB, and BpeB) (see Fig. 3). Only two aminoglycosides were assessed, however, since the cloned wild-type mexY gene restored aminoglycoside resistance in strain K3315 only partially (Table 1)—for some aminoglycosides (e.g., amikacin, neomycin), the wild-type gene provided such a modest increase in resistance (2-fold) that the impact of anything but a fully null mutation would not have been measurable. Why the plasmid-borne wild-type mexY gene failed to fully complement the mexY deficiency of strain K3315 with respect to aminoglycoside resistance while doing so for resistance to nonaminoglycosides is unclear. It is possible that the plasmid-encoded MexY is overproduced and so disrupts the cytoplasmic membrane, thereby sensitizing it to perturbation by aberrant polypeptides that are the eventual product of aminoglycoside action on the bacterial ribosome (29). In agreement with this explanation, several streptomycin-sensitive mutants lacking mutations in mexY but showing significantly increased MexY production as a result of vector backbone-borne mutations were also recovered in this study (data not shown).

Mapping of MexY mutations onto a three-dimensional model of MexY. To better understand how the aforementioned mutations compromise MexY-mediated aminoglycoside resistance, we constructed a three-dimensional model of trimeric MexY based on the asymmetrical AcrB trimer structure that includes one monomer each in the access, binding, and extrusion domains (PDB accession number 2HRT) (18). MexY and AcrB share an overall sequence identity of 51%, with an additional 19% of sequence similarity, rendering AcrB a suitable template for building a MexY structural model. The resultant asymmetric MexY trimer model (Fig. 2A) has an excellent geometric quality—95.0% of the modeled residues fall in the most favored regions of the Ramachandran plot, 4.2% in the additionally allowed region, and only 0.8% in the disallowed region, as validated by the PROCHECK (30) program. Mapping of the mexY mutations onto the MexY structure (mutations were mapped onto all three of the MexY monomers, although only the binding monomer is shown for illustrative purposes; Fig. 2B) revealed that mutations compromising resistance to the tested antimicrobials were scattered throughout the molecule, including within the

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**FIG 1** Expression of mutant MexY proteins in *P. aeruginosa* K3315. Whole-cell extracts of *P. aeruginosa* ΔmexY strain K3315 expressing plasmid pRK415-borne wild-type (WT) or mutant mexY genes (amino acid substitutions in MexY are indicated) were electrophoretically separated by SDS-PAGE, electroblotted, and developed with antibodies directed against the MexY protein. The MexY proteins, absent in strain K3315 harboring pRK415 without a cloned mexY gene (pRK415), are indicated by arrowheads. The migration positions of molecular mass markers are shown at left.
FIG 2  Mapping mutations impacting MexY-mediated antibiotic resistance on a 3-dimensional homology model of MexY. (A) An asymmetric trimer model of MexY constructed by homology modeling on the crystal structure of *E. coli* AcrB (PDB code 2HRT). Individual monomers are shown in space-fill formatting and are colored salmon red (loose/access conformation), gray (tight/binding conformation), and blue (open/extrusion conformation; mostly hidden behind the other 2 monomers). Structural details of the tight/binding monomer are shown in order to highlight the characteristic N- and C-terminal regions/subdomains of the docking domain (DN [yellow] and DC [purple], respectively), the porter domain (2 N-terminal [PN1, orange]/[PN2, pink] and 2 C-terminal [PC1, green]/[PC2, red] subdomains), and the transmembrane domain (transmembrane segments TM1-6 [light blue] and TM7-12 [dark blue], respectively) of RND transporters. The positions of the intermonomer vestibule and intramonomer cleft are also indicated. (B) Locations in the tight/binding MexY monomer of mutations (shown in space-fill formatting) that compromise MexY-mediated drug resistance. Relevant subdomains (as described for panel A) are highlighted in the corresponding colors. Structural models at left and right were rotated 50° counterclockwise and 100° clockwise, respectively, relative to the middle model in order to better illustrate the positions of various mutations. The dashed lines define the inner-membrane boundary. A cleft/opening, within which the putative proximal binding pocket occurs, is clearly seen in the structural model at left. A vestibule drug entry pathway is also highlighted in the leftmost structural model.
increased susceptibility of MexYR184H-expressing and therefore likely compromises trimerization as well. While the als. Similarly, the R184H mutation occurs within the hole into plains its inability to promote resistance to all tested antimicrobials (Table 1), consistent with the cytoplasm-proximal region of MexY; Fig. 3). Interestingly, P562 occurs within a region of MexY that in the corresponding region of AcrB is variably at the extreme lower edge of the cleft that appears to provide access of periplasmic substrates to the proximal binding pocket (24) or at the upper reaches of a so-called vestibule pathway (Fig. 2B; left structural model), specifically, vestibule pathway b (25), that is suggested to accommodate lipophilic pump substrates that are likely to partition into the outer leaflet of the inner membrane (25, 28).

