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Cell width dictates Type VI secretion tail length

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SUMMARY

The type VI secretion system (T6SS) is a multiprotein apparatus that injects protein effectors into target cells, hence playing a critical role in pathogenesis and in microbial communities [1–4]. The T6SS belongs to the broad family of contractile injection systems (CIS), such as *Myoviridae* bacteriophages and R-pyocins, that use a spring-like tail to propel a needle loaded with effectors [5,6]. The T6SS tail comprises an assembly baseplate on which polymerizes a needle, made of stacked Hcp hexamers, tipped by the VgrG-PAAR spike complex and wrapped by the contractile sheath made of TssB and TssC [7–13]. The T6SS tail is anchored to the cell envelope by a membrane complex that also serves as channel for the passage of the needle upon sheath contraction [14–16]. In most CIS, the length of the tail sheath is invariable, and is usually ensured by a dedicated protein called tape measure protein (TMP) [17–22]. Here we show that the length of the T6SS tail is constant in enteroaggregative *Escherichia coli* cells, suggesting that it is strictly controlled. By overproducing T6SS tail subunits we demonstrate that component stoichiometry does not participate to the regulation of tail length. The observation of longer T6SS tails when the apparatus is relocalized at the cell pole further shows that tail length is not controlled by a TMP. Finally, we show that tail stops its elongation when in contact with the opposite membrane, and thus that T6SS tail length is determined by the cell width.

RESULTS AND DISCUSSION

T6SS tail sheath length in EAEC.

The length of bacteriophage and other CIS tails such as that of anti-feeding prophages is strictly controlled [17-23]. To determine whether this is also the case for the T6SS, we

measured the length of T6SS sheaths in EAEC wild-type cells producing a functional fusion between the TssB tail subunit and the superfolder-Green Fluorescent Protein (TssB-sfGFP). The sfGFP-coding sequence was inserted on the chromosome, upstream the *tssB* stop codon. In this construct, the sfGFP sequence is in frame with the *tssB* gene, and the *tssB*-sfGFP fusion is under the control of the native *tssB* expression signals. Cells were grown in *sciI* inducing medium (SIM), a defined synthetic medium that avoids batch-to-batch composition variability and induces the expression of EAEC T6SS genes [24]. In agreement with the localization of T6SS MC along the cell body with an underrepresentation at the poles [15,25], we observed that T6SS sheaths assemble from one position on the cell body to the opposite membrane. To avoid measurements of the length of contracted sheaths or of sheaths under extension, time-lapse recordings were performed and only sheaths for which the elongation has been completed (i.e., when the sheath holds >1 min with the same length) were considered (Figure 1A). The distal extremity of these extended sheaths always co-localized with the TagA stopper protein (Figure 1B). Quantitative measurements of these T6SS tail length showed low disparities, with a length mean of $0.76 \pm 0.11 \mu\text{m}$ ($n = 150$) and a normal distribution (Figure 1C). We thus concluded that the length of the T6SS sheath is not randomly distributed, and hence that the arrest of T6SS sheath elongation is controlled. Based on this conclusion, we hypothesize that T6SS sheath length might be determined by (i) the number of available T6SS tail subunits, (ii) a tape measure protein, or (iii) the cell width.

T6SS tail sheath length is not regulated by tube/sheath components stoichiometry

Several reports have demonstrated that the length of some pilus-like structures is limited by the number of available pilin subunits. For example, the T2SS uses a periplasmic pseudo-pilus to expel the substrates in the external medium such as a piston or an Archimedes screw

[26]. By artificially increasing the number of pseudo-pilins, μm -long pili can be observed at the cell surface, suggesting that pseudo-pilus length is determined, in part, by the number of available subunits [27,28]. A strict control of the number of subunits in the cell by finely tuned gene expression and protein stability levels would prevent the costly synthesis of unnecessary subunits. To test whether T6SS sheath length might be controlled by the number of available tail subunits, we modified the stoichiometry balance by deregulating the levels of tube/sheath subunits, Hcp, TssB and TssC. TssA, which locates at the distal end of the growing sheath to coordinate the assembly of the tail tube/sheath [29-31], and the TagA stopper [31,32] were not included in the study as single TssA and TagA complexes are responsible for promoting T6SS tail elongation and arrest. *tssB-sfGFP*, and epitope-tagged *tssC* and *hcp*, were cloned into the pTrc99A vector, under the IPTG-inducible *ptrc* promoter. Pilot experiments showed that the production of TssB-sfGFP, TssC and Hcp can be tightly controlled by varying the IPTG inducer concentrations from 0 to 250 μM in the culture medium. However, although we do not know whether it results from protein aggregation or stoichiometric unbalance, we noticed that IPTG concentrations higher than 50 μM yielded non-functional T6SSs, as no dynamic sheath structures were observable by fluorescence microscopy. Nevertheless, with IPTG concentrations ranging from 0 to 10 μM , we observed an increase in TssB-sfGFP, TssC and Hcp protein levels up to ~ 14 -18 fold (Figure 2A) without affecting T6SS sheath formation and dynamics (Figure 2B). However, sheath length measurement analyses did not reveal striking differences (Figure 2B, 2C and Figure S1). Collectively, our results therefore argue against a direct correlation between the abundance of T6SS tail subunits and T6SS sheath length.

