

Figure S1. Local resolution estimate of subtomogram averaging results, using windowed FSC. Related to Figure 2.

(A) Averaged structure of the full pump colored by local resolution. (B) Fourier shell correlation plot of the full pump structure (7 Å, red); and the focused refinement results: TolC (8 Å, cyan); AcrB (10 Å, purple).

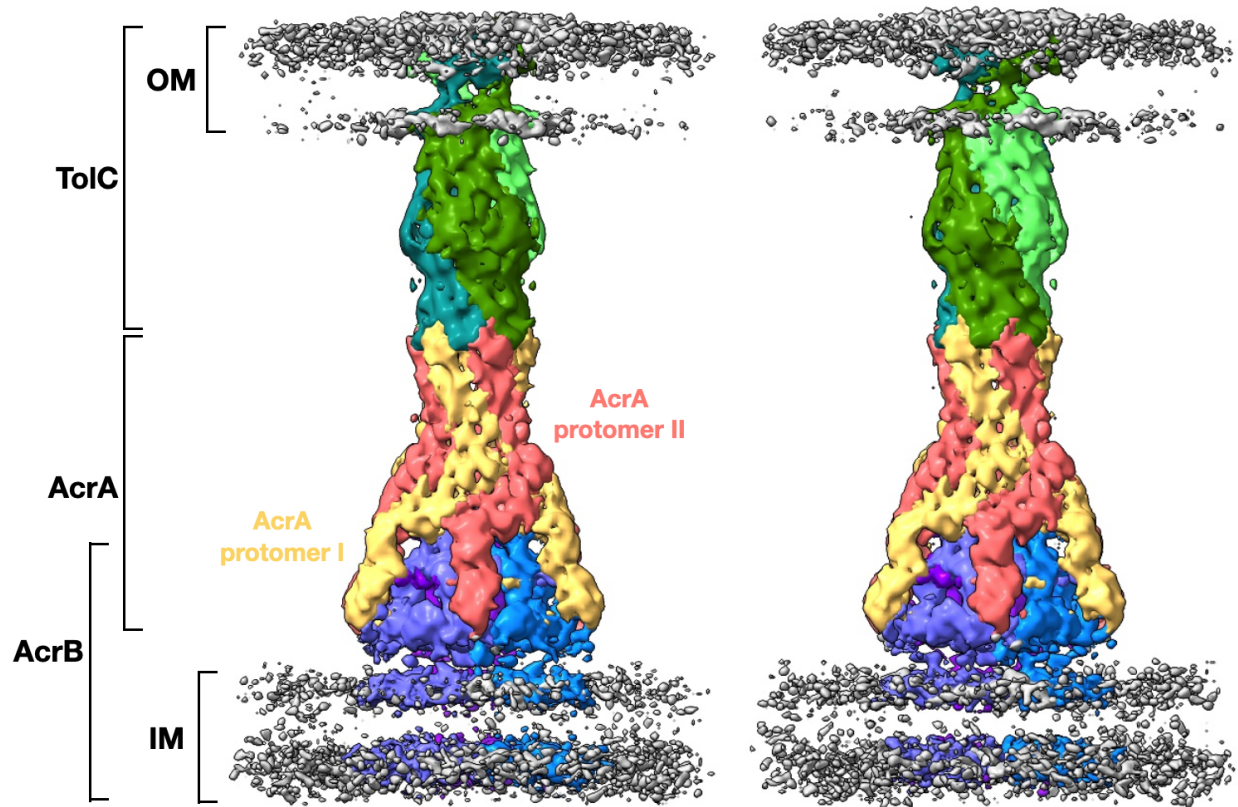


Figure S2. The two density maps generated separately based on the classification of TolC, representing two assembly models of the AcrAB-TolC efflux pump. Related to Figure 2.

Each of the TolC and AcrB protomers is colored uniquely. AcrA protomer I and protomer II are colored yellow and red, respectively. TolC has a 60-degree rotation relative to the AcrAB subcomplex between the two models. OM, outer membrane; IM, inner membrane.

