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

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Abstract

The type VI secretion system (T6SS) is an anti-bacterial weapon comprising a contractile tail anchored to the cell envelope by a membrane complex. The TssJ, TssL, and TssM proteins assemble a 1.7-MDa channel complex that spans the cell envelope, including the peptidoglycan layer. The electron microscopy structure of the TssJLM complex revealed that it has a diameter of ~18 nm in the periplasm, which is larger than the size of peptidoglycan pores (~2 nm), hence questioning how the T6SS membrane complex crosses the peptidoglycan layer. Here, we report that the MltE housekeeping lytic transglycosylase (LTG) is required for T6SS assembly in entericaggregative Escherichia coli. Protein–protein interaction studies further demonstrated that MltE is recruited to the periplasmic domain of TssM. In addition, we show that TssM significantly stimulates MltE activity in vitro and that MltE is required for the late stages of T6SS membrane complex assembly. Collectively, our data provide the first example of domestication and activation of a LTG encoded within the core genome for the assembly of a secretion system.

Keywords multiprotein assembly; peptidoglycan; protein complex; protein transport; secretion system

Subject Categories Microbiology, Virology & Host Pathogen Interaction; Structural Biology

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Introduction

The cell envelope of Gram-negative bacteria is crossed by multiprotein complexes that participate to the assembly of surface appendages (e.g., the flagellum) or serve as channels for the passage of large molecules such as pili, DNA, or protein effectors (e.g., piliation, conjugation, or secretion systems) [1]. These complexes are usually large and are anchored to both the inner and outer membranes [1]. However, the peptidoglycan layer represents a physical barrier for the assembly of these structures, as they are usually larger than peptidoglycan pores, estimated to have a diameter of ~2 nm [2]. Most of these systems have therefore evolved enzymes, called lytic transglycosylases (LTGs), that locally rearrange the cell wall [3–5]. LTGs cleave the glycan strands but have no action on peptide cross-links, therefore creating lateral separation of the peptidoglycan [6,7]. Endogeneous LTGs are involved in peptidoglycan synthesis, turnover, recycling, and daughter cell separation [7–9]. By contrast, the LTGs dedicated to specific cell-envelope spanning complexes are called specialized LTGs [3–5,8]. The activity of these enzymes needs to be tightly controlled to avoid peptidoglycan breaches and cell lysis [8,10]. In addition, the LTG activity should be spatially controlled to create sufficient space at the site of assembly. The spatial activation of specialized LTGs is secured by their recruitment to the site of assembly through interactions with one or several components of the apparatus. The recruitment of specialized LTGs to their cognate apparatus has been exemplified in the case of several cell-spanning machineries: The Rhodobacter sphaeroides SltF LTG is recruited to the flagellar FlgJ subunit [11,12], the PleA protein localizes at the cell pole in Caulobacter crescentus and is required for the assembly of the polar pilus and flagellar [13], the VirB1-like LTG is recruited to the VirB8-like protein in type IV secretion systems [4,14–19], and the EtgA LTG associates with the type III secretion system EscI rod component [4,20–22]. Interestingly, in a few cases, machine subunits comprise an additional domain with LTG activity, such as the flagellar rod FlgI protein [23–27] or the Bordetella pertussis T4SS PtlE subunit [28]. For several of these enzymes, it has been recently demonstrated that the transglycosylase activity is weak in vitro but stimulated in the presence of its partner, suggesting that binding to the cell-envelope spanning structure specifically activates the enzymatic activity and hence controls localized peptidoglycan hydrolysis. The activity of the T3SS EtgA LTG is enhanced by co-incubation with the Escl rod subunit [22]. In the case of the R. sphaeroides flagellum, the activity of SltF is modulated by both FlgB and FlgF [29].

