

# Engineered disulfide bonds support the functional rotation mechanism of multidrug efflux pump AcrB<sup>1</sup>

Brian McGillick, Swaminathan Lab

1. Seeger, M. A. *et al.* Engineered disulfide bonds support the functional rotation mechanism of multidrug efflux pump AcrB. *Nature structural & molecular biology* **15**, 199-205, doi:10.1038/nsmb.1379 (2008).

# Why I Think You Should Know About This Work

- This paper provides an interesting approach into how to elucidate dynamic protein movements from static structural information.

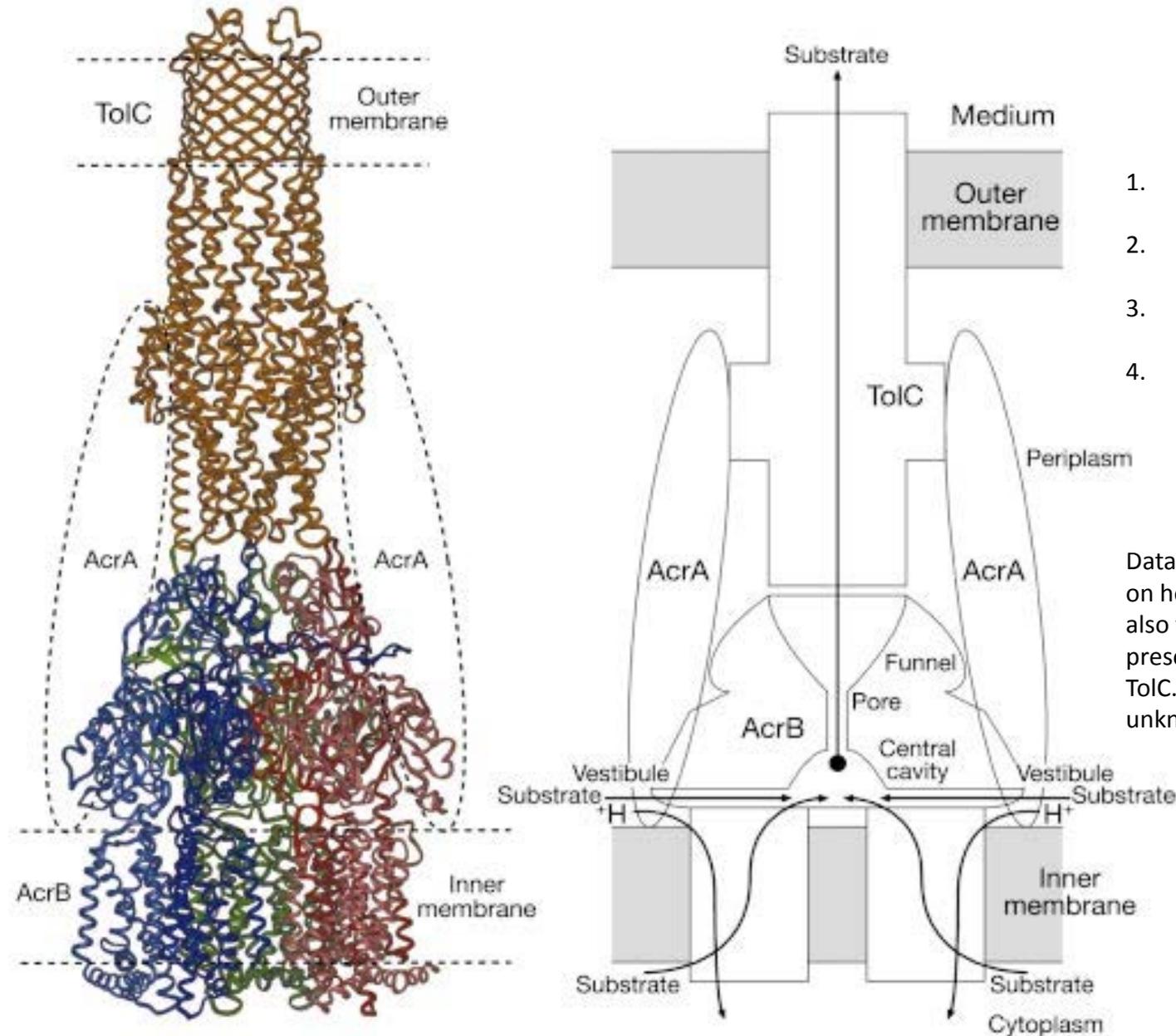
# Why I am Interested in This Work

- My lab is undertaking a new project involving *Burkholderia pseudomallei* efflux pumps AmrAB-OprA and BpeAB-OprB.
- The AcrA-AcrB-TolC complex is an analogous structure.
- Have been reading papers on different efflux pumps to gain insights into how to approach this new project.

# Background

- AcrA-AcrB-TolC complex is the major multidrug resistance efflux pump of E. Coli.
- The inner membrane protein, AcrB, is trimeric with both a symmetric and asymmetric trimer structure having been reported.
- Asymmetry of the trimer is the potential biologically relevant structure displaying stages of pump operation.

