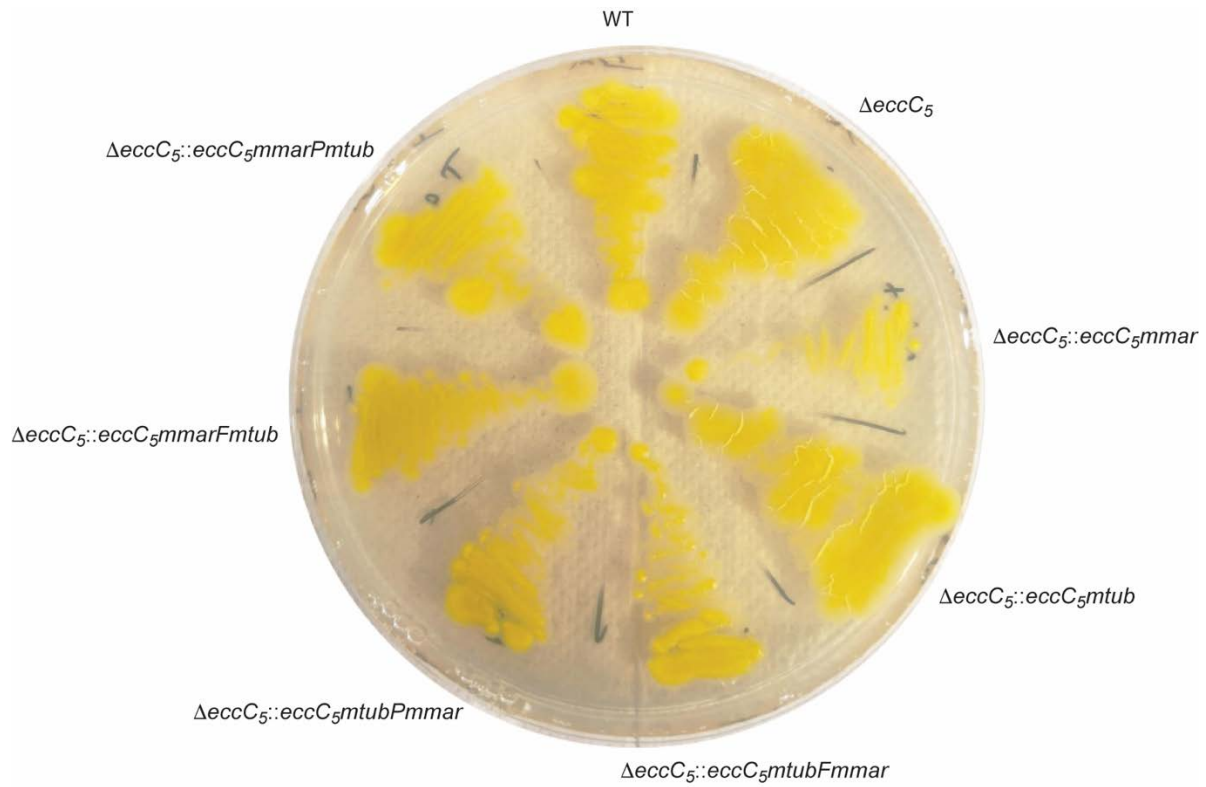
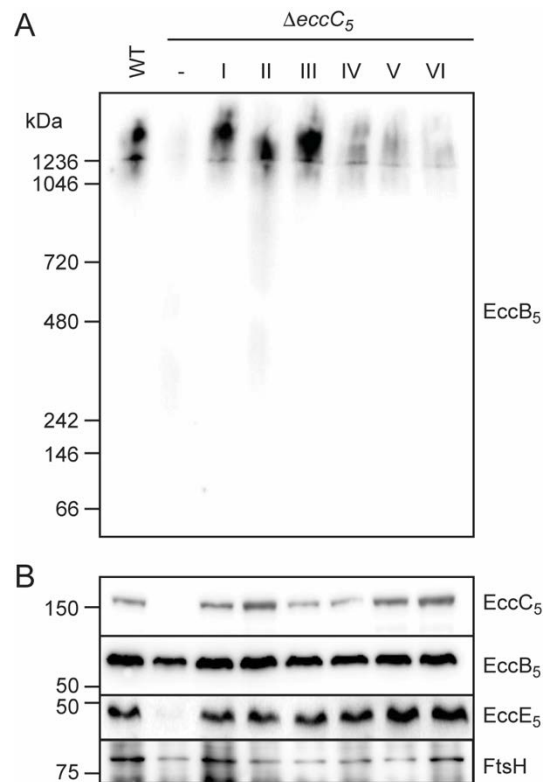


