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Interaction of the MexA and MexB Components of the MexAB-OprM Multidrug Efflux System of *Pseudomonas aeruginosa*: Identification of MexA Extragenic Suppressors of a T578I Mutation in MexB

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A T578I mutation in MexB compromised the protein's contribution to antimicrobial resistance and negatively impacted its interaction with MexA. Mutations causing single amino acid changes in the C-terminal domain of MexA (R221H, L245F, E254K, and V259I) suppressed the antimicrobial susceptibility of a MexB$_{T578I}$-expressing *Pseudomonas aeruginosa* strain and restored a MexA interaction with MexB$_{T578I}$. These data confirm the importance of the MexA C-terminal region in MexB binding and the likely significance of the region surrounding T587I of MexB in MexA interaction.

Pseudomonas aeruginosa is an opportunistic human pathogen characterized by an innate resistance to multiple antimicrobials (6), resistance increasingly attributable, at least in part, to the operation of broadly specific, multidrug efflux systems of the resistance-nodulation-division (RND) family (16). Several RND family multidrug efflux systems have been described in *Pseudomonas aeruginosa*, although the major system contributing to intrinsic multidrug resistance is encoded by the *mexAB-oprM* operon (16, 17). The MexAB-OprM efflux system consists of an inner membrane drug-proton antiporter (the RND component) (MexB), an outer membrane channel-forming component (OprM), and a periplasmic membrane fusion protein (MFP) (MexA) (16, 17). Crystal structures have been reported for MexA (2, 7) and OprM (1), and a MexB structure (11) has been derived from modeling on the available structure of the homologous AcrB protein (14), although details of pump assembly, including the identities of interacting domains of individual pump constituents, remain largely unknown.

In vivo interactions between MexA and MexB (12, 15) and MexA and OprM (12) have been confirmed, and the MexAB-OprM tripartite complex has been recovered from *P. aeruginosa* in the absence of cross-linking (12; D. Nehme and K. Poole, unpublished data). Interestingly, MexA association with MexB is dependent upon the presence of OprM (12, 15), although MexA-OprM association may be independent of MexB (12). Similarly, genetic (5) and biochemical (9, 23, 24) studies have confirmed in vivo interactions between AcrA, AcrB, and TolC in *Escherichia coli*, and an AcrAB-TolC complex is also recoverable from *E. coli* without prior cross-linking (23). A C-terminal domain of AcrA is implicated in the binding of this MFP to its cognate RND component, AcrB (4, 24), and while mutations in the corresponding region of MexA have been isolated and shown to abrogate MexA function (15), the importance of this region vis-à-vis MexB binding has not been established. The three-dimensional model of MexB identifies a region of the protein likely to be involved in MexA binding, and indeed, a mutation here (T578I) compromised MexB activity (11). To assess, then, the involvement of the MexA C-terminal domain in MexB binding, MexA suppressors of the T578I mutation in MexB were recovered and mapped. We report here the recovery of several C-terminal MexA suppressor mutations that restore binding to the MexB$_{T578I}$ protein in vivo.

The strains and plasmids used in this study are listed in Table 1. All bacterial strains were grown as indicated previously (15). Plasmids derived from pRK415 were maintained with tetracycline (10 μg/ml, *E. coli*; 30 μg/ml, *P. aeruginosa* K2275), while plasmids derived from pMMB206 were maintained with chloramphenicol (10 μg/ml, *E. coli*; 10 μg/ml, *P. aeruginosa* K2275). Plasmid pDN34 encoding MexB$_{T578I}$ was constructed by cloning a 4.5-kb EcoRI fragment from pJKM15 (11) carrying the *mexB*(*T578I*) gene into EcoRI-restricted pMMB206. Plasmid pDN39 encoding MexB$_{E864K}$ was similarly constructed by cloning the *mexB*(*E864K*) gene from pJKM16 (11) into EcoRI-restricted pMMB206. PCR was performed according to published protocols (15) except for the addition of 5% (vol/vol) dimethyl sulfoxide to the reaction mixture and the use of an annealing temperature of 60°C. DNA sequencing was performed by ACGT Corporation (Toronto, Ontario, Canada). The antimicrobial susceptibilities of *P. aeruginosa* K2275 and its plasmid-containing derivatives were assessed as described previously (15) using an isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mM)-supplemented growth medium. The expression of the MexA and MexB proteins in *P. aeruginosa* and *E. coli* strains was assessed following sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western immunoblotting of whole-cell extracts prepared from overnight LB cultures (19) with anti-MexA (15) and anti-MexB (22) antisera.

A T578I mutation in MexB compromises interaction with MexA. A previous study conducted in this lab identified a T578I mutation in MexB that severely compromised its ability to provide antibiotic resistance in a *P. aeruginosa* strain lacking a chromosomally encoded MexB protein; this mutation dramatically reduced the resistance of the strain to a wide range of MexA-MexB-OprM antimicrobial substrates (11) (Table 2).