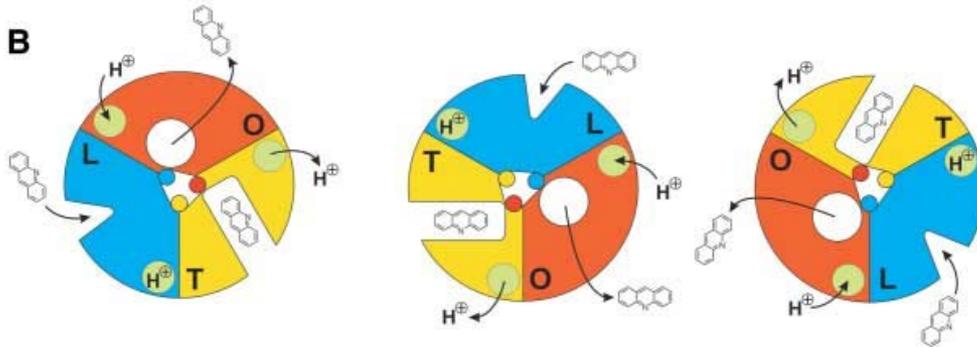
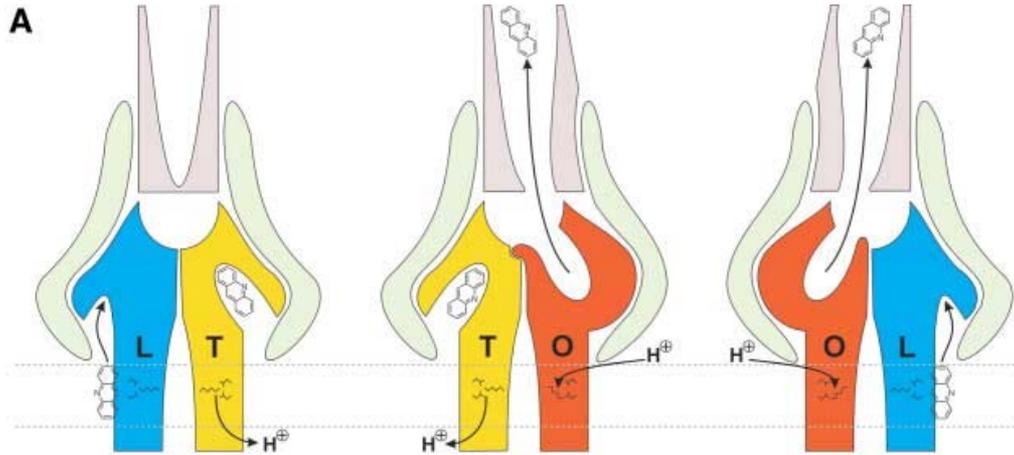
# Background-General Structure of AcrA-AcrB-TolC



1. TolC forms a pore through the outer membrane.
2. AcrB represents the pump mechanism of the system.
3. AcrA likely transmits allosteric movements from AcrB to TolC.
4. AcrB requires a proton motive force for its action.

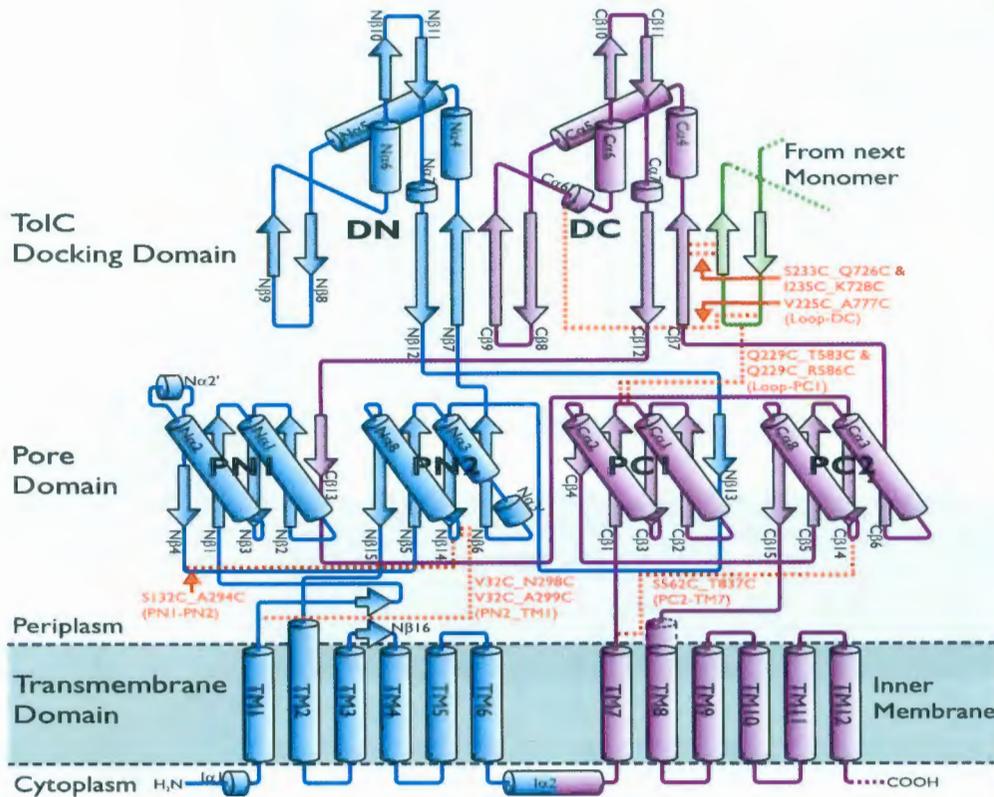
Data on AcrA location based primarily on homology modeling of MexA and also from some N and C regions of AcrA present in crystal structure of AcrB and TolC. Exact conformation however is unknown.

