Supplementary Information and Data

TABLE 1: Strains and plasmids

Strains/ plasmids	***Description***	***Source***
***E. coli* strains**		
NovaBlue	Source of DNA for PCR amplification of *macAB* and *tolC* genes – *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacIqZΔM15 Tn10*(Tetr)]	Novagen
C43(DE3)	Expression host for pET vectors - F- *ompT hsdSB* (*rB-mB-*) *gal dcm (DE3)*	1
BL21(DE3)	*F-*, omp*T*, hsd*Sβ(rβ-mβ-),* dcm, gal*, (DE3) ton*	Stratagene
KAM3(DE3)	Antibiotic sensitive host strain used for expression of pDuet vectors – *ΔacrB*	2
(*ΔtolC*)TG1	Antibiotic sensitive host strain used for expression of pDuet vectors – *ΔtolC*	4
(*ΔtolC*)TG1(DE3)	λDE3 lysogenic strain to enable T7 polymerase production for use with pET vectors	4
Plasmid cloning vectors		
pET21a	Expression of His-tagged proteins in *E. coli*	Novagen
pACYCDuet	Simultaneous expression of two proteins in *E. coli*	Novagen
Plasmid constructs		
pET-mtrD	*mtrD* cloned into pET21a(+) using NdeI-mtrD F and XhoI-mtrD R primers	4
pET-mtrE	*mtrE* cloned into pET21a(+) using NdeI-mtrE F and HindIII-mtrE R primers	4
pET-Δ34mtrC	*mtrC* mutant, encoding a derivative truncated at position 34, cloned into pET21a(+) using NdeI-Δ34-mtrC F and XhoI-mtrC R primers	4
pET-mtrC hairpin	*α*-helical hairpin domain of mtrC cloned into pET21a(+) using NdeI-mtrC hairpin F and NdeI-mtrC hairpin R primers	4
pACYCDuet-mtrC/mtrD	*mtrC* cloned into MCS1 of pACYCDuet, using BamHI-SalI F and mtrD cloned into MCS2 using NdeI-XhoI. primers.	This study
pACYCDuet-mtrC/mtrE	*mtrC* cloned into MCS1 of pACYCDuet using BamHI-mtrC F, HindIII-mtrC R, *mtrE* cloned into MCS2 of pACYCDuet using NdeI-mtrE F, KpnI-mtrE R.	4
pACYCDuet-mtrC/mtrD/mtrE	*mtrCmtrE* cloned into MCS1 of pACYCDuet using BamHI-mtrC F, EcoRI-mtrC-SD-R, EcoRI-mtrE -ATG F and SalI-mtrE R; *mtrD* cloned into MCS2 of pACYCDuet using NdeI-mtrD F and KpnI-mtrD R.	4
pACYCDuet-mtrC-S-tag	*NT-mtrC* cloned into pACYC using MtrC NT For Nde1 and MtrC NT Rev Xho1–S-tag fusion	4

Plasmid constructs for *in vivo* cross linking- TG1 *tolC* (DE3)	***Description***	***Source***
pACYC MtrC (C25S) MtrE (C21S) cys less + pET21-MtrD	Intrinsic cysteines in MtrC and MtrE were altered into serine for cysteine less control	This study
pACYC MtrC (C25S) MtrE (C21S/K390C) + pET21-MtrD	Cysteine inserted into MtrE inter protomer groove	This study
pACYC MtrC (C25S/E149C) MtrE (C21S/K390C) + pET21-MtrD	Cysteine inserted into MtrE inter protomer groove and MtrC hairpin domain E149C	This study
pACYC MtrC (C25S/D143C) MtrE (C21S/K390C) + pET21-MtrD	Cysteine inserted into MtrE inter protomer groove and MtrC hairpin domain D143C	This study
pACYC MtrC (C25S/E149C) MtrE (C21S) + pET21-MtrD	Cysteine inserted into MtrC hairpin domain E149C	This tudy
pACYC MtrC (C25S/D143C) MtrE (C21S) + pET21-MtrD	Cysteine inserted into MtrC hairpin domain D143C	This study

References

1. Miroux B, Walker J. (1996) *J. Mol Biol*. **260**:289-98.
2. Morita Y, Kodama K, Shiota S, Mine T, Kataoka A, Mizushima T, Tsuchiya T. (1998) *Antimicrob Agents Chemother*. **42**:1778-82.
3. Nagakubo S, Nishino K, Hirata T, Yamaguchi A. (2002) *J Bacteriol*. **184**:4161-7.
4. Janganan TK, Zhang L, Bavro VN, Matak-Vinkovic D, Barrera NP, Burton MF, Steel PG, Robinson CV, Borges-Walmsley MI, Walmsley AR. (2011) *J Biol Chem*. **286**:5484-93.