**Figure S1.** EspG<sub>5mtub</sub> complements secretion of a *M. marinum*  $\Delta$ espG<sub>5</sub>. Secretion analysis of *M. marinum* WT,  $\Delta$ espG<sub>5</sub> and  $\Delta$ espG<sub>5</sub> complemented with WT espG<sub>5mmar</sub> or espG<sub>5mtub</sub>. Proteins were visualized by SDS-PAGE and immunoblotting using antibodies against EsxN and PE\_PGRS proteins (ESX-5 substrates), GroEL2 (lysis and whole cell loading control) and EspG<sub>5</sub>.



**Figure S2.** Linker 2 of  $EccC_{5mtub}$  affects the macroscopic appearance of *M. marinum*. Colony morphology of *M. marinum* WT,  $\Delta eccC_5$  and  $\Delta eccC_5$  complemented with constructs depicted in Fig. 2B. Strains were streaked on 7H10 agar media supplemented with 10% Middlebrook OADC (BD Biosciences).



**Figure S3.** ESX-5 membrane complex analysis. (A) BN-PAGE and immunoblot analysis of DDM solubilized cell envelope fractions of *M. marinum* WT,  $\Delta eccC_5$  and  $\Delta eccC_5$  complemented with constructs depicted as in Fig. 2B. (B) SDS-PAGE and immunoblot analysis of the same set of samples as in A. Proteins were visualized using antibodies against EccB<sub>5</sub>, EccC<sub>5</sub>, EccE<sub>5</sub> (membrane proteins forming the ESX-5 membrane complex) and FtsH (membrane protein loading control).

A

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C5tb      RAEKLMENMGYRLVVKARTAGAAQAAG-VPNAVNLPAQAGLGYFRKSLEDIIRFQAEFLW 713
C5Mar     RAEKLMENMGYRLVVKARTAGAAQAAG-VPNAVNLPAQAGLGYFRKSLEDIIRFQAEFLW 714
TC0607    KLRGLDTHLSYRIGLRTFSAMESRVVLGVDPDAYELPPSPGNGYLKFATEPLVRFKAAYVS 692
           : . *  ::.**: **: : *  :...  **:* :***... *  **:: : *  ::**:* : :

C5TB      RDYFQPGVSIDGEE---APALVHSIDYIRPQLFTNSFTPLEVSVGGPDIEPVVAQPNGEV 770
C5Mar     RDYFQPGITVDGEE---APVLVHSIDYIRPQLFTNSFTPLEVTVGGPEIDKVVAHANGEV 771
TC0607    GPVDEEPQTRSEGPQIVRQVLPYLTDYIRPQVVEQQFPQPEQRA 734
           :   :   .   .   *   :   *   *   *   *   *   *   :   :   :   :   .