Docking domain mutations. The G216D mutation, which had the biggest negative impact on MexY-mediated drug resistance (Table 1), maps to a protruding “thumb” structure (Fig. 2B, middle structural model) within the periplasmic DN subdomain that inserts into a “hole” in the docking domain of the neighboring protoner (Fig. 2A). This structure has previously been implicated in the trimerization of RND components (31, 32) with a mutation in the MexYG216G-equivalent residue of P. aeruginosa MexB, G220, similarly compromising resistance to all antimicrobials (32) and mutations in the corresponding G217 (and neighboring) residue of E. coli AcrB compromising trimerization (31). Thus, MexYG216D is plausibly impaired in trimer assembly and this explains its inability to promote resistance to all tested antimicrobials. Similarly, the R184H mutation occurs within the hole into which the “thumb” structure of a neighboring protoner inserts and therefore likely compromises trimerization as well. While the increased susceptibility of MexYR184H-expressing P. aeruginosa to all tested antimicrobials is consistent with such a notion, the R184H mutation does not fully abrogate MexY-mediated resistance and so, unlike the G216D mutation, presumably does not fully block trimerization.

Periplasmic domain mutations. The R166C mutation maps to the periplasmic PN2 subdomain of MexY (Fig. 2B, rightmost structural model) in a region that, in AcrB (28), contacts the PN1’ subdomain of a neighboring monomer. This PN2-PN1’ intermonomeric contact appears to be central to the “functionally rotating” transport mechanism of multidrug RND transporters, whereby adoption of a tight or binding conformation by one protomer sterically facilitates (though the PN2-PN1’ contact) the cycling of its adjacent monomer to an “open” drug extrusion conformation (28). Thus, the R166C mutation may interfere with this cycling process in MexY. Still, the R166C mutation does not impact resistance to all antimicrobials (spectinomycin, norfloxacin, and cefepime MICs are unaffected; Table 1) and does not fully compromise MexY-mediated resistance to those agents that are impacted. Hence, if this mutation is affecting MexY operation, the impact is only partial and has, for some as-yet-unknown reason, differential impacts on the export of substrate antimicrobials. It is possible that the R166C mutation is also indirectly affecting substrate recognition/accommodation and that this may be the reason behind its substrate-specific impact.

