Supplementary materials for:

Engineered disulfide bonds support the functional rotation mechanism of multidrug efflux pump AcrB

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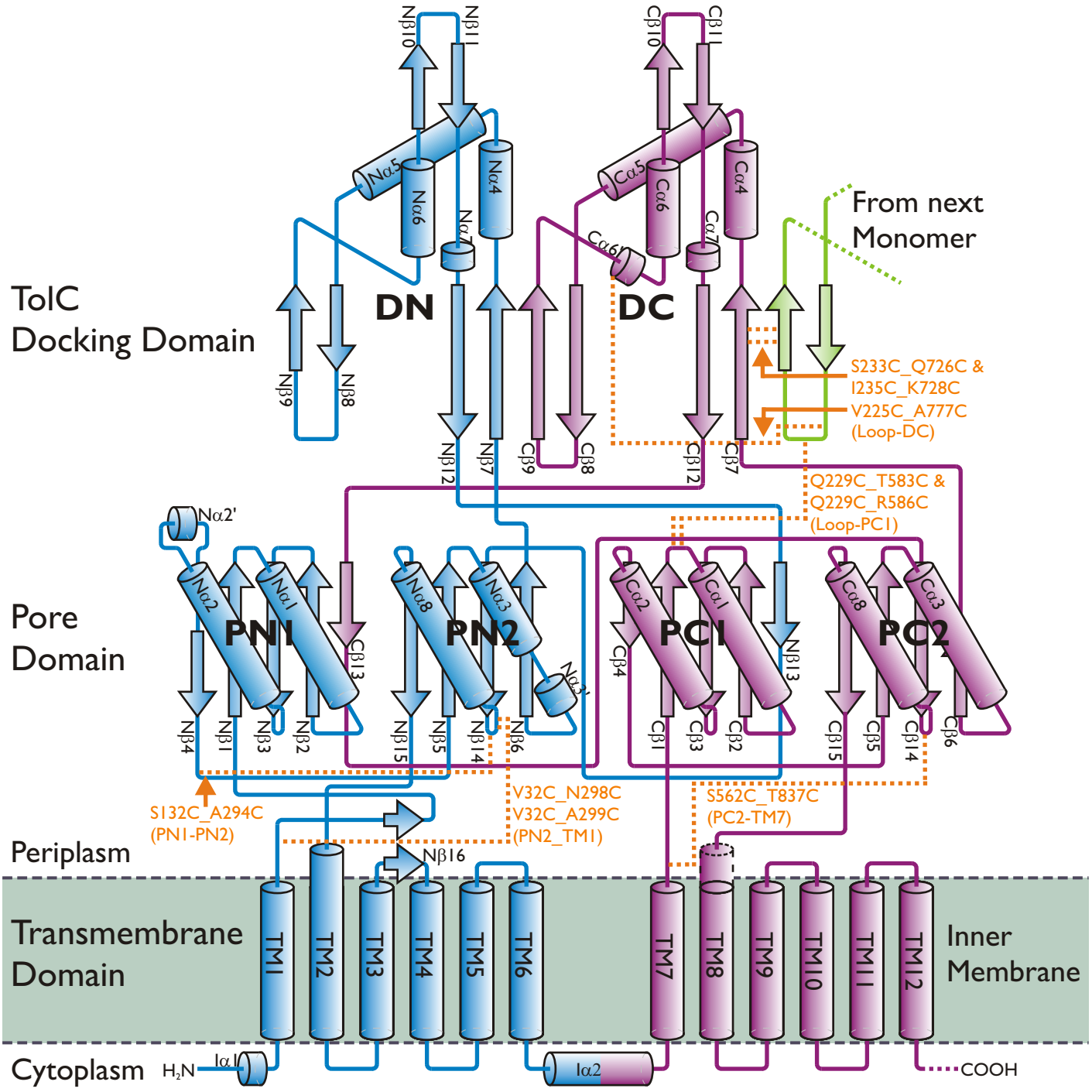
