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Site-Directed Mutagenesis Reveals Putative Substrate Binding Residues in the *Escherichia coli* RND Efflux Pump AcrB▽

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The *Escherichia coli* multidrug efflux pump protein AcrB has recently been cocrystallized with various substrates, suggesting that there is a phenylalanine-rich binding site around F178 and F615. We found that F610A was the point mutation that had the most significant impact on substrate MICs, while other targeted mutations, including conversion of phenylalanines 136, 178, 615, 617, and 628 to alanine, had smaller and more variable effects.

The *Escherichia coli* AcrB multidrug efflux pump is a member of the resistance-nodulation-division (RND) family and recognizes many chemically unrelated compounds, including various dyes and antibiotics (10, 11). AcrB cooperates with the membrane fusion protein AcrA and the TolC outer membrane protein.

While previous crystallographic studies with crystals grown in trigonal space group R32 described a symmetric AcrB trimer, recent studies of structures derived from monoclinic crystals described an asymmetric trimer in which each protomer was suggested to correspond to a distinct functional state of a proposed three-step transport cycle reminiscent of a peristaltic pump (9, 12, 13). In this model, the protomer in its binding or tight-state conformation forms a hydrophobic pocket defined by phenylalanines 136, 178, 610, 615, 617, and 628.

Analysis of doxorubicin- and minocycline-complexed AcrB crystals suggested that these two compounds interact with different residues of the binding protomer. Minocycline seemed to interact with F178, N274, and F615, while doxorubicin seemed to interact with Q176, F615 and F617 (9). Thus, it was proposed that the extremely broad substrate spectrum of AcrB could be explained by the flexible interaction of various ligands mostly with hydrophobic phenylalanines and to a minor degree with polar residues in the binding pocket.

Support for this model also came from several mutational studies which found that substrate specificity in RND efflux pumps is determined by residues in the periplasmic domain (2–4, 7, 8). A recent study found that the V610F mutation in the *E. coli* RND efflux pump YhiV, which is homologous to the V612F mutation in AcrB, leads to a 16-fold increase in the linezolid MIC compared to the MIC of the YhiUV-overproducing wild-type strain (2).

However, no systematic site-directed mutagenesis study of the phenylalanine residues that form the proposed hydrophobic binding pocket in AcrB has been described previously.

In the present study we constructed and tested such phenylalanine mutants to examine the functional role of hydrophobic residues in the proposed AcrB multidrug binding site. We used as the parental strain the previously described multidrug-resistant (*gyrA marR*) *acrB*-overexpressing *E. coli* K-12 strain 3-AG100 that was obtained after repeated exposure to a fluoroquinolone (5).

For site-directed mutagenesis the phage λ-based homologous recombination system (Red/ET counterselection Bac modification kit; GeneBridges, Heidelberg, Germany) was used to introduce an *rpsL-neo* cassette into the *acrB* gene of strain 3-AG100 (grown in Luria-Bertani broth) and to subsequently replace the cassette with an appropriate oligonucleotide (the sequences of the PCR primers and oligonucleotides that were obtained from Thermo Electron [Ulm, Germany] are shown in Table 1). Recombination events were confirmed by PCR and nucleotide sequencing of the *acrB* gene using standard techniques.

To confirm production of the mutant AcrB protein, we performed Western blotting using standard techniques. Most of the mutants exhibited a strong immunogenic response; the only exception was an F615A/F617A/F628A triple mutant which was excluded from further study due to insufficient AcrB expression (Fig. 1).

We used as a positive control strain F628F, which is a pseudomutant with MICs and ethidium bromide (EtBr) and phenylalanine-arginine β-naphthylamide (PAβN) accumulation properties corresponding to those of wild-type strain 3-AG100. F628A is characterized by a silent mutation from TTC to TTT (sequence shown in Table 1) that demonstrates that the site-directed mutagenesis technique has no inherent effect.