# Proposed Mechanism



- The conversion from the T monomer to the O monomer is accompanied by the release of a proton from the proton translocation site to the cytoplasm
- The structural changes in the T monomer create a hydrophobic pocket
- In the T monomer, the tunnel leads to residues of the O monomer PN1 subdomain, which operates as a plug for the tunnel exit.
- In the O conformation, the tilting of the PN1 subdomain opens an exit pathway from the binding pocket.

# Results

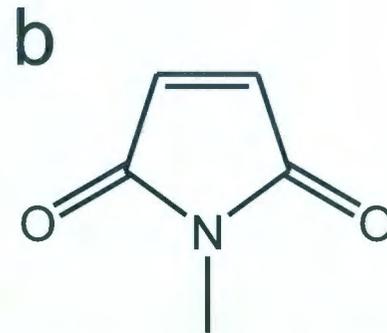
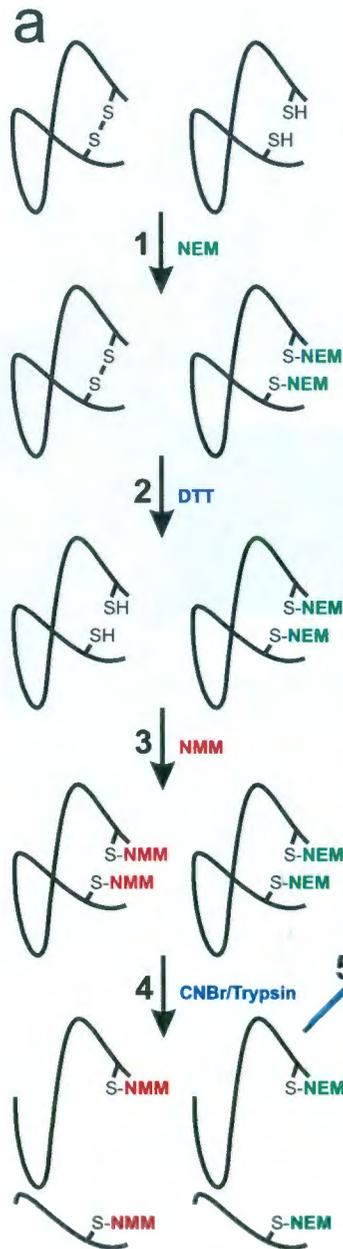


**Table 1 Distances of residues and degree of disulfide cross-linking**

Linked subdomains <sup>a</sup> cysteine residues	Distance between S <sub>y</sub> [Å] <sup>b</sup>			Disulfide cross- links [%] <sup>c</sup>
	L	T	O	
<i>PC2-TM7</i> (control) <sup>d</sup> R558C_E839C	16.2	14.0	10.3	-9.5 ± 4.7 <sup>e</sup>
<i>PC2-TM7</i> S562C_T837C	10.9	10.7	3.3	15.4 ± 1.3 <sup>e</sup>
<i>PN1-PN2</i> S132C_A294C	6.3	17.5	11.1	41.6 ± 0.6
<i>PN2-TM1</i> V32C_N298C V32C_A299C	7.2 9.5	3.5 4.8	7.0 11.9	41.3 ± 1.2 18.1 ± 1.0
<i>Loop<sup>d</sup>-DC</i> (functional control) S233C_Q726C I235C_K728C V225C_A777C	5.2 5.0 5.1	5.9 5.0 5.2	6.3 5.1 5.3	17.0 ± 0.4 23.8 ± 0.6 80.2 ± 1.3
<i>Loop-PC1</i> Q229C_T583C Q229C_R586C	5.8 5.5	7.4 7.8	6.4 6.7	69.4 ± 0.5 46.4 ± 0.5

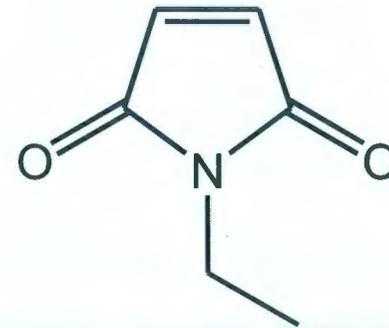
1. Loop and PC1 domain show strong cross linking as would be expected from proximity in crystal structure.
2. However, loop-DC domain showed relatively little crosslink. Possibly due to side chain rigidity in B-sheet.
3. Negative control is devoid of cross links.