C5TB      LESDDIEGGEDEDEEGVRTPKVGTVIDQLRKIKFEPYRLWQPPLTQPVAIDDLVNRFLG 830
C5Mar     VEEVEAEAEE---EGIRVPKVGTVIDQLRRINFEPYRLWQPPLTQPVAIDDLVNRFLG 827
TC0607    EENKSSES-----LFDVVVRQLAGHGPEPHQIWLPPLDVPPTLDELLPPLSP 781
           * . . * .   :   * : : * *   * * : : * * * * * * * : : * : : :

C5TB      RPWHKEYGS----ACNLVFPIGIDRPYKHDQPPWTVDTSGPGANVLILGAGGSGKTTAL 886
C5Mar     HPWQKEYGS----ARNLVFPIGIDRPYKHDQPPWTVDTSGPGSNVLILGAGGSGKTTAL 883
TC0607    SAAHGYTADGWEWRGRLHAVVGLVDRPFDQRRDPDYWLDLSGGAGHVGVAGGPQTGKSTML 841

```

B

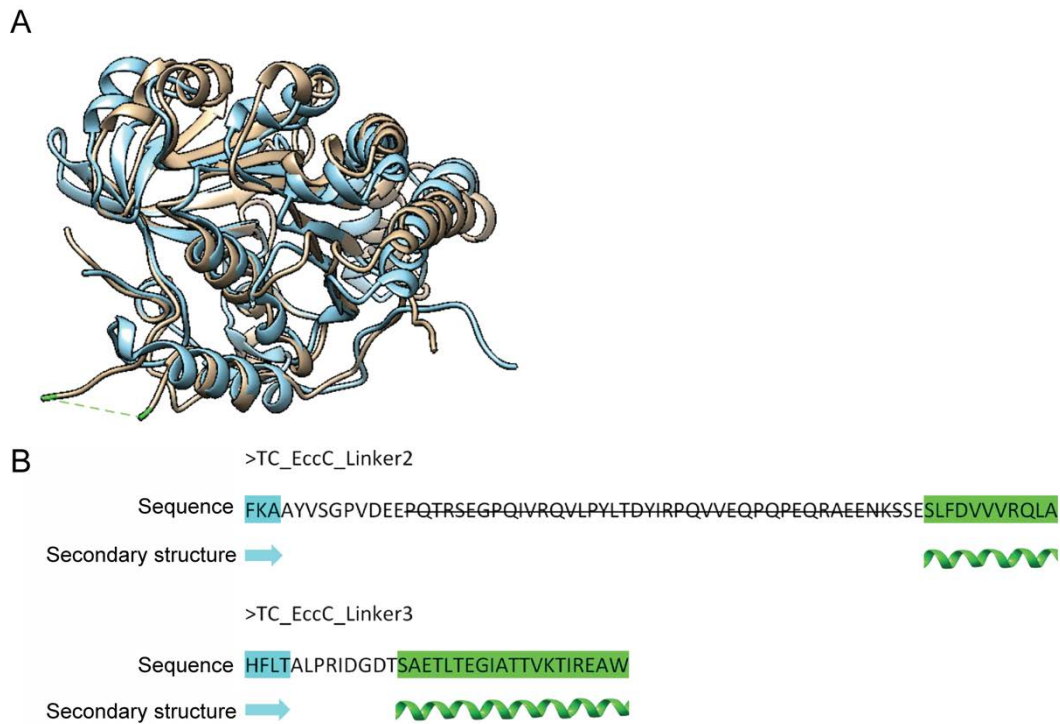
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EsxM_Mtub MASRFMTDPHAMRDMAGRFEVHAQTVEDEARRWASAQNISGAGWSGMAEATSLDTMTXM 60
EsxM_Mmar VTARFMTDPHAMRDMAGRFEMHAQTVEDEARKWASSQNIAGAGWSGMASATSLDTMGQM 60
EsxB_TC   MAPQSAVDRAAMAQAAQDIEQSANAIR-GMQNQLASAKDQLRSHWEGDASMAFEAVFNRF 59
           :: : . *  ** : *  : *  * : : .   .   * : : : :   : * . * . :   . : :

EsxM_Mtub NQAFRNIVNMLHGVRDGLVRDANNYEQQEQASQQILSS----- 98
EsxM_Mmar NTAFRNIVNMLHSVRDGLVRDANNYEQQEQASQQVLRG----- 98
EsxM_Mtub NQAFRNIVNMLHGVRDGLVRDANNYEQQEQASQQILSS----- 98
EsxB_TC   NEDFSRVLKALDGMHESLVQTRITYEAREEAAQSVNRVQALLNG 104
           * * . : : * . : : : * : :   . * * : * : * : * :