Transmembrane domain mutations. Four mexY mutations map to the transmembrane domains, with 2 being cytoplasm proximal (S16F and A960T; Fig. 2B, middle and leftmost structural model, respectively) and 2 being periplasm proximal (V339M and P562S; Fig. 2B, middle and leftmost structural models, respectively). All but S16 are conserved among RND transporters (Fig. 3), implying that they somehow contribute to basic pump operation. Still, only the A960T (and S16F) mutations are associated with reduced resistance to all tested antimicrobials (Table 1), consistent with the cytoplasm-proximal region of MexY playing a conserved role in RND pump operation. How these mutations impact MexY function is currently unclear, although, given that conformational changes occur within the TM region of RND transporters as they cycle between the different monomer states during drug export (17, 18), likely mediated by proton movement though the RND constituent (33, 34), it may be that the productive proton-driven conformational changes in the TM region are compromised to some extent by these mutations. Despite their conservation among RND multidrug transporters, mutations at V339 (V339M) and P562 (P562S) have a substrate-specific impact on MexY function, suggesting that, unlike the cytoplasm-proximal TM mutations, they are not compromising general pump operation. Similarly situated mutations in MexB (V928M and M395I) also impacted resistance to only a subset of antimicrobial substrates (32), although the reasons are unclear. Interestingly, P562 occurs within a region of MexY that in the corresponding region of AcrB is variably at the extreme lower edge of the cleft that appears to provide access of periplasmic substrates to the proximal binding pocket (24) or at the upper reaches of a so-called vestibule pathway (Fig. 2B; left structural model), specifically, vestibule pathway b (25), that is suggested to accommodate lipophilic pump substrates that are likely to partition into the outer leaflet of the inner membrane (25, 28). Binding of the detergent and AcrB substrate dodecyl-$\beta$-D-maltoside to this region of AcrB has previously been demonstrated (20), and D566 in AcrB (which corresponds to the P562-adjacent E563 residue in MexY) appears to be involved in ciprofloxacin binding (26) and is accessible to the AcrB substrate Bodipy FL maleimide (24). Thus, the region of MexY that encompasses P562 likely forms an entry pathway for certain MexXY-OprM substrate antimicrobials, explaining the selective impact of the P562S mutation on drug resistance. Given the proximity of MexYP562 to two entry pathways and the structure-altering nature of a P562S substitution, it is possible that both pathways are negatively impacted by this mutation. While one might expect the cleft pathway to be the route of choice for hydrophilic aminoglycosides, these agents do cross and therefore do interact with the IM, and given how little is known about this process, the possibility cannot be ruled out that these agents access the proximal binding pocket of MexY via the vestibule route.

Site-directed mutagenesis of a predicted substrate-binding pocket in MexY. The observation that some mexY mutations showed substrate-specific effects on resistance that included aminoglycosides suggests that the latter agents are actual substrates of MexY. In an attempt to better elucidate the molecular details of aminoglycoside recognition by MexY, the protein was aligned with other aminoglycoside-accommodating RND pump components (e.g., AcrD of E. coli [35], AmrB of Burkholderia pseudomallei [36], AdeB of Acinetobacter baumannii [37], and AxyY of Achromobacter xylosoxidans [38]; Fig. 3), as well as several RND components that do not accommodate aminoglycosides (Fig. 3). The intent was to identify residues that were uniquely conserved in aminoglycoside-accommodating RND pump components and, so, potentially contribute to aminoglycoside binding and/or export. No residues were specifically conserved in the aminoglycoside-accommodating pumps that were examined, although 4 of 6 of these had a conserved aspartate residue (D133 in MexY; Fig. 3). Interestingly, D133 occurs within a region of MexY that corresponds structurally to the proximal multisite binding pocket of E. coli AcrB (21) (see Fig. 2B). Moreover, the D133-equivalent residue in AcrB, S134 (Fig. 3), is one of the 4 key residues that coordinate the proximal binding pocket of this RND pump (21) and its mutation compromises AcrB-mediated drug resistance (21). MexY*D133 was, therefore, considered a candidate residue involved in aminoglycoside recognition/binding. The amino acid sequence alignment of RND proteins also revealed a number of residues that were uniquely present in a subset (e.g.,
MexY, AmrB, and AxyY) of the aminoglycoside-accommodating RND pumps. AmrB (66% identity) and AxyY (70% identity) exhibit the highest degree of homology to MexY among all the aminoglycoside-accommodating RND proteins (identity with the remainder is \( \approx 50\% \)), and while this may simply reflect a closer evolutionary relationship between these RND proteins (phylogenetic analysis shows that these protein clusters; data not shown), it may also be that there are multiple ways for RND pump components to recognize/accommodate aminoglycosides and that these three share a mechanism. Intriguingly, one of these conserved

