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Directed evolution of *Escherichia coli* AcrB may contribute to better understanding the mechanisms of action of known bacterial RND-type efflux pump inhibitors

Sabine Schuster, Samay Kohler, Annika Buck, Jürgen A. Bohnert, Winfried V. Kern

Department of Medicine, Center for Infectious Diseases & Travel Medicine, University Hospital, Freiburg

Background

- 1-(1-naphthylmethyl)-piperazine (NMP) and phenylalanine-arginine-β-naphthylamide (PAβN) are presumed RND-type efflux pump inhibitors (EPIs) [1, 2].
- It is hypothesized that they directly affect AcrB, the pumping part of the AcrAB-TolC efflux complex from *E.coli*.
- Various data from co-crystallization studies [3], computer simulations [4] and site-directed mutagenesis [5] give insights in substrate binding of RND pumps, but less information exists with respect to the mode of action of EPIs.

Objectives:

- We evaluate the usefulness of a directed evolution strategy by initially targeting the first periplasmic loop of AcrB for understanding EPI targets and mechanisms of action

Methods:

- In-vitro random mutagenesis with MutazymII® (Stratagene) error prone PCR adjusting an average mutation rate of 2 mutations per kb; mutagenesis products were introduced into the chromosome of the AcrB overexpressing *E.coli* strain 3AG100 by a two-step Red/ET® homologous recombination method. In short, at first the gene region corresponding to the first periplasmic loop of AcrB was replaced by a selection cassette and in a second step it was exchanged by error prone PCR products. Successful recombination was detectable by restored function of AcrAB-TolC.
- Appropriate drug/EPI combinations were used for pre-selection of mutants with putative reduced EPI efficacy. Phenotypes were further studied by MIC assays.
- Mutants of interest were sequenced. The impact of mutations was proved by site-directed mutagenesis and subsequent testing by MIC, accumulation and efflux assays.

	Selection plates (μg ml^{-1})				
	LIZ 90 + NMP 100	CAM 8 + NMP 100	NOV 16 + PAβN 25	NOV 32 + PAβN 25	CLA 16 + PAβN 25
Plated recombinants*	120,000	60,000	60,000	50,000	80,000
Colonies obtained from selection (growth 24 h)	144	24	–	–	34
Colonies obtained from selection (growth 48 h)	nd	nd	102	1	nd
MIC testet	144	21	102	1	34
Mutants with ≥ 4-fold reduced EPI activity (in combination with the drug used for selection)	128	2	6	1	–
Mutants with ≥ 4-fold reduced EPI activity in combination with ≥ 3 drugs	67	3	–	–	–
Mutants with mutations in acrB	40	5	4	–	–

Table 1: AcrB mutants (amino acid region 29-330) achieved from different selection conditions (drug +EPI). LIZ: Linezolid, CAM: Chloramphenicol, NOV: Novobiocine; CLA: Clarithromycine; nd: not detected.
*) *acrB* recombinants successfully repaired by Mutazymll® PCR products.

Results:

- The majority of mutants preselected on linezolid/NMP revealed an up to 4-fold decrease in EPI activity with one or more substrates (table 1, 3).
- 38 from 45 sequenced mutants harboured an alteration near the phenylalanine-rich putative substrate binding pocket most frequently from a non-polar to a polar amino acid.
- Gly288 was a key residue (fig. 1, table 2); Gly288Ser mutants generated by site-directed mutation using synonymic triplets yielded identical phenotypes.
- Gly141Asp-Asn282Tyr double mutants showed strong effects on NMP EPI efficacy with respect to linezolid MICs (fig.1, 2; table 3); interestingly, unlike the partial EPI resistance in linezolid assays, we observed enhanced ethidium accumulation with NMP (fig. 3).
- With drug/PAβN selection stable phenotypes with regard to reduced EPI activity were infrequent, and were associated with increased resistance against the drug itself rather than with EPI resistance.