T6SS sheath length is not regulated by a tape measure protein.

88 The length of contractile bacteriophage tails is dictated by a tape measure protein (TMP) [18-
89 20,33,34]. Such a ruler mechanism has been also evidenced or proposed for other CISs such
90 as antifeeding prophages and *Photorhabdus* virulence cassettes, or non-contractile structures
91 such as T3SS injectisome needles in *Yersinia*, or the bacterial hook in *Salmonella* [21,22,35-
92 38]. Although bacterial T6SSs have been shown to be structurally and functionally related to
93 contractile bacteriophages [6], and contrarily to R-pyocins, AFP, PVC or ACS gene clusters
94 [21,22,35], no gene encoding a potential TMP can be found within T6SS clusters [39]. In
95 addition, previous experiments showing T6SS tail sheath extension in *Vibrio cholerae*
96 spheroplasts [40] and in EAEC and *V. cholerae tagA* mutants [31,32] suggested that longer
97 tails assemble in the cell, hence arguing against a tape measure-like mechanism. To discard
98 this hypothesis, we sought to reposition the T6SS at the cell pole to follow the assembly of the
99 T6SS in the long axis of the cell body. In these conditions, the presence of a TMP would
100 cause an arrest of tail extension in the cytoplasm, whereas the tail will extend towards the
101 opposite cell pole or until no tail subunit is available for a TMP-independent mechanism.
102 T6SS biogenesis starts with the assembly of the membrane complex (MC) [7,15]. The T6SS
103 MC is composed of the TssJ outer membrane lipoprotein, and TssL and TssM inner
104 membrane proteins [14]. Its biogenesis starts with the positioning of TssJ and is pursued by
105 the sequential recruitment of TssM and TssL [15]. The MC initially positions along the cell
106 body with an underrepresentation at the cell pole [15,25]. To reposition T6SS MCs at the cell
107 pole, we fused TssM to the *Bacillus subtilis* polar/septal determinant DivIVA. This approach
108 has been successfully used to relocate the T4SS VirD4 coupling protein to the cell pole in
109 *Agrobacterium* cells [41]. A fragment encoding the *B. subtilis divIVA* gene was inserted on
110 the chromosome, downstream the *tssM* ATG start codon and in frame with the *tssM* coding
111 sequence, to engineer a *divIVA-tssM* fusion. Because TssL is the last component to be
112 recruited during MC biogenesis [15], we then imaged TssL fused to sfGFP in these cells.

Statistical analyses of sfGFP-TssL position, that hence reflects the localization of the fully-assembled T6SS MC [15], demonstrated that sfGFP-TssL forms ~32% of polar foci in presence of DivIVA-TssM, by contrast to the wild-type cells, with ~16% of polar foci (Figure 3A, 3B). Introduction of the *divIVA-tssM* fusion into *tssB-sfGFP* EAEC cells showed that few T6SS tails assemble from the pole suggesting that polar-localized MCs are less functional. However, the number of tail polymerizations starting from the cell pole was significantly increased with the relocation of the MC compared to wild-type cells. Statistical analyses showed that these extensions from the poles lead to significantly longer tails (Figure 3C), with a mean equal to $1.05 \pm 0.42 \mu\text{m}$ ($n = 75$). Sheaths that crossed the entire cell body with length up to $3.5 \mu\text{m}$ (corresponding to outliers in Figure 3C), and capable of contraction were readily observed (Figure 3D). Taken together, these data demonstrate that T6SS tails could be artificially extended by relocalization of the MC at the cell pole, and hence that sheath length is not regulated by a TMP-mediated ruler mechanism.

T6SS tail sheath length is dictated by the cell width.

In all the images recorded, we observed that sheaths extend towards and stop when in contact with the opposite membrane. As the membrane mechanically defines the cell limits, one may hypothesize that the distance between the two opposite membrane positions is sufficient to determine sheath length. Indeed, the measure of the distance between the two membranes in cells analyzed in Figure 1 showed a cell width average of $0.86 \mu\text{m} \pm 0.07$, which is comparable to the sheath length ($0.76 \mu\text{m} \pm 0.11$), specifically if we take into account the widths of the membranes, of the cytoplasmic portion of the MC, and of the BP [8,9,15,16]. To further investigate the impact of cell width on T6SS sheath length, we treated EAEC cells producing TssB-sfGFP with A22, a drug that causes cell shape defects by targeting the

cytoskeletal MreB protein. As expected, we observed a significant impact on cell morphology with a trend to cell rounding (Figure 4A). In these conditions, we observed T6SS sheaths with length varying from 0.7 to 2 μm (Figure 4A). However, when sheath length was plotted against cell width, a strict correlation is observed (Kendall's $\tau = 0.82$) (Figure 4B). In addition, the value of the slope, close to 1 ($m = 0.92$), demonstrates that sheath length is directly proportional to cell width, which is also confirmed by sheath length/cell width ratio calculations (Figure 4C). Similarly to wild-type cells [32], the distal extremity of these longer extended sheaths co-localized with TagA (Figure 4D). We therefore conclude that T6SS sheath length depends on cell width, and that T6SS sheath polymerization is arrested upon contact with the opposite membrane, likely by the TagA stopper.