Recently, we determined the structure of the 1.7-MDa type VI secretion system (T6SS) membrane complex from entericaggregative Escherichia coli (EAEC) using negative stain electron microscopy [30]. This complex spans the cell envelope, and its diameter was estimated to ~18 nm in the periplasm, suggesting that its proper insertion requires localized peptidoglycan rearrangement or degradation. However, no gene encoding LTG is encoded within T6SS gene clusters [31,32]. The T6SS is a sophisticated multiprotein
machine that is widely distributed in Gram-negative bacteria and responsible for the delivery of toxin effectors in both prokaryotic and eukaryotic cells, hence participating in bacterial competition and pathogenesis [33–42]. It is constituted of a cytoplasmic tail complex that is evolutionarily, structurally, and functionally related to contractile machines such as phages or pyocins [43–45]. The tail comprises an inner tube composed of Hcp hexamers stack on each other and wrapped into the contractile sheath formed by the polymerization of TssBC complexes [46–50]. The inner tube is tipped by the VgrG/PAAR needle complex that is used as a puncturing device to penetrate the target cell [47,51]. Once assembled, the sheath contracts and propels the Hcp/VgrG/PAAR needle complex, allowing effector delivery and target cell lysis [49,52–54]. The tail is built onto an assembly platform, the baseplate, constituted of the TssEFGK-VgrG subunits [55–57]. The baseplate docks to the membrane complex that both orients the tail toward the cell exterior and serves as channel for the passage of the Hcp/VgrG/PAAR needle complex [30,56,58,59]. The membrane complex is composed of the TssJ, TssL, and TssM proteins, each present in ten copies [30,60]. TssL and TssM are both inner membrane proteins, with soluble domains in the cytoplasm and periplasm, respectively [58,61,62]. TssJ is an outer membrane lipoprotein [63] that interacts with the C-terminal domain of the TssM periplasmic region [64]. The assembly of the membrane complex starts with the initial positioning of the TssJ lipoprotein and progresses inward with the ordered addition of TssM and TssL [30]. Once assembled, the membrane complex recruits the TssA protein and the baseplate complex prior to tail/sheath polymerization [56,65]. In addition to span the cell envelope, the membrane complex is anchored to the cell wall by an additional component TagL, or an additional domain fused to the C-terminus of TssL, that shares homology to peptidoglycan-binding proteins [60,66]. It is proposed that anchorage to the cell wall allows stabilization of the membrane complex, notably during sheath contraction. The negative stain electron microscopy structure of the EAEC TssJLM complex demonstrated that it is composed of a base comprising the cytoplasmic domains of TssL and TssM and forms a trans-envelope channel with ten arches and ten pillars constituted by the periplasmic domain of TssM and the TssJ lipoprotein [30]. The diameter of this complex in the periplasm is, however, incompatible with the size of peptidoglycan pores and we hypothesized that proper insertion or assembly of the T6SS membrane complex requires the action of a LTG. Recently, a study identified TagX, a T6SS-encoded peptidoglycan endopeptidase required for T6SS function in Actinobacter species [67]. However, the tagX gene is not conserved and the vast majority of T6SS gene clusters does not encode peptidoglycan hydrolases. This observation raised the question on how these type VI secretion systems deal with the peptidoglycan layer. Here, we provide evidence that the EAEC T6SS has domesticated the housekeeping MltE LTG for its assembly.

Results

Proper function of the EAEC T6SS requires the MltE housekeeping lytic transglycosylase

To test whether peptidoglycan remodeling is required for assembly of the T6SS, cells were treated with bulgecin A, a specific inhibitor of transglycosylases [68,69], and the release of Hcp in the culture supernatant, a marker of EAEC T6SS assembly and function [63], was probed by Western blot analyses. To avoid Hcp release by pre-assembled and active T6SS, we monitored the experiments in a strain deleted of the tssM gene but bearing a plasmid-borne wild-type tssM allele under the control of an inducible Tet promoter. In this strain, the basal expression of tssM is undetectable by Western blot and is not sufficient to support assembly of the T6SS. In the presence of inducer, the T6SS is assembled and hence Hcp was detected in the culture supernatant.

Figure 1. The LTG inhibitor bulgecin A prevents T6SS assembly.

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

B Hcp release assay. HA-tagged Hcp (HcpHA) release was assessed by separating cells (C) and cell-free culture supernatant (S) fractions from 10^8 wild-type (WT) cells before washing (before wash) and after washing and growth (after wash) in the absence (NT) or presence (bulgecin) of bulgecin A. Proteins were separated by 12.5% acrylamide SDS–PAGE and the periplasmic TolB protein (control for cell lysis) and HcpHA were immunodetected using anti-TolB (upper panel) and anti-HA (lower panel) antibodies. Molecular weight markers (in kDa) are indicated on the left. The experiment was performed in duplicate and a representative result is shown.
(Fig 1A). The addition of bulgecin A in the medium prior to tssM induction did not impact TssM production but prevented Hcp release (Fig 1A). This result demonstrates that the T6SS does not function when the activity of LTGs is inhibited, and suggests that the action of at least one endogenous LTG is required for the assembly of this apparatus. Using time-lapse fluorescence microscopy, we previously showed that the TssJLM membrane complex is used for several rounds of tail assembly and contraction [30]. To confirm this result, wild-type EAEC cells were washed to discard secreted Hcp proteins and resuspended in medium supplemented with bulgecin A, to prevent assembly of new T6SS membrane complexes. After 45 min of growth, the presence of Hcp in the supernatant was probed by Western blot analyses. We observed that Hcp was released, demonstrating that pre-assembled T6SS membrane complexes are not sensitive to treatment with bulgecin A (Fig 1B).

