Supplementary Tables

Conformational plasticity across phylogenetic clusters of RND multidrug efflux pumps and its impact on substrate specificity

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Table S1: Uniprot accession numbers of the sequences used for the sequence similarity analysis. The representative proteins of the HAE-1 RND transporter family in the transporter classification database (Saier et al. 2006) (accessed 18.08.2023) with addition of the BpeF and CmeB sequences were analysed. The protein sequences deposited in Uniprot (https://www.uniprot.org/, accessed: 18.08.2023) were used. Abbreviations: *Ec: Escherichia coli; Rr: Rhizobium radiobacter; Ab: Acinetobacter baumannii; Ng: Neisseria gonorrhoeae; Pa: Pseudomonas aeruginosa; Pp: Pseudomonas putida; Sm: Stenotrophomonas maltophilia; Ka: Klebsiella aerogenes; Bg: Burkholderia glumae; Cj: Campylobacter jejuni; Cc: Campylobacter coli; Bp: Burkholderia pseudomallei; St: Salmonella typhimurium; Vp: Vibrio parahaemolyticus; Ft: Francisella tularensis; Vc: Vibrio cholerae; Smr: Serratia marcescens; Rs: Ralstonia solanacearum; Abr: Alcanivorax borkumensis; Kp: Klebsiella pneumoniae; Ea: Erwinia amylovora; Hi: Haemophilus influenzae; Pf: Pseudomonas fluorescens*

Protein	**Uniprot ID**
Ec_AcrB	P31224
Ec_AcrF	P24181
Rr_IfeB	O68441
Ab_AdeE	Q8GKU1
Ng_MtrD	Q51073
Pa_MexB	P52002
Ec_AcrD	P24177
Pp_ArpB	Q9KJC2
Pp_TtgB	O52248
Pp_TtgE	Q9KWV4
Pp_TtgH	Q93PU4
Ec_MdtB	P76398
Ec_MdtC	P76399
Ec_MdtF	P37637
Sm_SmeW	B2FLY4
Pa_MexD	Q51396
Pa_MexF	Q9I0Y8
Pa_MexK	Q9HXW4
Pf_EmhB	Q6V6X8
Ka_EefB	Q8GC83
Bg_ToxH	Q4VSJ4
Pa_MexY	Q9ZNG8
Cj_CmeB	Q8RTE4
Cc_CmeB	A0A1L2IWC1

Bp_BpeB	Q6VV68
Bp_AmrB	O87936
St_GesB	Q8ZRG9
Vp_VmeB	Q2AAU3
Pa_TriC	Q9I6X4
Ft_AcrB	A0Q8A5
Ab_AdeJ	Q24LT7
Vc_VexF	A6P7H3
Smr_SdeB	Q84GI9
Pa_MexI	Q9HWH4
Pa_MexW	A0A6A9K223
Pa_MexQ	Q4LDT6
Pa_MexN	Q4LDT8
Vc_VexB	Q9KVI2
Vc_VexD	A6P7H1
Vc_VexK	Q9KRG9
Pa_MuxC	Q9I0V7
Pa_MuxB	Q9I0V6
Ab_AdeB	Q2FD70
Sm_SmeB	Q9RBY8
Sm_SmeE	Q9F240
Sm_SmeJ	A0A0U5D5D4
Ab_AdeG	Q2FD81
Rs_AcrB	Q8Y3H0
Abr_AcrB	Q0VQY6
Kp_OqxB	C5IZH1
Ea_AcrB	E3DBE3
Kp_AcrB	Q93K40
Hi_AcrB	Q57124
Bp_BpeF	Q63NK6

Table S2: AcrB substrates for the phenotype assays. The table lists the AcrB substrates used in the plate dilution assays (PDA) and for minimal inhibitory concentration (MIC) determination. The substrate class, the abbreviation used in the figures and the concentration used in the PDAs are indicated.

Substrate	Class	Abbreviation	Concentration [µg/mL]
Chloramphenicol	Phenicol	CAM	1.5
Thiamphenicol	Phenicol	TIA	12
Linezolid	Oxazolidinone	LIN	20
Tetracycline	Tetracycline	TET	0.3
Oxytetracycline	Tetracycline	OxTET	0.25
Tigecycline	Tetracycline	TIG	0.125
Chlortetracycline	Tetracycline	ClTET	0.2
Doxycycline	Tetracycline	DXC	0.5
Minocycline	Tetracycline	MIN	0.3
Dicloxacillin	Beta-Lactam (Penicillin)	DIC	36
Oxacillin	Beta-Lactam (Penicillin)	OXA	16
Piperacillin	Beta-Lactam (Penicillin)	PIP	0.075
Dodecyl-β-D-maltosid	Detergent	DDM	75
Sodium dodecyl sulfate	Detergent	SDS	70
Erythromycin	Macrolide	ERY	20
Clarythromycin	Macrolide	CLA	-
Novobiocin	Aminocoumarin	NOV	10
Fusidic acid	Steroid	FUA	12
Doxorubicin	Anthracycline	DOX	40
Hoechst33342	Dye	H33342	5
Tetraphenylphosphonium	Phosphonium cation	TPP	300