The susceptibilities of the different mutants to various antimicrobials and dyes and to the putative efflux pump inhibitors 1-naphthylmethylpiperazine (NMP) and PAβN were characterized by determining MICs in 96-well microtiter plates as described previously (1, 2, 6) and are shown in Table 2. EtBr

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TABLE 1. Oligonucleotides and primers used for Red/ET-recombination[a]

Oligonucleotide	Sequence (5′ → 3′)
Upper-oligoI (615–628)	ATCTGACCAAAGAAAAGAACAACGTTGAGTCGGTGTTCGCCGTTAACGGC*GGCCTGGTGATGATGGCGGGATCG*
Lower-oligoI (reverse complement orientation)	TCAACTTTGTTTTCTTCGCCCGGACGATCGGCCCAGTCCTTCAAGGAAAC*TCAGAAGAACTCGTCAAGAAGGCG*
Upper-oligoII (130–149)	TGGCGATGCCGTTGCTGCCGCAAGAAGTTCAGCAGCAAGGGGTGAGCGTT*GGCCTGGTGATGATGGCGGGATCG*
Lower-oligoII (reverse complement orientation)	ATGGCATCTTTCATATTCGCCGCCACGTAGTCGGAGATATCCTCCTGCGT*TCAGAAGAACTCGTCAAGAAGGCG*
Upper-oligoIII (175–194))	TGGCGGCGAATATGAAAGATGCCATCAGCCGTACGTCGGGCGTGGGTGAT*GGCCTGGTGATGATGGCGGGATCG*
Lower-oligoIII (reverse complement orientation)	TTCTGCGCTTTGATGGCGGTAATGACATCAACCGGCGTTAGCTGGAATTT*TCAGAAGAACTCGTCAAGAAGGCG*
Repair-oligo 1: rep-acrB-Phe610Ala	ACGTTGAGTCGGTG**GCA**GCCGTTAACGGCTTCGGCTTTGCGGGACGTGGTCAGAATACCGGTATTGCGTTCGTTTCCTTGAAGGACTGGGCCGATCGTCC
Repair-oligo 2: rep-acrB-Phe615Ala	ACGTTGAGTCGGTGTTCGCCGTTAACGGC**GCA**GGCTTTGCGGGACGTGGTCAGAATACCGGTATTGCGTTCGTTTCCTTGAAGGACTGGGCCGATCGTCC
Repair-oligo 3: rep-acrB-Phe617Ala	ACGTTGAGTCGGTGTTCGCCGTTAACGGCTTCGGC**GCA**GCGGGACGTGGTCAGAATACCGGTATTGCGTTCGTTTCCTTGAAGGACTGGGCCGATCGTCC
Repair-oligo 4: rep-acrB-Phe628Ala	ACGTTGAGTCGGTGTTCGCCGTTAACGGCTTCGGCTTTGCGGGACGTGGTCAGAATACCGGTATTGCG**GCA**GTTTCCTTGAAGGACTGGGCCGATCGTCC
Repair-oligo 5: rep-acrB-Phe628Phe	ACGTTGAGTCGGTGTTCGCCGTTAACGGCTTCGGCTTTGCGGGACGTGGTCAGAATACCGGTATTGCG**TTT**GTTTCCTTGAAGGACTGGGCCGATCGTCC
Repair-oligo 6: rep-acrB-Del615–617	GAACAACGTTGAGTCGGTGTTCGCCGTTAACGGC^^^^^^^^^GCGGGACGTGGTCAGAATACCGGTATTGCGTTCGTTTCCTTGAAGGACTGGGCCGATCGTCCGGGCG
Repair-oligo 7: rep-acrB-Phe136Ala	GCCGCAAGAAGTTCAGCAGCAAGGGGTGAGCGTTGAGAAATCATCCAGCAGC**GCA**CTGATGGTTGTCGGCGTTATCAACACCGATGGCACCATGACGCAGGAGGATATCTCCGACTACGTGGCGGCGA
Repair-oligo 8: rep-acrB-Phe178Ala	AGATGCCATCAGCCGTACGTCGGGCGTGGGTGATGTTCAGTTG**GCA**GGTTCACAGTACGCGATGCGTATCTGGATGAACCCGAATGAGCTGAACAAATTCCAGCTAACGCCGGTTGATGTCATTACCG
Forward primer for amplification of repair oligonucleotides 1–6	ATCTGACCAAAGAAAAGAACA<u>ACGTTGAGTCGGTG</u>
Reverse primer for amplification of repair oligonucleotides 1–6	<u>CGCCCGGACGATCGGCCCAGTCCTT</u>
Check-forward primer I	CCTTCTTGCCAGATGAGGAC
Check-reverse primer I	GCAGTACCCAGTTCCACGAT
Check-forward primer II	GTGCAGATCACCCTGACCTT
Check-reverse primer II	CGTTCTGCGCTTTGATGG
Check-forward primer III	ACCATGACGCAGGAGGATA
Check-reverse primer III	TAAGCTGTTGGCCTTTCACC