The EAEC 17-2 chromosome encodes 8 proteins with signature of LTGs. These include the soluble Slt70 and the membrane-bound MltA-E housekeeping lytic transglycosylases, as well as two putative LTGs: EtgA encoded within the T3SS gene island and the product of the EC042_2762 gene. To test the contribution of these proteins for the assembly of the EAEC T6SS, we generated individual knockout strains in each of these genes and tested the ability of these strains to support Hcp secretion. Western blot analyses of cell-free culture supernatants showed that Hcp release was abolished in the ΔmltE mutant was produced at levels comparable to wild-type MltE, cells producing MltEE64Q were unable to release Hcp and to provide a competitive advantage against other E. coli species [70]. Figure 3B shows that the number of GFP+ kanamycin-resistant E. coli K-12 prey cells recovered after co-culture with EAEC ΔmltE cells was 4-log higher compared to co-culture with EAEC wild-type cells. This effect is comparable to that observed for a ΔtssM mutant. The T6SS-phenotypes conferred by the mltE mutation were complemented by the production of a wild-type copy of MltE (Fig 3A and B). We then tested whether the activity of MltE is required for T6SS function. The crystal structure and the in vitro characterization of MltE revealed the importance of a glutamate residue, Glu-64, in the catalytic reaction [71,72]. Although the MltE64Q catalytic inactive mutant was produced at levels comparable to wild-type MltE, cells producing MltE64Q were unable to release Hcp and to provide a T6SS-dependent competitive advantage against E. coli K-12 (Fig 3A and B). Taken together, these results demonstrate that the assembly of the EAEC Sci-1 T6SS requires the activity of the MltE lytic transglycosylase.

**MltE is recruited and activated by TssM**

A number of LTGs, including that associated with T3SS, T4SS, and flagella, have been shown to interact with machine components to facilitate local peptidoglycan degradation at the site of assembly [11,16,22]. Based on the results presented above, we hypothesized that MltE should be recruited to the T6SS apparatus. MltE being an outer membrane lipoprotein facing the periplasm [73], we tested the interaction of a soluble form of MltE, sMltE, with the T6SS subunits or domains exposed in the periplasm. These include the soluble fragment of TssJ, the periplasmic domains of the TssM (TssM9p) and TagL (TagLP) proteins [30,58,60,63,64], as well as VgrG, which is proposed to fit inside the TssJLM complex channel at rest [30]. Bacterial two-hybrid analyses demonstrated that sMltE interacts with the TssM periplasmic domain (Fig 4A and B). This interaction is specific as sMltE does not interact with the other T6SS subunits tested, and TssMp does not interact with the seven other LTGs. The interaction of TssM9p with the full-length MltE lipoprotein was further confirmed by co-immunoprecipitation into the heterologous host E. coli K-12 (Fig 4C). These results define that MltE is recruited to the T6SS apparatus by binding directly to the TssM periplasmic region. The bacterial two-hybrid assay also showed that sMltE [640] variant interacts with TssM9p, demonstrating that this mutation does not interfere with MltE recruitment to TssM9p (Fig 4B). The TssM periplasmic region could be segmented into three sub-domains: Sub-domains 1 and 2 (amino acids 386–973) correspond to a region predicted to be essentially α-helical and are followed by the C-terminal sub-domain 3 (amino acids 974–1129) that folds as a β-sandwich-like structure [30]. Co-immunoprecipitations using two variants encompassing these regions (TssM966–973 and TssM972–1129) revealed that MltE binds to the α-helical sub-domains 1 + 2 (Fig 4C).