# Disulfide Cross-Link Quantification



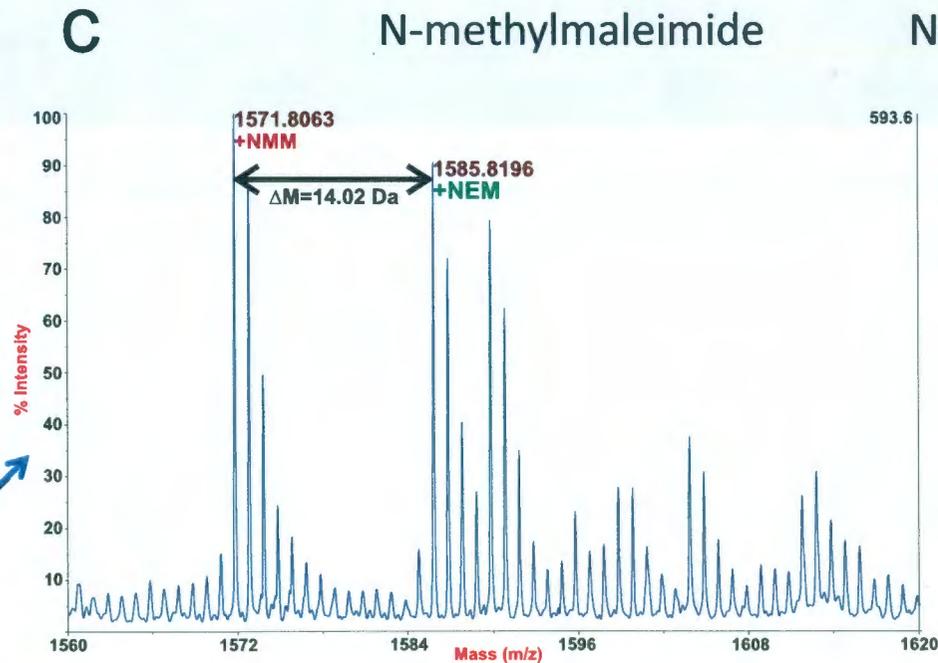
NMM (111.03 Da)

