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## Domestication of a housekeeping transglycosylase for assembly of a Type VI secretion system

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1 **activation of a LTG encoded within the core genome for the assembly of a secretion**  
2 **system.**

3

#### 4 **Introduction**

5 The cell envelope of Gram negative bacteria is crossed by multiprotein complexes that  
6 participate to the assembly of surface appendages (*e.g.*, the flagellum) or serve as channels for  
7 the passage of large molecules such as pili, DNA or protein effectors (*e.g.*, piliation,  
8 conjugation or secretion systems) [1]. These complexes are usually large and are anchored to  
9 both the inner and outer membranes [1]. However, the peptidoglycan layer represents a  
10 physical barrier for the assembly of these structures, as they are usually larger than  
11 peptidoglycan pores, estimated to have a diameter of ~ 2 nm [2]. Most of these systems have  
12 therefore evolved enzymes, called lytic transglycosylases (LTGs), that locally rearrange the  
13 cell wall [3-5]. LTGs cleave the glycan strands but have no action on peptide cross-links,  
14 therefore creating lateral separation of the peptidoglycan [6,7]. Endogeneous LTGs are  
15 involved in peptidoglycan synthesis, turnover, recycling and daughter cell separation [7-9].  
16 By contrast, the LTGs dedicated to specific cell-envelope spanning complexes are called  
17 specialized LTGs [3-5,8]. The activity of these enzymes needs to be tightly controlled to  
18 avoid peptidoglycan breaches and cell lysis [8,10]. In addition, the LTG activity should be  
19 spatially controlled to create sufficient space at the site of assembly. The spatial activation of  
20 specialized LTGs is secured by their recruitment to the site of assembly through interactions  
21 with one or several components of the apparatus. The recruitment of specialized LTGs to their  
22 cognate apparatus has been exemplified in the case of several cell-spanning machineries: the  
23 *Rhodobacter sphaeroides* SltF LTG is recruited to the flagellar FlgJ subunit [11,12], the PleA  
24 protein localizes at the cell pole in *Caulobacter crescentus* and is required for the assembly of  
25 the polar pilus and polar flagellum [13], the VirB1-like LTG is recruited to the VirB8-like  
26 protein in Type IV secretion systems [4,14-19], and the EtgA LTG associates with the Type  
27 III secretion system EscI rod component [4,20-22]. Interestingly, in a few cases, machine  
28 subunits comprise an additional domain with LTG activity, such as the flagellar rod FlgJ  
29 protein [23-27] or the *Bordetella pertussis* T4SS PtlE subunit [28]. For several of these  
30 enzymes, it has been recently demonstrated that the transglycosylase activity is weak *in vitro*  
31 but stimulated in presence of its partner suggesting that binding to the cell-envelope spanning

1 structure specifically activates the enzymatic activity and hence controls localized  
2 peptidoglycan hydrolysis. The activity of the T3SS EtgA LTG is enhanced by co-incubation  
3 with the EscI rod subunit [22]. In the case of the *R. sphaeroides* flagellum, the activity of SlfF  
4 is modulated by both FlgB and FlgF [29].

5         Recently, we determined the structure of the 1.7-MDa Type VI secretion system  
6 (T6SS) membrane complex from enteroaggregative *E. coli* (EAEC) using negative stain  
7 electron microscopy [30]. This complex spans the cell envelope, and its diameter was  
8 estimated to ~ 18 nm in the periplasm, suggesting that its proper insertion requires localized  
9 peptidoglycan rearrangement or degradation. However, no gene encoding LTG is encoded  
10 within T6SS gene clusters [31,32]. The T6SS is a sophisticated multiprotein machine that is  
11 widely distributed in Gram-negative bacteria and responsible for the delivery of toxin  
12 effectors in both prokaryotic and eukaryotic cells, hence participating in bacterial competition  
13 and pathogenesis [33-42]. It is constituted of a cytoplasmic tail complex that is evolutionarily,  
14 structurally and functionally related to contractile machines such as phages or pyocins [43-  
15 45]. The tail comprises an inner tube composed of Hcp hexamers stack on each other and  
16 wrapped into the contractile sheath formed by the polymerization of TssBC complexes [46-  
17 50]. The inner tube is tipped by the VgrG/PAAR complex that is used as a puncturing device  
18 to penetrate the target cell [47,51]. Once assembled, the sheath contracts and propels the  
19 Hcp/VgrG/PAAR needle complex, allowing effector delivery and target cell lysis [49,52-54].  
20 The tail is built onto an assembly platform, the baseplate, constituted of the TssEFGK-VgrG  
21 subunits [55-57]. The baseplate docks to the membrane complex that both orientates the tail  
22 towards the cell exterior and serves as channel for the passage of the Hcp/VgrG/PAAR needle  
23 [30,56,58,59]. The membrane complex is composed of the TssJ, TssL and TssM proteins,  
24 each present in ten copies [30,60]. TssL and TssM are both inner membrane proteins, with  
25 soluble domains in the cytoplasm and periplasm respectively [58,61,62]. TssJ is an outer  
26 membrane lipoprotein [63] that interacts with the C-terminal domain of the TssM periplasmic  
27 region [64]. The assembly of the membrane complex starts with the initial positioning of the  
28 TssJ lipoprotein and progresses inward with the ordered addition of TssM and TssL [30].  
29 Once assembled, the membrane complex recruits the TssA protein and the baseplate complex  
30 prior to tail/sheath polymerization [56,65]. In addition to span the cell envelope, the  
31 membrane complex is anchored to the cell wall by an additional component TagL, or an  
32 additional domain fused to the C-terminus of TssL, that shares homology to peptidoglycan-



1 binding proteins [60,66]. It is proposed that anchorage to the cell wall allows stabilization of  
2 the membrane complex, notably during sheath contraction. The negative stain electron  
3 microscopy structure of the EAEC TssJLM complex demonstrated that it is composed of a  
4 base comprising the cytoplasmic domains of TssL and TssM and forms a trans-envelope  
5 channel with ten arches and ten pilars constituted by the periplasmic domain of TssM and the  
6 TssJ lipoprotein [30]. The diameter of this complex in the periplasm is however incompatible  
7 with the size of peptidoglycan pores and we hypothesized that proper insertion or assembly of  
8 the T6SS membrane complex requires the action of a LTG. Recently, a study identified TagX,  
9 a T6SS-encoded peptidoglycan endopeptidase required for T6SS function in *Acinetobacter*  
10 species [67]. However, the *tagX* gene is not conserved and the vast majority of T6SS gene  
11 clusters does not encode peptidoglycan hydrolases. This observation raised the question on  
12 how these Type VI secretion systems deal with the peptidoglycan layer. Here, we show that  
13 the EAEC T6SS has domesticated the housekeeping MltE LTG for its assembly.

14

## 15 **Results**

16

### 17 **Proper function of the EAEC T6SS requires the MltE housekeeping lytic** 18 **transglycosylase.**

19 To test whether peptidoglycan remodeling is required for assembly of the T6SS, cells were  
20 treated with Bulgecin A, a specific inhibitor of transglycosylases [68,69], and the release of  
21 Hcp in the culture supernatant, a marker of EAEC T6SS assembly and function [63], was  
22 probed by western-blot analyses. To avoid Hcp release by pre-assembled and active T6SS, we  
23 monitored the experiments in a strain deleted of the *tssM* gene but bearing a plasmid-borne  
24 wild-type *tssM* allele under the control of an inducible Tet promoter. In this strain, the basal  
25 expression of *tssM* is undetectable by western-blot and is not sufficient to support assembly of  
26 the T6SS. In presence of inducer, the T6SS is assembled and hence Hcp was detected in the  
27 culture supernatant (Fig. 1A). The addition of Bulgecin A in the medium prior to *tssM*  
28 induction did not impact TssM production but prevented Hcp release (Fig. 1A). This result  
29 demonstrates that the T6SS does not function when the activity of LTGs are inhibited, and  
30 suggests that the action of at least one endogeneous LTG is required for the assembly of this

1 apparatus. Using time-lapse fluorescence microscopy, we previously showed that the TssJLM  
2 membrane complex is used for several rounds of tail assembly and contraction [30]. To  
3 confirm this result, wild-type EAEC cells were washed to discard secreted Hcp proteins, and  
4 resuspended in medium supplemented with Bulgecin A, to prevent assembly of new T6SS  
5 membrane complexes. After 45 min of growth, the presence of Hcp in the supernatant was  
6 probed by western-blot analyses. We observed that Hcp was released, demonstrating that pre-  
7 assembled T6SS membrane complexes are not sensitive to treatment with Bulgecin A (Fig.  
8 1B).

9 The EAEC 17-2 chromosome encodes 8 proteins with signature of LTGs. These  
10 include the soluble Slt70 and the membrane-bound MltA-E housekeeping lytic  
11 transglycosylases, as well as two putative LTGs: EtgA encoded within the T3SS gene island  
12 and the product of the EC042\_2762 gene. To test the contribution of these proteins for the  
13 assembly of the EAEC T6SS, we generated knock-out strains in each of these genes, and  
14 tested the ability of these strains to support Hcp secretion. Western-blot analyses of cell-free  
15 culture supernatants showed that Hcp release was abolished in the *mltE* strain, suggesting that  
16 the outer-membrane anchored MltE lipoprotein (EC042\_1244; gene accession number, GI:  
17 284920999) is necessary for T6SS function in EAEC and that no redundancy occurs between  
18 the EAEC LTGs for the assembly of the T6SS (Fig. 2 and Fig. 3A).

19 The EAEC Sci-1 T6SS has recently been shown to provide a competitive advantage  
20 against other *E. coli* species [70]. Fig. 3B shows that the number of GFP<sup>+</sup> kanamycin-resistant  
21 *E. coli* K-12 prey cells recovered after co-culture with EAEC *mltE* cells is 4-log higher  
22 compared to co-culture with EAEC wild-type cells. This effect is comparable to that observed  
23 for a  $\Delta$ *tssM* mutant. The T6SS<sup>-</sup> phenotypes conferred by the *mltE* mutation were  
24 complemented by the production of a wild-type copy of MltE (Fig. 3A and 3B). We then  
25 tested whether the activity of MltE is required for T6SS function. The crystal structure and  
26 the *in vitro* characterization of MltE revealed the importance of a glutamate residue, Glu-64,  
27 in the catalytic reaction [71,72]. Although the MltE<sup>E64Q</sup> catalytic inactive mutant was  
28 produced at levels comparable to wild-type MltE, cells producing MltE<sup>E64Q</sup> were unable to  
29 release Hcp and to provide a T6SS-dependent competitive advantage against *E. coli* K-12  
30 (Fig. 3A and 3B). Taken together, these results demonstrate that the assembly of the EAEC  
31 Sci-1 T6SS requires the activity of the MltE lytic transglycosylase.