TABLE 2: primers for mtr*CDE* constructions

Primers	
NdeI-mtrD F	**CATATG**GCTAAATTCTTTATCGACCGCCCCATTTTCG
XhoI-mtrD R	**CTCGAG**ATATTGTTTATCGTCCGAACCGGTTATACCCG
KpnI-mtrD R	**GGTACC**ATATTGTTTATCGTCCGAACCG
NdeI-mtrC F	**CATATG**GGCTTTTTATGCTTCTAAGGCGATGCGTGCG
XhoI-mtrC R	**CTCGAG**TTTCGCTTCAGAAGCAGGTTTGGCTTCAG
BamHI-mtrC F	**GGATCC**GGCTTTTTATGCTTCTAAGGCGATGCGTGCG
SalI-mtrC R	**GTCGAC**TTTCGCTTCAGAAGCAGGTTTGGCTTCAG
NdeI-mtrC-F s-tag	**CATATG**GGCGGGCAGCCTGCGGGTCGGGAA
Xho –mtrC-R	**CTCGAG**TTTCGCTTCAGAAGCAGGTTTGGCTTCAGATGCCGTC

TABLE 3: Primers used for mutagenesis and cysteine derivatives of *mtrC and mtrE*

MtrE Derivatives	**Primers**
K225E	For 5′ CCTTGATTGAATCTGCCGAGGCCGATTATGCCCATG 3′ Rev 5′ CATGGGCATAATCGGCCTCGGCAGATTCAATCAAGG 3′
R235E	For 5′ GCGCGCAGCGAGGAACAGGCGCGC 3′ Rev 5′ GCGCGCCTGTTCCTCGCTGCGCGC 3′
Y228F	For 5' CTTGATTGAATCTGCCAAAGCCGATTTTGCCCATGCCG 3′ Rev 5′ CGGCATGGGCAAAATCGGCTTTGGCAGATTCAATCAAG 3′
R239E	For 5′ CCGCGAACAGGCGGAGAATGCCTTGGCAAC 3′ Rev 5′ GTTGCCAAGGCATTCTCCGCCTGTTCGCGG 3′
K390E	For 5′ CGCGAGCAGCTGGATGAGGCCTATGACGCT 3′ For 5′ AGCGTCATAGGCCTCATCCAGCTGCTCGCG 3′
K397E	For 5′ CTATGACGCTTTAAGCGAGCAAAGCCGCGCCTC 3′ Rev 5′ GAGGCGCGGCTTTGCTCGCTTAAAGCGTCATAG 3′
MtrE cys mutants	
K225C	For 5' CTTGATTGAATCTGCCTGCGCCGATTATGCCCATGC 3' Rev 5' GCATGGGCATAATCGGCGCAGGCAGATTCAATCAAG 3'
R235C	For 5′ CCGCGCGCAGCTGCGAACAGGCG 3' Rev 5′ CGCCTGTTCGCAGCTGCGCGCGG 3′
R239C	For 5' CCGCGAACAGGCGTGCAATGCCTTGGCAAC 3' Rev 5' GTTGCCAAGGCATTGCACGCCTGTTCGCGG 3'
K390C	For 5' GCGCGAGCAGCTGGATTGCGCCTATGAC 3' Rev 5' GTCATAGGCGCAATCCAGCTGCTCGCGC 3'
Cys less C21S	For 5' GCCTTTGCATTGTCTGCCAGCACCATGATTCCTCAATAC 3' Rev 5' TATTGAGGAATCATGGTGCTGGCAGACAATGCAAAGGC 3'
MtrC cys mutants	
S141C	For 5' CGTTACAAACCGCTGGTTTGCGCCGATGCCATC 3' Rev 5' GATGGCATCGGCGCAAACCAGCGGTTTGTAACG 3'
D143C	For 5' CAAACCGCTGGTTTCCGCCTGTGCCATCAGTAAAC 3' Rev 5' GTTTACTGATGGCACAGGCGGAAACCAGCGGTTTG 3'
E149C	For 5' GCCGATGCCATCAGTAAACAATGCTACGATGCTGCG 3' Rev 5' CGCAGCATCGTAGCATTGTTTACTGATGGCATCGGC 3'
S146C	For 5' CTGGTTTCCGCCGATGCCATCTGTAAACAAGAGTACGAT 3' Rev 5' ATCGTACTCTTGTTTACAGATGGCATCGGCGGAAACCGA 3'
Cys less C25S	For 5' GCATTGGCACTGTCGTCTAGCGGTAAAGGC 3' Rev 5' GCCTTTACCGCTAGACGACAGTGCCAATGC 3'