**FIG 3**

Segments of amino acid sequence alignment of MexY and selected RND homologues in *P. aeruginosa* and other Gram-negative bacteria. The amino acid sequences of MexY and the indicated RND homologues were aligned using the multiple-sequence-alignment program T-coffee (55). Only those aligned sequences that encompassed the MexY residues whose mutation compromised drug resistance (indicated with an arrowhead and labeled) are shown. Residues that are conserved in other RND homologues are highlighted in bold lettering. Amino acid sequences were obtained from the NCBI Protein database (GenBank accession no. NP_250708 [MexY_ *P. aeruginosa* PAO1], EGP45231 [AxyY_ *Achromobacter xylosoxidans* AXX-A], YP_108402 [AmrB_ *Burkholderia pseudomallei* K96243], CAJ77844 [AdeB_ *Acinetobacter baumannii* AYE], YP_001972001 [SmeZ_ *Stenotrophomonas maltophilia* K279a], NP_416965 [AcrD_ *Escherichia coli* K-12 MG1655], NP_250708 [MexD_ *P. aeruginosa* PAO1], NP_249117 [MexB_ *P. aeruginosa* PAO1], NP_414995 [AcrB_ *E. coli* K-12 MG1655], and YP_006275722 [BpeB_ *B. pseudomallei* 1026b]). MexY, AxyY, AmrB, AdeB, SmeZ, and AcrD have been linked to aminoglycoside resistance.

 MexY, AmrB, and AxyY) of the aminoglycoside-accommodating RND pumps. AmrB (66% identity) and AxyY (70% identity) exhibit the highest degree of homology to MexY among all the aminoglycoside-accommodating RND proteins (identity with the remainder is \( \approx 50\% \)), and while this may simply reflect a closer evolutionary relationship between these RND proteins (phylogenetic analysis shows that these protein cluster; data not shown), it may also be that there are multiple ways for RND pump components to recognize/accommodate aminoglycosides and that these three share a mechanism. Intriguingly, one of these conserved
residues, Y613 (Fig. 3), whose unique presence in these three proteins was confirmed in a more extensive comparison involving all “confirmed to be resistance-mediating” RND proteins from *P. aeruginosa*, *E. coli*, and *B. pseudomallei* (22 proteins in total; data not shown), was also identified in the vicinity of the proposed proximal binding pocket of MexY (Fig. 2B), on the tip of the switch-loop (20) that separates the proximal and distal binding pockets. Mutations of the switch-loop in AcrB have been shown to compromise AcrB-mediated resistance to erythromycin and doxorubicin, apparently owing to mutation-driven structural changes in the loop interfering with the binding of these larger substrates to the proximal binding pocket (20, 21). MexY Y613 was, thus, possibly involved in aminoglycoside binding as well. Finally, a lysine residue unique to MexY (K79) was identified within the putative MexY proximal binding pocket (Fig. 2B) and a role for it in aminoglycoside binding was also proposed. The chosen candidate residues were individually mutated to alanine on plasmid pCL10, and the impact on MexY-mediated antimicrobial resistance of *P. aeruginosa* strain K3315 was assessed. MexY Y613 was also mutated to serine, which is the residue at this position in *E. coli* AcrB and the one which has been implicated in erythromycin binding (21). It was reasoned that, should D133 contribute to substrate binding and MexY-mediated antimicrobial resistance, then the change to the AcrB-equivalent serine might specifically compromise aminoglycoside binding/resistance but retain erythromycin binding/resistance, while the alanine substitution would compromise binding of resistance to both.

All site-directed mutation-bearing *mexY* genes yielded wild-type levels of MexY protein (Fig. 1, bottom). As shown in Table 1 (bottom), both mutations of D133 reduced resistance to all agents tested, with the exception of spectinomycin, where an increase in resistance was noted. These observations suggest that the mutationally altered binding pocket is better able to accommodate spectinomycin while concomitantly being less able to accommodate the other substrates, confirming a role for the putative MexY proximal binding pocket and D133 specifically in substrate (including, importantly, aminoglycoside) recognition and MexY-mediated antimicrobial resistance. Interestingly, the Y613A mutation compromised resistance to the aminoglycoside/aminocyclitol agents but not erythromycin (Table 1), initially suggesting that it might specifically impact the former agents only. In cells treated with chloramphenicol to induce chromosomal *mexX*, however, this mutation also compromised cefepime resistance but not norfloxacin resistance (Table 1). These data are consistent with the loop functioning directly in the binding/recognition of these agents or, more likely, the Y613A mutation impacting the disposition of the switch-loop, sterically hindering access of these agents to the proximal binding pocket, as proposed, for example, for the switch-loop mutations in *E. coli* AcrB (20, 21). This further supports the idea of the putative binding pocket of MexY playing a significant role in substrate (including aminoglycoside) recognition/accommodation. In contrast to the results reported above, mutation of MexY K79 failed to compromise resistance to any agent tested and, in fact, enhanced resistance to several agents, including a number of aminoglycosides and spectinomycin (Table 1). The reason for this is unclear, though this result highlights once more the significance of this putative proximal binding pocket of MexY in recognition of antimicrobials, including aminoglycoside. The fact that native MexY retains a residue that is less than optimal for drug accommodation/resistance gives further evidence that antimicrobials are not the intended substrates for this RND efflux system.