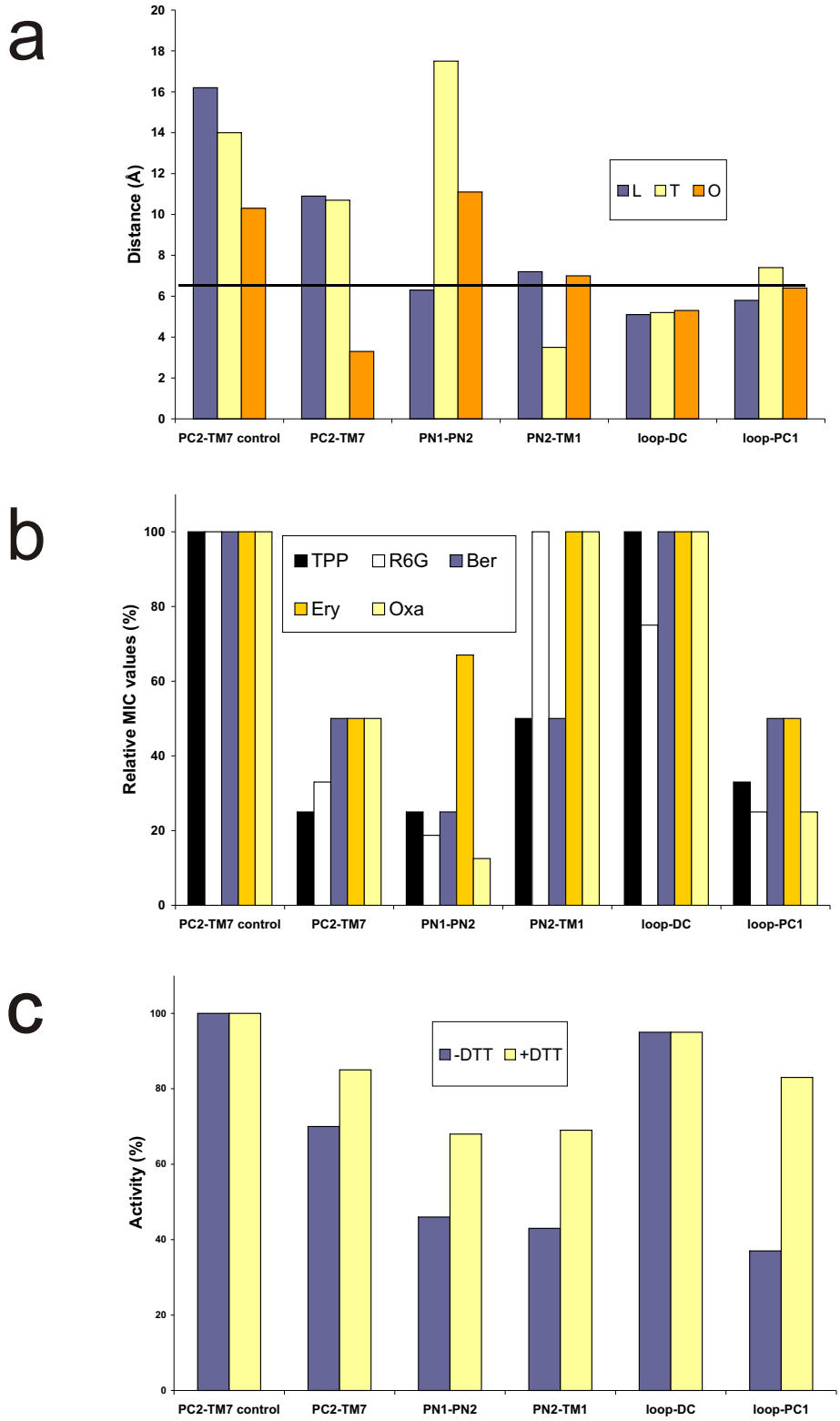
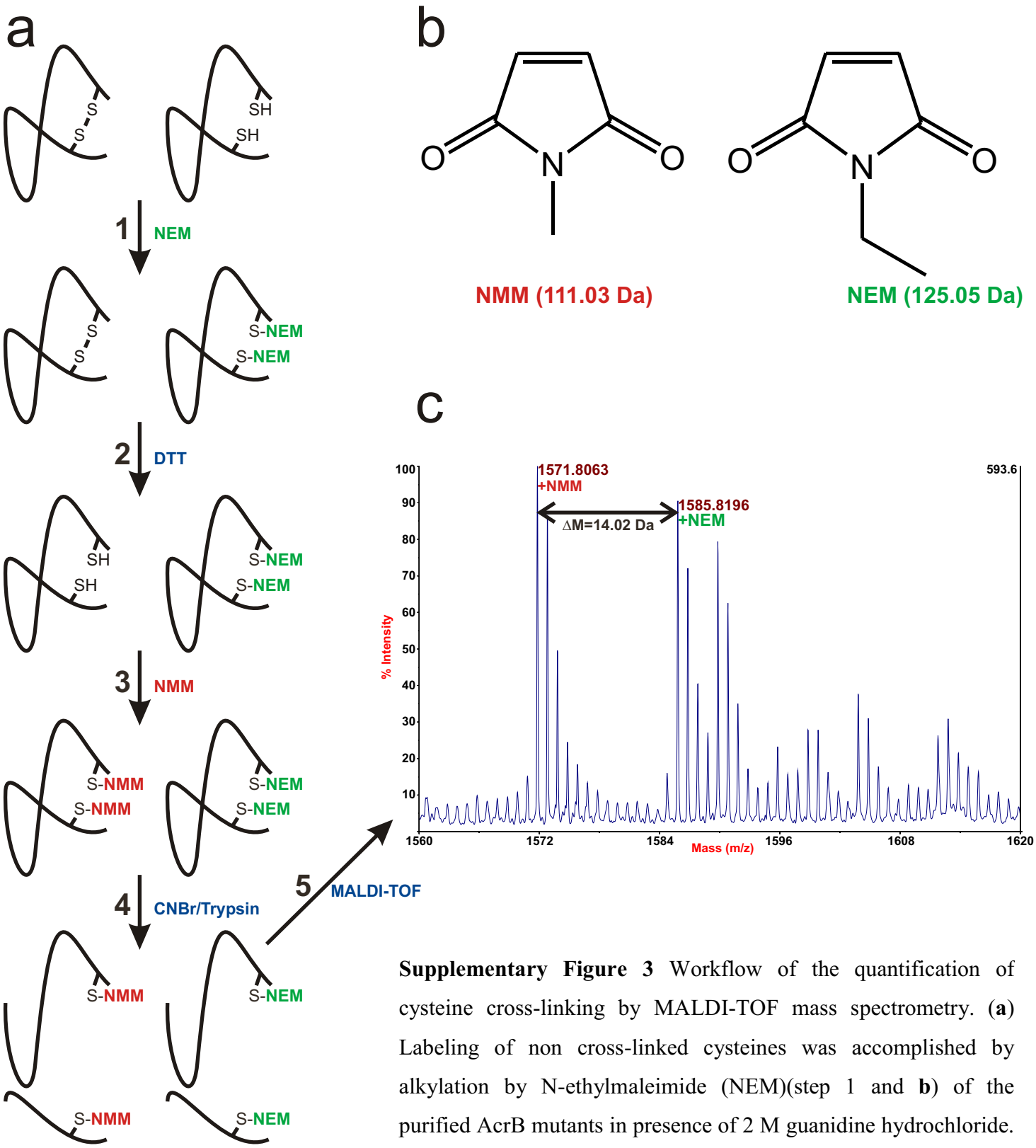