Figure Legends

Suppl. Fig. 1 – ITC analyses of the interaction of MtrC with MtrE K225E/R239E**.** 200 μM NT-MtrC was titrated into **(A)** 15 μM MtrE and **(B)** 15 μM MtrE K225E/R239E in a VP-ITC microcalorimeter and the heat exchange determined at 25^{o}C. In each case, the upper panel shows the raw energy changes during the titration, while the lower panel represents the derived integrated total energy change as a function of the molar ratio (based on the molecular weight of the monomeric protein) of the interactants. Non-linear regression fitting of the data (shown as a solid line through the data points in the lower panel) to a monophasic one-site model yielded the following thermodynamic parameters for the interaction: the interaction of Nt-MtrC with MtrE was characterized by a K_a, ΔH and ΔS of 1.0 ($\pm$ 0.50) x 10^5 M^{-1}, -6.8 ($\pm$ 3.6) x 10^3 cal.mol^{-1} and -0.01 cal.mol^{-1}.K^{-1}; while the interaction of Nt-MtrC with MtrE K225E/R239E was characterized by a K_a, ΔH and ΔS of 2.72 ($\pm$ 0.13) x 10^5 M^{-1}, -5.2 ($\pm$ 0.34) x 10^5 cal.mol^{-1} and -151 cal.mol^{-1}.K^{-1}.

Suppl. Fig. 2 – An SDS-PAGE analysis of the stability of the MtrC-MtrE complexes trapped upon cross-linking MtrC as dimers. 8 μM MtrC E149C was cross-linked with 1 mM BMH in the presence of MtrE C21S to trap complexes of MtrC dimers with trimeric MtrE (as shown). In control experiments, MtrC E149C was mixed with MtrE without BMH (lane 1) and MtrC was cross-linked with BMH in the absence of MtrE C21S (lane 12); revealing that MtrC E149C does not stabilizes complex formation in the absence of BMH and that BMH cross-linking of MtrC E149C only yields the dimer. The samples were then treated with 50 mM DTT, urea at the indicated concentration (e.g. 3M – 8M) and/or boiled at 100 ^{o}C for 5 mins as indicated (e.g. additions/ applied conditions are indicated by a +). For each gel lane, 20 μl of each protein (8μM) was loaded onto the gel.