Concluding remarks

In this study, we have shown that the T6SS tails in EAEC cells have a defined length. Our further analyses demonstrated that the length of the T6SS tail is not determined by a tape measure protein or by the number of available tail tube/sheath subunits, but rather by the distance between the baseplate and the opposite membrane. This mechanism contrasts with the TMP-dependent regulation found in most contractile tailed machines. However it is in agreement with the fact that no putative TMP-encoding genes are encoded on T6SS gene clusters [5,39]. In TMP-dependent contractile tails, it has been shown that a strict correlation exists between the number of TMP residues and the length of cognate sheaths [21,22]. An extrapolation for the T6SS would mean that a sheath of 0.76 μm corresponds to a TMP of $\sim 5,000$ amino-acids. Such a protein is not encoded within T6SS gene clusters. Finally, the observation that DivIV-mediated relocalisation of the MC to the cell pole yields extra-long sheath structures demonstrates that no TMP is involved. The assembly of extra-long sheaths

also suggests that the number of subunits necessary to assemble a tail tube/sheath is not limiting in the bacterial cytoplasm, and hence that sheath length is not regulated by the number of available tail subunits. Indeed, this hypothesis was also discarded by artificially modulating the levels of T6SS tail subunits, demonstrating that it does not influence tail sheath length. This results is also in agreement with the recent measurement of T6SS subunits abundance demonstrating that tail tube/sheath components are not limiting in *Acinetobacter baylyi*, *V. cholerae*, or *P. aeruginosa* cells [42]. All our observations therefore argue for an arrest of sheath polymerization at the opposite membrane. This hypothesis is likely, as altering the distance between the two membranes by using A22, an antagonist of the MreB morphological determinant, showed a strict correlation between membrane width and sheath length. We conclude that tail tube/sheath polymerization stops when it hits the opposite membrane. In most cases, the assembly of the T6SS tubular structure starts perpendicular to the membrane and hence, the length of the sheath correlates with the cell width. However, when T6SS tail assembly starts non-perpendicularly, its polymerization will proceed until it touches the opposite membrane boundary. This model is also consistent with the recent identification of TagA, a membrane-bound protein that binds to the distal extremity of the growing tail and stops tube/sheath polymerization in EAEC and *V. cholerae* [31,32]. However, TagA is not a universal stopper as recordings of sheath dynamics of TagA⁺ T6SS (*V. cholerae*) or T6SSs lacking TagA (*Acinetobacter baylyi*, *P. aeruginosa* H1, *S. enterica* Typhimurium, *Francisella novicida*) showed that sheaths extend to and stop at the opposite membrane [40, 42-45]. It would be interesting to define how T6SS sheath polymerization is stopped in bacterial species lacking TagA.

In conclusion, T6SS has evolved to use a mechanism of tail length control distinct from other CIs. We thus propose a model in which T6SS tail length is determined by the distance to the opposite membrane. In EAEC, and likely other TagA⁺ species, proper arrest of

tail tube/sheath polymerization is mediated by the TagA stopper by binding to the TssA cap protein once the distal end of the sheath hits the opposite membrane. .

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Authors contributions

Y.G.S. and E.C. designed research; Y.G.S., T.D. and E.C. performed research; Y.G.S. performed statistical analyses; Y.G.S., T.D., and E.C. analyzed data; L.J. provided expertise and feedback; Y.G.S. and E.C. wrote the paper with contributions from T.D. and L.J.

Declaration of interests

The authors declare no competing interests.

209 **Legend to Figures**

210 **Figure 1. Statistical measure of the EAEC T6SS sheath length.** (A) Representative
211 fluorescence micrograph of EAEC cells producing TssB-sfGFP and labelled with FM4-64
212 (upper panel, phase channel; lower panel, merge of GFP and FM4-64 channels). Scale bar, 1
213 μm . (B) Representative fluorescence micrograph of EAEC cells producing TssB-CFP and
214 YFP-TagA, and labeled with FM4-64 (upper panel, phase channel; lower panel, merge of
215 CFP, YFP, and FM4-64 channels). The open arrowheads point T6SS extended sheaths in
216 contact with the TagA stopper. Scale bar, 1 μm . (C) Violin plot representation of the sheath
217 length in wild-type cells. The distribution of the sheath lengths is represented by the outer
218 shape. The bold horizontal bar represents the median value (median = 0.76 μm); the lower
219 and upper boundaries of the internal box plot correspond to the 25th and 75th percentiles
220 respectively; whiskers extend 1.5 times the interquartile range from the 25th and 75th
221 percentiles. The number of measured sheaths ($n=150$) is indicated above.