32 **MltE is recruited and activated by TssM.**

1 A number of LTGs, including that associated with T3SS, T4SS and flagella, have been shown  
2 to interact with machine components to facilitate local peptidoglycan degradation at the site  
3 of assembly [11,16,22]. Based on the results presented above, we hypothesized that MltE  
4 should be recruited to the T6SS apparatus. MltE being an outer-membrane lipoprotein facing  
5 the periplasm [73], we tested the interaction of a soluble form of MltE,  $s$ MltE, with the T6SS  
6 subunits or domains exposed in the periplasm. These include the soluble fragment of TssJ, the  
7 periplasmic domains of the TssM (TssM<sub>P</sub>) and TagL (TagL<sub>P</sub>) proteins [30,58,60,63,64], as  
8 well as VgrG, which is proposed to fit inside the TssJLM complex channel at rest [30].  
9 Bacterial two-hybrid analyses demonstrated that  $s$ MltE interacts with the TssM periplasmic  
10 domain (Fig. 4A-B). This interaction is specific as  $s$ MltE does not interact with the other  
11 T6SS subunits tested, and TssM<sub>P</sub> does not interact with the seven other LTGs. The interaction  
12 of TssM<sub>P</sub> with the full-length MltE lipoprotein was further confirmed by co-immune-  
13 precipitation into the heterologous host *E. coli* K-12 (Fig. 4C). These results define that MltE  
14 is recruited to the T6SS apparatus by binding directly to the TssM periplasmic region. The  
15 bacterial two-hybrid assay also showed that the  $s$ MltE<sup>E64Q</sup> variant interacts with TssM<sub>P</sub>,  
16 demonstrating that this mutation does not interfere with MltE recruitment to TssM<sub>P</sub> (Fig. 4B).  
17 The TssM periplasmic region could be segmented into three sub-domains: sub-domains 1 and  
18 2 (amino-acids 386-973) correspond to a region predicted to be essentially  $\alpha$ -helical, and are  
19 followed by the C-terminal sub-domain 3 (amino-acids 974-1129) that folds as a  $\beta$ -sandwich-  
20 like structure [30]. Co-immune-precipitations using two variants encompassing these regions  
21 (TssM<sub>386-973</sub> and TssM<sub>972-1129</sub>) revealed that MltE binds to the  $\alpha$ -helical sub-domains 1+2  
22 (Fig. 4C).

23 MltE is a non-processive *endo*-transglycosylase, which is considered to have a  
24 relatively low peptidoglycan hydrolase activity *in vivo* compared to other LTGs [73]. Indeed,  
25 peptidoglycan hydrolysis assays showed that purified soluble form of MltE,  $s$ MltE, is  
26 significantly less active compared to lysozyme (Fig. 5A and 5B; initial rate of  $s$ MltE=  
27  $0.45 \times 10^{-3}$  AU/min/nmol). However, the activity of the T3SS-associated EtgA protein, has  
28 been shown to be modulated via its interaction with the T3SS rod component EscI to avoid  
29 unspecific peptidoglycan lysis [22]. We therefore tested whether  $s$ MltE is activated once  
30 bound to TssM<sub>P</sub>. Figures 5A and 5B show that incubation of  $s$ MltE with TssM<sub>P</sub> stimulated the  
31 activity of  $s$ MltE 7-fold (initial rate of  $s$ MltE in presence of TssM<sub>P</sub>=  $3.15 \times 10^{-3}$  AU/min/nmol).

1 Control experiments showed that TssM<sub>P</sub>, the <sub>s</sub>MltE<sup>E64Q</sup>:TssM<sub>P</sub> complex or the <sub>s</sub>MltE:TssM<sub>P</sub>  
2 complex in presence of Bulgecin A, have no significant activity (Fig. 5A and 5B).

### 3 **MltE is required for oligomerisation of the TssM protein**

4 The assembly of the T6SS is an ordered process in which the different subunits of the  
5 apparatus are sequentially recruited to the site of assembly. The assembly starts with the  
6 initial positioning of the TssJ lipoprotein and progresses by the addition of TssM and TssL,  
7 and the polymerization of TssJLM complexes to yield the membrane complex [30]. The  
8 cytoplasmic TssA protein then binds to the TssJM or TssJLM complex and recruits the  
9 baseplate, prior to tail polymerization [56,65,74]. To define at which stage of this biogenesis  
10 pathway the activity of MltE is necessary, we first assayed the TssJ-TssM and TssL-TssM  
11 interactions in the WT strain and its isogenic  $\Delta mltE$  mutant. As previously published [64],  
12 TssJ and TssL co-precipitates with TssM. Figures 6A and 6B respectively show that TssJ-  
13 TssM and TssL-TssM interactions are not affected by the absence of MltE. The latter stages  
14 of T6SS membrane complex biogenesis is the polymerization of the TssJLM complex [30].  
15 The multimerization of TssM and its complexes with TssJ and TssL could be visualized in  
16 wild-type cells after *in vivo* chemical cross-linking using bis-(sulfosuccinimidyl)-suberate  
17 (BS<sup>3</sup>) (Fig. 6C). Although TssJM and TssML complexes are still assembled in  $\Delta mltE$  cells or  
18  $\Delta mltE$  cells producing the MltE<sup>E64Q</sup> catalytic MltE mutant, no cross-linked TssM-TssM  
19 species were observed in these cells (Fig. 6C). Assembled TssJLM complexes can be  
20 observed directly in cells using a chromosomal fusion between TssM and a fluorescent  
21 reporter such as GFP [30]. Fluorescence microscopy recordings show that GFP<sup>TssM</sup> forms  
22 fluorescent clusters at the cell periphery in wild-type cells (Fig. 6D). However, no focus was  
23 observable in  $\Delta mltE$  cells or  $\Delta mltE$  cells producing MltE<sup>E64Q</sup> (Fig. 6D). Taken together, these  
24 results suggest that local peptidoglycan hydrolysis by MltE is not required for formation of  
25 TssJLM hetero-trimers but rather is necessary for assembly of the TssJLM core complex.

26

### 27 **Concluding remarks**

28 In this work, we observed that treatment of EAEC cells with the LTG inhibitor bulgecin A  
29 prevents assembly of the Sci-1 T6SS. Systematic deletion of genes encoding LTG or putative  
30 LTG coupled to phenotypic assays demonstrated that the housekeeping MltE LTG is required

1 for Sci-1 T6SS function, as Hcp release in the culture supernatant was abolished in  $\Delta mltE$   
2 cells. In addition,  $\Delta mltE$  cells presented a decreased T6SS-dependent antagonist activity  
3 against *E. coli* K-12. The EAEC strain used in this study, 17-2, encodes a second T6SS, Sci-2  
4 that belongs to the T6SS-3 family [75]. No dedicated LTG is encoded within this cluster, and  
5 it will be thus interesting to define whether MltE - or an another host LTG - is required for the  
6 assembly of the Sci-2 T6SS.

7 We further showed that MltE is recruited to the site of assembly of the T6SS  
8 membrane complex by interacting with the  $\alpha$ -domains 1 and 2 of the TssM periplasmic  
9 region. In addition, we showed that the presence of the periplasmic domain of TssM  
10 stimulates the LTG activity of MltE 7-fold *in vitro*. These results are comparable to the  
11 enteropathogenic *E. coli* EscI T3SS rod component that binds and stimulates the EtgA LTG  
12 or the. EtgA is a specialized LTG, encoded and co-regulated with the T3SS gene cluster  
13 [21,22,76], a situation that is common in cell-envelope spanning machines such as flagella,  
14 Type IV pili, T3SS or T4SS [3,5]. By contrast, with few exceptions [67], no peptidoglycan  
15 hydrolase is encoded within T6SS gene clusters. Therefore, assembly of the T6SS membrane  
16 complex requires hijacking of an host LTG to locally rearrange the cell wall. The *mltE* gene is  
17 also present in *E. coli* K-12 strains lacking T6SS, in which it participates to peptidoglycan  
18 homeostasis [10,73]. The EAEC Sci-1 T6SS has therefore re-routed MltE for its own  
19 assembly. However, the observation that T6SS gene clusters have been horizontally  
20 transferred between species suggests that each strain may have domesticated different host  
21 LTG. Another example of domestication of non-specialized LTG is the recruitment of MltD  
22 to anchor the *Helicobacter pylori* flagellum [77].

23 The recruitment of MltE by TssM therefore spatially controls the activity of MltE at  
24 close proximity to the site of assembly of the T6SS. Interestingly, MltE has a relatively weak  
25 activity on *E. coli* peptidoglycan compared to other LTGs [73]. However, Fibriansah *et al.*  
26 noted that MltE is more active on the peptidoglycan of *Micrococcus luteus*, which differs  
27 from that of *E. coli* by the nature of the peptide stems. They proposed that MltE activity either  
28 requires the activity of an amidase to cleave the peptidoglycan peptide moieties or that its  
29 conformation is modulated by protein partners [72]. The coordinated action of amidases and  
30 LTGs has been documented, notably during sporulation in *Bacillus subtilis* [78]. Although it  
31 would be interesting to test whether amidases are required for the assembly of the T6SS, the  
32 observation that TssM enhances the activity of MltE *in vitro* suggests that TssM helps MltE to

1 bypass the presence of peptide stems. TssM might displace the peptide stem to avoid steric  
2 hindrance and to increase accessibility of MltE to the glycan strand, or might induce a  
3 conformational change in MltE hence increasing its affinity for its substrate.

4 Our results also defined that MltE is required for the late stages of the assembly of the  
5 T6SS membrane complex (Fig. 7). The biogenesis of the T6SS membrane complex begins  
6 with the positioning of the TssJ outer membrane lipoprotein (Fig. 7a) and the recruitment of  
7 (i) TssM and (ii) TssL (Fig. 7b-c) prior to multimerization (Fig. 7d) [30]. The assembled  
8 TssJLM membrane complex is constituted of five dimers of TssJLM heterotrimeric  
9 complexes (Fig. 7d) [30]. We showed that the absence of MltE does not interfere with the  
10 interaction between TssJ and TssM, as well as between TssM and TssL. However, we did not  
11 detect TssM dimers in  $\Delta mltE$  cells or in cells producing a catalically-inactive MltE LTG. We  
12 therefore propose that MltE is recruited to TssM prior to multimerization (Fig. 7). This  
13 hypothesis means that the monomeric periplasmic domain of TssM can cross the cell wall to  
14 interact with TssJ, and that local rearrangement of the peptidoglycan is necessary for the  
15 polymerization of TssJLM hetero-trimers.