N-methylmaleimide

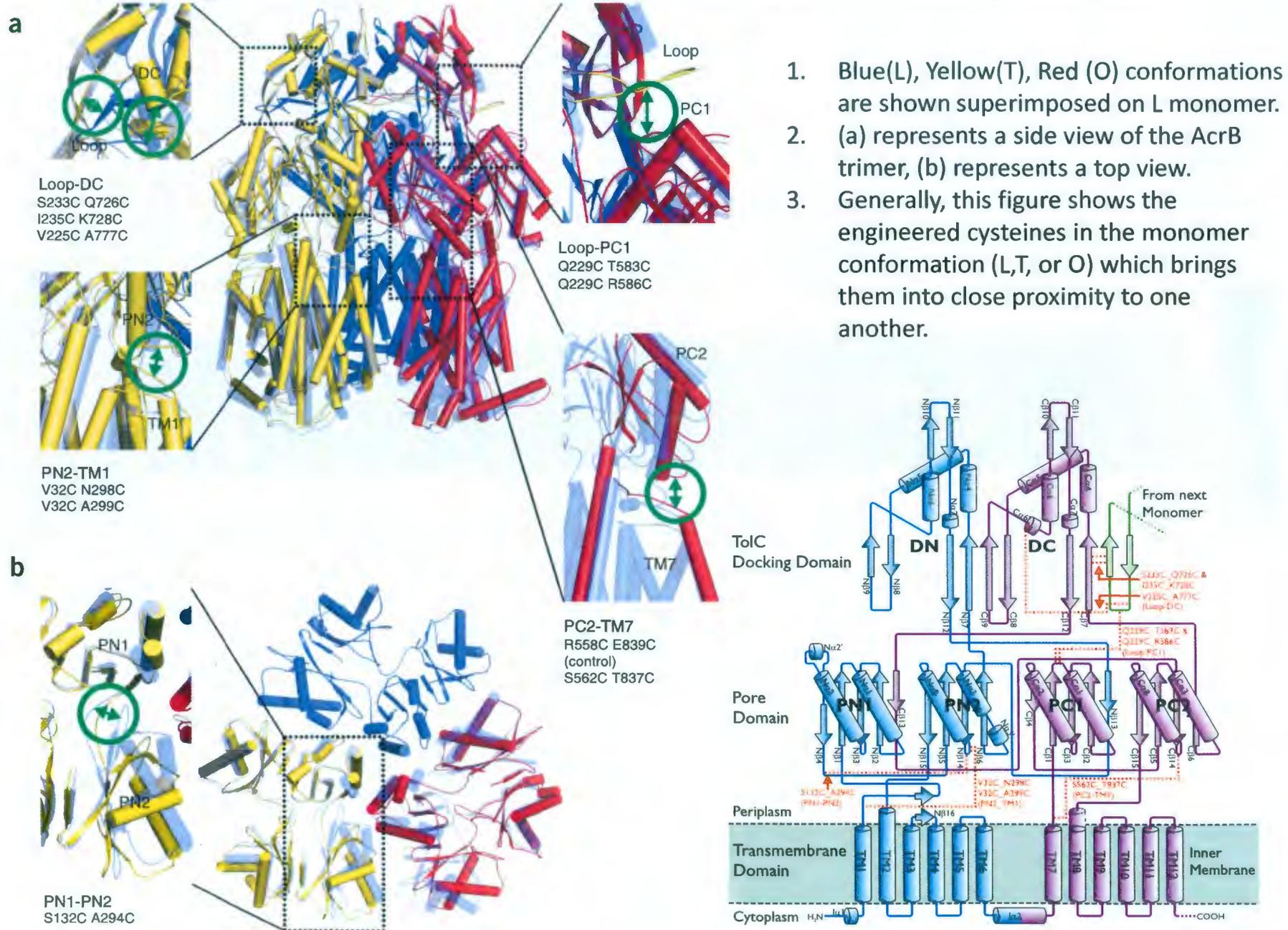


NEM (125.05 Da)