[a] The upper and lower oligonucleotides include the primer sequences for amplification of the *rpsL-neo* cassette (indicated by italics). The 5′ parts of the oligonucleotides are homologous to the corresponding *acrB* regions upstream and downstream (nucleotides 1793 to 1842 and 1885 to 1934 for exchange region I, nucleotides 338 to 387 and 448 to 497 for region II, and nucleotides 473 to 522 and 583 to 632 for region III). The exchanged nucleotide triplets in the repair oligonucleotides are indicated by bold type (e.g., GTT is changed to TTT at nucleotides 1834 to 1836 in *acrB*). The underlined sequences in the amplification primers are the priming parts for the repair oligonucleotides, which have to be elongated. The Check-forward and Check-reverse primers are used to confirm successful exchange of the *rpsL-neo* cassette and to sequence the modified region of *acrB* (check PCR product for *acrB* region I, nucleotides 1685 to 2030; check PCR product for region II, nucleotides 262 to 634; check PCR product for region III, nucleotides 442 to 691).

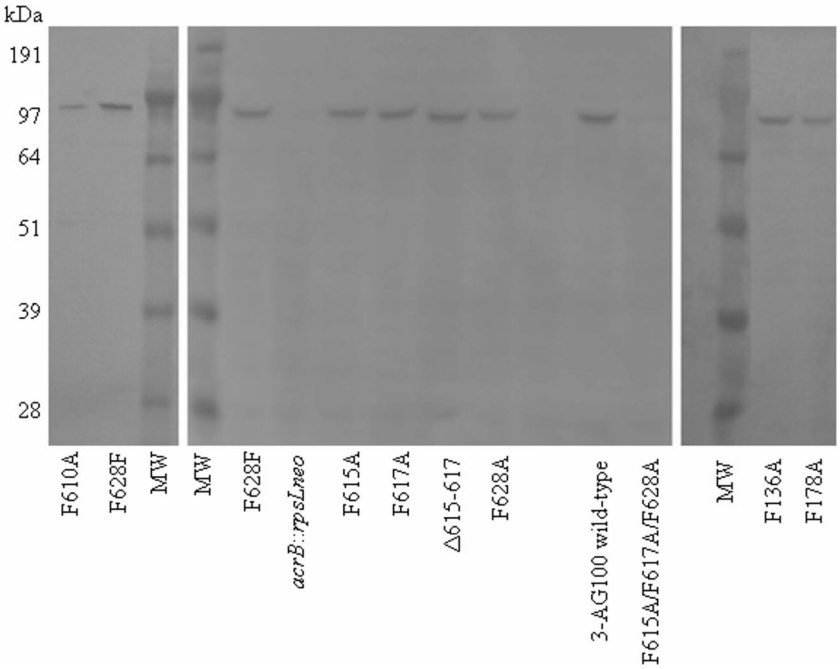

FIG. 1. Western blot analysis of mutant AcrB production. Total protein extracts of *E. coli* 3-AG100 mutants (14 μg protein) were separated by NuPAGE Novex bis-Tris (Invitrogen, California) gel electrophoresis and probed with polyclonal anti-AcrB antibodies. Lanes MW contained molecular weight markers.

(external concentration, 2.5 μM) and PAβN (external concentration, 200 μM) fluorescence accumulation assays were carried out at least in duplicate for 30 min using our previously described protocol (2). Both EtBr and PAβN are excellent substrates of AcrAB-TolC and were chosen since they are structurally diverse; thus, the recognition by the AcrB binding pocket was assumed to be mediated by different residues. EtBr is a nonspecific DNA intercalator which, upon binding to its target structure, causes enhancement of fluorescence, while the intrinsically low-fluorescence compound PAβN is cleaved by esterases, yielding the highly fluorescent compound β-naphthylamine as described previously in a study using the related substrate Ala-Nap (naphthylamide) (6). The results obtained are shown in Fig. 2a and 2b. We also used an EtBr concentration of 25 μM and a PAβN concentration of 20 μM and obtained similar results (data not shown).

The complete disruption of *acrB* by the *rpsL-neo* cassette led to a highly drug-susceptible phenotype and dramatic increases in EtBr and PAβN accumulation. The positive control pseudomutant F628F displayed no changes in the MIC assays or in the dye accumulation assays. Novobiocin was the only drug whose MIC was consistently markedly reduced for every single mutant. The F136A, F178A, F615A, F617A, and F628A mutations had very variable effects on substrate MICs. In addition to the susceptibility of the mutants to novobiocin, the MICs of oxacillin and the macrolides tested were also reduced in all but the F617A mutant. F178A markedly increased the susceptibility to linezolid, and the F628A mutation was found to reduce the MICs of Hoechst 33342, pyronine Y, and minocycline more than 4-fold and to increase EtBr and PAβN accumulation ~2-fold after 30 min.