16 Taken together, our results provide evidence that the EAEC Sci-1 T6SS has  
17 domesticated an endoneous LTG to allow the proper assembly and insertion of the cell-  
18 spanning complex.

19

## 20 **Materials and Methods**

### 21 **Bacterial strains, growth conditions and chemicals**

22 The strains used in this study are listed in Table EV1. *Escherichia coli* K-12 strains DH5 $\alpha$ , W3110,  
23 BTH101 and BL21(DE3)/MC1061 were used for cloning procedures, co-immune-precipitation,  
24 bacterial two-hybrid and protein purification respectively. Enteroaggregative *E. coli* (EAEC) strains  
25 used in this work are isogenic derivatives of the wild-type O3:H2 17-2 strain. *E. coli* K-12 and EAEC  
26 cells were routinely grown in LB broth at 37°C, with aeration. For induction of the *sci-1* T6SS gene  
27 cluster, cells were grown in Sci-1-inducing medium (SIM: M9 minimal medium supplemented with  
28 glycerol (0.2%), vitamin B1 (1  $\mu$ g/mL), casaminoacids (40  $\mu$ g/mL), LB (10% v/v)) [79]. Plasmids and  
29 mutations were maintained by the addition of ampicillin (100  $\mu$ g/mL for K-12, 200  $\mu$ g/mL for EAEC),  
30 kanamycin (50  $\mu$ g/mL for K-12, 50  $\mu$ g/mL for chromosomal insertion on EAEC, 100  $\mu$ g/mL for  
31 plasmid-bearing EAEC) or chloramphenicol (40  $\mu$ g/mL). Gene expression was induced by the

1 addition of iso-propyl- $\beta$ -D-thio-galactopyranoside (IPTG, Sigma-Aldrich, 0.2 mM for 1 hour), L-  
2 arabinose (Sigma-Aldrich; 0.005% for 0.5 hour for complementation assays, 0.2% for 1 hour for co-  
3 immuno-precipitation) or anhydrotetracyclin (AHT; IBA Technologies; 0.2  $\mu$ g/mL for 45 min).  
4 Bulgecin A (a kind gift of Mathilde Bonis and Ivo G. Boneca (Institut Pasteur Paris)) was used at 50  
5  $\mu$ g/mL or 100  $\mu$ g/mL for *in vitro* or *in vivo* inhibition experiments respectively.

## 6 **Strain construction**

7 Deletions of genes *mltA* (EC042\_3012, gene accession identifier (GI): 284922752), *mltB*  
8 (EC042\_2894, GI: 284922637), *mltC* (EC042\_3170, GI: 284922906), *mltD* (EC042\_0224, GI:  
9 284920001), *mltE* (EC042\_1244, GI: 284920999), *slt70* (EC042\_4889, GI: 284924571),  
10 EC042\_2762 (GI: 284922508) and *etgA* (EC042\_3052, GI: 284922791) were engineered on the  
11 EAEC 17-2 chromosome using the modified one-step inactivation procedure [80] using  $\lambda$  red  
12 recombinase expressed from pKOBEG [81] as previously described [63]. The *mltE* gene was deleted  
13 from the 17-2 strain producing the GFP-TssM fusion protein expressed from the chromosomal native  
14 locus [30] using the same procedure. The kanamycine cassette from plasmid pKD4 [80] was amplified  
15 with oligonucleotides carrying ~ 50-nucleotide extensions homologous to regions adjacent to the target  
16 gene (custom primers, synthesized by Eurogentec, are listed in Table EV1). The Polymerase Chain  
17 Reaction (PCR) product was column purified (PCR and Gel Clean up, Promega) and electroporated  
18 into competent cells. Kanamycin resistant clones were selected and the insertion of the kanamycin  
19 cassette at the targeted site was verified by PCR. The kanamycin cassette was then excised using  
20 plasmid pCP20 [80] and the final strain was verified by PCR.

## 21 **Plasmid construction**

22 PCR were performed with a Biometra thermocycler, using the Pfu Turbo DNA polymerase  
23 (Stratagene; La Jolla, CA). Plasmids and oligonucleotides are listed in Table EV1. Constructions of  
24 pOK-Hcp<sub>HA</sub>, pUC-Hcp<sub>FLAG</sub>, pIBA-TssM<sub>FL</sub>, pIBA-TssM<sub>P</sub>, pMS-TssJ<sub>HA</sub> and pETG20A-TssM<sub>P</sub> have  
25 been previously described [60,63,64]. Plasmids pBADnLIC-sMltE and pBADnLIC-sMltE-E64Q have  
26 been previously described [72] and have been kindly provided by Andy-Mark Thunnissen (University  
27 of Groningen, The Netherlands). All pASK-IBA4 and bacterial two-hybrid vectors and the pBAD-  
28 MltE<sub>V</sub> plasmid, encoding the C-terminally VSV-G-tagged full-length MltE protein under the control of  
29 the arabinose promoter (in the pBAD33 vector), have been constructed by restriction-free cloning  
30 [82]. Briefly, the gene of interest fused to 5' and 3'-extensions annealing to the target vector was  
31 amplified and used as oligonucleotides for a second PCR using the target vector as template. The  
32 E64Q point mutation was inserted into pBAD-MltE<sub>V</sub> and pT18-MltE by site-directed mutagenesis

1 using complementary oligonucleotides bearing the desired mutation. All constructs have been verified  
2 by PCR and DNA sequencing (MWG).

### 3 **T6SS phenotypic assays and GFP-TssM fluorescence microscopy recordings**

4 Hcp release and anti-bacterial competition assays were performed as previously described [63,70]. For  
5 the Hcp release assay with bulgecin A treatment, the experiment was performed in a  $\Delta tssM$  strain  
6 carrying plasmid pASK-IBA37-TssM, allowing AHT-dependent inducible expression of the *tssM*  
7 gene and producing HA-tagged Hcp. Cells were grown in SIM until  $A_{600} \sim 0.4$  and treated - or not -  
8 with bulgecin A (100  $\mu$ M). After 40 min, *tssM* expression was induced by the addition of AHT and the  
9 culture was further grown for 45 minutes. Cell pellets and supernatants were fractionated by  
10 centrifugation. The final supernatant fraction samples were obtained by filtration on 0.25  $\mu$ M-PES  
11 membranes and TCA precipitation as previously published [63]. Controls were performed to verify  
12 that bulgecin A treatment did not interfere with TssM production. Controls for cell lysis were  
13 performed by immuno-detecting the periplasmic TolB protein. For treatment of cells with pre-  
14 assembled TssJLM complexes, wild-type 17-2 cells producing HA-tagged Hcp were grown in SIM to  
15  $A_{600} \sim 0.5$ , and the cells and supernatant fractions were separated as described above. Cells were  
16 washed in SIM and resuspended in SIM supplemented- or not - with bulgecin A (100  $\mu$ M). After  
17 further growth for 45 min, cells and supernatant fractions were separated as described above.  
18 Fluorescence microscopy recordings were performed as previously published [30]. All experiments  
19 have been done at least in duplicate and a representative result is shown. Statistical analysis of anti-  
20 bacterial competition assays was performed by Student's *t*-test. Significant differences were defined as  
21  $p < 0.05$  (\*),  $p < 0.01$  (\*\*), and  $p < 0.001$  (\*\*\*)).

### 22 **Protein production and purification**

23 The periplasmic domain of TssM was purified from *E. coli* BL21(DE3) cells carrying the pETG20A-  
24 TssM<sub>p</sub> plasmid and the native protein was obtained after cleavage of the Thioredoxin-6 $\times$ His N-  
25 terminal extension by the tobacco etch virus (TEV) protease, as previously published [64]. The soluble  
26 MltE protein and its E64Q variant were purified from *E. coli* MC1061 cells carrying the pBADnLIC-  
27 sMltE or pBADnLIC-sMltE-E64Q vector as previously published [72].

### 28 **Peptidoglycan hydrolysis assays**

29 *Preparation of the peptidoglycan fraction.* The peptidoglycan fraction from the JE5505 *lpp* strain was  
30 prepared as previously published [83], resuspended in phosphate-buffered saline and treated with 200  
31  $\mu$ g/mL amylase (Sigma-Aldrich) for 2 hours at 37°C. *Remazol brilliant blue assay.* This protocol for  
32 the peptidoglycan hydrolysis assay has been modified from a published protocol [84]. The purified



1 peptidoglycan was washed with distilled water, resuspended in 200 mM NaOH and labeled with 25  
2 mM Remazol brilliant blue (RBB, Sigma-Aldrich) for 14 hours at 37°C, and washed four times with  
3 distilled water. RBB-labeled peptidoglycan was incubated with the 50 µg of protein of interest for 30  
4 min or 4 hours in PBS buffer, and the reaction was quenched by the addition of 50 µg/mL Bulgecin A.  
5 After ultra-centrifugation for 40 min at 68,000×g, the absorbance of the supernatant was measured at  
6 595 nm. *Peptidoglycan turbidity assay*. This peptidoglycan hydrolysis assay has been performed as  
7 previously published [72] using a suspension of 0.25 mg/mL of purified *Micrococcus luteus*  
8 peptidoglycan (Sigma-Aldrich) in MES 50 mM pH6.0, NaCl 200 mM ( $A_{600}=0.57 \pm 0.04$ ). The  
9 turbidity at 600 nm was measured every 20 minutes after addition of 50 µg (~2.48 nmol) of  $\sigma$ MltE. For  
10 experiments in presence of TssM<sub>p</sub>, a 1:2 ( $\sigma$ MltE:TssM<sub>p</sub>) molar ratio have been used. The initial rate  
11 was measured as the slope of the initial linear curve (expressed in absorbance units/min/nmol). For all  
12 peptidoglycan hydrolysis assays, controls were performed with buffer, lysozyme or in presence of 50  
13 µg/mL Bulgecin A. The assays have been performed in triplicate and a representative experiment is  
14 shown.