222 **Figure 2. T6SS tail tube/sheath subunits levels do not determine T6SS sheath length.** (A)
223 Western-blot analyses of tail tube/sheath component abundance. 2×10^8 cells of $\Delta tssBC\Delta hcp$
224 cells producing TssB-sfGFP (TssB_{sfGFP}), 6 \times His-tagged TssC (TssC_{6His}) and FLAG-tagged
225 Hcp (Hcp_{FLAG}) from the pTrc-B_{GFP}-C_{6H}-Hcp_F grown in presence of 0.4% of glucose or of the
226 indicated concentration of IPTG were subjected to 12.5% acrylamide SDS-PAGE and
227 immunodetected with anti-GFP, -His, -FLAG and -EF-Tu monoclonal primary antibodies and
228 secondary antibodies coupled to AlexaFluor[®] 680. The EF-Tu cytoplasmic elongation factor
229 is used as loading control. The increased fold values compared to the glucose condition,
230 measured by densitometric quantification of the band signal intensities and relative to the
231 loading control, are indicated below. Molecular weight markers (in kDa) are indicated on left.
232 (B-C) Representative fluorescence microscopy recordings (B) and statistical analyses of
233 sheath length measurements (C) of $\Delta tssBC\Delta hcp$ cells producing Hcp_{FLAG}, TssB-sfGFP and

TssC_{6His} in presence of 0.4% glucose or of the indicated concentration of IPTG, and labelled with FM4-64 (left panels, phase channel; right panels, merge of GFP and FM4-64 channels). Scale bar, 2 μ m. In the violin plot representation of the statistical analysis, the distribution of the sheath length is represented by the outer shape. The bold horizontal bar represents the median; the lower and upper boundaries of the internal box plot correspond to the 25th and 75th percentiles respectively; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles. Outliers are shown as black dots. Statistical significance from three independent assays ($n = 100$ for each conditions) relative to the glucose condition is indicated above the plots (ns, non-significative; ***, $p < 0.001$; two-tailed Student's t -test). A comparison of tail tube/sheath subunits levels and sheath lengths is shown in Figure S1.

Figure 3. Sheath length is not controlled by a tape measure protein. Representative fluorescence microscopy recordings of EAEC cells producing sfGFP-TssL in WT (*A*) or DivIV-TssM (*B*) cells, and labelled with FM4-64 (upper panels, phase channel; lower panels, merge of GFP and FM4-64 channels). White and blue arrowheads indicate TssL foci with body or polar localizations, respectively. Scale bar, 2 μ m. The spatial repartition of sfGFP-TssL foci is shown on right, as a projection of foci from $n = 152$ and $n = 151$ WT and DivIVA-TssM cells, respectively, on a single cell (from blue to yellow, see heatmap color chart on right of panel *A*). The percentage of sfGFP-TssL foci with polar localization is indicated below. (*C*) Violin plot representation of sheath length measurements in DivIVA-TssM cells producing TssB-sfGFP (red). An example of a representative microscopy field is shown in the inset (scale bar, 2 μ m). The distribution of sheath length in WT cells (shown in Figure 1*B*) is reported in transparency for comparison (blue). The distribution of the sheath length is represented by the outer shape. The bold horizontal bar represents the median value (median = 0.93 μ m and 0.76 μ m for DivIVA-TssM and WT cells, respectively); the lower and upper boundaries of the internal box plot correspond to the 25th and 75th percentiles respectively;

whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles. Outliers are shown as black dots. The number of measured sheaths ($n=75$) is indicated below. Statistical significance relative to WT cells is indicated above the plots (***, $p < 0.001$; one-tailed Wilcoxon's t -test). (D) Fluorescence microscopy time-lapse recordings of DivIVA-TssM cells producing TssB-sfGFP highlighting polar T6SS sheath assembly (white arrowhead) and contraction (blue arrowhead). Scale bar, 2 μm .

Figure 4. Cell width dictates T6SS sheath length. (A) Representative fluorescence micrographs of EAEC cells producing TssB-sfGFP in absence (upper panel) or in presence of the MreB inhibitor A22 (lower panel), and labelled with FM4-64 (upper panel, phase channel; lower panel, merge of GFP and FM4-64 channels). Scale bar, 2 μm . (B) Plot representation showing the relationship between sheath length and cell width in absence (blue) and presence of A22 (orange). The dashed line represents the mean of the WT cell width (mean = 0.87 μm). Value of the slope (m) is indicated on right. Kendall's $\tau = 0.82$. (C) Dotplot of the ratios between sheath length and the width of the corresponding cell from cells grown in absence (blue) or presence of A22 (orange). Horizontal bars represent the mean in each condition. The values of the means and standard deviations are indicated above the plots. The number of analyzed cells (n) is indicated below. (D) Representative fluorescence micrograph of EAEC cells producing TssB-CFP and YFP-TagA in presence of the MreB inhibitor A22, and labelled with FM4-64 (left panel, phase channel; right panel, merge of CFP, YFP, and FM4-64 channels). The open arrowheads point T6SS extended sheaths in contact with the TagA stopper. Scale bar, 2 μm .