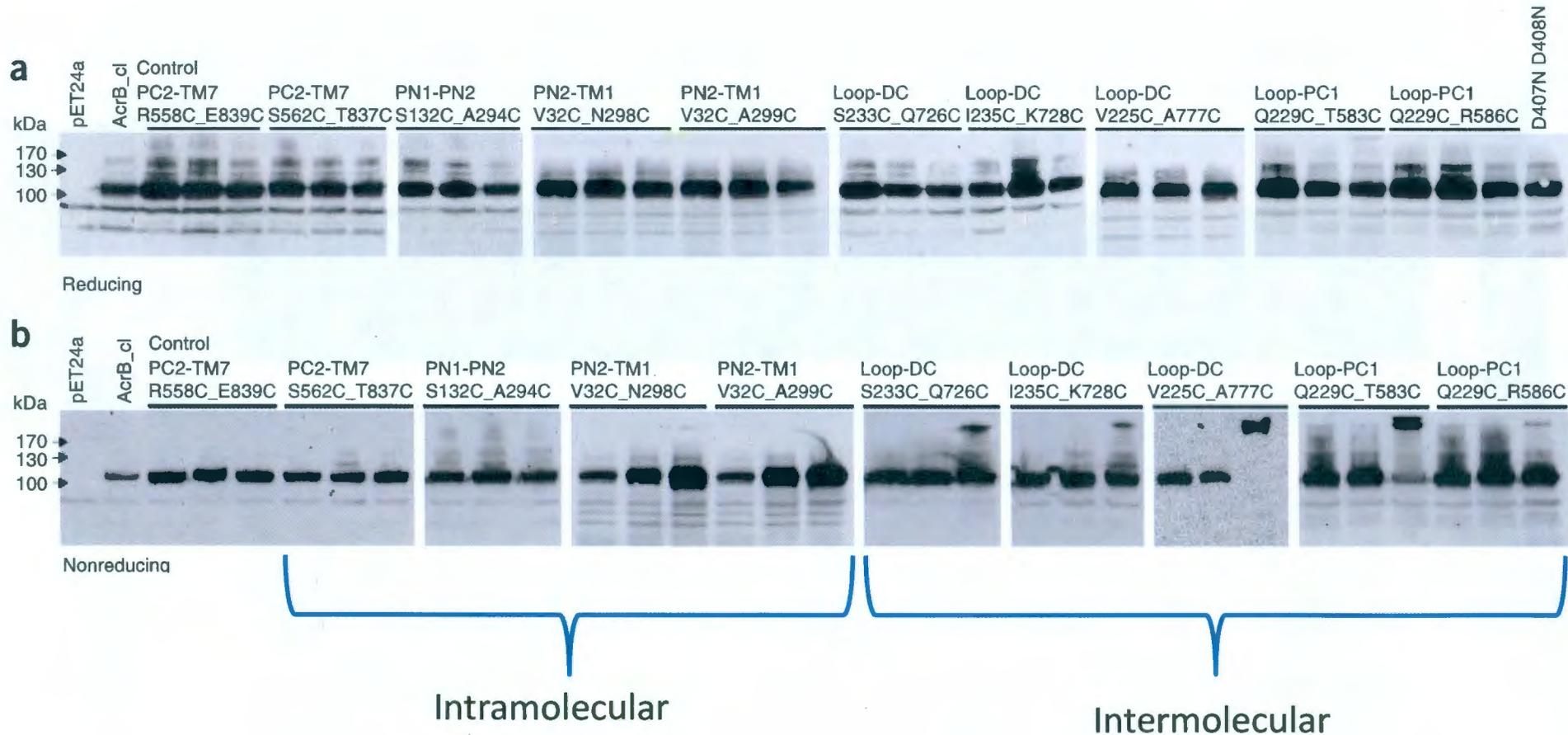
N-ethylmaleimide



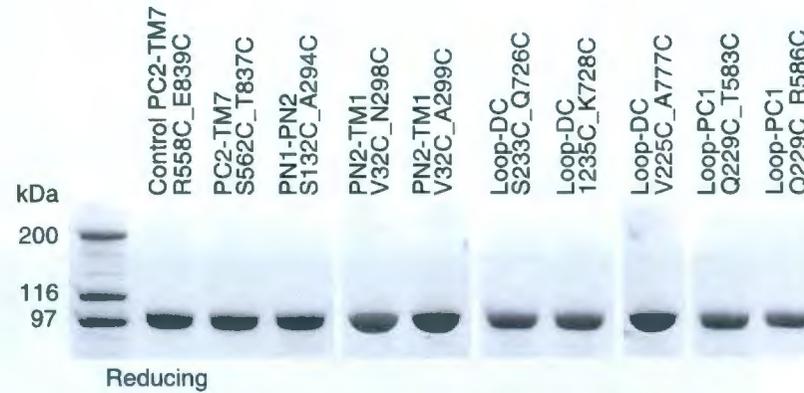
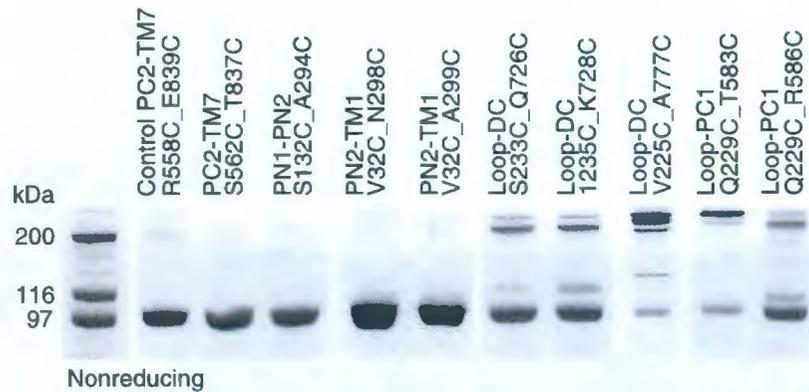
# Engineered Disulfide Bond Locations