Table S3: Minimal inhibitory concentration (MIC) values. BW25133 Δ*acrB* cells expressing AcrB wildtype (wt) or V612 variants were exposed to a serial dilution of a toxic substrate. The inactive D407N was used as negative control. The MIC value corresponds to the first dilution step at which no growth was detected. The table shows the mean MIC value for at least three biological replicates in µg/mL.

	wt	D407N	V612F	V612W	V612N	V612A
Chloramphenicol	4	1-2	8	8	8	8
Thiamphenicol	32	16	64	32-64	64	32
Linezolid	128	8	256	256	128-256	256
Tetracycline	1	0.25-0.5	1	1	1	1
Oxytetracycline	0.5	0.25	1	1	1	1
Tigecycline	0.25	0.125	0.125-0.25	0.25	0.25	0.25
Chlortetracycline	1	0.25	1	1	1	1
Doxycycline	2	0.25	2	2	2	2
Minocycline	4-16	0.25-8	0.5-2	16	16	16
Dicloxacillin	256	8-32	128	128	128	256
Oxacillin	128	2-8	64	64-128	64	128
Piperacillin	0.5	0.0625-0.125	0.125-0.25	0.25-0.5	0.25-0.5	0.5
SDS	>8192	64	64	64-128	>8192	>8192
Erythromycin	128	8	64	64	64	64-128
Clarythromycin	64-128	4-16	32-64	64	32	64
Novobiocin	8-16	1-4	2-8	8	8	16-32
Fusidic acid	64-128	8-16	32-64	32-64	32	64
Hoechst33342	4-8	1-2	2-4	2	4	4

Table S4: Estimated binding free energies for the docking poses in figure S5. ΔGb gives the total binding free energy **in kcal/mol.** The contributions from each individual residue involved in the interactions are indicated. Values are coloured from highest (white) to lowest (grey). Abbreviations: MIN – minocycline, DOX- doxorubicin, ERY – erythromycin, CAM – chloramphenicol.

MIN

	ΔGb	S48	Q176	L177	F178	G179	S180	E273	N274	I277	A279	S287	F610	V/F/W612	F615	R620
wt	-28.2	-0.7		-1.4	-6.3	-4.2	-1.3	-1.5	-5.7	-5.8	-0.7	-0.6	-0.8	-1.5	-2.4	-7.8
V612F	-28.7	-1.0	-1.1	-3.0	-6.4	-7.1	-2.6		-4.3	-5.2	-1.0		-0.6	-2.1	-1.0	
V612W	-25.6	-3.5	-0.7	-1.8	-5.8	-4.0	-2.3	-1.3	-5.3	-5.0	-0.7			-2.3	-1.5	

DOX

	ΔGb	T44	S46	Q89	S128	E130	S132	S133	S134	F136	Q176	F178	G179	I277	F610	V/F/W612	F615	F617	R620	F628
wt	-53.5	-1.1	-5.9	-3.8	-6.7	-7.8				-1.3	-5.2	-5.5		-1.9	-1.0	-2.0	-2.3	-0.7		
V612F	-46.4		-2.2	-3.4	-2.2	-4.9			-1.1		-5.8	-7.8	-1.2	-3.4	-0.8	-4.0	-3.1		-1.8	-1.1
V612W	-54.2	-2.9		-5.1		-5.3	-7.7	-1.7	-5.7	-2.1	-3.6	-1.9				-3.5	-5.9	-1.0		

ERY

	ΔGb	T44	Q89	T91	E130	S132	S133	S134	F136	Q176	F178	K292	V/F/W612	F615	F617	R620	E673
wt	-50.3	-2.0	-7.0	-2.4	-2.5	-7.7	-2.4	-12.1	-2.2	-3.8	-1.7	-5.7		-5.2	-3.6	-1.8	-2.1
V612F	-52.1	-2.1	-6.9	-2.3	-2.5	-7.6	-2.6	-12.1	-2.1	-3.8	-1.7	-5.6	-2.2	-5.2	-3.6	-1.7	-2.1
V612W	-52.8	-2.1	-6.9	-2.3	-2.5	-7.6	-2.6	-12.1	-2.1	-3.8	-1.6	-5.6	-2.9	-5.5	-3.6	-1.6	-2.1