MltE is a non-processive endo-transglycosylase, which is considered to have a relatively low peptidoglycan hydrolase activity _in vivo_ compared to other LTGs [73]. Indeed, peptidoglycan hydrolysis assays showed that the purified soluble form of MltE, sMltE, is significantly less active compared to lysozyme (Fig 5A and B; initial rate of sMltE = 0.45 × 10⁻³ AU/min/μmol). However, the activity of the T3SS-associated EtgA protein has been shown to be modulated via its interaction with the T3SS rod component EscI to avoid unspecific peptidoglycan lysis [22]. We therefore tested whether sMltE is activated once bound to TssM9p. Figure 5A and B shows that

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**Figure 3. The MltE LTG is required for T6SS function.**

Hcp release assay. FLAG-tagged Hcp (HcpFL) release was assessed by separating cells (C) and cell-free culture supernatant (S) fractions from 10⁵ cells of the indicated strains. Proteins were separated by 12.5% acrylamide SDS–PAGE and TolB and HcpFL were immunodetected using anti-TolB (upper panel) and anti-FLAG (lower panel) antibodies. Molecular weight markers (in kDa) are indicated on the left. The experiment was performed in triplicate and a representative result is shown.
incubation of $\Delta$MltE with TssM+ stimulated the activity of $\Delta$MltE sevenfold (initial rate of $\Delta$MltE in the presence of TssM+ = $3.15 \times 10^{-9}$ AU/min/nmol). Control experiments showed that TssM+, the $\Delta$MltE^ES40:TssM+ complex or the $\Delta$MltE:TssM+ complex in the presence of bulgecin A, has no significant activity (Fig 5A and B).

**MltE is required for oligomerization of the TssM protein**

The assembly of the T6SS is an ordered process in which the different subunits of the apparatus are sequentially recruited to the site of assembly. The assembly starts with the initial positioning of the TssJ lipoprotein and progresses by the addition of TssM and TssL, and the polymerization of TssJLM complexes to yield the membrane complex [30]. The cytoplasmic TssA protein then binds to the TssM or TssJLM complex and recruits the baseplate, prior to tail polymerization [56,65,74]. To define at which stage of this biogenesis pathway the activity of MltE is necessary, we first assayed the TssJ-TssM and TssL-TssM interactions in the WT strain and its isogenic $\Delta$mltE mutant. As previously published [64], TssJ and TssL co-precipitate with TssM. Figure 6A and B, respectively, shows that the TssJ-TssM and TssL-TssM interactions are not affected by the absence of MltE. The latter stages of T6SS membrane complex biogenesis is the polymerization of the TssJLM complex [30]. The multimerization of TssM and its complexes with TssJ and TssL could be visualized in wild-type cells after in vivo chemical cross-linking using bis-(sulfosuccinimidyl)-suberate (BS3) (Fig 6C). Although TssJ and TssM complexes are still assembled in $\Delta$mltE cells or $\Delta$mltE cells producing the MltE^ES40 catalytic MltE mutant, no cross-linked TssJ-TssM or TssM-TssL species were observed in these cells (Fig 6C). Assembled TssJLM complexes can be observed directly in cells using a chromosomal fusion between TssM and a fluorescent reporter such as GFP [30]. Fluorescence microscopy recordings show that GFPTssM forms fluorescent clusters at the cell periphery in wild-type cells (Fig 6D). However, no focus was observable in $\Delta$mltE cells or $\Delta$mltE cells producing MltE^ES40 (Fig 6D). Taken together, these results suggest that local peptidoglycan hydrolysis by MltE is not required for formation of TssJLM heterotrimers but rather is necessary for assembly of the TssJLM core complex.

**Discussion**

In this work, we observed that treatment of EAEC cells with the LTG inhibitor bulgecin A prevents assembly of the Sci-1 T6SS. Systematic deletion of genes encoding LTG or putative LTG coupled to phenotypic assays demonstrated that the housekeeping MltE LTG is required for Sci-1 T6SS function, as Hcp release in the culture supernatant was abolished in $\Delta$mltE cells. In addition, $\Delta$mltE cells presented a decreased T6SS-dependent antagonist activity against *E. coli* K-12. The EAEC strain used in this study, 17-2, encodes a second T6SS, Sci-2 that belongs to the T6SS-3 family [75]. No dedicated LTG is encoded within this cluster, and it will be thus interesting to define whether MltE—or an another host LTG—is required for the assembly of the Sci-2 T6SS.