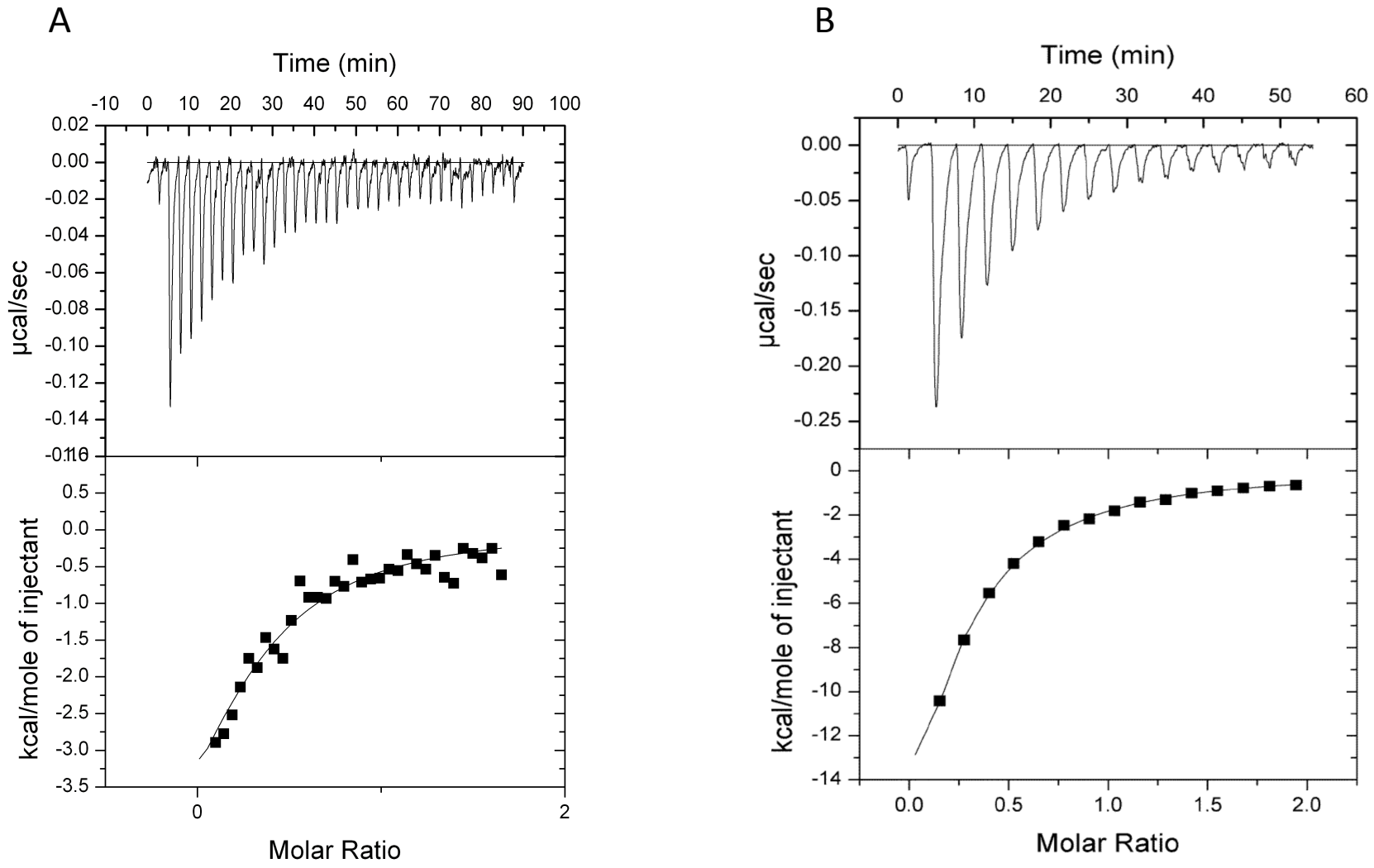
Suppl. Fig. 3 – An SDS-PAGE gel analysis of the cross-linking of NT MtrC D143C, NT MtrC E149C and MtrE C21S K225C with 1 mM BMH was undertaken with the following samples: Mr-standard (lane 1), MtrE (lane 2), MtrC (lane 3), MtrE/MtrC (lane 4), MtrC D143C (lane 5), MtrC D143C/MtrE (lane 6), MtrC E149C (lane 7), MtrC E149C/MtrE (Lane 8), MtrE K225C (lane 9), MtrC D143C/MtrE K225C (lane 10) and MtrC E149C/MtrE K225C (lane 11). Complexes of MtrC and MtrE (denoted 1-6), identified by MS, are indicated by arrows. Note that cys-free MtrE C21S and MtrC NT derivatives were used throughout, so that cross-linking could only occur through the inserted cysteines. For each gel lane, 20 μl of an 8 μM or (when two proteins were combined) 16 μM protein/ 1 mM BMH solution was loaded onto the gel. The positions of relevant Mr markers are shown on the gel.

Suppl. Fig. 4 – MtrD form trimers. An SDS-PAGE gel analysis of MtrD, in which 8 μM MtrD was run on a gel in the absence and presence of 1 mM BMH. Wild-type MtrD, with a single cysteine, forms trimers that are further stabilized by cross-linking with BMH.

Suppl. Fig. 5 – Schematic diagram showing the topology of the assembled MtrCDE tripartite pump. (A) Schematic representation of the 4 α-helices of each subunit (colored green, orange and blue) of the trimeric MtrE, with the position of the intra- and inter-protomer grooves identified. **(B)** Schematic representation of the assembled tripartite pump, in which MtrD interacts with MtrE, whilst MtrC binds across the surface of these proteins to stabilize their interaction. The α-helical coiled-coil/hairpin domain of MtrC interacts with the intra- and inter-protomer grooves of MtrE, so that two MtrC are bound per MtrE subunit, and the β-domain of MtrC interacts with MtrD, probably at a site within each subunit and at the interface of the subunits of MtrD, to give an assembly with a 3:6:3 stoichiometry. The planes of the membranes are only to illustrate that MtrD and MtrE interact with the inner- and outer-membranes respectively, rather than to show the precise sites of interaction. Only the β-barrel domain of MtrE, and α-helical domain of MtrD are expected to be integrated into the membranes; whilst the α-helical domain of MtrE and the globular headpiece of MtrD span the periplasm and it is these domains across which MtrC binds.