STAR METHODS

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Eric Cascales (cascales@imm.cnrs.fr). There are no restrictions to the availability of reagents.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Strains used in this study are listed in Table S1. *Escherichia coli* DH5 α (New England Biolabs) or CC118 λ pir have been used for cloning procedures. Enteroaggregative *E. coli* (EAEC) strains used in this work are isogenic derivatives of the wild-type O3:H2 17-2 strain. The wild-type 17-2 strain and its TssB-sfGFP [46], sfGFP-TssL [15], TssB-CFP/YFP-TagA (this work), and DivIVA-TssM (this work) derivatives have been used for *in vivo* studies. *E. coli* cells were routinely grown in Lysogeny broth (LB) broth at 37°C, with aeration. For induction of the *sciI* T6SS gene cluster, cells were grown in SciI-inducing medium [SIM: M9 minimal medium supplemented with glycerol 0.25 %, vitamin B1 200 $\mu\text{g.mL}^{-1}$, casaminoacids 40 $\mu\text{g.mL}^{-1}$, MgCl_2 2 mM, CaCl_2 0.1 mM, and LB (10% v/v)] [24]. Plasmids and chromosomal deletions and insertions were maintained by the addition of kanamycin (50 $\mu\text{g.mL}^{-1}$), or ampicillin (100 $\mu\text{g.mL}^{-1}$). Gene expression from pTrc99A derivative plasmids was induced at $A_{600 \text{ nm}} \approx 0.4$ by the addition of 2-10 μM of isopropyl- β -D-thiogalactopyranoside (IPTG, Sigma-Aldrich). The MreB antagonist S-(3,4-dichlorobenzyl)isothioureahydrochloride (known as A22 [47], purchased from Sigma-Aldrich) has been added to the culture at the concentration of 10 $\mu\text{g.mL}^{-1}$ for 2 hours prior to analyses. Membranes were stained with 10 $\mu\text{g.mL}^{-1}$ of the N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino) phenyl) hexatrienyl) pyridinium dibromide dye (FM4-64, Molecular Probes, Life technologies).

308 METHOD DETAILS

309 **Plasmid construction.** All plasmids used in this study are listed in Table S2.
310 Oligonucleotides used for plasmid construction are listed in Table S3. pTrc99A [48] and
311 pKD4 [49] plasmid derivatives were engineered by ligation-free restriction-free cloning. PCR
312 amplification were performed in a Biometra thermocycler using the Q5[®] DNA polymerase
313 (New England Biolabs). Briefly, the fragment of interest was amplified and then used as
314 oligonucleotides for a second PCR for amplification of the target plasmid. For construction of
315 pTrc-B_{GFP}-C_{6His}, a fragment encoding *tssB-sfGFP* and *tssC-6×His* was amplified from EAEC
316 17-2 TssB-sfGFP [46] genomic DNA using primers
317 *GGATAACAATTTACACAGGAAACAGACCATGAGCAGTTCGTTTCAGAATGAAATCC*
318 *C* and
319 *CCCGGGTACCGAGCTCGAATTCTTAATGGTGATGGTGATGATGCGCTTTTGCCTTC*
320 *GGCATCTGC* (6×His tag sequence underlined, sequence annealing on the target pTrc99A
321 plasmid [48] italicized), and inserted into pTrc99A. The *hcp* gene was amplified from EAEC
322 17-2 genomic DNA using primers
323 *GCGCATCATCACCATCACCATTAAGAATTaggaggtattacaccATGGCAATTCCAGTTTA*
324 *TCTGTGGCTG* and
325 *GGATCCCCGGGTACCGAGCTCGTTACTTGTCATCGTCATCTTTATAATCCGCGGTGG*
326 *TACGCTCACTCC* (FLAG tag sequence italicized, sequence annealing on the target pTrc-
327 B_{GFP}-C_{6H} plasmid underlined, Shine-Dalgarno ribosome-binding site in lower case), and
328 inserted into pTrc-B_{GFP}-C_{6His} to yield pTrc-B_{GFP}-C_{6H}-Hcp_F. For construction of plasmid
329 pKD4-DivIVA-Nt allowing chromosomal insertion of *divIVA* at the 5'-end of genes, the
330 *divIVA* gene was amplified from *Bacillus subtilis* str. 168 using primers
331 *GGAACTTCGGAATAGGAACTAAGGAGGATATTCATATGCCATTAACGCCAAATG*
332 *ATATTCACAACAAGAC* and

333 GCTGACATGGGAATTAGCCATGGTCCCCCTCCGCCGGCCGCTGCTTCCTTTTCCTCA
334 AATACAGCGTCGACTTC (3×Ala-3×Gly sequence linker italicized, sequence annealing on
335 the target pKD4 plasmid underlined), and inserted into plasmid pKD4 [49]. For construction
336 of plasmid pKD4-CFP-Ct allowing chromosomal insertion of *ecfp* at the 3'-end of genes, the
337 *ecfp* gene was amplified from the pTrc99A-ecfp vector (gift from Leon Espinosa, LCB,
338 Marseille, France) using primers
339 GATTGCAGCATTACACGTCTTGAGCGATTGCAGCGGCCGGCGGAGGGGTGAGCAA
340 GGGCGAGGAGC and
341 GAACTTCGAAGCAGCTCCAGCCTACACTTACTTGTACAGCTCGTCCATGCCG
342 (3×Ala-3×Gly sequence linker italicized, sequence annealing on the target pKD4 plasmid
343 underlined), and inserted into plasmid pKD4 [49]. For construction of plasmid pKD4-YFP-Nt
344 allowing chromosomal insertion of *eyfp* at the 5'-end of genes, the *eyfp* gene was amplified
345 from the pCBP-EYFP vector (gift from Emmanuelle Bouveret, Institut Pasteur, Paris, France)
346 using primers
347 CGGAATAGGAACTAAGGAGGATATTCATATGGTGAGCAAGGGCGAGGAGC and
348 CGGCTGACATGGGAATTAGCCATGGTCCCCCTCCGCCGGCCGCTGCCTTGTACAGC
349 TCGTCCATGCCGAGAG (3×Ala-3×Gly sequence linker italicized, sequence annealing on
350 the target pKD4 plasmid underlined), and inserted into plasmid pKD4 [49]. All plasmids were
351 verified by colony-PCR and DNA sequencing (Eurofins genomics).