We further showed that MltE is recruited to the site of assembly of the T6SS membrane complex by interacting with the $\alpha$-domains 1

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

A Hcp release assay. FLAG-tagged Hcp (Hcp^FL) release was assessed by separating cells (C) and cell-free culture supernatant (S) fractions from $10^8$ WT, $\Delta$mltE cells or $\Delta$mltE cells producing wild-type (mltE+) or E64Q mutant (mltE^E64Q) VSV-G-tagged MltE (MltE^V) from arabinose-inducible plasmids. Proteins were separated by 12.5% acrylamide SDS-PAGE and ToIB, Hcp^FL, and MltE^V were immunodetected using anti-ToIB (upper panel), anti-FLAG (middle panel), and anti-VSV-G (lower panel) antibodies, respectively. Molecular weight markers (in kDa) are indicated on the left. The experiment was performed in triplicate and a representative result is shown.

B Anti-bacterial activity. *Escherichia coli* K-12 prey cells (W3110 gfp^+, kan^R) were mixed with the indicated attacker cells, spotted onto SC-1 inducing medium (SIM) agar plates, and incubated for 4 h at 37°C. The image of a representative bacterial spot and the average and standard deviation (n = 3) of the relative fluorescence of the bacterial mixture (in arbitrary units, AU) are shown in the upper graph. The number of recovered *E. coli* prey cells (counted on selective kanamycin medium) is indicated in the lower graph (in log10 of colony-forming units (cfu)). The black, dark gray, and light gray circles indicate values from three independent assays, and the average is indicated by the bar. The experiment was performed in triplicate and a representative result is shown. Asterisks indicate significant differences compared to the wild-type attacker strain (NS, non-significant, **P < 0.001; Student’s t-test).
and 2 of the TssM periplasmic region. In addition, we showed that the presence of the periplasmic domain of TssM stimulates the LTG activity of MltE sevenfold in vitro. These results are comparable to the enteropathogenic E. coli EscI T3SS rod component that binds and stimulates the EtgA LTG. EtgA is a specialized LTG, encoded and co-regulated with the T3SS gene cluster [21,22,76], a situation that is common in cell-envelope spanning machines such as flagella, type IV pili, T3SS, or T4SS [3,5]. By contrast, with few exceptions [67], no peptidoglycan hydrolase is encoded within T6SS gene clusters. Therefore, assembly of the T6SS membrane complex requires hijacking of an host LTG to locally rearrange the cell wall. The mltE gene is also present in E. coli K-12 strains lacking T6SS, in which it participates to peptidoglycan homeostasis [10,73]. The EAEC Sci-1 T6SS has therefore re-routed MltE for its own assembly. However, the observation that T6SS gene clusters have been horizontally transferred between species suggests that each strain may have domesticated different host LTGs. Another example of domestication of non-specialized LTG is the recruitment of MltD to anchor the Helicobacter pylori flagellum [77].

The recruitment and stimulation of MltE by TssM therefore spatially controls the activity of MltE at close proximity to the site of assembly of the T6SS. Interestingly, MltE has a relatively weak activity on E. coli peptidoglycan compared to other LTGs [73]. However, Fibriansah et al [72] noted that MltE is more active on the peptidoglycan of Micrococcus luteus, which differs from that of E. coli by the nature of the peptide stems. They proposed that MltE

Figure 4. MltE interacts with the TssM periplasmic domain.
A, B Bacterial two-hybrid assay. BTH101 reporter cells producing the indicated proteins or domains fused to the T18 or T25 domain of the Bordetella adenylate cyclase were spotted on X-gal indicator plates. The blue color of the colony reflects the interaction between the two proteins. TolB and Pal are two proteins known to interact but unrelated to the T6SS or the MltE proteins. The experiment was performed in triplicate and a representative result is shown.
C Co-immunoprecipitation assay. The solubilized lysates from 2 × 1013 Escherichia coli K-12 W3110 cells co-expressing the indicated FLAG-tagged TssM variants (exported in the periplasm) and VSV-G-tagged MltE protein (Total, T) were subjected to immunoprecipitation on anti-FLAG-coupled agarose beads. The immunoprecipitated material (IP) was subjected to 12.5% acrylamide SDS-PAGE and immunodetected with anti-FLAG (upper panel, TssM domains) and anti-VSV-G (lower panel, MltE) antibodies. Molecular weight markers (in kDa) are indicated on the left. The experiment was performed in triplicate and a representative result is shown.
Figure 5. TssM<sub>p</sub> increases MltE peptidoglycan hydrolase activity.

