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Site-Directed Mutagenesis Reveals Amino Acid Residues in the *Escherichia coli* RND Efflux Pump AcrB That Confer Macrolide Resistance▽

The *Escherichia coli* AcrB efflux pump is a resistance-nodulation-division (RND) pump that recognizes many unrelated compounds (9, 10). AcrB forms a complex with AcrA and TolC and is the single most important contributor to multidrug resistance in *E. coli*. Crystallographic models suggest that AcrB forms an asymmetric trimer in which each protomer corresponds to a distinct functional state of a proposed three-step transport cycle (8, 11, 12). Substrate specificity is predominantly determined by residues situated in the periplasmic loops of RND efflux pumps (2, 3, 4, 6, 7). Doxorubicin and minocycline could be cocrystalized with AcrB in a putative binding pocket, consisting of F136, F178, F610, F615, F617, and F628 (8). This suggests that the broad substrate spectrum of AcrB is the result of flexible interaction of ligands with mostly hydrophobic phenylalanines and, to a minor degree, with polar residues in the binding pocket.

We have recently examined the role of AcrB binding pocket phenylalanines (1) and now opted to identify nonaromatic residues that might determine drug-protein interaction specificity. Our strategy was to generate binding pocket hybrids of broad-spectrum RND efflux pumps with similar but not identical antibiotic resistance profiles. An AcrB/MexB binding pocket hybrid was considered an attractive model system, since both proteins are broad-spectrum RND pumps and share 70% sequence identity but display certain differences in their antibiotic resistance.

E. coli cells expressing only MexAB-OprM were shown to confer higher levels of resistance to cinoxacin than those expressing AcrAB-TolC, while susceptibility to ethidium bromide (EtBr) and two macrolides was found to be significantly increased (13). The same study concluded from various N-terminal AcrB/C-terminal MexB hybrids that the region defined by residues 612 to 849 contributes to the specificity toward these drugs.

We replaced the AcrB residues 615 to 628 with the homologous MexB sequence from *Pseudomonas aeruginosa* (AcrB-615-628MexB). The rationale was to choose an AcrB part that is situated within the 612-to-849 region and contains a significant portion of the putative substrate binding pocket. It contains three binding pocket phenylalanines (F615, F617, F628), conserved in AcrB and MexB, and displays a 36% sequence variation in nonaromatic residues (Fig. 1).

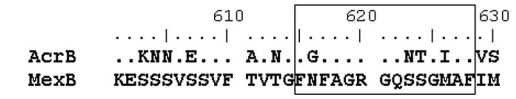

FIG. 1. AcrB and MexB sequence comparison (amino acid residues 600 to 630). AcrB residues that are identical to MexB residues are depicted as a dot. The box marks the region from residue 615 to 628 used for site-directed mutagenesis.

We used as the parental strain the multidrug-resistant (*gyrA marR*) *acrB*-overexpressing *E. coli* K-12 strain 3-AG100 (5).

Site-directed mutagenesis of strain 3-AG100 and confirmation by DNA sequencing were performed as described elsewhere (1, 2).

MIC assays (2) were carried out on 96-well plates, and the results are given in Table 1.

The complete disruption of *acrB* led to a highly drug-susceptible phenotype. The AcrB-615-628MexB hybrid displayed a highly specific reduction in macrolide resistance (MR).

To identify the residue, which was responsible for the reduction in MR, we reintroduced single AcrB-specific mutations (N616G, S623N, and M626I) into AcrB-615-628MexB. AcrB-615-628MexB-N616G was found to completely restore the AcrB-specific MR. AcrB-615-628MexB-S623N partially restored the clarithromycin and erythromycin but not the azithromycin resistance. The AcrB-615-628MexB-M626I mutant did not regain MR.

TABLE 1. MICs of different pump substrates from AcrB mutants of *E. coli* 3-AG100

Mutant[a]	MIC (μg/ml)[b]													
	Erythromycin	Clarithromycin	Azithromycin	Clindamycin	Novobiocin	Levofloxacin	Moxifloxacin	Minocycline	Chloramphenicol	EtBr	Pyronin Y	Hoechst 33342	Linezolid	Oxacillin
AcrB-F628F (pseudomutant)	512	512	64	256	512	1	2	4	8	512	32	4	512	512
acrB::*rpsLneo*	**4**	**4**	**0.5**	**4**	**4**	**0.06**	**0.06**	**0.125**	**1**	**16**	**0.5**	**0.125**	**16**	**0.5**
AcrB-615-628MexB	**128**	**64**	**16**	256	512	1	2	2	16	512	16	4	1,024	256
AcrB-615-628MexB-N616G	256	512	64	ND	512	1	ND	2	8	512	64	ND	1,024	256
AcrB-615-628MexB-S623N	256	128	16	ND	1,024	1	ND	2	16	512	32	ND	1,024	512
AcrB-615-628MexB-M626I	**128**	**64**	**8**	ND	512	1	ND	4	8	512	32	ND	1,024	256
AcrB-I626M	512	512	32	ND	512	1	ND	4	8	1,024	32	ND	512	512
AcrB-G616N	**128**	**128**	**16**	ND	512	1	ND	4	8	1,024	32	ND	512	256
AcrB-N623S-Q624S	ND	512	ND	ND	512	1	ND	4	8	1,024	32	ND	512	256

[a] F628F is a pseudomutant with MICs corresponding to wild-type strain 3-AG100, which was generated to demonstrate that the site-directed mutagenesis technique (1, 2) has no inherent effect. AcrB-615-628MexB is a 3-AG100-derived strain where AcrB amino acid residues 615 to 628 were replaced with the homologous MexB residues.

[b] MICs that differ ≥fourfold from that of *E. coli* 3-AG100 are shown in boldface type. ND, not determined.

To prove that G616 and not I626 is responsible for the AcrB-specific MR, we introduced the G616N mutation into the wild-type AcrB sequence of strain 3-AG100 and could reproduce the phenotype of the AcrB-615-628MexB hybrid. In contrast, the I626M mutation in AcrB had no relevant effect, supporting the specificity of residue 616. The AcrB-N623S-Q624S mutant revealed no distinctive phenotype.

We conclude that residues at *E. coli* AcrB position 616 determine the level of MR, with glycine leading to a macrolide-resistant and asparagine leading to a macrolide-sensitive phenotype. No nonmacrolide antibiotic MICs were affected by the MexB-specific mutation G616N. Other mutations have been found near the suggested binding pocket that have an impact on macrolide MICs (6, 14) but are not as highly specific as the one that we have characterized in this study.

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