CAM

	ΔGb	S134	F136	V139	Q151	Q176	L177	F178	G179	I277	I278	A279	P326	Y327	V571	M573	F610	V/F/W612	F615	F617	I626	F628	L668	V672
wt	-29.6	-7.1	-3.0											-3.3	-1.7	-2.4			-1.5	-1.1	-0.6	-5.4	-1.5	-1.9
V612F	-33.6				-1.4	-4.6	-3.7	-6.1	-2.8	-4.9	-0.8	-1.6					-0.7	-3.6	-1.8		-0.8	-1.0		
V612W	-30.6			-1.4				-2.3		-1.4			-0.9	-1.5		-0.8		-8.1	-5.3	-1.4	-1.4	-4.7		

Table S5: Structural comparison of the TTO structure of OqxB (8zxs, this study) with the TTT structure of OqxB (7cz9) and LTO structure of AcrB (4dx5)

		AcrB(4dx5)			OqxB (7cz9)		
		Chain A (L)	Chain B (T)	Chain C (O)	Chain A (T)	Chain B (T)	Chain C (T)
OqxB (8zxs)	Chain A	2.95	2.81	3.50	0.81	1.06	0.89
	Chain B	3.27	2.69	3.49	0.85	0.92	0.99
	Chain C	3.72	3.77	2.39	2.51	2.40	2.49

The RMSD (root mean square deviation) in Å is calculated between aligned pairs of the backbone Cα atoms in each monomer by LSQKAB in CCP4 program suite.

Table S6: Data collection and refinement statistics crystallographic structures AcrB V612W

	AcrB V612W MIY (TTT) PDB 9FE2	AcrB V612W apo (TTT) PDB 9FE3
Data collection		
Space group	I 2 3	I 2 3
Cell dimensions		
a, *b*, *c* (Å)	227.49, 227.49, 227.49	227.43, 227.43, 227.43
α, β, γ (°)	90.00, 90.00, 90.00	90.00, 90.00, 90.00
Resolution (Å)	46.44 - 1.89 (1.96 - 1.89)	44.60 - 2.30 (2.38 - 2.30)
R_{sym} or R_{merge}	0.01656 (1.971)	0.01593 (2.029)
$I / \sigma I$	21.91 (0.32)	19.98 (0.33)
Completeness (%)	96.99 (70.06)	97.68 (77.33)
Redundancy	2.0 (2.0)	2.0 (2.0)
Refinement		
Resolution (Å)	46.44 - 1.89 (1.96 - 1.89)	44.60 - 2.3 (2.38 - 2.3)
No. reflections	150429 (10838)	84315 (6626)
R_{work} / R_{free}	0.2239 / 0.2455	0.2535 / 0.2909
No. atoms		
Protein	9499	9073
Ligand/ion	33	0
Water	384	6
B-factors		
Protein	56.84	97.59
Ligand/ion	83.56	-
Water	53.54	65.66
R.m.s. deviations		
Bond lengths (Å)	0.012	0.003
Bond angles (°)	1.19	0.65

*Values in parentheses are for highest-resolution shell.

Table S7: Data collection and refinement statistics crystallographic structures AcrB V612F

	AcrB V612F MIY (TTT) PDB 9FHC	AcrB V612F apo (TTT) PDB 9FE4
Data collection		
Space group	I 2 3	P 3 2 1
Cell dimensions		
a, *b*, *c* (Å)	227.46, 227.46, 227.46	134.41, 134.41, 190.97
α, β, γ (°)	90.00, 90.00, 90.00	90.00, 90.00, 120.00
Resolution (Å)	29.87 - 2.20 (2.279 - 2.20)	49.70 - 2.80 (2.90 - 2.80)
R_{sym} or R_{merge}	0.1293 (0.9247)	0.09319 (3.095)
I / σI	27.16 (4.31)	20.19 (0.90)
Completeness (%)	94.51 (67.04)	99.73 (99.79)
Redundancy	24.9 (21.4)	11.0 (10.3)
Refinement		
Resolution (Å)	29.87 - 2.20 (2.279 - 2.20)	49.70 - 2.80 (2.90 - 2.80)
No. reflections	93142 (6569)	49611 (4859)
R_{work} / R_{free}	0.2080 / 0.2380	0.2696 / 0.3030
No. atoms		
Protein	9115	7843
Ligand/ion	33	0
Water	281	0
B-factors		
Protein	40.76	110.83
Ligand/ion	39.25	-
Water	33.70	-
R.m.s. deviations		
Bond lengths (Å)	0.008	0.009
Bond angles (°)	1.27	1.21

*Values in parentheses are for highest-resolution shell.