The observation that mutations in regions of MexY that correspond to a previously defined drug export pathway(s) in AcrB (vestibule/cleft drug entry pathway and proximal drug binding pocket) impact resistance to aminoglycosides is consistent with these unusual hydrophilic RND pump substrates using a common drug export pathway versus one unique to these agents. Thus, the failure of AcrB and other RND pumps to accommodate aminoglycosides presumably stems simply from their lacking the necessary binding constituents in their entry/export pathways to accommodate aminoglycosides.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** The bacterial strains and plasmids used in this study are described in Table 2. Bacterial cells were cultured in Luria broth (LB-broth) and on Luria agar (L-agar), with antibiotics as necessary, at 37°C. Plasmid pEX18Tc and its derivatives were maintained or selected in *Escherichia coli* with 10 µg/ml tetracycline. Plasmid pRK415 and its derivatives were maintained or selected with 10 (in *E. coli*) or 50 (in *Pseudomonas aeruginosa*) µg/ml tetracycline.

**DNA methods.** Standard protocols were used for restriction endonuclease digestion, ligation, transformation, and agarose gel electrophoresis, as described by Sambrook and Russell (39). Plasmid and chromosomal DNA was prepared as described before (40). DNA fragments used for cloning were cloned from agarose gels using a Wizard SV gel and PCR cleanup system (Fisher Scientific, Ltd., Nepean, Canada). CaCl₂-dependent *E. coli* (41) and electrocompetent *P. aeruginosa* (42) cells were prepared as previously described. Oligonucleotide (PCR primer) synthesis was carried out by Integrated DNA Technologies (Corvalle, ILA). Nucleotide sequencing was carried out by ACGT Corp. (Toronto, Canada) using universal and custom primers (Table 3).

**Construction of a mexY deletion strain of *P. aeruginosa***. To introduce an in-frame deletion of *mexY* into wild-type *P. aeruginosa* PAO1 strain K767, a pEX18Tc-based deletion construct was first prepared by sequentially cloning PCR-amplified 1-kb DNA fragments corresponding to the regions upstream and downstream of *mexY* into pEX18Tc. The *mexY* upstream and downstream regions were individually amplified using primers mexYUP-F and mexYUP-R and primers mexYDN-F and mexYDN-R, respectively. Amplification was achieved in a 50-µl reaction mixture formulated as described before (40) and heated for 3 min at 98°C, followed by 35 cycles of 0.5 min at 98°C, 0.5 min at 68°C, and 0.5 min at 72°C, before finishing with 10 min at 72°C. The resultant Δ*mexY* deletion vector, pCL8, was mobilized into *P. aeruginosa* strain K767 from *E. coli* S17-1 (40), and tetracycline-resistant transconjugants were recovered and patched onto sucrose as described before (40). Sucrose-resistant colonies were then screened for chromosomal deletion of *mexY* using colony PCR (43) with 2.5 U Taq polymerase in 10% (vol/vol) dimethyl sulfoxide (DMSO). Colony PCR was carried out using primers mexYscreen-F and mexYscreen-R at a 0.6 µM final concentration and deoxynucleoside triphosphates (dNTPs) at a 0.2 mM final concentration in a 10-µl reaction volume, with the mixture heated for 3 min at 95°C, followed by 35 cycles of 0.5 min at 95°C, 0.5 min at 59.1°C, and 4 min at 72°C, before finishing with 10 min at 72°C.