```

**Figure S4.** Divergence of Linker 2 sequences. (A) Alignment of the linker 2 region from *M. tuberculosis* H37Rv EccC<sub>5</sub>, *M. marinum* M EccC<sub>5</sub> and *T. curvata* EccC. The C-terminal part of NBD1 and the N-terminal part of NBD2 are highlighted in blue. The sequences that were swapped between constructs EccC<sub>5</sub><sub>mmar</sub>P<sub>mtub</sub> and EccC<sub>5</sub><sub>mtub</sub>P<sub>mmar</sub> (partial linker 2 swap) are depicted in bold. The sequences that were swapped between constructs EccC<sub>5</sub><sub>mmar</sub>F<sub>mtub</sub> and EccC<sub>5</sub><sub>mtub</sub>F<sub>mmar</sub> (full linker 2 swap) are underlined. The missing structural feature of the linker 2 in the *T. curvata* EccC crystal structure (Rosenberg et al., 2015) is stroked through. (B) Sequence alignment between EsxB of *T. curvata* and EsxM from *M. marinum* and *M. tuberculosis*. Alignments were produced using Clustal Omega.



**Figure S5.** Comparison of linker 2 and linker 3. (A) Superimposition of NBD1 (light brown) and NBD2 (light blue) from *T. curvata* (Rosenberg et al., 2015), emphasizing the missing structural feature in the linker 2 region, in green. (B) linker 2 and linker 3 loop sequences with the adjacent upstream  $\beta$ -sheet (blue) and downstream  $\alpha$ -helix (green). The missing structural feature is stroked through in the linker 2 sequence.



**Figure S6.** Sequence alignment of Ecc<sub>5</sub> of *M. tuberculosis* H37Rv (C5tub), Ecc<sub>5</sub> of *M. marinum* M (C5mar), and Ecc<sub>5</sub> of *T. curvata* (TC0607). The missing features in the crystal structure of *T. curvata* EccC are stroked through, including the linker 2 region (Rosenberg *et al.*, 2015). Transmembrane domains are highlighted in red and that are important for substrate binding to NBD3 are in red. The underlined sequence represents the “Full linker 2”, which was swapped between the two Ecc<sub>5</sub> homologs. The sequence corresponding to the “Partial linker 2” swapped region is depicted in bold. The alignment was produced using Clustal Omega.