Table S8: Data collection and refinement statistics crystallographic structures AcrB V612N

	AcrB V612N (LTO) PDB 9FHG	AcrB V612N (TTT) PDB 9FHJ
Data collection		
Space group	P 21 21 21	I 2 3
Cell dimensions		
a, *b*, *c* (Å)	145.81, 161.40, 245.41	228.65, 228.65, 228.65
α, β, γ (°)	90.00, 90.00, 90.00	90.00, 90.00, 90.00
Resolution (Å)	49.44 - 3.00 (3.11 - 3.00)	41.74 - 3.55 (3.68 - 3.55)
R_{sym} or R_{merge}	0.06477 (0.8325)	0.01899 (2.092)
I / σI	6.86 (1.03)	15.67 (0.33)
Completeness (%)	99.75 (99.71)	93.89 (45.09)
Redundancy	2.0 (2.0)	2.0 (2.0)
Refinement		
Resolution (Å)	49.44 - 3.00 (3.11 - 3.00)	41.74 - 3.55 (3.68 - 3.55)
No. reflections	115982 (11502)	22677 (1078)
R_{work} / R_{free}	0.2239 / 0.2829	0.2719 / 0.3260
No. atoms		
Protein	25972	9086
Ligand/ion	0	0
Water	0	0
B-factors		
Protein	79.72	195.91
Ligand/ion	-	-
Water	-	-
R.m.s. deviations		
Bond lengths (Å)	0.009	0.004
Bond angles (°)	1.14	0.68

*Values in parentheses are for highest-resolution shell.

Table S9: Data collection and refinement statistics crystallographic structure OqxB

	OqxB (TTO) PDB 8ZXS
Data collection	
Space group	P 21 21 21
Cell dimensions	
a, *b*, *c* (Å)	121.39, 165.94, 249.03
α, β, γ (°)	90.00, 90.00, 90.00
Resolution (Å)	49.34 - 2.75 (2.80 - 2.75)
R_{sym} or R_{merge}	0.082 (>1.0)
I / σI	12.9 (0.9)
Completeness (%)	99.65 (95.19)
Redundancy	7.33 (7.30)
Refinement	
Resolution (Å)	49.34 - 2.75
No. reflections	130594 (6157)
R_{work} / R_{free}	0.2363 / 0.2877
No. atoms	
Protein	
Ligand/ion	
Water	
B-factors	124,7
Protein	
Ligand/ion	
Water	
R.m.s. deviations	
Bond lengths (Å)	0,005
Bond angles (°)	0,888

*Values in parentheses are for highest-resolution shell.

Table S10: Cryo-EM data collection, refinement and validation statistics

	OqxB (O*O*O*) EMD-50334 PDB 9FDZ	AcrB V612F (O) EMD-50332 PDB 9FDQ	AcrB V612W (O) EMD-50331 PDB 9FDP
Data collection and processing			
Magnification	105000	130000	105000
Voltage (kV)	300	300	300
Electron exposure (e–/Å^2)	50	60	50
Defocus range (μm)	-0.8 to -2.4	-0.5 to -3.0	-0.8 to -3.5
Pixel size (Å)	0.837	0.68	0.837
Symmetry imposed	no	no	no
Initial particle images (no.)	2703412	732894	856118
Final particle images (no.)	543928	120564	81754
Map resolution (Å)	2.86	3.47	3.3
FSC threshold	0.143	0.143	0.143
Refinement			
Initial model used (PDB code)	AlphaFold	4dx5	4dx5
Model resolution (Å)	2.86	3.4	3.3
FSC threshold	0.143	0.143	0.143
Map sharpening *B* factor (Å^2)	-	-	-
Model composition			
Non-hydrogen atoms	23736	7853	7857
Protein residues	3120	1033	1033
Ligands	0	0	0
B factors (Å^2)			
Protein	49.18/217.07/114.41	14.15/112.77/38.75	14.15/112.77/38.75
Ligand	-	-	-
R.m.s. deviations			
Bond lengths (Å)	0.004	0.002	0.002
Bond angles (°)	0.951	0.549	0.507
Validation			
MolProbity score	1.10	1.18	1.15
Clashscore	3.10	3.91	3.59
Poor rotamers (%)	0.59	0.00	0.00
Ramachandran plot			
Favored (%)	98.78	99.22	99.32
Allowed (%)	1.22	0.78	0.68
Disallowed (%)	0.00	0.00	0.00