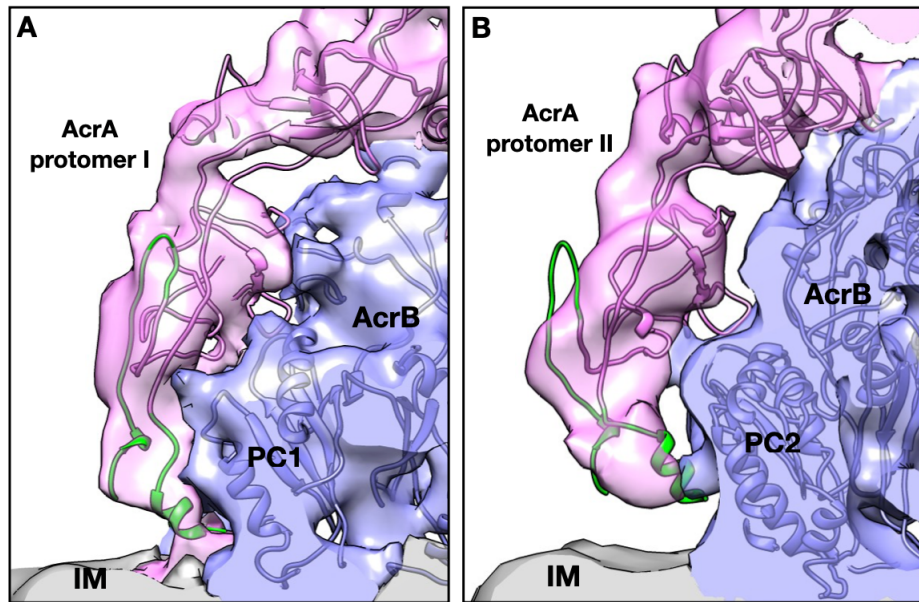


Figure S3. Side view of the interactions between AcrA and AcrB. Related to Figure 3.

(A) shows AcrA protomer I; and (B) shows AcrA protomer II. The *de novo* model for the AcrA termini colored green. IM, inner membrane.

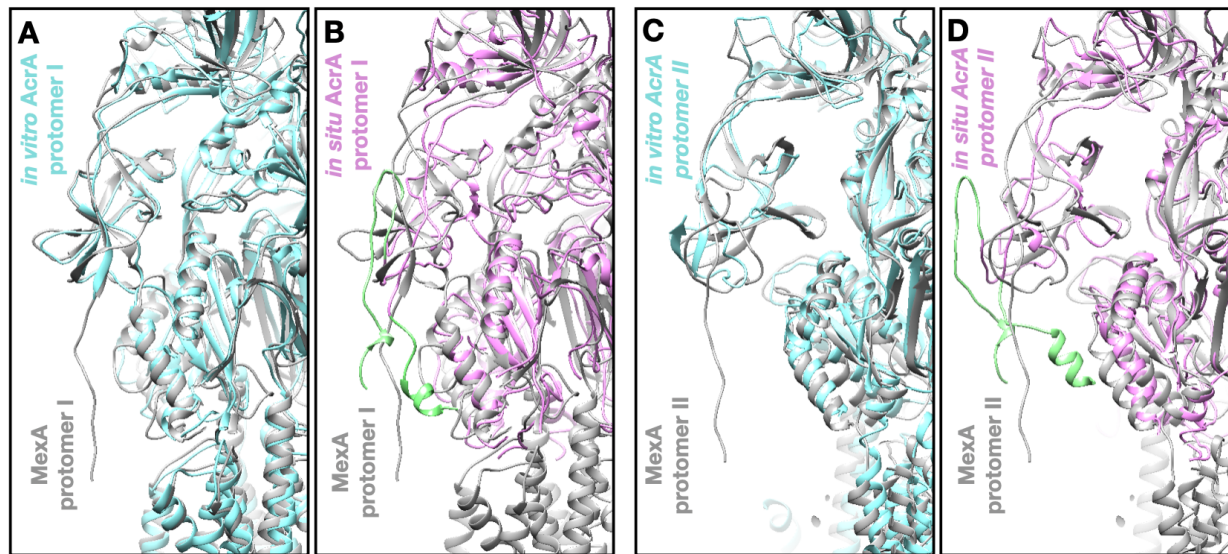


Figure S4. Comparison of the cryo-EM structure of MexAB-OprM assembled on a nanodisc (6TA6) (grey), the cryo-EM structure of purified AcrAB-TolC (5O66) (cyan), and the *in situ* structure of AcrAB-TolC (this study) (pink). Related to Figure 4.

From left to right are showing the superimpositions of MexA protomer I with *in vitro* AcrA protomer I (A), MexA protomer I with *in situ* AcrA protomer I (B), MexA protomer II with *in vitro* AcrA protomer II (C), and MexA protomer II with *in situ* AcrA protomer II (D).

Table S1. List of TolC channel states and AcrAB subcomplex existence in different structural models from previous studies or this study. Related to Figure 2.

Environment	Conformational state	TolC state	AcrAB	Source	Identifier
<i>in vitro</i>	isolated TolC	closed	N/A	Koronakis et al., 2000	PDB: 1EK9
	full pump+inhibitor	open	N/A	Wang et al., 2017	PDB: 5NG5
	full pump+antibiotics	open	N/A	Wang et al., 2017	PDB: 5O66
	crosslinked AcrAB-TolC	closed	N/A	Wang et al., 2017	PDB: 5VS5
	full pump apo state	open	N/A	Du et al., 2014	EMD-5915
<i>In situ</i>	full pump+inhibitor	open	Non-exist	This study	This study
	full pump+antibiotics	closed	exist	Shi et al., 2019	EMD-0532

Inhibitor: MBX3132 (binds to AcrB and locks the pump in the transporting state); antibiotics: puromycin; crosslinked AcrAB-TolC: the full pump assembly stabilized through the disulfide-bond linked AcrAB, generated by introducing cysteine-substitutions (AcrA-S273C and AcrB-S258C).