<b>Walker B</b>		
EccCab1_M_tub	TLFVVVDEFAELLQSHPDFIGLFDRIICRVGRSLRVHLLLATQ	SLQTGGVVRIDKLEPNLTY 652
EccCab1_M_marinum	TLFVVVDEFAELLQSHPDFIGLFDRIICRVGRSLRVHLLLATQ	SLQTGGVVRIDKLEPNLTY 652
EccC2_M_tub	VLLVIVDEYLELFANHKKWIDLIHIGQEGRGANVFFMLGGQ	RLDLSS--LQKVKSNIAF 687
EccC3_M_marinum	TLFVVVADEFITMLADHPEYAEFLFDYVARKGRSFRIHILFASQ	TLDVVGK--IKDIDKNTSY 654
EccC3_M_tub	TLFVVVADEFITMLADHPEYAEFLFDYVARKGRSFRIHILFASQ	TLDVVGK--IKDIDKNTAY 649
EccC4_M_marinum	TLFIIVDEFAELLSQHPDFAEVFVAIGRLGRSLGMHLLLASQ	RLEEEGR--LRGLEAHLISY 584
EccC4_M_tub	ILFIIVDEFSELLSQHPDFVDVFLAIGRVGRSLGMHLLLASQ	RLDEGR--LRGLETHLSY 594
EccC5_M_marinum	MLVVVIDEFYEWFRIMPTAVDVLDSIGRQGRAYWIHLMMASQ	TIESR---AEKLMENMGY 666
EccC5_M_tub	MLVVVIDEFYEWFRIMPTAVDVLDSIGRQGRAYWIHLMMASQ	TIESR---AEKLMENMGY 665
	*.: : **:	: : : : * . : : : : * : :
EccCab1_M_tub	RIALRTTSSHESKAVIGTPEAQYITNK--ESGVGFL-RVGMEDPVKFFSTFYISGPYMPPA	709
EccCab1_M_marinum	RIALRTTSSHESKAVIGTPEAQYITNK--ESGVGFL-RVGMEDPIKFFSTLYISGPYVPPA	709
EccC2_M_tub	RIALRAESGDDSRVIGSDAAYHLPK--ENGFALL-KVGPRLDEPFRCFYLSAPFVVPK	744
EccC3_M_marinum	RIGLKVASASVSRQIIGVEDAYHIEAGKEHKGVGFLVPAPGAAPIRFRSTYVDGIYEPQP	714
EccC3_M_tub	RIGLKVASPSVSRQIIGVEDAYHIEAGKEHKGVGFLVPAPGATPIRFRSTYVDGIYEPQP	709
EccC4_M_marinum	RVCLKTLSAIESRTALGTDAFELPNT---PGSGLL-SSPTAELTRFETAFVSGPVPAGP	640
EccC4_M_tub	RMCLKTWSASESRNVLTQDAYQLPNT---PGAGLL-QTGTGELIRFQTAFAVSGPLRRAS	650
EccC5_M_marinum	RLVLKARTAGA-AQAAGVNPVAVNLPQAQ---AGLGYF-RKSLEDIRFQAEFLWRDYFQPG	721
EccC5_M_tub	RLVLKARTAGA-AQAAGVNPVAVNLPQAQ---AGLGYF-RKSLEDIRFQAEFLWRDYFQPG	720
	*: * . : :	* * : : * . : : * : :
EccCab1_M_tub	AGVETNGEA-GGPGQQT-----RQAARIHRFTAAPVLEEAP-----	745
EccCab1_M_marinum	T-AETNGDG-SGPSTQFA-----KRALQIREFTAAPVLEEAL-----	744
EccC2_M_tub	KKEVART-----IDMTLTQPRLYDWQYQPLDAADAEA---LATAAAAADAEPEDEF-----	790
EccC3_M_marinum	QTKTRVVHN-----IPEPKLFTATAVEPDPDPTV--I SEV	746