A Remazol Brilliant Blue assay. The absorbance of supernatants from the reaction containing purified and RBB-labeled Escherichia coli peptidoglycan and the indicated protein (50 µg) was measured at λ = 595 nm after incubation for 0.5 or 4 h at 37°C. The results shown are the average and standard deviation from triplicate reactions (n = 3). Asterisks indicate significant differences compared to the buffer (NS, non-significant; **P < 0.01; ***P < 0.001; Student’s t-test). The supernatant of the reaction after 4 h of incubation is shown on bottom.

B Peptidoglycan hydrolysis. The decrease of the absorbance of the Micrococcus luteus peptidoglycan suspension in the presence of the indicated protein (50 µg) was measured at λ = 600 nm at 37°C over time. The results shown are the average and standard deviation from triplicate reactions (n = 3).
activity either requires the activity of an amidase to cleave the peptidoglycan peptide moieties or that its conformation is modulated by protein partners. The coordinated action of amidases and LTGs has been documented, notably during sporulation in *Bacillus subtilis* [78]. Although it would be interesting to test whether amidases are required for the assembly of the T6SS, the observation that TssM enhances the activity of MltE in vitro suggests that TssM helps MltE to bypass the presence of peptide stems. TssM might displace the peptide stem to avoid steric hindrance and to increase accessibility of MltE to the glycan strand, or might induce a conformational change in MltE, hence increasing its affinity for its substrate.

Our results also defined that MltE is required for the late stages of the assembly of the T6SS membrane complex (Fig 7). The biogenesis of the T6SS membrane complex begins with the positioning of the TssJ outer membrane lipoprotein (Fig 7, step a) and the recruitment of (i) TssM and (ii) TssL (Fig 7, steps b and c) prior to multimerization (Fig 7, step d) [30]. The assembled TssJLM membrane complex is constituted of five dimers of TssJLM heterotrimeric complexes (Fig 7, step d) [30]. We showed that the absence of MltE does not interfere with the interaction between TssJ and TssM, as well as between TssM and TssL. However, we did not detect TssM dimers in ΔmltE cells or in cells producing a catalytically inactive MltE LTG. We therefore propose that MltE is recruited to TssM prior to multimerization (Fig 7). This hypothesis suggests that the monomeric periplasmic domain of TssM can cross the cell wall to interact with TssJ, and that local rearrangement of the peptidoglycan is necessary for the polymerization of TssJLM heterotrimers.

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

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

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

C BS3 cross-linking assay. $2 \times 10^9$ cells of the indicated strain producing FLAG-tagged TssM (with the exception of ΔtssM cells) were treated (+) or not (−) with the BS3 cross-linker agent. After the cross-linking reaction, cells were boiled in Laemmli buffer and total proteins were subjected to 7% acrylamide SDS–PAGE and immunodetected with anti-FLAG antibodies. The TssM protein (pTssM) and its complexes (*TssM-TssJ; **TssM-TssL) are indicated on the right as well as the TssM dimer (arrow). Molecular weight markers (in kDa) are indicated on the left. The experiment was performed in triplicate and a representative result is shown.

D Fluorescence microscopy. Recordings showing TssM localization using the chromosomally encoded sfGFP-tssM fusion in wild-type (WT) or ΔmltE cells or ΔmltE cells producing the wild-type (mltE$^+$) or catalytic variant (mltE$^{E64Q}$) MltE protein. Scale bars are 1 μm. The experiment was performed in triplicate and a representative result is shown.
Materials and Methods