# Single and Double Cysteine Mutants

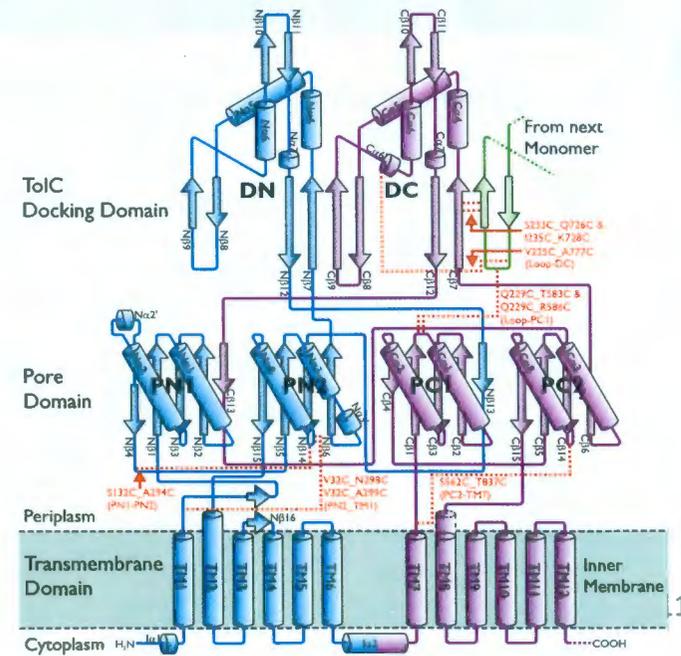


# Purified AcrB Constructs



intermolecular

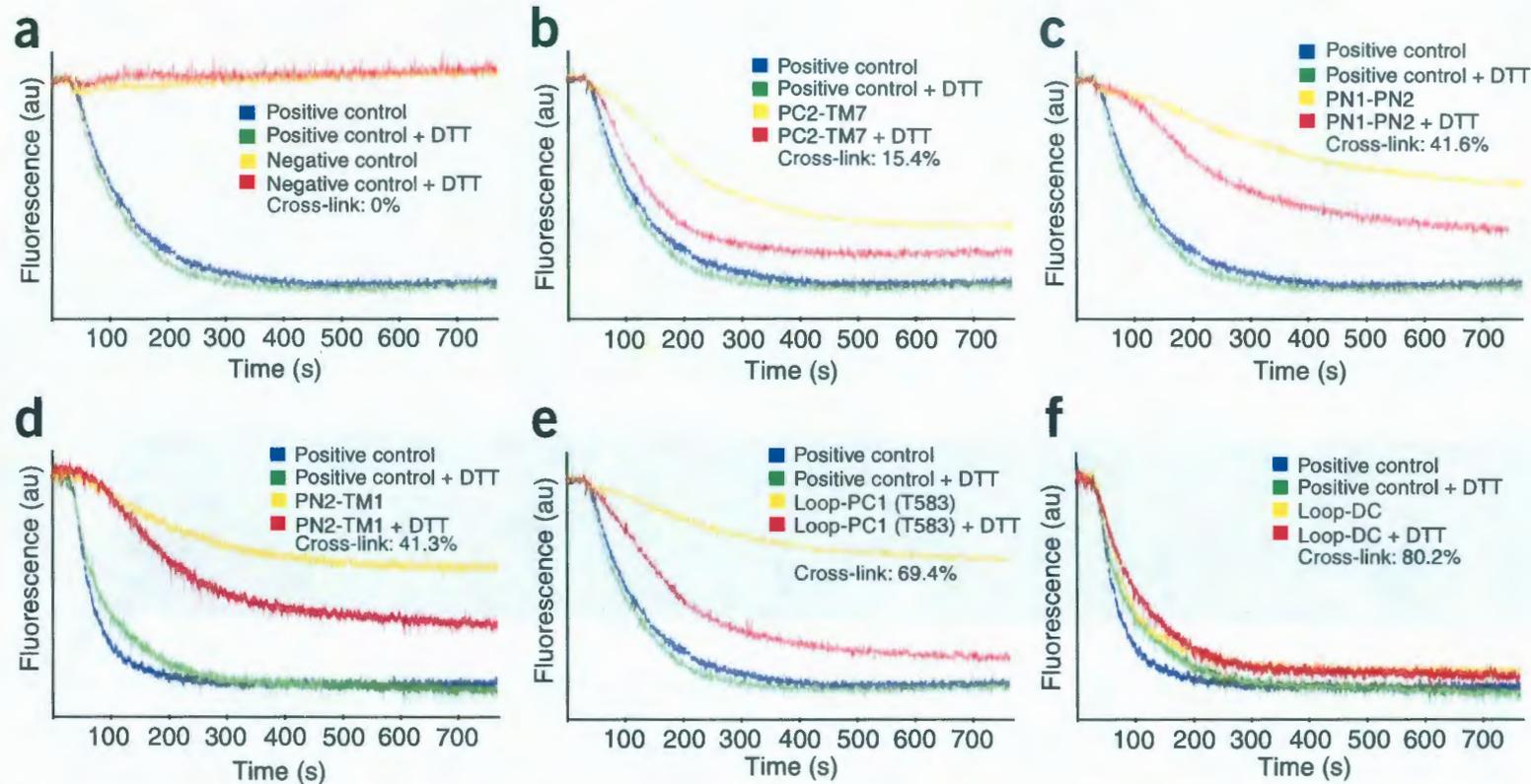
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exposure of R558C (and E839C) at the protein surface, which could facilitate intermolecular cross-linking in the *E. coli* cell.



# MIC Data

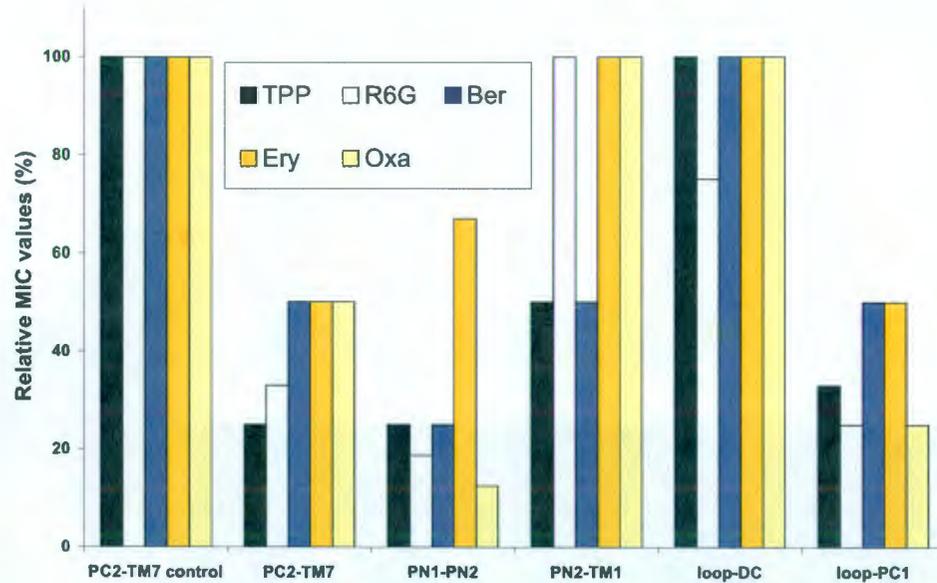
Plasmid <sup>a</sup>	Tetraphenyl-	Rhodamine	Erythro-			Tetraphenyl-	Rhodamine	Erythro-			
	phosphonium	6G	Berberine	mycin		Oxacillin	phosphonium	6G	Berberine		mycin
pET24a	3.125	2	64	4	2	<i>loop<sup>c</sup>-DC</i>					
pET24acrB_cl	400	128	1024	64	128	S233C	400	128	1024	64	128
						Q726C	400	128	1024	32-64	128
						S233C_Q726C	200-400	128	1024	32-64	128
<i>PC2-TM7 control</i>											
R558C	400	128	1024	64	128	I235C	400	128	1024	64	128
E839C	400	128	1024	64	128	K728C	400	128	1024	32-64	128
R558C_E839C	400	128	1024	64	128	I235C_K728C	400	64-128	1024	64	128
<i>PC2-TM7</i>											
S562C	200	64-128	512	32-64	64	V225C	400	64-128	1024	64	128
T837C	400	128	1024	64	64-128	A777C	400	64-128	1024	64	128
S562C_T837C	<b>50</b>	32	256	16-32	32	V225C_A777C	400	64	1024	64	128
<i>PN1-PN2</i>						<i>loop-PC1</i>					
S132C	200	128	1024	64	64-128	Q229C	400	128	1024	64	128
A294C	400	128	1024	32-64	128	T583C	100-200	32-64	512	32	64
S132C_A294C	<b>50</b>	<b>16-32</b>	<b>256</b>	32	<b>8-16</b>	Q229C_T583C	50	<b>8-16</b>	256	16	<b>16</b>
<i>PN2-TM1</i>						R586C	400	64-128	1024	32	64-128
V32C	400	64	1024	64	128	Q229C_R586C	100-200	32	512	16-32	32-64
N298C	50	16	256	16	16						
V32C_N298C	25	16	128	16	16						
A299C	400	128	1024	64	128						
V32C_A299C	400	64	1024	64	64						