#### 15 **Bacterial two-hybrid assay**

16 The adenylate cyclase-based bacterial two-hybrid technique [85] was used as previously published  
17 [86]. Briefly, the proteins to be tested were fused to the isolated T18 and T25 catalytic domains of the  
18 *Bordetella* adenylate cyclase. After introduction of the two plasmids producing the fusion proteins into  
19 the reporter BTH101 strain, plates were incubated at 30°C for 24 hours. Three independent colonies  
20 for each transformation were inoculated into 600 µL of LB medium supplemented with ampicillin,  
21 kanamycin and IPTG (0.5 mM). After overnight growth at 30°C, 10 µL of each culture were dropped  
22 onto LB plates supplemented with ampicillin, kanamycin, IPTG and 5-bromo-4-chloro-3-indolyl-β-D-  
23 galactopyranoside (X-Gal) and incubated for 16 hours at 30 °C. Controls include interaction assays  
24 with TolB and Pal, two protein partners unrelated to the T6SS. The experiments were done at least in  
25 triplicate and a representative result is shown.

#### 26 **Co-immune-precipitation**

27  $10^{11}$  exponentially growing cells producing the proteins of interest were harvested, and resuspended in  
28 buffer TN (Tris-HCl 20 mM pH8.0, NaCl 100 mM) supplemented with protease inhibitors (Complete,  
29 Roche), lysozyme (100 µg/mL) and DNase (100 µg/mL) and broken by three passages at the French  
30 press (1000 psi). Unbroken cells were discarded by centrifugation for 15 min at 3,000×g and the total  
31 cell extract was mixed with an equal volume of 2×CellLytic™ B Cell Lysis reagent (Sigma-Aldrich)  
32 and incubated for 1 hour with strong shaking. The insoluble material was discarded by centrifugation  
33 for 45 min at 60,000×g and the supernatant from  $2 \times 10^{10}$  cells was incubated overnight at 4°C with  
34 anti-FLAG M2 affinity beads (Sigma-Aldrich). Beads were then washed three times with

1 1×CellLytic™ in buffer TN. The total extract and immunoprecipitated material were resuspended and  
2 boiled in Laemmli loading buffer prior to analyses by SDS-PAGE and immunoblotting. The  
3 experiments were done in triplicate and a representative result is shown.

#### 4 ***In vivo* BS3 cross-linking assay**

5 2×10<sup>9</sup> exponentially growing cells were harvested, washed with sodium phosphate (SP) buffer  
6 (NaH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub> 10 mM pH7.4), resuspended in 1 mL of SP supplemented with 0.5 mM bis (3-  
7 sulfo-N-hydroxy-succinimide ester) suberate (BS<sup>3</sup>; Sigma-Aldrich). After incubation at room  
8 temperature for 25 min, the cross-linking reaction was quenched by the addition of Tris-HCl pH8.0  
9 (100 mM final concentration). Cross-linked cells were resuspended and boiled in non-reducing  
10 Laemmli loading buffer prior to analyses by SDS-PAGE and immunoblotting. The experiments were  
11 done in triplicate and a representative result is shown.

#### 12 **Miscellaneous**

13 For Western-blot analyses, cell extracts or precipitated proteins were resuspended in Laemmli buffer  
14 and boiled for 10 min. Proteins were separated by SDS-PAGE and transferred onto nitrocellulose  
15 membranes. Immunoblots were probed with anti-VSV-G (clone P5D4, Sigma-Aldrich), anti-FLAG  
16 (clone M2, Sigma-Aldrich), anti-HA (clone HA-7, Sigma-Aldrich) monoclonal antibodies, or anti-  
17 TolB polyclonal antibodies (laboratory collection) and anti-rabbit or anti-mouse secondary antibodies  
18 coupled to the alkaline phosphatase. Immunostaining was achieved in sodium phosphate buffer  
19 (pH9.0) supplemented with MgCl<sub>2</sub> 10mM, 5-bromo-4-chloro-3-indolyl-phosphate 40 µg/mL and  
20 nitro-blue tetrazolium chloride 40 µg/mL.

21

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12

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28 **Author contributions.** Y.S. and E.C. designed the research and conceived the study; Y.S. and E.C.  
29 performed the experiments; E.C. wrote the manuscript.

30 **Additional Information.** Table EV1 lists the strains, plasmids and oligonucleotides used in this study  
31 is available. Correspondence and requests for material should be addressed to E.C.

32 **Competing interests.** The authors declare no competing financial interests.



## 1 Legend to Figures

### 2 **Figure 1. The LTG inhibitor Bulgecin A prevents T6SS function.**

3 **A** Hcp release assay. HA-tagged Hcp (Hcp<sub>HA</sub>) release was assessed by separating cells (C) and  
4 cell-free culture supernatant (S) fractions from 10<sup>9</sup> wild-type (WT),  $\Delta tssM$  cells or  $\Delta tssM$  cells  
5 carrying the AHT-inducible FLAG-tagged *tssM*-borne plasmid (*tssM*<sup>+</sup>) treated (bulgecin) or not  
6 (NT) with bulgecin A prior to *tssM* gene induction. Proteins were separated by 12.5%-  
7 acrylamide SDS-PAGE and the periplasmic TolB protein (control for cell lysis), Hcp<sub>HA</sub> and  
8 FLTssM were immunodetected using anti-TolB (middle panel), anti-HA (lower panel) and anti-  
9 FLAG (upper panel) antibodies. Molecular weight markers (in kDa) are indicated on the left.  
10 The experiment was performed in duplicate and a representative result is shown.

11 **B** Hcp release assay. HA-tagged Hcp (Hcp<sub>HA</sub>) release was assessed by separating cells (C) and  
12 cell-free culture supernatant (S) fractions from 10<sup>9</sup> wild-type (WT) cells before washing cells  
13 (before wash) and after washing and growth (after wash) in absence (NT) or presence (bulgecin)  
14 of bulgecin A. Proteins were separated by 12.5%-acrylamide SDS-PAGE and the periplasmic  
15 TolB protein (control for cell lysis) and Hcp<sub>HA</sub> were immunodetected using anti-TolB (upper  
16 panel) and anti-HA (lower panel) antibodies. Molecular weight markers (in kDa) are indicated  
17 on the left. The experiment was performed in duplicate and a representative result is shown.

### 18 **Figure 2. The MltE LTG is required for T6SS function.**

19 Hcp release assay. FLAG-tagged Hcp (Hcp<sub>FL</sub>) release was assessed by separating cells (C) and  
20 cell-free culture supernatant (S) fractions from 10<sup>9</sup> cells of the indicated strains. Proteins were  
21 separated by 12.5%-acrylamide SDS-PAGE and TolB and Hcp<sub>FL</sub> were immunodetected using  
22 anti-TolB (upper panel) and anti-FLAG (lower panel) antibodies. Molecular weight markers (in  
23 kDa) are indicated on the left. The experiment was performed in triplicate and a representative  
24 result is shown.

### 25 **Figure 3. The MltE peptidoglycan hydrolase activity is required for T6SS function.**

26 **A** Hcp release assay. FLAG-tagged Hcp (Hcp<sub>FL</sub>) release was assessed by separating cells (C) and  
27 cell-free culture supernatant (S) fractions from 10<sup>9</sup> WT,  $\Delta mltE$  cells or  $\Delta mltE$  cells producing  
28 wild-type (*mltE*<sup>+</sup>) or E64Q mutant (*mltE*<sup>E64Q</sup>) VSV-G-tagged MltE (MltE<sub>V</sub>) from arabinose-  
29 inducible plasmids. Proteins were separated by 12.5%-acrylamide SDS-PAGE and TolB, Hcp<sub>FL</sub>  
30 and MltE<sub>V</sub> were immunodetected using anti-TolB (upper panel), anti-FLAG (middle panel) and  
31 anti-VSV-G (lower panel) antibodies, respectively. Molecular weight markers (in kDa) are

1 indicated on the left. The experiment was performed in triplicate and a representative result is  
2 shown.

3 **B** Anti-bacterial activity. *E. coli* K-12 prey cells (W3110 *gfp*<sup>+</sup>, kan<sup>R</sup>) were mixed with the  
4 indicated attacker cells, spotted onto Sci-1 inducing medium (SIM) agar plates and incubated  
5 for 4 hours at 37°C. The image of a representative bacterial spot and the average and standard  
6 deviation ( $n=3$ ) of the relative fluorescence of the bacterial mixture (in arbitrary unit, AU) are  
7 shown in the upper graph. The number of recovered *E. coli* prey cells (counted on selective  
8 kanamycin medium) is indicated in the lower graph (in log<sub>10</sub> of colony-forming unit (cfu)). The  
9 black, dark grey and light grey circles indicate values from three independent assays, and the  
10 average is indicated by the bar. The experiment was performed in triplicate and a representative  
11 result is shown. Asterisks indicate significant differences compared to the wild-type attacker  
12 strain (NS, non significant; \*\*\*,  $p < 0.001$ ).

13 **Figure 4. MltE interacts with the TssM periplasmic domain.**

14 **A-B** Bacterial two-hybrid assay. BTH101 reporter cells producing the indicated proteins or domains  
15 fused to the T18 or T25 domain of the *Bordetella* adenylate cyclase were spotted on X-Gal  
16 indicator plates. The blue color of the colony reflects the interaction between the two proteins.  
17 TolB and Pal are two proteins known to interact but unrelated to the T6SS or the MltE proteins.  
18 The experiment was performed in triplicate and a representative result is shown.

19 **C** Co-immunoprecipitation assay. The solubilized lysates from  $2 \times 10^{10}$  *E. coli* K-12 W3110 cells  
20 co-producing the indicated FLAG-tagged TssM<sub>P</sub> variants (exported in the periplasm) and VSV-  
21 G-tagged MltE protein (Total, T) were subjected to immune precipitation on anti-FLAG-  
22 coupled agarose beads. The immunoprecipitated material (IP) was subjected to 12.5%-  
23 acrylamide SDS-PAGE and immunodetected with anti-FLAG (upper panel, TssM domains) and  
24 anti-VSV-G (lower panel, MltE) antibodies. Molecular weight markers (in kDa) are indicated  
25 on the left. The experiment was performed in triplicate and a representative result is shown.

26 **Figure 5. TssM<sub>P</sub> increases MltE peptidoglycan hydrolase activity.**

27 **A** Remazol brilliant blue assay. The absorbance of supernatants from the reaction containing  
28 purified and RBB-labelled *E. coli* peptidoglycan and the indicated protein (50 µg) were  
29 measured at  $\lambda=595$  nm after incubation for 0.5 or 4 hours at 37°C. The results shown are the  
30 average and standard deviation from triplicate reactions ( $n=3$ ). Asterisks indicate significant  
31 differences compared to the buffer (NS, non significant; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ). The  
32 supernatant of the reaction after 4 hours of incubation is shown on bottom.