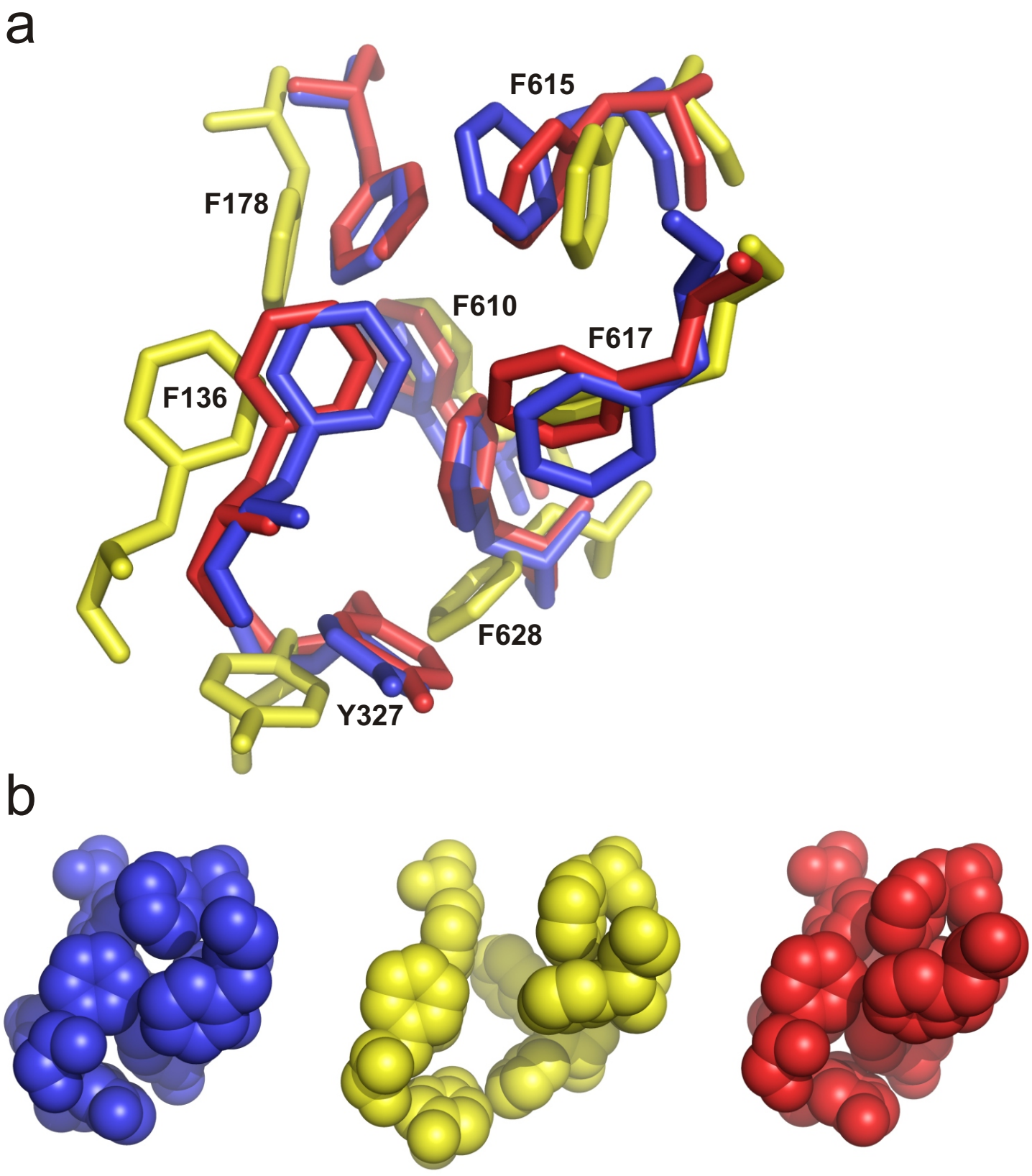
Supplementary Figure 1 AcrB monomer secondary structure scheme (residues 2-1033). The pore domain contains four subdomains, PN1, PN2, PC1 and PC2. The TolC docking domain has two subdomains, DN and DC. TM, transmembrane helices; Nα, Nβ, Cα and Cβ are α-helices and β-sheets of the N-terminal part or the C-terminal part of the periplasmic domain. Iα2 is the cross-α-helix at the cytoplasmic side. N- and C-terminal halves are depicted in blue and magenta, respectively. The intermonomer connecting loop from the adjacent monomer (hairpin structure protruding from one monomer into the next) is depicted in green. The locations of the disulfide bridges are indicated by dashed orange lines. The cysteine substitutions on the subdomains (in brackets) are indicated in orange.