# N-Phenylnaphthylamine (NPN) Efflux Assay



1. NPN fluoresces strongly in hydrophobic environment of bacterial inner membrane.
2. Disulfide cross link reduction restores pump activity.
3. Negative control in panel a is an inactivating mutant.

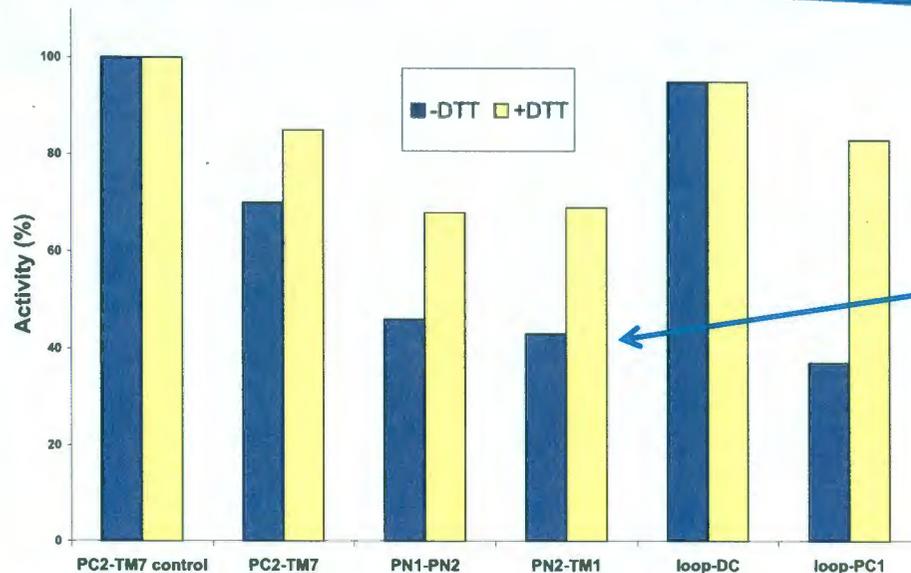
# MIC and Efflux Assay Correlation



- PC2-TM7 mutant had a relatively low level of cross linking, yet showed somewhat high MIC reduction.

PC2-TM7				
S562C_T837C	10.9	10.7	3.3	15.4 ± 1.3

- Disulfide bridge only possible (< ~6.4 Å in O conformation)
- Possible explanation that O conformation is locked while L and T can shift back and forth.



Assay and MIC values do not agree with PN2-TM1 cross link. Possibly specific to NPN since some but not all substances showed reduced MIC in this mutant.

# Conclusions

- Disulfide bonds can be formed between locations too far for bridge formation in the symmetrical AcrB structure.
- Disulfide bridges do not form between regions too distant from one another in asymmetric model.
- Disulfide bridge formation lowers MIC of several antibiotics.
- MIC value can be recovered by reducing the disulfide bonds with DTT
- All of this data suggests that allosteric movements, like those observed in the asymmetric crystal structure, are necessary for proper efflux pump operation.

# Take-Home Message

- This paper provides biochemical data to explain structural data from crystallographic study.
- Provides evidence that the asymmetric trimer is the functional biological unit and not an artifact of crystallization.
- If the original symmetric structure was the biological unit, it is likely that cross linking would not be extensively observed and that any cross links present would have little functional impact.