	Frequency
Gly288Ser	**15**
Gly288Met	**3**
Gly288Cys	**1**
Ala279Thr	**6**
Gly141Asp	**5**
Gly141Asp-Asn282Tyr	**2**

Table 2: Number of detected mutations (from 45 drug/NMP selected mutants) at frequently altered aminos acid sites of AcrB

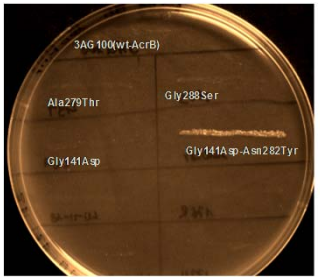

Fig. 2: Mutants streaked on a selective agar plate (LB containing 90 μg mL^{-1} linezolid + 100 μg mL^{-1} NMP)

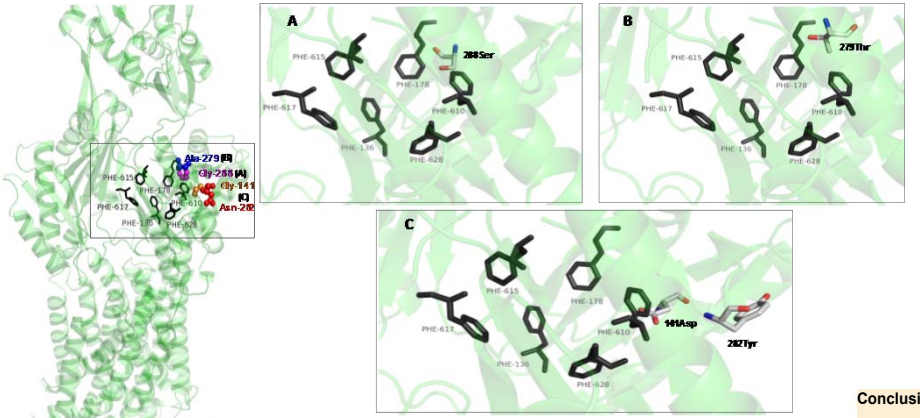

Fig. 1: Most frequently occurring mutations Gly288Ser (A) and Ala279Thr (B) and the double mutation Gly141Asp-Asn282Tyr (C). The putative binding pocket phenylalanines seen from the periplasmic outer face of the binding state AcrB protomer (PDB: 2HRT chain B; www.pdb.org) are indicated as black sticks.

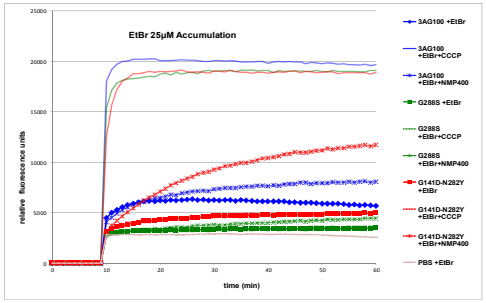

Fig. 3: Ethidium bromide (EtBr) accumulation assay in absence and presence of 400μM NMP (100 μg mL^{-1}). CCCP (200μM) was used as control for maximum accumulation. EtBr was added at min 9. 3AG100: wild-type AcrB strain.

Strain	Oxacillin	+ NMP	Linezolid	+ NMP	Novobiocine	+ NMP	EtBr	+ NMP
3AG100(wt-AcrB)	512	64	1024	32	2048	256	1024	128
A279T	512	64	1024	64	256	128	1024	256
G288S	256	64	1024	64	256	64	1024	256
N282Y	512	128	1024	32	512	128	nd	nd
G141D	256	128	1024	64	1024	256	512	128
G141D-N282Y	256	64	1024	**256**	1024	128	512	64

Table 3: MICs (μg mL^{-1}) in the absence and presence of NMP of site-directed generated mutants harbouring mutations detected by directed evolution. nd: not detected; wt: wild type.

Conclusions:

- Our in-vitro random mutagenesis studies targeting the first periplasmic loop of AcrB revealed mutants with prominent (mostly substrate specific) altered NMP efficacy, whereas with PAβN we failed to generate significant "EPI resistance" so far.
- Directed evolution targeting an MDR transporter is an adequate method for contributing data potentially valuable for rational EPI design. Pursuing this strategy, more rounds of random mutagenesis should follow. Furthermore, advanced studies have to comprise the entire pump as well as further selection conditions.

Correspondence: sabine.schuster@uniklinik-freiburg.de

References:

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