Suppl. Fig. 6 – Comparison of the EM generated pictures of the average of selected images of MtrC (top panels) and MacA (bottom panels) particles before (left panel) and after (right panel) the imposition of 6-fold symmetry. The box size is 280 Å. The images indicate that both MtrC and MacA form hexameric ring-structures, with outer inner-diameters of approximately 55 Å and 34 Å, respectively.

Suppl. Fig. 1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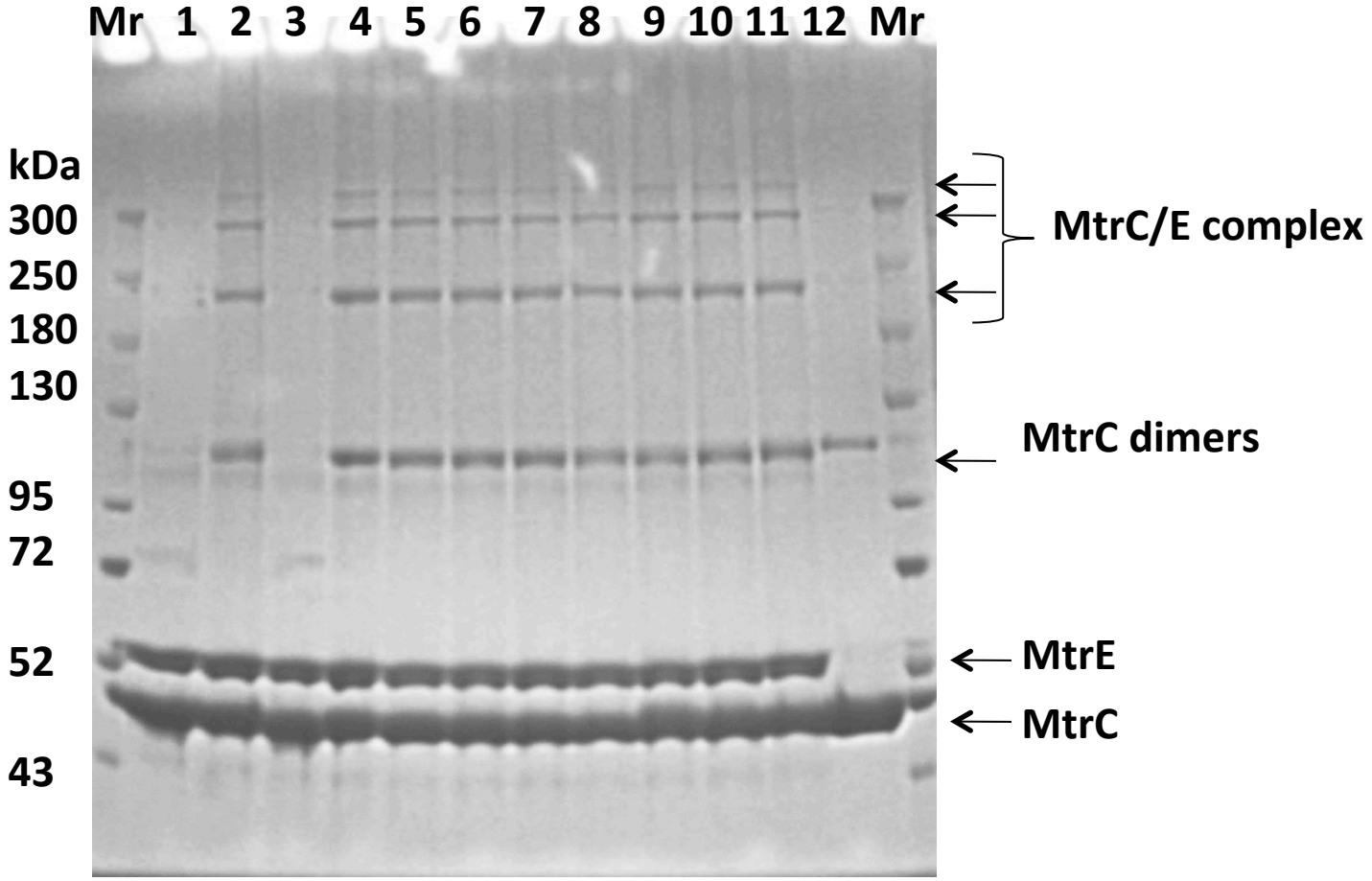
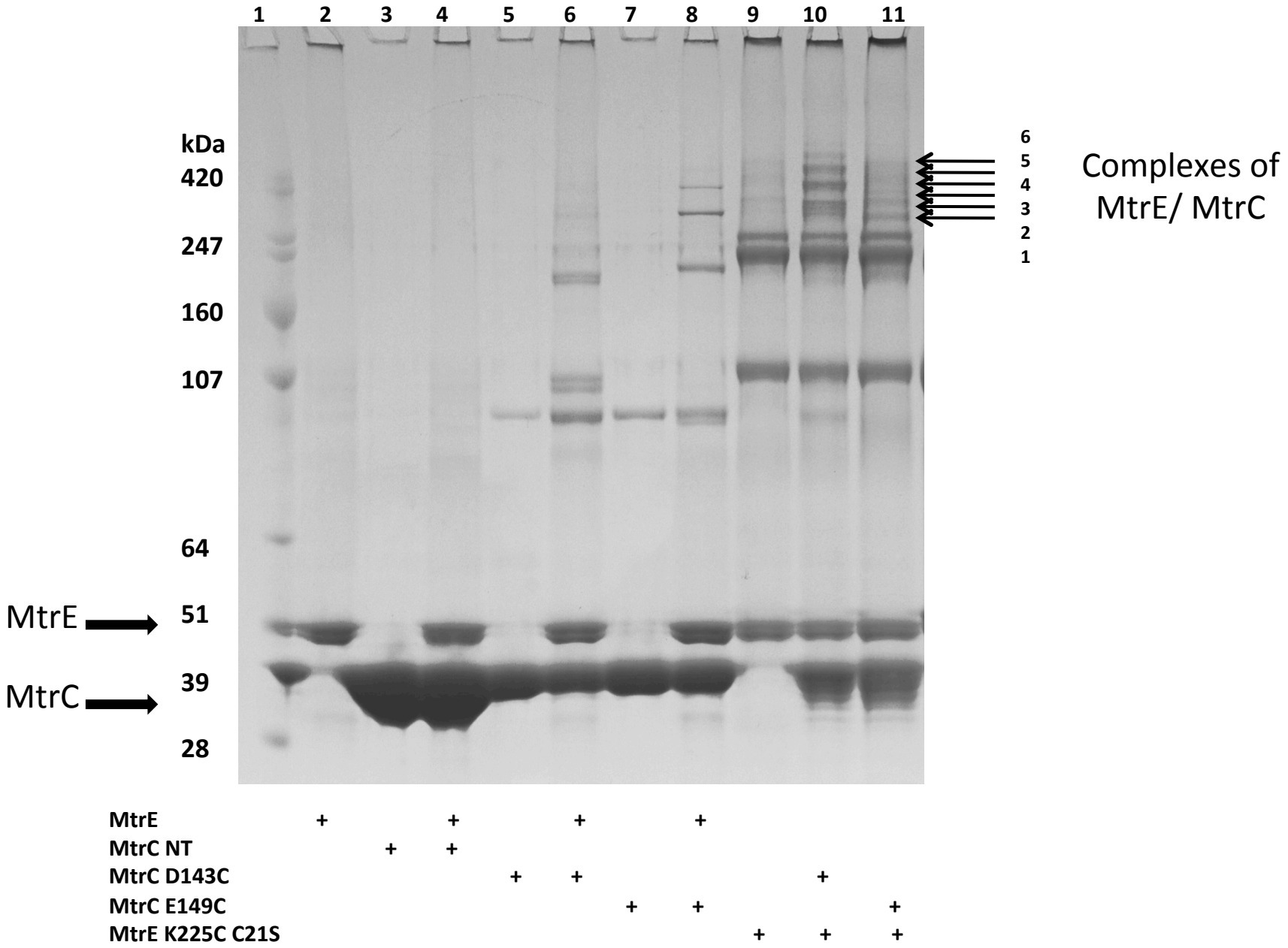
Time (min)
µcal/sec
kcal/mole of injectant
Molar Ratio
B
Time (min)
µcal/sec
kcal/mole of injectant
Molar Ratio

Suppl. Fig. 2



BMH	-	+	-	+	+	+	+	+	+	+	+	+	
DTT	-	-	+	+	+	+	+	+	-	-	-	+	
Boil	-	-	+	-	+	+	+	+	-	-	-	+	
Urea	-	-	5	-	-	3	5	8	3	5	8	8	
MtrE C21S	+	+	+	+	+	+	+	+	+	+	+	-	
MtrC E149C	+	+	+	+	+	+	+	+	+	+	+	+	