1 **B** Peptidoglycan hydrolysis. The decrease of the absorbance of the *M. luteus* peptidoglycan  
2 suspension in presence of the indicated protein (50  $\mu$ g) was measured at  $\lambda=600$  nm at 37°C over  
3 time. The experiment was performed in triplicate and a representative result is shown.

4 **Figure 6. MltE is required for TssM multimerization.**

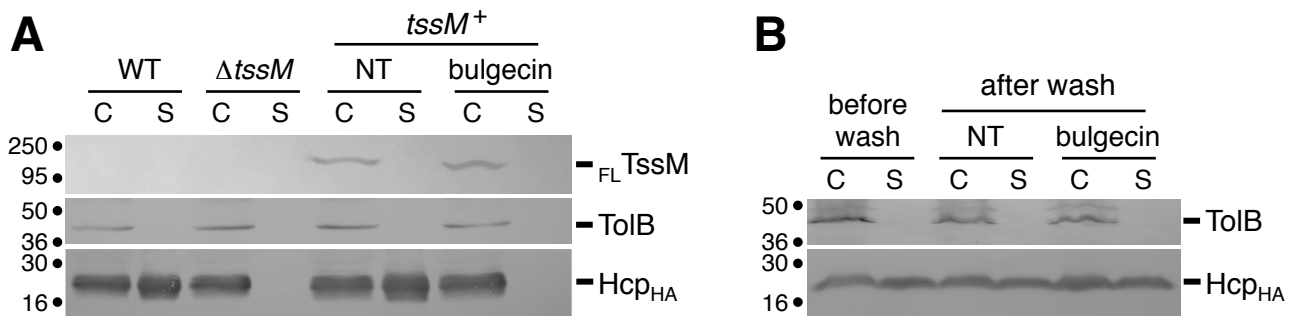
5 **A-B** Co-immunoprecipitation assay. The solubilized lysates from  $2 \times 10^{10}$  EAEC wild-type or  $\Delta mltE$   
6 cells producing FLAG-tagged TssM ( $_{FL}$ TssM) and/or HA-tagged TssJ (TssJ $_{HA}$ , panel A) or TssL  
7 (TssL $_{HA}$ , panel B) (Total, T) were subjected to immune precipitation on anti-FLAG-coupled  
8 agarose beads. The immunoprecipitated material (IP) was subjected to 12.5%-acrylamide SDS-  
9 PAGE and immunodetected with anti-FLAG (upper panel, TssM) and anti-HA (lower panel,  
10 TssJ or TssL) antibodies. Molecular weight markers (in kDa) are indicated on the left. The  
11 experiments were performed in triplicate and a representative result is shown.

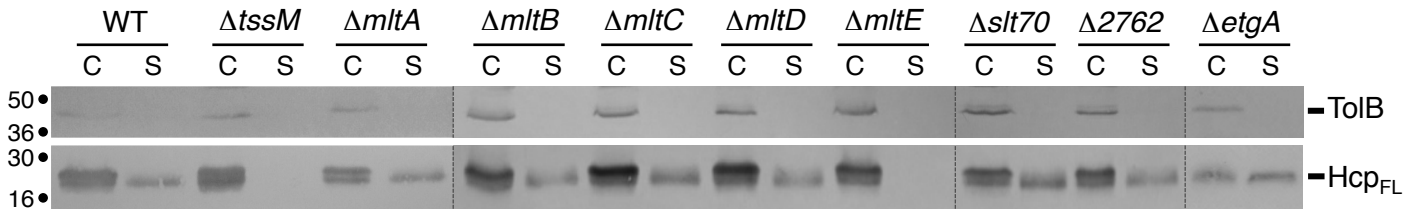
12 **C** BS<sup>3</sup> cross-linking assay.  $2 \times 10^9$  cells of the indicated strain producing FLAG-tagged TssM (with  
13 the exception of  $\Delta tssM$  cells) were treated (+) or not (-) with the BS<sup>3</sup> cross-linker agent. After  
14 the cross-linking reaction, cells were boiled in Laemmli buffer and total proteins were subjected  
15 to 7%-acrylamide SDS-PAGE and immunodetected with anti-FLAG antibodies. The TssM  
16 protein ( $_{FL}$ TssM) and its complexes (\*, TssM-TssJ; \*\*, TssM-TssL) are indicated on the right as  
17 well as the TssM dimer (arrow). Molecular weight markers (in kDa) are indicated on the left.  
18 The experiment was performed in triplicate and a representative result is shown.

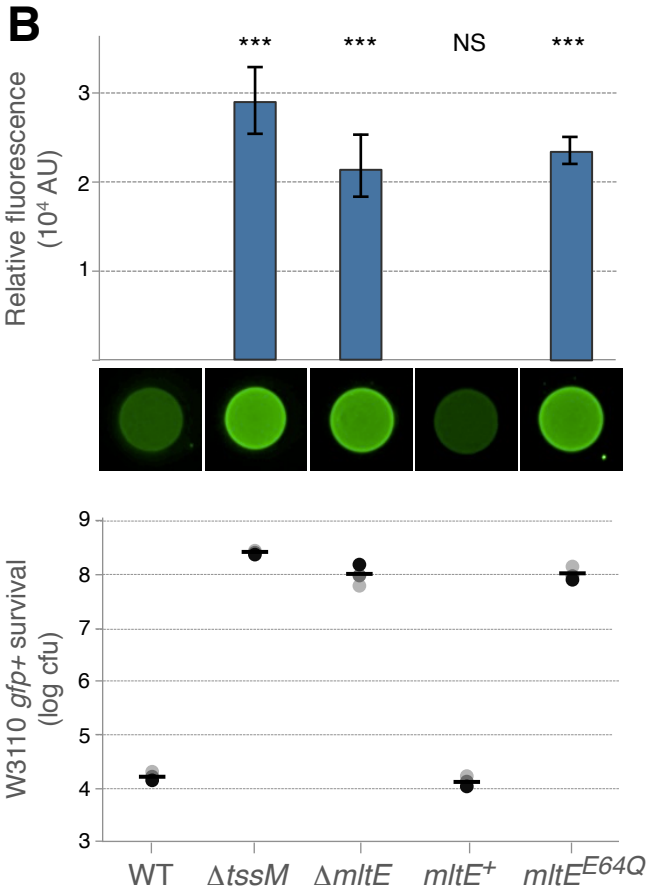
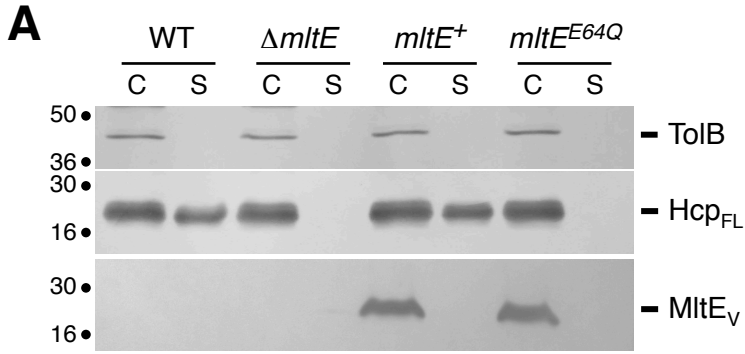
19 **D** Fluorescence microscopy. Recordings showing TssM localization using the chromosomally-  
20 encoded *sfGFP-tssM* fusion in wild-type (WT) or  $\Delta mltE$  cells or  $\Delta mltE$  cells producing the  
21 wild-type (*mltE*<sup>+</sup>) or catalytic variant (*mltE*<sup>E64Q</sup>) MltE protein. Scale bars are 1  $\mu$ m. The  
22 experiment was performed in triplicate and a representative result is shown.

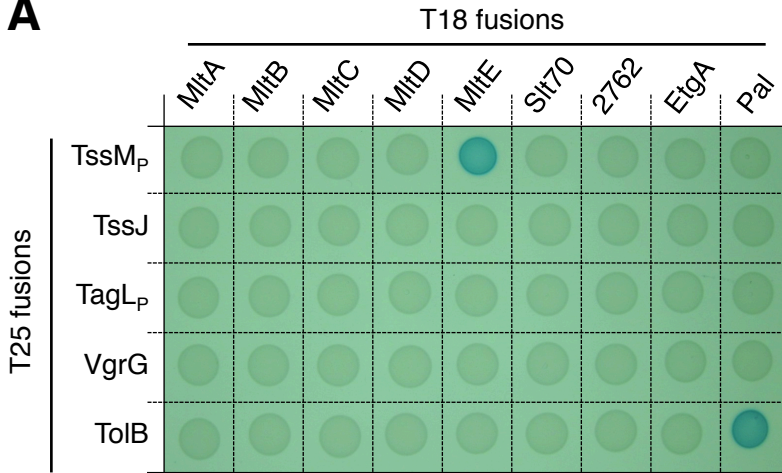
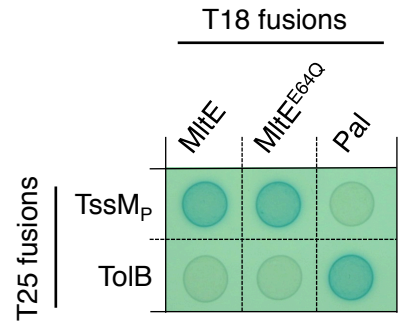
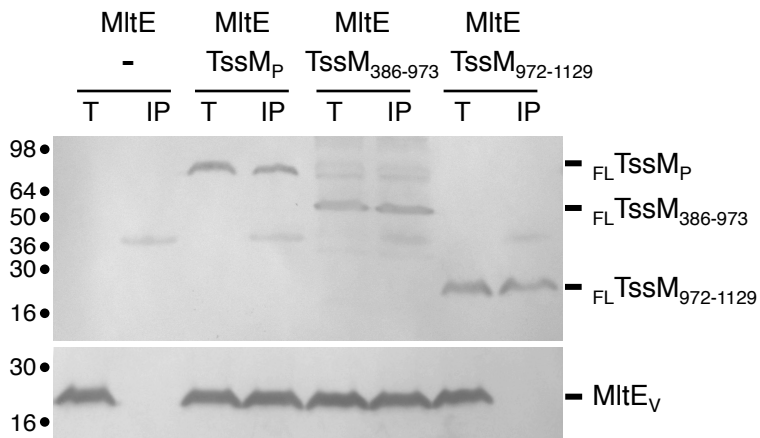
23 **Figure 7. Schematic model of the assembly of the TssJLM complex.**