Supplementary Figure 2 Overview on (**a**) distances (in Å) between the Sγ atoms of the introduced cysteines at the indicated monomer (L, T or O). The black line represents 6.4 Å distance. (**b**) relative MIC values (in %) of the double cysteine AcrB mutants compared to the single cysteine mutant exhibiting the lowest MIC value(s). (**c**) Effect of DTT on the NPN efflux activity (in %). Relative values were calculated from the NPN fluorescence signal displayed at 600 s after addition of glucose. The AcrB cysteine double mutants displayed on the X-axes are: PC2-TM7 control (R558C_E839C); PC2-TM7 (S562C_T837C); PN1-PN2 (S132C_A294C); TM1-PN2 (V32C_N298C); loop-DC (V225C_A777C) and loop-PC1 (Q229C_T583C). Abbreviations: TPP: Tetraphenyl-phosphonium, R6G: Rhodamine 6G, Ber: Berberine, Ery: Erythromycin, Oxa: Oxacillin.



Supplementary Figure 3 Workflow of the quantification of cysteine cross-linking by MALDI-TOF mass spectrometry. (**a**) Labeling of non cross-linked cysteines was accomplished by alkylation by N-ethylmaleimide (NEM)(step 1 and **b**) of the purified AcrB mutants in presence of 2 M guanidine hydrochloride. In a second step (2), disulfide bonds were reduced by DTT followed by removal of DTT and modification of the liberated cysteines by N-methylmaleimide (NMM)(step 3 and **b**). Mutants were digested with CNBr and in some cases in addition with trypsin (step 4). (**c**) Peptides were analyzed by MALDI-TOF mass spectrometry and signal to noise ratios of the peaks of the NEM and NMM modified peptides, exhibiting a mass difference of 14 Da, were quantified. A MALDI-TOF mass spectrum of peptides originating from the NEM and NMM modified AcrB_cl_S132C_A294C mutant is displayed as an example.



Supplementary Figure 4 Structural changes in the periplasmic drug binding site. (**a**) Stick representation of the superimposed hydrophobic drug binding site residues F136, F178, Y327, F610, F615, F617 and F628 in the L (blue), T (yellow) or O (red) conformation. (**b**) Space-fill models of the hydrophobic drug binding site residues. In the T monomer (yellow) the hydrophobic drug binding pocket is in an open state and closed in monomers L (blue) and O (red).

Supplementary Table 1 Distances of Nα2 helix (pore helix) residues in the symmetric (pdb ID: 1IWG) or asymmetric trimeric AcrB structure (pdb ID: 2GIF)[a]. Distances expected to facilitate disulfide cross-linking are indicated in bold.

	Distance between Cγ (Å)			
	Symmetric trimer	Asymmetric trimer		
Residue		**L-T**	**T-O**	**O-L**
D101	11.9	11.8	11.9	10.6
V105	**3.0**	**3.7**	**4.2**	**4.3**
Q106	11.9	12.3	12.8	13.7
N109	**4.3**	8.5	8.2	11.7
Q112	**4.8**	**5.9**	**5.8**	9.6
P116	8.5	9.1	13.8	14.0

[a] In the symmetric trimer, distances are valid for all three monomers. For the asymmetric structure, the values are given for residues located on the L and T monomer (L-T), T and O monomer (T-O) or on the O and L monomer (O-L).

Supplementary Methods

Construction of *acrB* knock-out

The *acrB* gene on the chromosome of *E. coli* BW25113 was deleted using the method described by Datsenko and Wanner[1]. The primers used for this deletion were 5'-GCCTAATTTCTTTATCGATCGCCCGATTTTTGCGTGGGTGGTGTAGGCTGGAG CTGCTTC-3' and 5'-ATCCTGAGTTGGTGGTTCAATTACTCCTTAATGTTCGTAGCATATGAATATCC TCCTTAG-3'. Nucleotides were removed after position 43 of the *acrB* gene till 50 bp downstream of the stop codon. The deleted *acrB* locus was marked by genes conferring either kanamycin or chloramphenicol resistance. Positive clones were verified by PCR, western blotting and drug susceptibility tests. For elimination of the polar effects on expression of *acrA*, the antibiotic resistance gene was removed by the pCP20-encoded recombinase[1]. Drug resistance of *E. coli* BW25113Δ*acrB* could be fully restored by constitutive "leaky" expression of *acrB* from the pET24acrB$_{His}$ plasmid, although the level of AcrB protein was about 4 times lower compared to chromosomally expressed *acrB* levels as estimated from Western blot analysis (not shown).

N-Phenylnaphtylamine (NPN) efflux assay

NPN is an uncharged lipophilic molecule with weak fluorescent properties in aqueous environments but is strongly fluorescent in a non-polar environment. NPN is a substrate of the AcrA/AcrB/TolC efflux pump[2,3]. Cultures of *E. coli* BW25113Δ*acrB* harboring pET24acrB_cl with and without cysteine mutations were grown in LB Kan^{50} (40 ml, 37°C/280 rev min^{-1}) to a final OD_{600} between 0.85 and 1.1, harvested and resuspended in 50 mM KP_i pH 7, 1 mM $MgSO_4$ (4 ml). Cells were de-energized in the presence of carbonyl cyanide m-chlorophenylhydrazone (CCCP; 200 µM final concentration) for 10 min on ice. CCCP was removed by washing the cells three times with 50 mM KP_i pH 7, 1 mM $MgSO_4$ (4 ml). Fluorescence measurements were performed with a Shimadzu Spectrofluorophotometer RF-5001PC (**Fig. 3a,b,c,e**) or a Cary Eclipse Fluorescence spectrophotometer (**Fig. 3d,f**) and samples were stirred in a 2 ml cuvette at RT. Fluorescence was measured at excitation of 355 nm and emission of 420 nm with 5 nm