352 **Strain construction.** Strains were engineered by λ -red recombination [49] using plasmid
353 pKOBEG [50] and PCR products (oligonucleotides listed in Table S3). Briefly, a kanamycin
354 cassette was amplified from plasmids pKD4, pKD4-DivIVA-Nt, pKD4-CFP-Ct, or pKD4-
355 YFP-Nt, using oligonucleotides carrying 50-nucleotide extensions homologous to regions
356 adjacent to the gene of interest. Cassette allowing deletion of the *hcp* gene was generated
357 using the pKD4 vector template with primers

358 TCCCCTGCGCCGGAAGAGGGCGCATCAGAAAACATAACGGAGTAATTTTTGTGT
359 AGGCTGGAGCTGCTTCG and
360 TGAAGAAAAAATAAAAAATGACGGACAGGATGCCCTGTCCGTCCGGCAGAACATA
361 TGAATATCCTCCTTAGTTCC (50-bp sequence annealing on the 5' and 3' regions of the
362 deleted gene underlined). For chromosomal insertion of DivIVA at the *tssM* locus, the
363 DivIVA cassette was generated using the pKD4-DivIVA-Nt vector with primers
364 TTCTCATCCGGAGAAGAACATTTTATCAGTACTGTTACATCAGGAAACCAGAATG
365 AATAACGATTGTGTAGGCTGGAGCTGCTTCGAAGTTCCTATAC and
366 CACACCAATAAATACAATCCCCGGTCGCCCAAAGCGACCAGACAGACAGGCCAG
367 TTTATTCCTCCGCCGGCCGCTGC (50-bp sequence annealing on the 5' and 3' regions of
368 the *tssM* ATG start codon underlined). For chromosomal insertion of CFP at the *tssB* locus,
369 the *cfp* cassette was generated using the pKD4-CFP-Ct vector with primers
370 CCGGCACTGAGTCAGACGCTGCGTGATGAACTGCGTGCACTGGTGCCGAAAAG
371 GCGGCAGCGGCCGGCGGAGGG and
372 GCAACGTTCTTTTCTTTCTGTACAGACATCAGCATTTTCTCTCGTAATCCGTTAAA
373 CATATGAATATCCTCCTTAGTTCCTATTCCGAAGTTCC (50-bp sequence annealing on
374 the 5' and 3' regions of the *tssB* TAA stop codon underlined). For chromosomal insertion of
375 YFP at the *tagA* locus, the *yfp* cassette was generated using the pKD4-YFP-Nt vector with
376 primers
377 CTTTTCGTCACGTGTTAATCATGATTTAATACAGCAACACCGAATCTGCCGCGATTG
378 TGTAGGCTGGAGCTGCTTCGAAGTTCCTATAC and
379 TCCGGTAATGACCGGGGGTCACCACCGGTTTTTCAGTTTCACTTCAGAAGTCCCTC
380 CGCCGGCCGCTGC (50-bp sequence annealing on the 5' and 3' regions of the *tagA* ATG
381 start codon underlined). Cassette amplicons were column-purified (NucleoSpin® Gel and PCR
382 clean-up, Macherey Nagel), and 600 ng were electroporated into electrocompetent target cells

($\Delta tssBC$ for Δhcp ; 17-2, sfGFP-TssL and TssB-sfGFP for DivIVA; 17-2 for YFP-TagA; YFP-TagA for TssB-CFP), kanamycin-resistant clones were selected and verified by colony-PCR. When possible, kanamycin cassettes were excised by the FRT-specific FLP recombinase using vector pCP20 [49].

SDS-PAGE, Western-blotting, imaging and quantification analyses. Standard methods were used for sodium dodecyl-sulfate poly-acrylamide gel electrophoresis (SDS-PAGE) and protein transfer on nitrocellulose membranes. Membranes were probed with anti-GFP (clone 7.1, Roche), anti-His (clone His1, Sigma), anti-FLAG (clone M2, Sigma), and anti-EF-Tu (clone mAb900, HyCult Biotech) monoclonal antibodies, and goat anti-mouse secondary antibodies coupled to AlexaFluor[®] 680 (Invitrogen). Images were recorded at $\lambda=700$ nm using an Odyssey[®] infrared imaging system (LI-COR Biosciences). Image analyses were performed with the ImageJ processing program using the Fiji interface [51], as previously described [52]. Briefly, the image was first converted to grayscale in .jpg format. The rectangle tool of ImageJ was used to select a rectangular area of the size corresponding to the lane width, in order to cover the minimal area to contain the whole of the largest band. The same frame was used to select each TssB, TssC, Hcp or EF-Tu band. For each selection, the number of pixels was calculated. A control region with no band was also selected to subtract the background. The number of pixels of each band, subtracted from the background, was then divided by the EF-Tu loading control intensity at the same IPTG concentration, to compensate for loading differences. The fold-change relative to the glucose sample was then calculated.