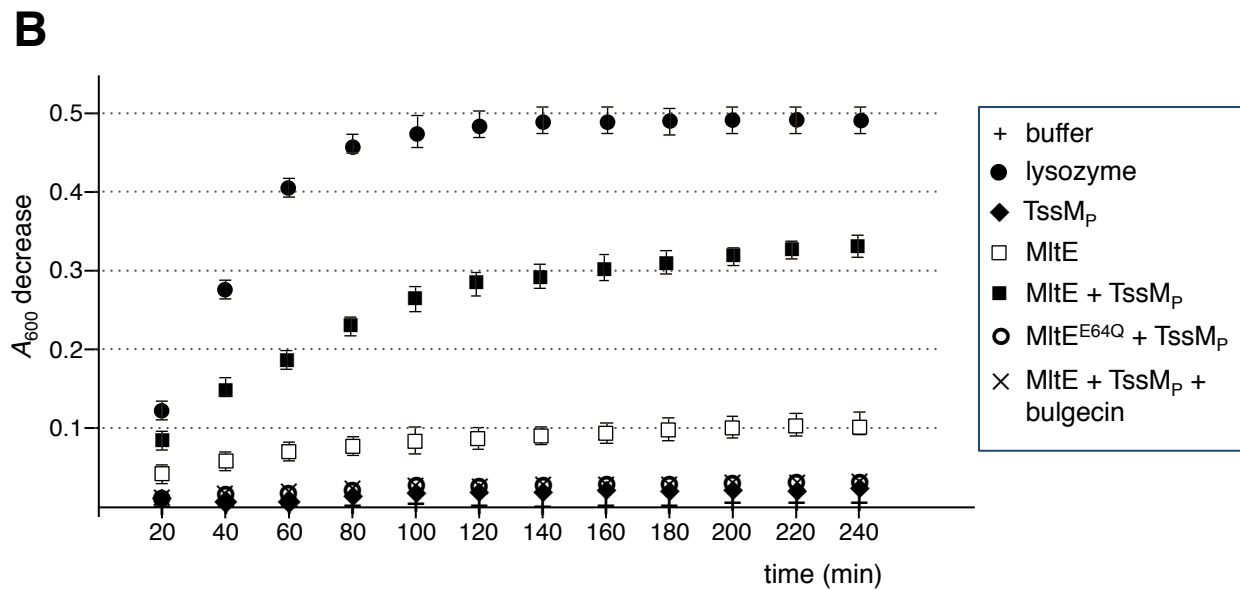
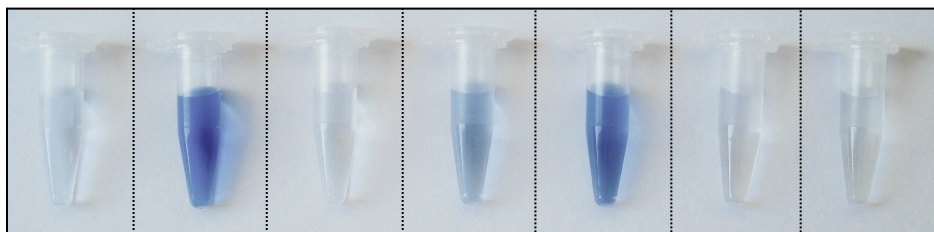
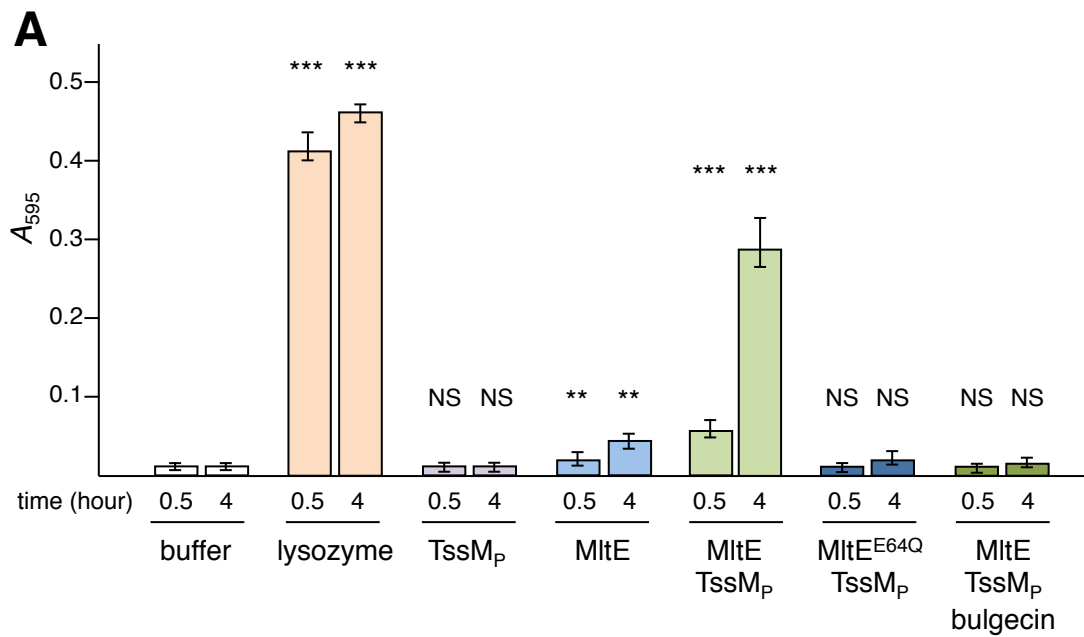
24 Biogenesis of the TssJLM complex begins with the initial positioning of the TssJ outer  
25 membrane (OM) lipoprotein (a) and the sequential recruitment of the TssM (b) and TssL (c)  
26 inner membrane (IM) proteins. TssM binds to the TssJ lipoprotein via its C-terminal  $\beta$ -domain  
27 3 (dark blue) and recruits the MltE LTG (green ball) via its  $\alpha$ -domain 1+2 (light blue). The  
28 TssM-mediated activation of MltE creates localized degradation in the cell wall (PG) allowing  
29 polymerization of the TssJLM complex (d). The crystal and electron microscopy structures are  
30 shown (TssJ, PDB:3RX9 [64]; TssM<sub>p</sub>-TssJ complex, PDB:4Y7O [30]; TssL cytoplasmic  
31 domain, PDB: 3U66 [87]; TssJLM complex, EMD:2927 [30]).



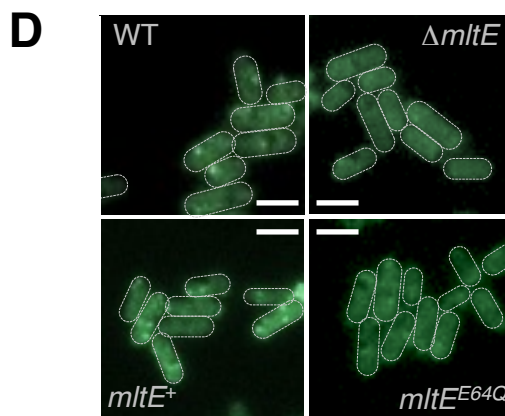
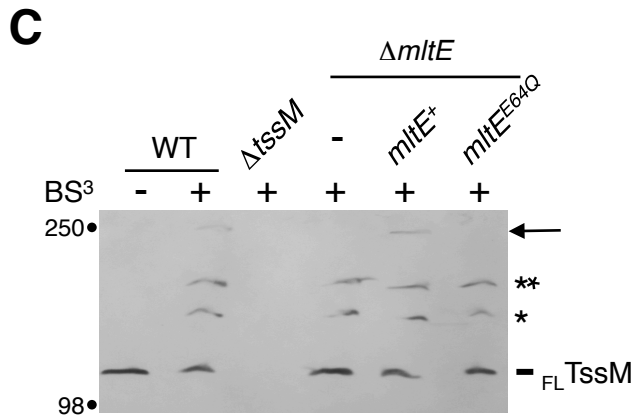
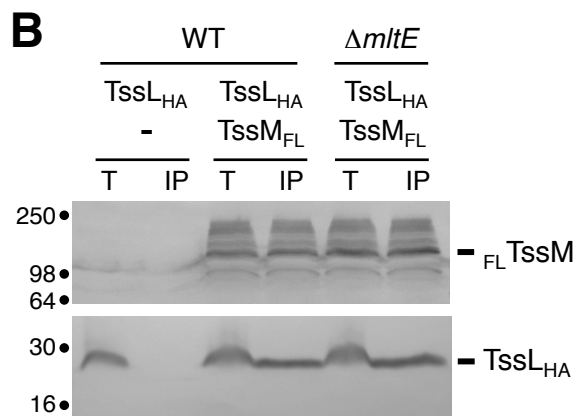
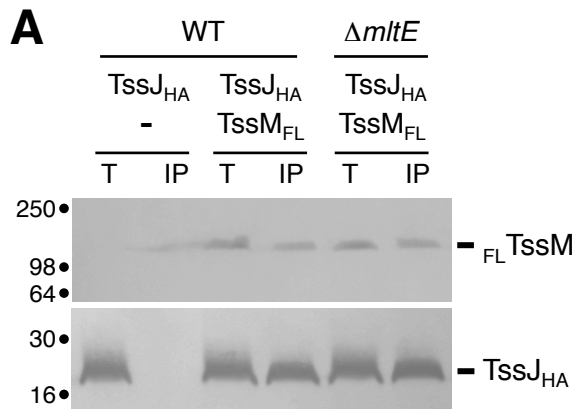