The crystallographic structure of the asymmetric AcrB trimer suggests that the main interactions between substrates and protein are due to an ensemble of phenylalanines mediating hydrophobic interactions, which might explain the extremely broad substrate specificity (Fig. 3). Surprisingly, although the AcrB cocrystallization with doxorubicin and minocycline suggested that there is a strong interaction of these substrates with F178 and F615, the F615A and F178A mutations had no measurable impact on the MICs of these two substrates. Deletion of amino acids 615 to 617 was associated with minor changes in the susceptibility to minocycline and some changes in the MICs of macrolides and oxacillin.

The lack of correlation between the changes in susceptibility to doxorubicin and minocycline and the model derived from cocrystallization might have been due to redundancy of phenylalanines in the binding pocket. Mutating or deleting only one or even two phenylalanines might only lead to a (slight) reorientation of the substrate and use of other phenylalanines as hydrophobic interaction partners without generally compromising substrate capture. However, bulkier substrates, like the macrolides or novobiocin, might be unable to adapt properly in the altered environment of the pocket and might be affected more by a single mutation.

To test this hypothesis, we generated the F615A/F617A/F628A triple phenylalanine mutant; however, since we obtained only a weak Western blot band (Fig. 1), suggesting that the level of expression of the mutant AcrB protein was low, we did not include this mutant in further analyses.

The F610A mutant, however, displayed dramatically en-

TABLE 2. MICs of different pump substrates for AcrB mutants of *E. coli* 3-AG100[a]

Mutation	MIC (μg/ml)																			
	Oxacillin	Doxorubicin	Novobiocin	Clarithromycin	Erythromycin	Azithromycin	Clindamycin	Pyronin Y	Linezolid	Minocyclin	EtBr	Levofloxacin	Ciprofloxacin	Hoechst 33342	Propidium iodide	PAβN	Chloramphenicol	Tetracycline	NMP	Spectinomycin	Gentamicin
F628F wild type (pseudomutation)	>256	>256	512	512	512	64	256	32	512	4	>256	1	0.5	4	>512	>400	8	4	400	32	8
acrB::*rpsL-neo*	0.5	2	4	4	4	0.5	4	0.5	16	0.125	16	0.06	0.03	0.25	128	50	1	1			
F136A	64		64	64	64	16															
F178A	32		16	64	128	16			64					0.5							
F610A	64	128	32	16	64	2	32	2		0.25	128	0.13	0.06		256	100	2	1			
F615A	64		64	32	32																
F617A			128																		
Δ615–617	64		32	128	128					1											
F628A	128		128	64		16		8		1				1							

[a] Gentamicin, spectinomycin, and NMP are not substrates of AcrB and were used as controls. For the mutants only the MICs that were ≥4-fold different from the wild-type MIC (F628F pseudomutant) are shown. The substrates and control substances are in order (from left to right) based on the differences in the MICs between the wild type (pseudomutant F628F) and the inactivation mutant (*acrB*::*rpsL-neo*).