Fluorescence microscopy and statistical analyses. Cells were grown in SIM to a $A_{600\text{ nm}} \approx 0.6\text{--}0.8$, harvested and resuspended in fresh SIM to a $A_{600\text{ nm}} \approx 10$. For inhibition of MreB function, cells grown in SIM were treated at $A_{600\text{ nm}} \approx 0.3$ for 2 hours with $10\text{ }\mu\text{g.mL}^{-1}$ of A22 prior to data acquisition, as previously published [53]. For membrane staining, cells were

labelled with FM4-64 ($10 \mu\text{g.mL}^{-1}$) for 2 min prior to centrifugation. Concentrated cell mixtures were spotted on a thin pad of SIM supplemented with 2% agarose, or 2% agarose and A22 ($10 \mu\text{g.mL}^{-1}$), covered with a cover slip, and incubated for 20-30 min at room temperature before microscopy acquisition. Fluorescence microscopy was performed on a Nikon Eclipse Ti microscope equipped with an Orcaflash 4.0 LT digital camera (Hamamatsu) and a perfect focus system (PFS) to automatically maintain focus so that the point of interest within a specimen is always kept in sharp focus at all times despite mechanical or thermal perturbations. All fluorescence images were acquired with a minimal exposure time to minimize bleaching and phototoxicity effects. Exposure times were typically 30 ms for phase contrast, 200 ms for TssB-sfGFP, 300 ms for TssB-CFP, 1 s for sfGFP-TssL, 1.5 s for YFP-TagA, and 50 ms for FM4-64. The experiments were performed at least in triplicate and a representative result is shown. Images were analyzed using ImageJ (<http://imagej.nih.gov/ij/>) and the MicrobeJ v5.11y plugin (<http://www.microbej.com/>) [54].

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses of microscopy images were performed with several representative fields from at least three independent biological replicates, using Excel and the R software environment. The number of measured cells or events (n) is indicated on each figure. Differences in sheath length between groups were examined by unpaired parametric Student t test or nonparametric Wilcoxon t test. Relationship between sheath length and cell width was examined by Mann-Kendall test. Significance was defined by $p < 0.001$ (***) and $p < 0.0001$ (****).

430 **DATA AND CODE AVAILABILITY**

431 This study did not generate datasets and codes.

432

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434 **REFERENCES**

- 435 1. Cherrak, Y, Flaugnatti, N, Durand, E, Journet, L, and Cascales, E. (2019). Structure and
436 activity of the type VI secretion system. *Microbiol. Spectrum* 7, PSIB-0031-2019.
- 437 2. Coulthurst, S. (2019). The Type VI secretion system: a versatile bacterial weapon.
438 *Microbiol. 165*, 503–515.
- 439 3. Durand, E., Cambillau, C., Cascales, E., and Journet, L. (2014). VgrG, Tae, Tle, and
440 beyond: the versatile arsenal of Type VI secretion effectors. *Trends Microbiol.* 22, 498–
441 507.
- 442 4. Chassaing, B., and Cascales, E. (2018). Antibacterial weapons: targeted destruction in the
443 microbiota. *Trends Microbiol.* 26, 329–338.
- 444 5. Sarris, P.F., Ladoukakis, E.D., Panopoulos, N.J., and Scoulica, E.V. (2014). A phage tail-
445 derived element with wide distribution among both prokaryotic domains: a comparative
446 genomic and phylogenetic study. *Genome Biol. Evol.* 6, 1739–1747.
- 447 6. Taylor, N.M.I., van Raaij, M.J., and Leiman, P.G. (2018). Contractile injection systems of
448 bacteriophages and related systems. *Mol. Microbiol.* 108, 6–15.
- 449 7. Brunet, Y.R., Zoued, A., Boyer, F., Douzi, B., and Cascales, E. (2015). The type VI
450 secretion TssEFGK-VgrG phage-like baseplate is recruited to the TssJLM membrane
451 complex via multiple contacts and serves as assembly platform for tail tube/sheath
452 polymerization. *PLoS Genet.* 11, e1005545.
- 453 8. Nazarov, S., Schneider, J.P., Brackmann, M., Goldie, K.N., Stahlberg, H., and Basler, M.
454 (2018). Cryo-EM reconstruction of Type VI secretion system baseplate and sheath distal
455 end. *EMBO J.* 37, e97103.
- 456 9. Cherrak, Y., Rapisarda, C., Pellarin, R., Bouvier, G., Bardiaux, B., Allain, F., Malosse,
457 C., Rey, M., Chamot-Rooke, J., Cascales, E., *et al.* (2018). Biogenesis and structure of a
458 type VI secretion baseplate. *Nat. Microbiol.* 3, 1404–1416.
- 459 10. Brunet, Y.R., Hénin, J., Celia, H., and Cascales, E. (2014). Type VI secretion and
460 bacteriophage tail tubes share a common assembly pathway. *EMBO Rep.* 15, 315–321.
- 461 11. Leiman, P.G., Basler, M., Ramagopal, U.A., Bonanno, J.B., Sauder, J.M., Pukatzki, S.,
462 Burley, S.K., Almo, S.C., and Mekalanos, J.J. (2009). Type VI secretion apparatus and

463 phage tail-associated protein complexes share a common evolutionary origin. *Proc. Natl.*
464 *Acad. Sci. U. S. A.* *106*, 4154–4159.