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Properties or genotype[a]	Source or reference
P. aeruginosa		
K870	Spontaneous Sm^r derivative of wild-type strain PAO1	18
K2275	K870 Δ*mexR* Δ*mexAB*	15
K1589	K870 Δ*mexR* Δ*mexB*	8
E. coli		
DH5α	φ80d*lac*ZΔ*M15* Δ(*lacZYA-argF*) *endA1 recA1*	3
S17-1	*thi pro hsdR recA* Tra^+	21
Plasmids		
pRK415	*P. aeruginosa-E. coli* shuttle cloning vector; Tc^r	10
pDN3	pRK415::*mexA*	15
pDN30	pRK415::*mexA*(*R221H*)	This study
pDN31	pRK415::*mexA*(*L245F*)	This study
pDN32	pRK415::*mexA*(*E254K*)	This study
pDN33	pRK415::*mexA*(*V259I*)	This study
pJKM15	pRK415::*mexB*(*T578I*)	11
pJKM16	pRK415::*mexB*(*E864K*)	11
pMMB206	*P. aeruginosa-E. coli* shuttle cloning vector; Cm^r	13
pDN25	pMMB206::*mexB*-His	15
pDN38	pMMB206::*mexA-mexB*-His	This study
pDN34	pMMB206::*mexB*(*T578I*)	This study
pDN35	pMMB206::*mexB*(*T578I*)-His	This study
pDN36	pMMB206::*mexA-mexB*(*T578I*)-His	This study
pDN37	pMMB206::*mexA*(*L245F*)-*mexB*(*T578I*)-His	This study
pDN39	pMMB206::*mexB*(*E864K*)	This study

[a] Tc^r, tetracycline resistance; Cm^r, chloramphenicol resistance; *mexB*-His, *mexB* gene was engineered to produce a MexB protein with a C-terminal hexahistidine tag; *mexB*(*T578I*)-His, *mexB* gene encoding MexB with a T578I substitution was engineered to produce a MexB protein with a C-terminal hexahistidine tag. Amino acid changes in the MexA or MexB proteins encoded by the indicated plasmids are shown in parentheses.

Interestingly, T578 occurs in a region of MexB that corresponds to a region in the homologous AcrB protein of the *E. coli* AcrAB-TolC pump implicated in interaction with its MFP component, AcrA (14). Accordingly, we predicted that $MexB_{T578I}$ was compromised in its ability to provide antibiotic resistance because it was unable to interact with MexA. To assess this directly, *P. aeruginosa* K2275 expressing plasmid-encoded MexA and wild-type MexB-His (histidine-tagged MexB) (from pDN38) or MexA and $MexB_{T578I}$-His (from pDN36) was extracted with detergent, and the extracts were incubated with Ni-nitrilotriacetic acid (Ni-NTA) agarose beads (QIAGEN, Mississauga, Ontario, Canada) to recover the histidine-tagged MexB proteins as described previously (15). Corecovery of MexA (assessed using immunoblotting) was then used as a measure of MexA binding to the corresponding MexB protein in vivo (15). The *mexB*(*T578I*) gene was first histidine tagged by excising the 3′ end of wild-type *mexB*-His from plasmid pDN25 as a KpnI-HindIII fragment and cloning it into KpnI-HindIII-restricted pDN34 to yield pDN35. This effectively swapped the untagged 3′ end of *mexB*(*T578I*) with a His-tagged 3′ end. To introduce the wild-type *mexA* gene into plasmids pDN25 and pDN35, the *mexA* gene was amplified from plasmid pDN3 using primers JT-28-EcoRI (5′-GAATTCGAATTCGAA TGTAAGTATTTTGCCTGC-3′; tandem EcoRI sites under-

TABLE 2. Antibiotic susceptibility of *P. aeruginosa* K2275 expressing mutant MexA and MexB proteins[a]

Plasmids	MexAB proteins expressed[b]	MIC (μg/ml) for[c]:			
		CAR	NOV	NAL	CEF
pDN3, pDN25	$MexA_{WT}$, $MexB_{WT}$	128	512	128	8
pDN3, pDN34	$MexA_{WT}$, $MexB_{T578I}$	4	32	16	1
pDN30, pDN34	$MexA_{R221H}$, $MexB_{T578I}$	128	256	128	8
pDN31, pDN34	$MexA_{L245F}$, $MexB_{T578I}$	128	256	128	8
pDN32, pDN34	$MexA_{E254K}$, $MexB_{T578I}$	128	256	128	8
pDN33, pDN34	$MexA_{V259I}$, $MexB_{T578I}$	128	256	128	8
pDN30, pDN39	$MexA_{R221H}$, $MexB_{E864K}$	2	16	8	1
pDN31, pDN39	$MexA_{L245F}$, $MexB_{E864K}$	2	16	8	1
pDN32, pDN39	$MexA_{E254K}$, $MexB_{E864K}$	2	16	8	1
pDN33, pDN39	$MexA_{V259I}$, $MexB_{E864K}$	2	16	8	1
pDN3, pDN39	$MexA_{WT}$, $MexB_{E864K}$	2	16	8	1

[a] *P. aeruginosa* K2275 harboring the indicated plasmids was used to perform antibiotic susceptibility testing as described in the text. IPTG was included in the growth medium to induce MexB expression from pMMB206-derived plasmids.