Bacterial strains, growth conditions and chemicals

The strains used in this study are listed in Appendix Table S1. *Escherichia coli* K-12 strains DH5α, W3110, BTH101, and BL21 (DE3)/MC1061 were used for cloning procedures, co-immunoprecipitation, bacterial two-hybrid assay, and protein purification, respectively. Enterocaggregative *E. coli* (EAEC) strains used in this work are isogenic derivatives of the wild-type O3:H2 17-2 strain. *E. coli* K-12 and EAEC cells were routinely grown in LB broth at 37°C, with aeration. For induction of the sim-1 T6SS gene cluster, cells were grown in Sci-1-inducing medium [SIM: M9 minimal medium supplemented with glycerol (0.2%), vitamin B1 (1 μg/ml), casaminoacids (40 μg/ml), LB (10% v/v)] [79]. Plasmids and mutations were maintained by the addition of ampicillin (100 μg/ml for K-12, 200 μg/ml for EAEC), kanamycin (50 μg/ml for K-12, 50 μg/ml for chromosomal insertion on EAEC, 100 μg/ml for plasmid-bearing EAEC), or chloramphenicol (40 μg/ml). Gene expression was induced by the addition of iso-propyl-β-D-thio-galactopyranoside (IPTG, Sigma-Aldrich, 0.2 mM for 1 h), L-arabinose (Sigma-Aldrich; 0.005% for 0.5 h for complementation assays, 0.2% for 1 h for co-immunoprecipitation), or anhydrotetracycline (AHT; IBA Technologies; 0.005% for 0.5 h for complementation assays, 0.2% for 1 h for co-immunoprecipitation).

Strain construction

Deletions of genes *mltA* (EC042_3012, gene accession identifier (GI): 284922752), *mltB* (EC042_2894, GI: 284922637), *mltC* (EC042_3170, GI: 284922906), *mltD* (EC042_0224, GI: 284920001), *mltE* (EC042_1244, GI: 284920999), *slt70* (EC042_4889, GI: 284924571), EC042_2762 (GI: 284922508), and *etgA* (EC042_3052, GI: 284922791) were engineered on the EAEC 17-2 chromosome using the modified one-step inactivation procedure [80] using λ red recombinase expressed from pKOBEG [81] as previously described [63]. The *mltE* gene was deleted from the 17-2 strain producing the cffpTssM fusion protein expressed from the chromosomal native locus [30] using the same procedure. The kanamycin cassette from plasmid pKD4 [80] was amplified with oligonucleotides carrying ~50 nucleotide extensions homologous to regions adjacent to the target gene (custom primers, synthesized by Eurogentec, are listed in Appendix Table S1). The polymerase chain reaction (PCR) product was column purified (PCR and Gel Clean up, Promega) and electro-porated into competent cells. Kanamycin-resistant clones were selected and the insertion of the kanamycin cassette at the targeted site was verified by PCR. The kanamycin cassette was then excised using plasmid pCP20 [80] and the final strain was verified by PCR.

Plasmid construction

PCR was performed with a Biometra thermocycler, using the Pfu Turbo DNA polymerase (Stratagene; La Jolla, CA, USA). Plasmids and oligonucleotides are listed in Appendix Table S1. Constructions of pOK-HcpHAla, pUC-HcpFLAG, pIBA-TssMΔ, pIBA-TssMϕ, pMS-TssJHA, and pETG20A-TssMϕ have been previously described [60,63,64]. Plasmids pBADnLIC-sMltE and pBADnLIC-sMltE-E64Q have been previously described [72] and have been kindly provided by Andy-Mark Thunnissen (University of Groningen, the Netherlands). All pASK-IBA4 and bacterial two-hybrid vectors and the pBAD-Mlteϕ plasmid, encoding the C-terminally VSV-G-tagged full-length MltE protein under the control of the arabinoise promoter (in the pBAD33 vector), have been constructed by restriction-free cloning [82]. Briefly, the gene of interest fused to 5′ and 3′...
extensions annealing to the target vector was amplified and used as oligonucleotides for a second PCR using the target vector as template. The E64Q point mutation was inserted into pBAD-MtIE<sub>α</sub> and pT18-MtIE by site-directed mutagenesis using complementary oligonucleotides bearing the desired mutation. All constructs have been verified by PCR and DNA sequencing (MWG).

**T6SS phenotypic assays and GFPtssM fluorescence microscopy recordings**

Hcp release and anti-bacterial competition assays were performed as previously described [63,70]. For the Hcp release assay with bulgecin A treatment, the experiment was performed in a ΔtssM strain carrying plasmid pASK-IBA37-TssM, allowing AHt-dependent inducible expression of the tssM gene and producing HA-tagged Hcp. Cells were grown in SIM until A600nm -0.4 and treated—or not—with bulgecin A (100 μM). After 40 min, tssM expression was induced by the addition of αHT and the culture was further grown for 45 min. Cell pellets and supernatants were fractionated by centrifugation. The final supernatant fraction samples were obtained by filtration on 0.25-μM PES membranes and TCA precipitation as previously published [63]. Controls were performed to verify that bulgecin A treatment did not interfere with TssM production. Controls for cell lysis were performed by immunodetecting the periplasmic TolB protein. For treatment of cells with pre-assembled TssJLM complexes, wild-type 17-2 cells producing HA-tagged Hcp were grown in SIM to A600nm -0.5, and the cells and supernatant fractions were separated as described above. Cells were washed in SIM and resuspended in SIM supplemented or not with bulgecin A (100 μM). After further growth for 45 min, cells and supernatant fractions were separated as described above. Fluorescence microscopy recordings were performed as previously published [30]. All experiments have been done at least in duplicate and a representative result is shown. Statistical analysis of antibacterial competition assays was performed by Student’s t-test. Significant differences were defined as *P < 0.05, **P < 0.01, and ***P < 0.001.