Suppl. Fig. 3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kDa
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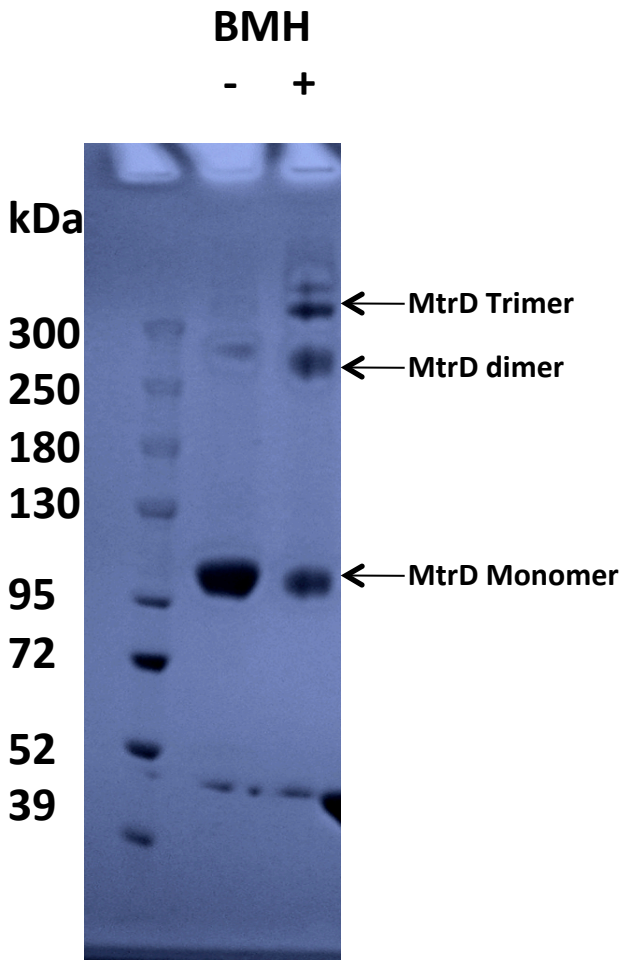
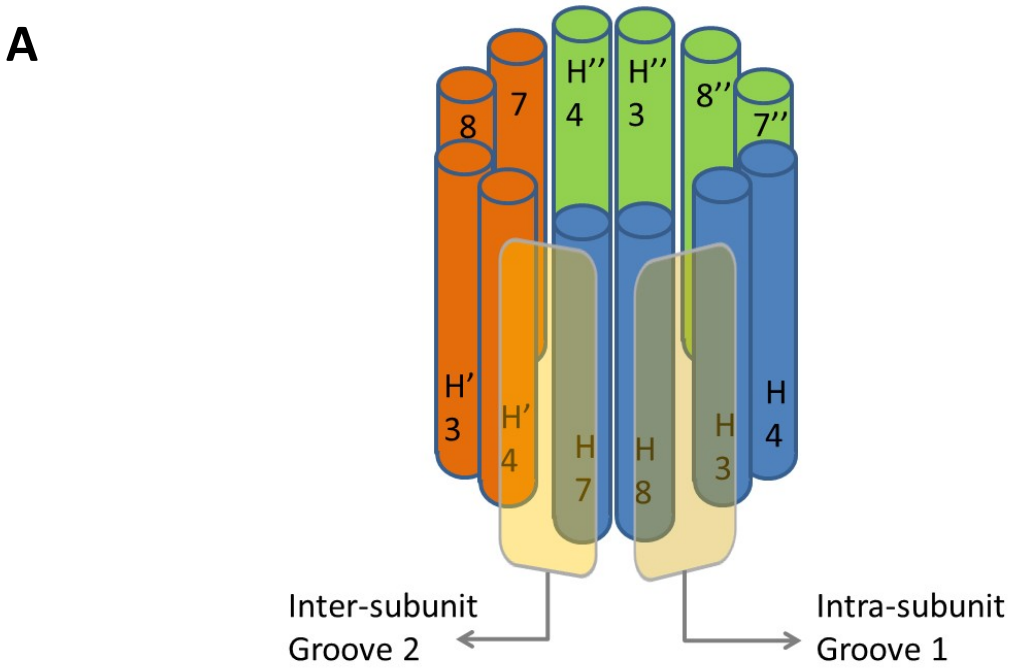
247
160
107
64
51
39
28
MtrE
MtrC
6
5
4
3
2
1
Complexes of MtrE/ MtrC
MtrE + + + +
MtrC NT + +
MtrC D143C + + +
MtrC E149C + + +
MtrE K225C C21S + + +