EccC3_M_tub	TAKAVVVQS-----VPEPKLFTAAAVEPDPDPTV--IADT	741
EccC4_M_marinum	PDADSV-AL-----PAAVRPFTSEATGDRV-----GG	667
EccC4_M_tub	PSAVHP-VA-----PPSVRPFTHAAAPVTA-----GP	677
EccC5_M_marinum	ITVDGEEAPVLVHSIDYIRPQLFTNSFTPLEVTVGGEIDKVVVAHANGEVVEVEA-----	777
EccC5_M_tub	VSIDGEEAPALVHSIDYIRPQLFTNSFTPLEVTVGGPDIEPVVAQPNGEVLESDDIEGGE	780
	. :	
<b>Start EccCb1</b>		
EccCab1_M_tub	TPMTAEPEVRTLREVLDQLGTA-ESRAYKMWLPPLTNPVPLNELIARDR-----R-	795
EccCab1_M_marinum	TPMTAEPEVRTLREVILDQLSTV-ESRAYKMWLPPLVDPTPLDELVARDR-----R-	794
EccC2_M_tub	LYYDDGFKKKKIVDVLRESLYNPHRSPRRPWLPAPLEDEPEVDRLVAAYRGKPPWHVDYQG	850
EccC3_M_marinum	EEEHVPGPPRKLIIATIGEQLARY-GPQAPRLWLPPLDEPIPLSTLLATAG-----VA	797
EccC3_M_tub	DEQEPADPPRKLIIATIGEQLARY-GPRAPQLWLPPLDETIPLSAALARAG-----VG	792
EccC4_M_marinum	ADGADTATGPTVLQTVLDRIAGH-GPRAHQVWLPPLDRAPALASLLSDGA-----AE	718
EccC4_M_tub	VGGTAEVPTPTVLHVAVLDRLVGH-GPAAHQVWLPPLDEPPMLGALLRDAE-----PA	728
EccC5_M_marinum	EAEHEGIRVPKVGTVIIDQLRRI-NFEPYRLWQPPLTQPVAIDDLVNRFLGHPWQKEYGS	836
EccC5_M_tub	DEDEEGVRTPKVGTVIIDQLRRI-KFEPYRLWQPPLTQPVAIDDLVNRFLGRPWHKEYGS	839
	. : . : : :	: * ** : :
<b>Walker A</b>		
EccCab1_M_tub	-QPLRFALGIMDEPRRHLQDVWGVDVSGAGNIGIGGAPQTGKSTLLQTMVMSAAATHSP	854
EccCab1_M_marinum	-QPLRFALGIMDEPRRHLQDVWGVDVSGAGNIGIGGAPQTGKSTLLQTLVMSAAATHSP	853
EccC2_M_tub	NPGLMFPVGMIDIPESQVVHAVDA--LRSNIIIVGAKQRGKTTTLMALMCSAATMYTP	908
EccC3_M_marinum	QRQWRWPLGEIDKPFEMRRDPLVFDATSSAGNLVIHGGPKSGKSTALQSFMLSAASLHSP	857
EccC3_M_tub	PRQWRWPLGEIDRPFEMRRDPLVFDARSSAGNMVIHGGPKSGKSTALQTFILSAASLHSP	852
EccC4_M_marinum	HAELAVPIGVVDRPFDQSRPTLIDLTAAAGNVAVVVGAPQSGKSTTLSTLITALAATHDP	778
EccC4_M_tub	QAEAVPIGIVDRPFEQSRVPLTIDLSGAAGNVAVVVGAPQTGKSTALRTLIMALAATHDA	788
EccC5_M_marinum	ARNLVFPIGVIDRPFKHDQPPWTVDTSGPGSNVLIILGAGGSGKTTALQTLISSAALHTTP	896
EccC5_M_tub	ACNLVFPIGIIDRPFYKHDQPPWTVDTSGPGANVLIILGAGGSGKTTALQTLICSAALHTTP	899
	: * : * * :	: * : * . * * * * : : : * : :