slits. Cell density was adjusted to OD_{600} of 0.2 and NPN (10 µM final concentration, dissolved in methanol) and if required DL-dithiothreitol (DTT; 10 mM final concentration) was added. Incorporation of NPN into the inner membrane was followed until saturation was reached (6 min). Cells were re-energized by addition of glucose (0.2% (w/v) final concentration) and the fluorescence signal was followed for another 12 min. The fluorescence signal was normalized to the same value for all measurement at the time point of glucose addition. The trace generated with *E. coli* BW25113Δ*acrB* harboring pET24a showed some fluorescence increase upon re-energization. This slight fluorescence signal increase was subtracted from all other measured traces.

Sample preparation for quantification of disulfide bridges by mass spectrometry

An overview of the workflow is presented in **Supplementary Fig. 3**. Membranes of *E. coli* C43 (DE3) containing overproduced cysteine double mutants of AcrB_cl were solubilized with 2% (w/v) n-dodecyl-β-D-maltoside (DDM) in presence of N-ethylmaleimide (NEM, Sigma; 10 mM final concentration) for 2 hours at 18 °C. After high spin centrifugation (1 hour at 145000xg) the detergent extract was applied on a Ni^{2+}-NTA gravity flow column with 1 ml bed volume (Qiagen). The bulk protein was washed away with 3 ml buffer A (10 mM KP_i, pH 7.8, 10 mM imidazole, pH 8, 200 mM NaCl, 10% (w/v) glycerol, 0.05% (w/v) Cyclohexyl-n-hexyl-β-D-maltoside (CHM) and 2 M guanidine hydrochloride) supplemented with 10 mM NEM. The modification of the free thiol groups by NEM was allowed to proceed for 30 minutes at room temperature and at 10, 20 and 25 minutes buffer A containing 10 mM NEM (0.5 ml) was added to the column. NEM was washed away with buffer A (8 ml) and the disulfide linkages were reduced during 10 minutes by addition of buffer A (3 ml) containing DL-dithiothreitol (DTT; 10 mM final concentration). Excess DTT was washed away with buffer A (6 ml) and the newly generated free thiol groups were modified during 30 minutes by addition of buffer A containing 10 mM N-methylmaleimide (NMM) (3 ml). At 10, 20 and 25 minutes buffer A containing 10 mM NMM (0.5 ml) was added to the column. The purification of the AcrB_cl mutants was completed by washing with 50 mM imidazole, pH 7, 200 mM NaCl, 10% (w/v) glycerol, 0.05% (w/v) CHM and 2 M guanidine hydrochloride (25 ml) and a stepwise elution with the same buffer but containing 200

mM instead of 50 mM imidazole pH 7. In order to determine the completeness of the reduction of the disulfide bonds and of the modification reactions by maleimides, the S233C_Q726C mutant was reduced on the Ni^{2+}-NTA column by DTT as a first step and then treated as described above (modification with NEM, reduction with DTT and modification with NMM, results see below). To eliminate measurement mistakes caused by the physical differences between NEM and NMM, the protocol was also carried out for all mutants with the NMM modification as the first and the NEM modification as the second step, respectively. Purified and maleimide modified AcrB_cl mutants (10 µg) were diluted in ddH_2O (300 µl, final volume) and precipitated by centrifugation with trichloroacetic acid (TCA, 9.2% (w/v) final concentration). Cyanogen bromide (CNBr, Fluka) digestion of the precipitated protein and further sample preparation was carried out as described[4] with some modifications. Briefly, the TCA precipitate was washed twice with 95% (v/v) ethanol (150 µl) and subsequently with acetone (150 µl). The air dried pellet was dissolved in trifluoroacetic acid (TFA, 48 µl) containing 500 fold molar excess CNBr (40 mM) per methionine of the corresponding AcrB_cl mutant. After addition of ddH_2O (12 µl, 20% (v/v) final concentration) the samples were incubated in the dark for at least 18 hours at RT to complete digestion. Subsequently, ddH_2O (180 ml) was added and the samples were freeze-dried. For all AcrB_cl constructs except for R558C_E839C, S562C_T837C and V225C_A777C, a subsequent tryptic digest was necessary in order to generate suitably sized peptides for MALDI-TOF analysis. For this, the lyophilized CNBr digested peptides were dissolved in 50% (v/v) acetonitrile (ACN, 50 µl) and 10 µl thereof containing approximately 2 µg of peptides was transferred to a new tube and lyophilized. The peptides were digested in 50 mM $(NH_4)_2CO_3$ (20 µl) containing 5 µg ml^{-1} trypsin (trypsin:AcrB ratio 1:20 (w/w)) for 3 hours at 37°C. After digestion, the samples were lyophilized. Finally, peptides of all preparations were washed and freeze-dried three times in 50% (v/v) ACN (30 µl). CNBr and tryptic digests of every mutant were prepared in triplicate. For the PC2-TM7 (S562C_T837C) and PC2-TM7 control ((R558C_E839C) mutants, the first NEM modification was followed by a Tris(2-carboxyethyl)phosphine (10 mM final concentration) reduction step and incubation with 0.5 mM [^{14}C]NEM for 2 h. Protein was retrieved using EZview™ Red HIS-Select HC