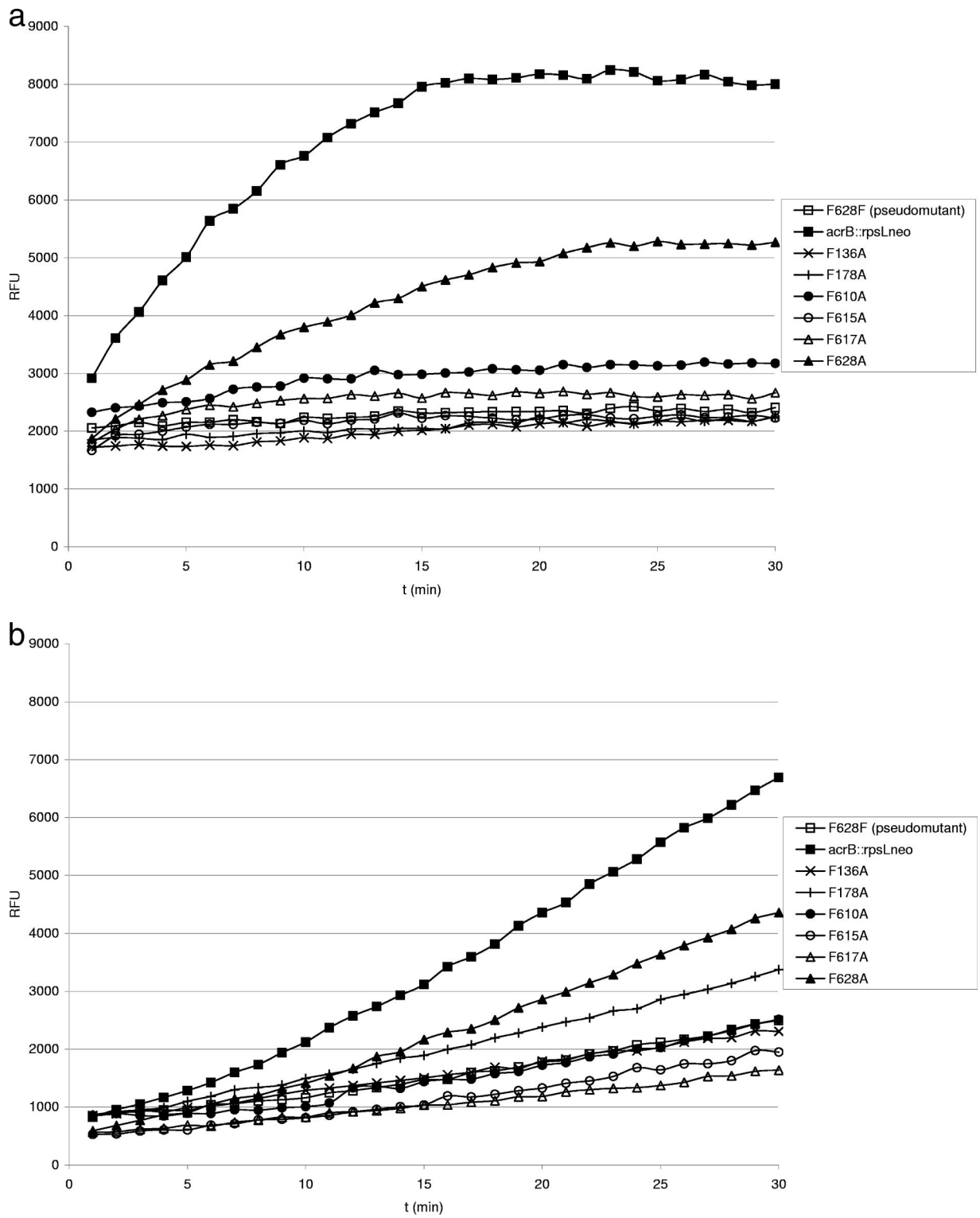

FIG. 2. Increases in EtBr (a) and PAβN (b) fluorescence in AcrB phenylalanine mutants compared to pseudomutant AcrB strain F628F. Fluorescence was recorded for 30 min after addition of 2.5 μM EtBr or 200 μM PAβN. The values are means of at least duplicate experiments. RFU, relative fluorescence units.

hanced susceptibility to almost all AcrB substrates tested (but not to aminoglycosides and NMP, which are not AcrB substrates), although the absolute changes varied considerably for different substrates. In contrast, the F610A mutation increased EtBr accumulation only moderately and did not affect PAβN accumulation. This difference might have been due to the different time windows between the MIC and fluorescence experiments. The dramatic impact on substrate MICs indicates

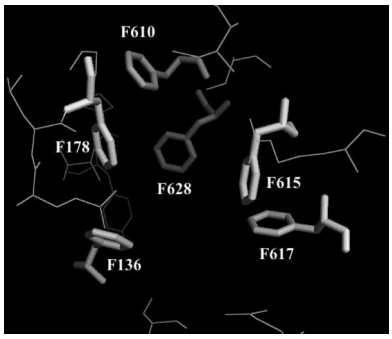

FIG. 3. AcrB binding pocket based on the "tight" monomer 2GIF structure coordinates (12). Phenylalanines are indicated by sticks. The image was generated using the molecular visualization software PyMol (http://pymol.sourceforge.net).

that the F610 residue has a special role in the substrate extrusion process, although the exact mechanism remains unclear. The other targeted mutations, including conversion of phenylalanines 136, 178, 615, 617, and 628 to alanine, generally had smaller effects on substrate susceptibility and presumably efflux function and binding, and the effects were variable depending on the substrate.

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