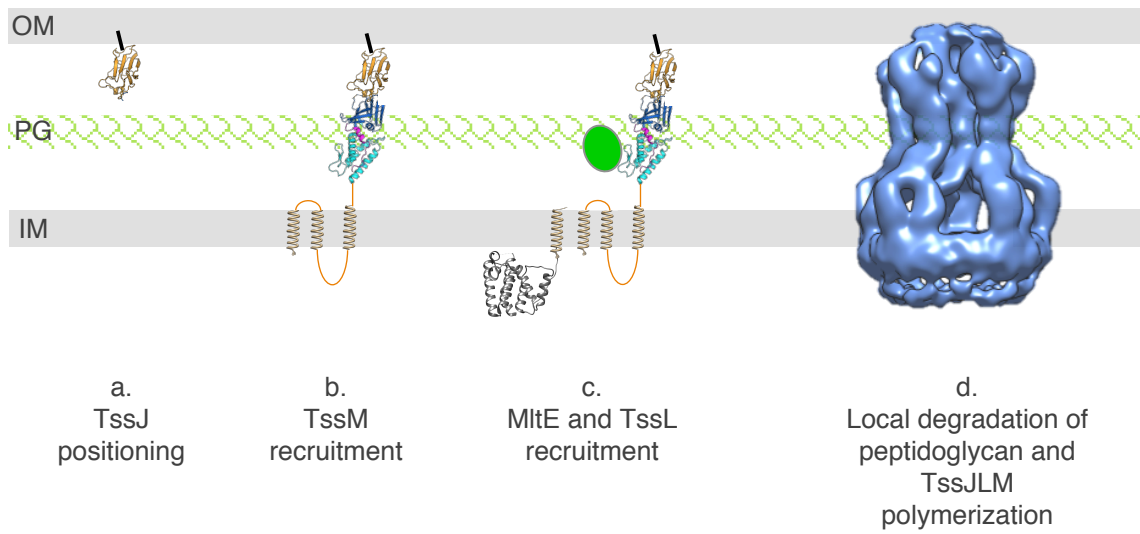


**A****B****C**









## **SUPPLEMENTAL DATA**

**Domestication of a housekeeping transglycosylase for assembly of a Type VI secretion system.**

Y.G. Santin and E. Cascales

## Supplemental Table S1. Strains, plasmids and oligonucleotides used in this study.

### Strains

Strains	Description and genotype	Source
<i>E. coli</i> K-12		
DH5 $\alpha$	F-, $\Delta(\text{argF-lac})$ U169, <i>phoA</i> , <i>supE44</i> , $\Delta(\text{lacZ})$ M15, <i>relA</i> , <i>endA</i> , <i>thi</i> , <i>hsdR</i>	New England Biolabs
W3110	F-, lambda- IN( <i>rrnD-rrnE</i> )1 <i>rph-1</i>	Laboratory collection
BTH101	F-, <i>cya-99</i> , <i>araD139</i> , <i>galE15</i> , <i>galK16</i> , <i>rpsL1</i> ( <i>Str r</i> ), <i>hsdR2</i> , <i>mcrA1</i> , <i>mcrB1</i> .	Karimova <i>et al.</i> , 2005
BL21(DE3)	F-, <i>fhuA2</i> , <i>lon</i> , <i>ompT</i> , <i>gal</i> ( $\lambda$ int::( <i>lacI</i> :: <i>PlacUV5</i> :: <i>T7 gene1</i> ), <i>dcm</i> , $\Delta$ <i>hsdS</i>	New England Biolabs
MC1061	F-, <i>araD139</i> $\Delta(\text{ara-leu})$ , <i>galE15</i> , <i>galK16</i> , $\Delta(\text{lac})$ X74, <i>rpsL</i> ( <i>Str<sup>R</sup></i> ) <i>hsdR2</i> <i>mcrAB1</i>	New England Biolabs
Enteroaggregative <i>E. coli</i>		
17-2	WT enteroaggregative <i>Escherichia coli</i>	Arlette Darfeuille-Michaud
17-2 $\Delta$ <i>tssM</i>	17-2 deleted of the <i>tssM</i> gene of the <i>sciI</i> T6SS gene cluster	Aschtgen <i>et al.</i> , 2010
17-2 $\Delta$ <i>mltA</i>	17-2 deleted of the <i>mltA</i> gene	This study
17-2 $\Delta$ <i>mltB</i>	17-2 deleted of the <i>mltB</i> gene	This study
17-2 $\Delta$ <i>mltC</i>	17-2 deleted of the <i>mltC</i> gene	This study
17-2 $\Delta$ <i>mltD</i>	17-2 deleted of the <i>mltD</i> gene	This study
17-2 $\Delta$ <i>mltE</i>	17-2 deleted of the <i>mltE</i> gene	This study
17-2 $\Delta$ <i>slt70</i>	17-2 deleted of the <i>slt70</i> gene	This study
17-2 $\Delta$ 2762	17-2 deleted of the <i>EC042_2762</i> gene	This study
17-2 $\Delta$ <i>etgA</i>	17-2 deleted of the <i>etgA</i> gene	This study
17-2 sfGFP-TssM	sfGFP inserted after the start codon of the <i>tssM</i> gene in 17-2	Durand <i>et al.</i> , 2015
17-2 $\Delta$ <i>mltE</i> sfGFP-TssM	17-2 sfGFP-TssM deleted of the <i>mltE</i> gene	This study

## Plasmids

Vectors	Description	Source
<b>Vectors for chromosomal insertions</b>		
pKD4	One-step gene inactivation vector, Kan <sup>R</sup>	Datsenko & Wanner, 2000
pKOBEG	Recombination vector, phage $\lambda$ <i>rec<math>\gamma</math><math>\beta</math><math>\alpha</math></i> operon under the control of the pBAD promoter, Cm <sup>R</sup>	Chaveron <i>et al.</i> , 2000
<b>Expression vectors</b>		
pUA66- <i>rrnB</i>	<i>P<sub>rrnB</sub> :: gfpmut2</i> transcriptional fusion in pUA66, Kan <sup>R</sup>	Zaslaver <i>et al.</i> , 2006
pASK-IBA37(+)	cloning vector, <i>P<sub>tet</sub></i> , f1 origin, Amp <sup>R</sup>	IBA Technology
pASK-IBA37-FLAG-TssM	<i>sci-1 tssM</i> carrying N-terminal FLAG tag cloned into pASK-IBA37(+)	Aschtgen <i>et al.</i> , 2010
pASK-IBA4	cloning vector, <i>P<sub>tet</sub></i> , <i>OmpA</i> signal sequence, f1 origin, Amp <sup>R</sup>	IBA Technology
pASK-IBA4-TssMp	<i>sci-1 tssM</i> periplasmic domain (aa 386-1129), cloned into pASK-IBA4, N-terminal FLAG epitope	Felisberto-Rodrigues <i>et al.</i> , 2011
pASK-IBA4-TssM <sub>386-973</sub>	<i>sci-1 tssM</i> periplasmic fragment (aa 386-973), cloned into pASK-IBA4, N-terminal FLAG epitope	Felisberto-Rodrigues <i>et al.</i> , 2011
pASK-IBA4-TssM <sub>972-1129</sub>	<i>sci-1 tssM</i> periplasmic fragment (aa 972-1129), cloned into pASK-IBA4, N-terminal FLAG epitope	Felisberto-Rodrigues <i>et al.</i> , 2011
pMS600	cloning vector, pOK12 derivative, <i>Plac</i> , P15A origin, Kan <sup>R</sup>	Aschtgen <i>et al.</i> , 2008
pMS-Hcp <sub>HA</sub>	<i>sci-1 hcp</i> gene cloned into pMS600, C-terminal HA epitope	Aschtgen <i>et al.</i> , 2010
pMS-TssJ <sub>HA</sub>	<i>sci-1 tssJ</i> gene cloned into pMS600, C-terminal HA epitope	Aschtgen <i>et al.</i> , 2008
pMS-TssL <sub>HA</sub>	<i>sci-1 tssL</i> gene cloned into pMS600, C-terminal HA epitope	Aschtgen <i>et al.</i> , 2012
pUC12	cloning vector, <i>Plac</i> , ColE1 origin, Amp <sup>R</sup>	Norlander <i>et al.</i> , 1983
pUC-Hcp <sub>FLAG</sub>	<i>sci-1 hcp</i> gene cloned into pUC12, C-terminal FLAG epitope	Aschtgen <i>et al.</i> , 2008
pBAD33	cloning vector, <i>Para</i> , <i>araC</i> , p15A origin, Cm <sup>R</sup>	Guzman <i>et al.</i> , 1995
pBAD33-MltE <sub>VSV-G</sub>	<i>mltE</i> gene cloned into pBAD33, C-terminal VSV-G epitope	This study
pBAD33-MltE <sup>E64Q</sup>	<i>mltE</i> Glu64-to-Gln mutation introduced in pBAD33-MltE <sub>VSV-G</sub> , C-terminal VSV-G epitope	This study
pETG20A	Gateway® destination cloning vector, <i>PT7</i> , N-terminal 6×His tag, TEV cleavage sequence, ColE1 origin, Amp <sup>R</sup>	Arie Gerlof
pETG20A-TssMp	<i>sci-1 tssM</i> periplasmic domain (aa 386-1129), cloned into pETG20A	Felisberto-Rodrigues <i>et al.</i> , 2011
pBADnLIC	cloning vector, <i>Para</i> , <i>araC</i> , N-terminal 10×His tag, TEV cleavage sequence, P15A origin, Amp <sup>R</sup>	Geertsma & Poolman, 2007
pBADnLIC-sMltE	<i>mltE</i> gene lacking its signal sequence cloned into pBADnLIC	Fibriansah <i>et al.</i> , 2012
pBADnLIC-MltE <sup>E64Q</sup>	<i>mltE</i> Glu64-to-Gln mutation introduced in pBADnLIC-MltE	Fibriansah <i>et al.</i> , 2012
<b>Bacterial two-hybrid vectors</b>		
pT18-FLAG	bacterial two hybrid vector, ColE1 origin, <i>Plac</i> , T18 fragment of <i>Bordetella pertussis</i> CyaA, Amp <sup>R</sup>	Battesti & Bouveret, 2008

pT18-MltA	<i>mltA</i> gene lacking its signal sequence cloned downstream T18 in pT18-FLAG	This study
pT18-MltB	<i>mltB</i> gene lacking its signal sequence cloned downstream T18 in pT18-FLAG	This study
pT18-MltC	<i>mltC</i> gene lacking its signal sequence cloned downstream T18 in pT18-FLAG	This study
pT18-MltD	<i>mltD</i> gene lacking its signal sequence cloned downstream T18 in pT18-FLAG	This study
pT18-MltE	<i>mltE</i> gene lacking its signal sequence cloned downstream T18 in pT18-FLAG	This study
pT18-MltE <sup>E64Q</sup>	<i>mltE</i> Glu64-to-Gln mutation introduced in pT18-MltE	This study
pT18-Slt70	<i>slt70</i> gene lacking its signal sequence cloned downstream T18 in pT18-FLAG	This study
pT18-2762	<i>EC042_2762</i> gene lacking its signal sequence cloned downstream T18 in pT18-FLAG	This study
pT18-EtgA	<i>etgA</i> gene lacking its signal sequence cloned downstream T18 in pT18-FLAG	This study
pT18-Pal	<i>pal</i> gene lacking its signal sequence cloned downstream T18 in pT18-FLAG	Battesti & Bouveret, 2008
pT25-FLAG	bacterial two hybrid vector, P15A origin, <i>Plac</i> , T25 fragment of <i>Bordetella pertussis</i> CyaA, Kan <sup>R</sup>	Battesti & Bouveret, 2008
pT25-VgrG	<i>sci-1 vgrG</i> gene cloned downstream T25 in pT25-FLAG	Zoued <i>et al.</i> , 2013
pT25-TssJ <sub>sol</sub>	<i>sci-1 tssJ</i> gene lacking its signal sequence cloned downstream T25 in pT25-FLAG	Zoued <i>et al.</i> , 2013
pT25-TssMp	<i>sci-1 tssM</i> periplasmic domain (amino-acids 386-1129) cloned downstream T25 in pT25-FLAG	Zoued <i>et al.</i> , 2013
pT25-TagLp	<i>sci-1 tagL</i> periplasmic domain (amino-acids 352-576) cloned downstream T25 in pT25-FLAG	This study
pTolB-T25	<i>tolB</i> gene cloned upstream T25 in pT25-FLAG	Battesti & Bouveret, 2008