**Figure S7.** Alignment of all EccC homologs of *M. marinum* M and *M. tuberculosis* H37Rv. Part of the linker 2 region, which shows no sequence homology and overlaps largely with the missing structural feature of the *T. curvata* EccC and the “Partial linker 2 swap” constructs, is highlighted in grey. the C-terminal part of NBD1 and the N-terminal part of NBD2 are highlighted in blue. Walker A and B motifs are highlighted in green. The first residue of EccCb1 is depicted in red. The alignment was produced using Clustal Omega.

**Table S1.** List of plasmids used in this study.

<b>Nr.</b>	<b>Plasmid</b>	<b>Features</b>	<b>Source</b>
1.	pMV-espG <sub>5mtub</sub>	<i>hsp60</i> promotor, L5 <i>attP</i> & <i>aph</i> , <i>oriE</i> , <i>hyg</i> <sup>R</sup>	This study
2.	pMV-espG <sub>5mmar</sub>	<i>hsp60</i> promotor, L5 <i>attP</i> & <i>aph</i> , <i>oriE</i> , <i>hyg</i> <sup>R</sup>	Phan et al., 2017
3.	pMV-eccC <sub>5mtub</sub>	<i>hsp60</i> promotor, L5 <i>attP</i> & <i>aph</i> , <i>oriE</i> , <i>kan</i> <sup>R</sup>	This study
4.	pMV-eccC <sub>5mmar</sub>	<i>hsp60</i> promotor, L5 <i>attP</i> & <i>aph</i> , <i>oriE</i> , <i>kan</i> <sup>R</sup>	Ates et al., 2015
5.	pMV-eccC <sub>5mtub</sub> F <sub>mmar</sub>	<i>hsp60</i> promotor, L5 <i>attP</i> & <i>aph</i> , <i>oriE</i> , <i>kan</i> <sup>R</sup>	This study
6.	pMV-eccC <sub>5mtub</sub> P <sub>mmar</sub>	<i>hsp60</i> promotor, L5 <i>attP</i> & <i>aph</i> , <i>oriE</i> , <i>kan</i> <sup>R</sup>	This study
7.	pMV-eccC <sub>5mmar</sub> F <sub>mtub</sub>	<i>hsp60</i> promotor, L5 <i>attP</i> & <i>aph</i> , <i>oriE</i> , <i>kan</i> <sup>R</sup>	This study
8.	pMV-eccC <sub>5mmar</sub> P <sub>mtub</sub>	<i>hsp60</i> promotor, L5 <i>attP</i> & <i>aph</i> , <i>oriE</i> , <i>kan</i> <sup>R</sup>	This study



**Table S2.** List of primers used in this study.

<b>Nr.</b>	<b>Name</b>	<b>Sequence (5' - 3')</b>
1.	EsxMtub_Xmnl_F	GACTGAAAGAATTCCAT ATGGCCTCACGTTTTAT GAC
2.	EspG5tub_HindIII_R	GACTAAGCTTTTCATACTCTGCTGTGTGTTTTTC
3.	EccC5tub_Xmnl_F	GACTGAAAGAATTCCAT ATGAAGCGT GGTTTTGCCC
4.	EccC5tub_HindIII_R	GACTAAGCTTCTACCGACGCACCTCGG
5.	C5tub_MunI.fw	CCC GCAATTGTT CACCAACTCATT CAC
6.	C5tub_interswap.fw	GAAGAAGGCATCCGAGT GCCGAAGGTT GGGAC GGT
7.	C5_swap_1_FW 69C	ATATGGCCGGCAGGGCCGT GCCTATTG
8.	C5_swap_1_RV 67C	GTCGATCGCGACGGGCTG
9.	C5_swap_2_FW 62C	CCCGTCGCGATCGACGACTTG
10.	C5_swap_2_RV 65C	CTGATCACCGCGGCCATG
11.	C5 mar Tb full domain 1 FW	ATCCGGAGGAAAGAATTCTATTGCCGCAAGGCCCGACG
12.	C5 mar Tb full domain 1 RV	GTCGATGCTGACGCCGGGCTGGAAGTAGTCC
13.	C5 mar Tb full domain 2 FW	GGCGTCAGCATCGACGGCGA
14.	C5 mar Tb full domain 2 RV	ACCGGTTGACCAGGTCGTCGATGGCGAC
15.	C5 mar Tb full domain 3 FW	GACCTGGTCAACCGGTTCTCTG
16.	C5 mar Tb full domain 3 RV	CGACATCGATAAGCTTTTACTTTTCGCACCTCGGTC
17.	C5 mar partial TB interdom 1 RV	GAACGAGTTGGTAAACAAC TGC GGGCGAAT G
18.	C5 mar partial TB interdom 2 FW	CATTGCCCCGAGTTGTTTACCAACTCGTTC
19.	C5 mar partial TB interdom 2 RV	GTACCCACCTTGGGGGTGCGCACCCCTC
20.	C5 mar partial TB interdom 3 FW	GCACCCCAAGGTGGGTACCGTGATC