**Cloning of mexY**. Plasmid pCL10, a pRK415 derivative carrying the *mexY* gene and 58 bp of upstream sequence, was constructed using a two-step cloning approach. First, the *mexY* gene (coding sequence only) was PCR amplified from the chromosome of K767 using primers mexY-3138-F and mexY-3138-R and cloned into pEX18Tc as a HindIII-BamHI-restricted fragment. The PCR conditions were as described above for generation of the *mexY* deletion fragments but using instead an annealing temperature of 70°C and an extension time of 100 s. The 58-bp upstream sequence (carrying the ribosome-binding site) was amplified from the chromosome of K767 also as described above but with a 30-s extension.
time, using primers mexY-58-F and MexY-58-R, and cloned into the aforementioned mexY-carrying pEX18Tc derivative as a HindIII-SalI-restricted fragment to create plasmid pCL9. The mexY gene together with its 58 bp of upstream sequence was then excised from pCL9 as a HindIII-BamHI fragment and cloned into pRK415 to yield pCL10.

Hydroxylamine mutagenesis of mexY. Ten micrograms of mexY-carrying pCL10 was mutagenized with hydroxylamine hydrochloride (460 mM) for 30 min at 70°C as previously described (44). Eighty micro- 

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| Table 2 | Bacterial strains and plasmids used in this study |
| --- | --- |
| **Strain or plasmid** | **Description** |
| **E. coli** strains |  |
| DH5α | q80lacZΔM15 endA1 recA1 hsdR17 (rK−mK−) supE44 thi-1 gyrA96 relA1 F− Δ(lacZYA-argF) U169 |
| S17-1 | thi pro hsdR recA Tra |
| **P. aeruginosa** strains |  |
| K767 | PAO1 prototroph wild type |
| K1525 | K767ΔmexXY |
| K3315 | K767ΔmexY |
| Plasmids |  |
| pEX18Tc | Broad-host-range gene replacement vector; sacB Tcr |
| pCL8 | pEX18Tc::mexY |
| pCL9 | pEX18Tc::mexY |
| pRK415 | P. aeruginosa-E. coli shuttle cloning vector; Tcr |
| pCL10 | pRK415::mexY<sub>WT</sub> |
| pCL11 | pRK415::mexY<sub>S16F</sub> |
| pCL12 | pRK415::mexY<sub>R166C</sub> |
| pCL13 | pRK415::mexY<sub>R340A</sub> |
| pCL14 | pRK415::mexY<sub>Y339M</sub> |
| pCL15 | pRK415::mexY<sub>Y460T</sub> |
| pCL16 | pRK415::mexY<sub>K79A</sub> |
| pCL17 | pRK415::mexY<sub>D133A</sub> |
| pCL18 | pRK415::mexY<sub>D133S</sub> |
| pCL19 | pRK415::mexY<sub>Y613A</sub> |
| pCL20 | pRK415::mexY<sub>V625</sub> |
| pCL21 | pRK415::mexY<sub>Y613A</sub> |

| **a** | Tcr, tetracycline resistance; WT, wild type. |

| Table 3 | Oligonucleotides used in this study |
| --- | --- |
| **Primer** | **Oligonucleotide sequence (5′ → 3′)**<sup>a</sup> | **Source** |
| mexYUP-F | ATTAAGAGCTCTACCGCCAGGCTG | This study |
| mexYUP-R | GCTGCATCTAGATCGTAGCGTTCTC | This study |
| mexYDN-F | AGTCGATCTAGATGCCCCTAGCGAAAC | This study |
| mexYDN-R | GCAGACAAGCTTCTGGCCGACTATC | This study |
| mexYscreen-F | TGTTCCGCAATCCGCATC | This study |
| mexYscreen-R | GCGTAGCCGATCATGTC | This study |
| mexY-3138-F | GGCTCGAAGCTTATGGCTCGTTTCTTC | This study |
| mexY-3138-R | ATCTAGGATCTCAGGCTTGCCTCCGTCG | This study |
| mexY-D133A-F | TGGAGAAGGCGGCCACCAGCATCCAGCTGAT | This study |
| mexY-D133A-R | ATCAGCTGGATGCTGCCGCCGCCTTCTCCA | This study |
| mexY-Y613A-F | GGCGGCTTCAGCCTGCACGGACGCGC | This study |
| mexY-Y613A-R | CAGTCCCTGCATCAATTGCT | This study |

<sup>a</sup> Restriction endonuclease cleavage sites are underlined. Site-directed mutations are bolded and italicized.
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