## Oligonucleotides

Name	Destination	Sequence (5' to 3')
For chromosomal mutant strain construction <sup>a</sup>		
5-Del-mltA-DW	<i>mltA</i> gene deletion	CGGTTTGTTATCTTCGTTGCGCCTTATTTTTTAACTGAAGAAGAGAACATGTGTAGGCTGGAGCTGCTTCG
3-Del-mltA-DW	<i>mltA</i> gene deletion	TTACCCCTCACCCTGTCATATCCGTA AAAACGGCATA CAGAATATCACACATATGAATATCCTCCTTAGTTC
5-Del-mltB-DW	<i>mltB</i> gene deletion	TGATGCTTTACCATACTTGCCCTGGTTGAATCTGTTAAATGGACCCCTCTGTGTAGGCTGGAGCTGCTTCG
3-Del-mltB-DW	<i>mltB</i> gene deletion	AAAAGCTGATTAGCCAGAGGGAAGCTCACGCTCCCTCTTGCAAATAGCATATGAATATCCTCCTTAGTTC
5-Del-mltC-DW	<i>mltC</i> gene deletion	GCACGCCTCCGGCAACTTGCATAAAAACAACACAACACGCACCCGGATGTGTAGGCTGGAGCTGCTTCG
3-Del-mltC-DW	<i>mltC</i> gene deletion	TGTGGATAACATTTTTGCCCTGAGCATCGTCAGGGGCGGTTAATGGAACATATGAATATCCTCCTTAGTTC
5-Del-mltD-DW	<i>mltD</i> gene deletion	TCCGTTCCGCGTTATGATCGGTCTCTTTTAAAGCAACTATTGACACACACTGTGTAGGCTGGAGCTGCTTCG
3-Del-mltD-DW	<i>mltD</i> gene deletion	AAATAAAAAAGGCACCGGGGAATCGGTGCCTTTTTATTATCTGGTTGCATATGAATATCCTCCTTAGTTC
5-Del-mltE-DW	<i>mltE</i> gene deletion	TGTGCCGTTGTCACCTCAACGGCGATTCCAGGCTATAAGGATAGAAGAATGTGTAGGCTGGAGCTGCTTCG
3-Del-mltE-DW	<i>mltE</i> gene deletion	CTCTCGAGCGGGAAGCCCGGGAGAAAGCGGACAAAGTGC GCGACTGATCATATGAATATCCTCCTTAGTTC
5-Del-slt70-DW	<i>slt70</i> gene deletion	TTACGCGGCATGACGCTGCATTGATGATTTACACTTAGAGGATGCGCTTTGTGTAGGCTGGAGCTGCTTCG

3-Del-slt70-DW	<i>slt70</i> gene deletion	<u>CCGGTTGTA</u> CTCGCTAAAGAGTACGATAGCATATCATAAACGTGCGGACATATGAATATCCTCCTTAGTTC
5-Del-2762-DW	<i>EC042_2762</i> gene deletion	<u>TTATTACGTTTTTTCAAGCTGGGACGCGCACGACACAGAGAATTA</u> ACTAATGTGTAGGCTGGAGCTGCTCG
3-Del-2762-DW	<i>EC042_2762</i> gene deletion	<u>ATGCGCCGCCAGGAAATTA</u> AAGCGCAGAAAAAAGCGCGATCCTCGACGGACATATGAATATCCTCCTTAGTTC
5-Del-etgA-DW	<i>etgA</i> gene deletion	<u>GATTATATTAGTATCTCGTTCC</u> TTTCCTTCAATCCTACACATAAAAAATATTGTGTAGGCTGGAGCTGCTTCG
3-Del-etgA-DW	<i>etgA</i> gene deletion	<u>AGCTTTACGTATGGGTGTT</u> TGCACTATATAAAAAAAGAGGCTTTAGGCCATATGAATATCCTCCTTAGTTC

**For plasmid construction**<sup>b,c</sup>

5-pBAD-MltE <sub>VSV-G</sub>	insertion of <i>mltE</i> into pBAD33	<u>CTCTCTACTGTTTTCTCCATACCCGTTTTTTTTGGGCTAGCAGGAGGTATTACACCATGAAATT</u> AAGATGGTTTTGCCTTTTTGATTGTG
3- pBAD-MltE <sub>VSV-G</sub>	insertion of <i>mltE</i> into pBAD33	<u>GGTCGACTCTAGAGGATCCCCGGGTACCTTATTTTCCTAATCTATTCATTTCAATATCTGTATAC</u> ATCGCGTCCAGTGCCTGC
T18N-5-MltA	insertion of signal sequence-less <i>mltA</i> into pT18-FLAG	<u>CGCCACTGCAGGGATTATAAAGATGACGATGACAAGTCTTCCAAACCAAC</u> CGATCGCG
T25T18N-3-MltA	insertion of signal sequence-less <i>mltA</i> into pT18-FLAG	<u>CGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCTAGTTAGCCGCTA</u> AAGACGTTACCTGCG
T18N-5-MltB	insertion of signal sequence-less <i>mltB</i> into pT18-FLAG	<u>CGCCACTGCAGGGATTATAAAGATGACGATGACAAGAGCAGCAAGCCAA</u> AACCTACTGAG
T25T18N-3-MltB	insertion of signal sequence-less <i>mltB</i> into pT18-FLAG	<u>CGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCTAGTTACTGCACG</u> CGCGCCAG
T18N-5-MltC	insertion of signal sequence-less <i>mltC</i> into pT18-FLAG	<u>CGCCACTGCAGGGATTATAAAGATGACGATGACAAGTCGACGACCAAAA</u> AAGGCGATACC
T25T18N-3-MltC	insertion of signal sequence-less <i>mltC</i> into pT18-FLAG	<u>CGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCTAGTTATCGGCGG</u> CGGTAGGATTTTTG
T18N-5-MltD	insertion of signal sequence-less <i>mltD</i> into pT18-FLAG	<u>CGCCACTGCAGGGATTATAAAGATGACGATGACAAGCAGAGTACCGGCA</u> ACGTTCAAC
T25T18N-3-MltD	insertion of signal sequence-less <i>mltD</i> into pT18-FLAG	<u>CGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCTAGTTAGGAATCT</u> GGCATGTTGTTGTTTTTACAAAC
T18N-5-MltE	insertion of signal sequence-less <i>mltE</i> into pT18-FLAG	<u>CGCCACTGCAGGGATTATAAAGATGACGATGACAAGTCATCAAAGCATGA</u> CTACACGAACCC
T25T18N-3-MltE	insertion of signal sequence-less <i>mltE</i> into pT18-FLAG	<u>CGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCTAGTTACATCGCGT</u> CCAGTGCCTG
T18N-5-Slt70	insertion of signal sequence-less <i>slt70</i> into pT18-FLAG	<u>CGCCACTGCAGGGATTATAAAGATGACGATGACAAGGCGCGAGCCGACT</u> CACTG
T25T18N-3-Slt70	insertion of signal sequence-less <i>slt70</i> into pT18-FLAG	<u>CGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCTAGTTAGTAACGA</u> CGTCCCCATCCGTG

T18N-5-2762	insertion of signal sequence-less <i>EC042_2762</i> into pT18-FLAG	<u>CGCCACTGCAGGGATTATAAAGATGACGATGACAAGGCTCTCTGG</u> CCATCCATTC
T25T18N-3-2762	insertion of signal sequence-less <i>EC042_2762</i> into pT18-FLAG	<u>CGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCTAGTTAAT</u> TTGTTTCTCTTCACTCCCTTTCCTGG
T18N-5-EtgA	insertion of signal sequence-less <i>etgA</i> into pT18-FLAG	<u>CGCCACTGCAGGGATTATAAAGATGACGATGACAAGGCCAGTAGCGCTTG</u> CTTTAATGAAGC
T25T18N-3-EtgA	insertion of signal sequence-less <i>etgA</i> into pT18-FLAG	<u>CGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCTAGTTATTTGCTA</u> AAGCCTTACGCTTGTCTATTTTC
T25N-5-TagLp	insertion of <i>tagL<sub>352-576</sub></i> fragment into pT25-FLAG	<u>GGCGGGCTGCAGATTATAAAGATGACGATGACAAGCGGCTGGTTCGCAGC</u> GTG
T25T18N-3-TagLp	insertion of <i>tagL<sub>352-576</sub></i> fragment into pT25-FLAG	<u>CGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCTAGTTACTCCGTT</u> ATGTTTTCTGATGCGCC

**For site-directed mutagenesis<sup>d</sup>**

A-MltE-E64Q	Glu64-to-Gln mutation in <i>mltE</i>	GGCGATTATCGCTATCCAATCGGGTGGTAATCC
B-MltE-E64Q	Glu64-to-Gln mutation in <i>mltE</i>	GGATTACCACCCGAT <b>T</b> GGATAGCGATAATCGCC

<sup>a</sup> Sequences corresponding to the downstream and upstream regions of the gene to be deleted underlined

<sup>b</sup> Sequence annealing on the target plasmid underlined.

<sup>c</sup> FLAG or VSV-G epitope coding sequence *italicized*.

<sup>d</sup> Mutagenesized codon in **Bold**.