**Protein production and purification**

The periplasmic domain of TssM was purified from *E. coli* BL21 (DE3) cells carrying the pETG20A-TssM<sub>α</sub> plasmid and the native protein was obtained after cleavage of the thioredoxin-6-6-His N-terminal extension by the tobacco etch virus (TEV) protease, as previously published [64]. The soluble MtIE protein and its E64Q variant were purified from *E. coli* MC1061 cells carrying the pBADnLIC-sMtIE or pBADnLIC-sMtIE-E64Q vector as previously published [72].

**Peptidoglycan hydrolysis assays**

**Preparation of the peptidoglycan fraction**

The peptidoglycan fraction from the JE5505 lpp strain was prepared as previously published [83], resuspended in phosphate-buffered saline, and treated with 200 μg/ml amylase (Sigma-Aldrich) for 2 h at 37°C.

**Remazol Brilliant Blue assay**

This protocol for the peptidoglycan hydrolysis assay has been modified from a published protocol [84]. The purified peptidoglycan was washed with distilled water, resuspended in 200 mM NaOH and labeled with 25 mM Remazol Brilliant Blue (RBB, Sigma-Aldrich) for 14 h at 37°C, and washed four times with distilled water. RBB-labeled peptidoglycan was incubated with the 50 μg of protein of interest for 30 min or 4 h in PBS buffer, and the reaction was quenched by the addition of 50 μg/ml bulgecin A. After ultra-centrifugation for 40 min at 68,000 g, the absorbance of the supernatant was measured at 595 nm.

**Peptidoglycan turbidity assay**

This peptidoglycan hydrolysis assay has been performed as previously published [72] using a suspension of 0.25 mg/ml of purified *M. luteus* peptidoglycan (Sigma-Aldrich) in MES 50 mM pH 6.0, NaCl 200 mM (A<sub>600</sub>nm = 0.57 ± 0.04). The turbidity at 600 nm was measured every 20 min after addition of 50 μg (~2.48 nmol) of MtIE. For experiments in the presence of TssM<sub>α</sub>, a 1:2 (MtIE: TssM<sub>α</sub>) molar ratio has been used. The initial rate was measured as the slope of the initial linear curve (expressed in absorbance units/min/nmol). For all peptidoglycan hydrolysis assays, controls were performed with buffer, lysozyme, or in the presence of 50 μg/ml bulgecin A. The assays have been performed in triplicate and a representative experiment is shown.

**Bacterial two-hybrid assay**

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

**Co-immunoprecipitation**

10<sup>11</sup> exponentially growing cells producing the proteins of interest were harvested and resuspended in buffer TN (Tris–HCl 20 mM pH 8.0, NaCl 100 mM) supplemented with protease inhibitors (Complete, Roche), lysozyme (100 μg/ml) and DNase (100 μg/ml), and broken by three passages at the French press (1,000 psi). Unbroken cells were discarded by centrifugation for 15 min at 3,000 g and the total cell extract was mixed with an equal volume of 2× CellLytic™ B Cell Lysis reagent (Sigma-Aldrich) and incubated for 1 h with strong shaking. The insoluble material was discarded by centrifugation for 45 min at 60,000 g and the supernatant from 2 × 10<sup>11</sup> cells was incubated overnight at 4°C with anti-FLAG M2 affinity beads (Sigma-Aldrich). Beads were then washed three times with 1× CellLytic™ in buffer TN. The total extract and immunoprecipitated material were resuspended and boiled in Laemmli loading buffer prior to analyses by SDS-PAGE and immunoblotting.
The authors declare that they have no conflict of interest.

Author contributions

YGS and EC designed the research and conceived the study; YGS and EC performed the experiments; and EC wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

References