Suppl. Fig. 4


BMH
-
+
kDa
300
250
180
130
95
72
52
39
MtrD Trimer
MtrD dimer
MtrD Monomer

Suppl. Fig. 5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7
H''
4
H''
3
8''
7''
H'
3
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H
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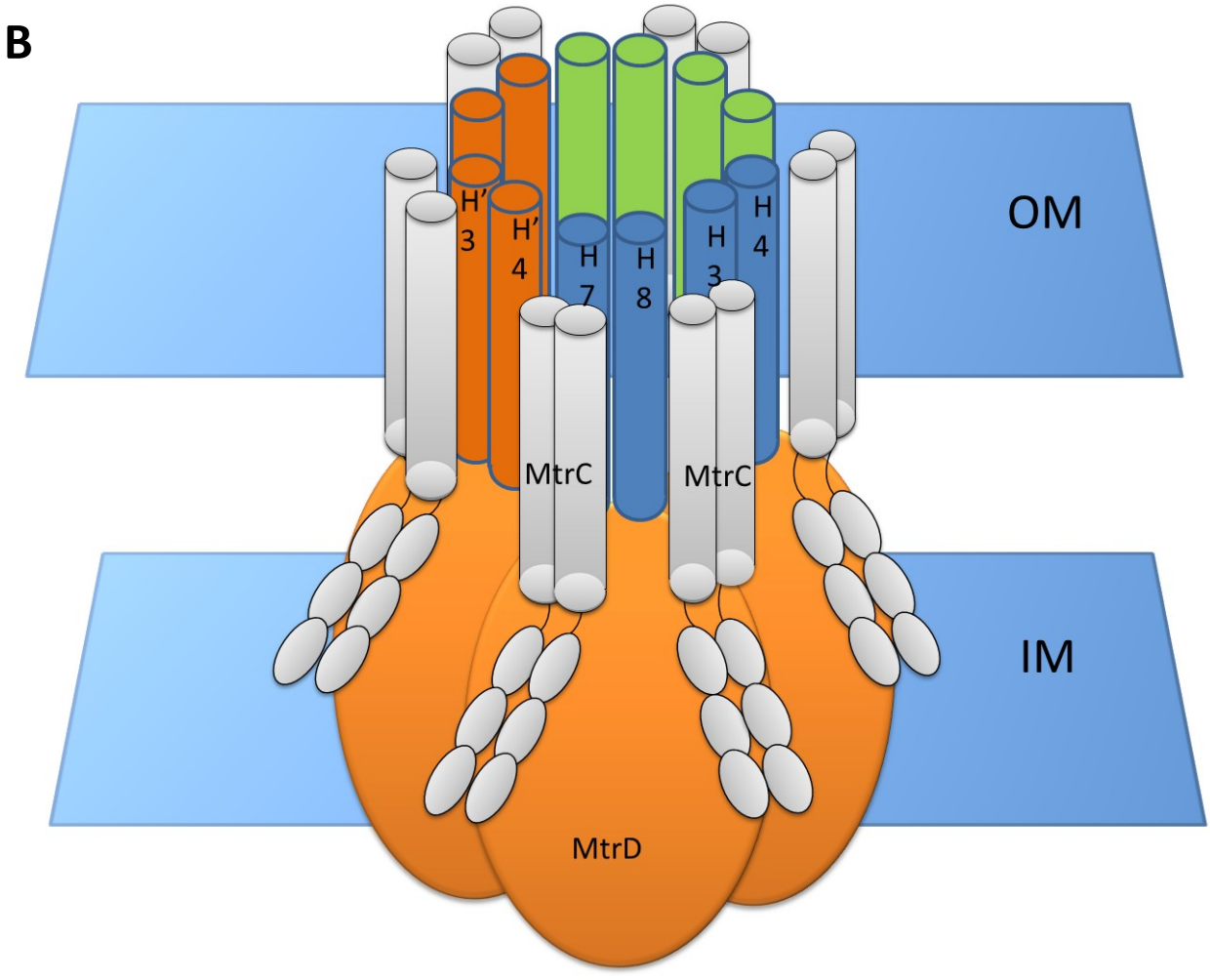
H
4
Inter-subunit
Groove 2
Intra-subunit
Groove 1


B
H'
3
H'
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H
7
H
8
H
3
H
4
OM
MtrC
MtrC
IM
MtrD

Suppl. Fig. 6