Nickel Affinity Gel (Sigma) and the amount of protein bound [^{14}C]NEM determined via liquid scintillation counting.

Mass spectrometry measurements by MALDI-TOF

Molecular masses of most of the mutants were determined on a 4700 Proteomics Analyzer from Applied Biosystems, a MALDI-TOF instrument equipped with reflector. The peptides of the mutants V32C_N298C and V32C_A299C were measured on a 4800 *Plus* MALDI TOF/TOF Analyzer from Applied Biosystems. All measurements were performed at the Functional Genomics Center Zurich (FGCZ). The peptides of the mutants V32C_N298C, V225C_A777C, Q229C_T583C and Q229C_T586C had to be further purified using ZipTip C18 resins (Millipore). All peptides were spotted using α-cyano-4-hydroxycinnamic acid (HCCA) as matrix. For those samples not requiring ZipTip purification, the peptides (2 µg or 10 µg) were dissolved in 67% (v/v) ACN, 0.1% (v/v) TFA (30 µl or 150 µl) and 1 µl thereof was mixed with 67% (v/v) ACN, 0.1% (v/v) TFA, 5 mg ml^{-1} HCCA (4 µl). The mixture was spotted four times using 0.8 µl per spot. In case ZipTips were used, the peptides were dissolved in 0.1% (v/v) TFA (10 µl), immobilized on the ZipTip resin, washed twice with 0.1% (v/v) TFA and eluted in 66% (v/v) ACN, 0.1% (v/v) TFA, 5 mg ml^{-1} HCCA (5 µl). From these samples, 4x 0.8 µl were spotted.

Quantification of the MALDI-TOF spectra

For quantification of the cysteine containing peptides (2 per cysteine double mutant), only the peak showing the highest signal-to-noise ratio was used. The masses of the quantified peaks were as follows (the first number stands for the NMM, the second for the NEM modified peptide, respectively). S562C_T837C: 1624.76 Da/1638.78 Da, S132C_A294C: 1571.78 Da/1585.80 Da, V32C_N298C: 2713.34 Da/2727.36 Da, V32C_A299C: 1571.78 Da/1585.8 Da, S233C_Q726C: 1526.77 Da/1540.79 Da, I235C_K728C: 1500.72 Da/1514.74 Da, V225C_A777C: 979.42 Da/993.44 Da, Q229C_T583C and Q229C_R586C: 1485.74 Da/1499.76 Da. For the R558C_E839C mutant, only the NEM modified peak (1610.78 Da) could be observed because the NMM modified peak (1596.76 Da) was obscured by a strong peak (1595.84 Da) originating

from CNBr digest of AcrB. The signal to noise ratios (S/N ratios) of the peaks of interest were exported and used for the calculations. The percentage of cysteine bridges relative to the total cysteines was calculated by dividing the S/N ratio of the peak of the NMM-modified peptide (or of the NEM-modified peptide in case NEM was used as the second modification agent) by the sum of S/N ratios of the peaks of the NEM- and NMM-modified peptides. Since every NEM/NMM modified cysteine double mutant was digested three times independently and the peptides were spotted four times, twelve values for the estimation of the SS-bond formation were obtained. The standard deviation of these values was less than 4% for all mutants. The numbers derived from both successions of maleimide modification were averaged. One control sample was first reduced prior to both maleimide labeling steps (S233C_Q726C, see above) in order to determine the background value for the modification procedure used. In this way, a modification background of 3.77% was determined, which was subtracted from all determined values.

References

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