[b] The MexA and MexB proteins expressed from the indicated plasmids are shown, with mutations indicated in subscript. $MexA_{WT}$, wild-type MexA.

[c] CAR, carbenicillin; NOV, novobiocin; NAL, nalidixic acid; CEF, cefoperazone.

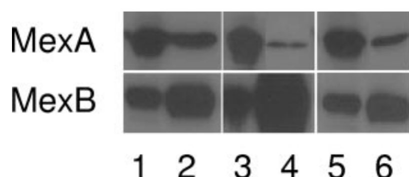

FIG. 1. Western immunoblot assessing in vivo binding of wild-type MexA and $MexA_{L245F}$ to $MexB_{T578I}$-His. Cell envelopes from *P. aeruginosa* strain K2275 carrying plasmid pDN38 (pMMB206::*mexA-mexB*-His) (lanes 1 and 2), pDN36 [pMMB206::*mexA-mexB*(*T578I*)-His] (lanes 3 and 4), and pDN37 [pMMB206::*mexA*(*L245F*)-*mexB* (*T578I*)-His] (lanes 5 and 6) were extracted as described in the text. Triton X-100-soluble extracts of cell envelope preparations were incubated with Ni-NTA agarose and Triton X-100-soluble cell envelope extracts (odd-numbered lanes), and elution fractions off Ni-NTA agarose (even-numbered lanes) were immunoblotted and developed with antibodies to MexA (top panel) and MexB (bottom panel).

lined) and JT-27 (5′-GAGCTCGAGCTCGATCACCCACGCG AAAATGG-3′). The PCR product was digested with EcoRI, freeing a *mexA*-containing 730-bp fragment from the PCR product, and cloned into EcoRI-restricted pDN25. The resulting vector, pDN38, carried the wild-type *mexA* gene upstream of *mexB*-His, with both genes under the control of the resident p*lac* promoter of pDN38. The same *mexA*-containing fragment was cloned into pDN35 upstream of the *mexB*(*T578I*)-His gene of this vector, yielding pDN36 in which *mexA* and *mexB*(*T578I*)-His were similarly controlled by p*lac*. Plasmids pDN38 and pDN36 were subsequently mobilized into *P. aeruginosa* K2275 from *E. coli* DH5α using a triparental mating procedure (25) and plasmid-containing isolates selected on chloramphenicol (10 μg/ml) and imipenem (0.5 μg/ml; to counterselect donor and helper *E. coli* strains). As seen previously, MexA was readily recovered together with wild-type MexB-His on Ni-NTA agarose beads (Fig. 1, lane 2, top panel), confirming the ability of these proteins to interact in vivo. In contrast, very little MexA was recovered together with $MexB_{T578I}$-His (Fig. 1, lane 4, top panel), despite the even higher level of $MexB_{T578I}$-His recovered from the Ni-NTA agarose beads in this experiment compared with that of wild-type MexB-His (Fig. 1, bottom panel, compare lanes 2 and 4). Clearly, $MexB_{T578I}$-His was less able to bind MexA than its wild-type counterpart was.

Isolation of MexA suppressors of $MexB_{T578I}$. To assess further the significance of T578 vis-à-vis MexB interaction with MexA and, possibly, to identify regions or residues of MexA important for this interaction, attempts were made to recover MexA suppressors of $MexB_{T578I}$. Thus, *mexA*-carrying plasmid pDN3 was submitted to hydroxylamine chemical mutagenesis as described previously (15). The pool of mutagenized plasmids was introduced into *E. coli* S17-1 via electroporation (20) and mobilized into *P. aeruginosa* K2275 (Δ*mexR* Δ*mexAB*) harboring the $MexB_{T578I}$-encoding plasmid pDN34 via conjugation (15). Selection of suppressor mutations was performed by spreading the conjugation mixture on LB agar containing tetracycline (10 μg/ml), carbenicillin (20 μg/ml), imipenem (0.5 μg/ml; as counterselection against the donor *E. coli*), and 1 mM IPTG [to induce transcription of *mexB*(*T578I*)]. *P. aeruginosa* K2275 expressing the plasmid-encoded $MexB_{T578I}$ (i.e., a MexA-$MexB_{T578I}$-OprM pump) is unable to grow in the presence of 20 μg/ml carbenicillin, while *P. aeruginosa* expressing a wild-type functional MexAB-OprM system can. Therefore, potential MexA suppressors would restore growth of K2275(pDN34) on carbenicillin. Of several transconjugants carrying possible MexA suppressors, four showed increased resistance to several antimicrobials known to be substrates for MexAB-OprM (Table 2), consistent with these transconjugants harboring a MexAB-OprM pump with restored activity. Isolation of the mutagenized pDN3 from each of these transconjugants and their subsequent reintroduction into *P. aeruginosa* K2275 confirmed that restored multidrug resistance in K2275 was indeed dependent upon the mutagenized *mexA* gene in each instance. Nucleotide sequencing of these *mexA* genes confirmed single mutations in each of the genes producing single amino acid changes in MexA (R221H, L245F, E254K and V259I; Table 2). The MexA suppressor mutations were, however, specific to $MexB_{T578I}$ and did not rescue the hypersusceptibility phenotype attributable to a MexB mutation (E864K; Table 2) situated in the predicted vestibule region of the MexB trimer (11). A representative MexA suppressor, *mexA*(*L245F*), was introduced into plasmid pDN35 as described above for wild-type *mexA*, using plasmid pDN31 as a

A

R221H
ANAMATVQQLDPIYVDVTQPSTALLRLRRELASGQLE**R**AGDNAAKVSLK 232

L245F **E254K** **V259I**
LEDGSQYPLEGR**L**EFSEVSVD**E**GTGS**V**TIRAVFPNPNNELLPGMFVHAQ 281

LQEGVKQKAILAPQQGVTRDLKGQATALVVNAQNKVELRVIKADRVIGD 330

B

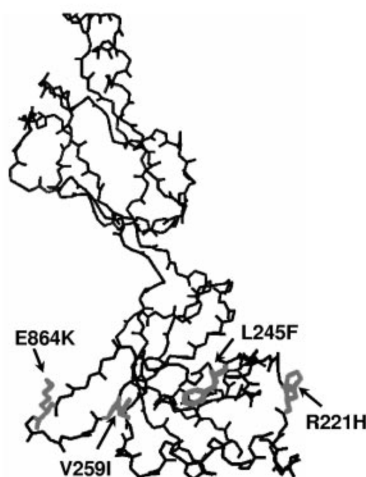

FIG. 2. Mutations in MexA that suppress the antibiotic susceptibility phenotype of $MexB_{T578I}$-expressing *P. aeruginosa*. (A) Linear sequence of the MexA C-terminal region (residues 184 to 330). Suppressor mutations are highlighted above the corresponding residue, which is shown in bold type in the MexA sequence shown. Previously identified (15) residues in MexA whose mutation compromised MexA function are underlined. The region of MexB that aligns with a proposed AcrB-binding domain of AcrA (4) is italicized. (B) Three-dimensional structure of monomeric $MexA_{29-259}$ (PDB identification number 1VF7; http://www.ncbi.nlm.nih.gov) with the mutated residues indicated in gray.

template for PCR amplification of the gene, and the resultant vector, pDN37, encoding both $MexA_{L245F}$ and $MexB_{T578I}$-His, was mobilized into *P. aeruginosa* K2275. As expected, $MexA_{L245F}$ showed markedly improved binding to $MexB_{T578I}$-His (Fig. 1, lane 6) relative to wild-type MexA.

MexA suppressor mutations map near the putative MexB-binding domain. A C-terminal domain of AcrA was previously shown to be instrumental in the interaction of this MFP component of the AcrAB-TolC efflux system with AcrB, its cognate RND partner (4). Interestingly, the four suppressor mutations isolated in the course of this study map very close to the corresponding region of MexA, with three of the changes (L245F, E254K, and V259I) within 30 amino acids of this region (Fig. 2A). This region was previously implicated in MexB binding and was the site of several amino acid changes that negatively impacted MexA function (15) (Fig. 2A). Interestingly, too, the mutant residues all map to a common face of the MexA structure (Fig. 2B), a face that in light of the above data may well be involved in MexB interaction. Still, as these occur outside the proposed interaction domain and upstream of mutations previously shown to impact MexA function, it may also be that these mutations have instead a common influence on the disposition of the "downstream" MexB-binding region (not resolved in the available MexA crystal structure), indirectly facilitating improved interaction with the $MexB_{T578I}$ protein. In possible agreement with this, the suppressor MexAs were still functional with wild-type MexB (i.e., the cloned *mexA* suppressor genes complemented the multidrug-susceptible phenotype of a Δ*mexA P. aeruginosa* strain) (data not shown).

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