465 12. Basler, M., Pilhofer, M., Henderson, G.P., Jensen, G.J., and Mekalanos, J.J. (2012). Type
466 VI secretion requires a dynamic contractile phage tail-like structure. *Nature* *483*, 182–186.

467 13. Kudryashev, M., Wang, R.Y.-R., Brackmann, M., Scherer, S., Maier, T., Baker, D.,
468 DiMaio, F., Stahlberg, H., Egelman, E.H., and Basler, M. (2015). Structure of the Type
469 VI secretion system contractile sheath. *Cell* *160*, 952–962.

470 14. Aschtgen, M.-S., Gavioli, M., Dessen, A., Lloubès, R., and Cascales, E. (2010). The SciZ
471 protein anchors the enteroaggregative *Escherichia coli* Type VI secretion system to the
472 cell wall. *Mol. Microbiol.* *75*, 886–899.

473 15. Durand, E., Nguyen, V.S., Zoued, A., Logger, L., Péhau-Arnaudet, G., Aschtgen, M.-S.,
474 Spinelli, S., Desmyter, A., Bardiaux, B., Dujeancourt, A., *et al.* (2015). Biogenesis and
475 structure of a type VI secretion membrane core complex. *Nature* *523*, 555–60.

476 16. Rapisarda, C., Cherrak, Y., Kooger, R., Schmidt, V., Pellarin, R., Logger, L., Cascales,
477 E., Pilhofer, M., Durand, E., and Fronzes, R. (2019). In situ and high-resolution cryo-EM
478 structure of a bacterial type VI secretion system membrane complex. *EMBO J.* *38*,
479 e100886.

480 17. Katsura, I., and Hendrix, R.W. (1984). Length determination in bacteriophage lambda
481 tails. *Cell* *39*, 691–698.

482 18. Katsura, I. (1990). Mechanism of length determination in bacteriophage lambda tails. *Adv.*
483 *Biophys.* *26*, 1–18.

484 19. Abuladze, N.K., Gingery, M., Tsai, J., and Eiserling, F.A. (1994). Tail length
485 determination in bacteriophage T4. *Virology* *199*, 301–310.

486 20. Belcaid, M., Bergeron, A., and Poisson, G. (2011). The evolution of the tape measure
487 protein: units, duplications and losses. *BMC Bioinformatics* *12*, S10.

488 21. Rybakova, D., Schramm, P., Mitra, A.K., and Hurst, M.R.H. (2015). Afp14 is involved in
489 regulating the length of anti-feeding prophage (Afp). *Mol. Microbiol.* *96*, 815–826.

490 22. Böck, D., Medeiros, J.M., Tsao, H.-F., Penz, T., Weiss, G.L., Aistleitner, K., Horn, M.,
491 and Pilhofer, M. (2017). In situ architecture, function, and evolution of a contractile
492 injection system. *Science* *357*, 713–717.

493 23. Vianelli, A., Wang, G.R., Gingery, M., Duda, R.L., Eiserling, F.A., and Goldberg, E.B.
494 (2000). Bacteriophage T4 self-assembly: localization of gp3 and its role in determining
495 tail length. *J. Bacteriol.* *182*, 680–688.

496 24. Brunet, Y.R., Bernard, C.S., Gavioli, M., Lloubès, R., and Cascales, E. (2011). An
497 epigenetic switch involving overlapping *fur* and DNA methylation optimizes expression
498 of a type VI secretion gene cluster. *PLoS Genet.* *7*, e1002205.

- 499 25. Santin, Y.G., Camy, C.E., Zoued, A., Doan, T., Aschtgen, M.-S., and Cascales, E. (2019).
500 Role and recruitment of the TagL peptidoglycan-binding protein during Type VI secretion
501 system biogenesis. *J. Bacteriol.* *201*, e00173-19.
- 502 26. Nivaskumar, M., and Francetic, O. (2014). Type II secretion system: a magic beanstalk or
503 a protein escalator. *Biochim. Biophys. Acta* *1843*, 1568–1577.
- 504 27. Sauvonnet, N., Vignon, G., Pugsley, A.P., and Gounon, P. (2000). Pilus formation and
505 protein secretion by the same machinery in *Escherichia coli*. *EMBO J.* *19*, 2221–2228.
- 506 28. Durand, E., Bernadac, A., Ball, G., Lazdunski, A., Sturgis, J.N., and Filloux, A. (2003).
507 Type II protein secretion in *Pseudomonas aeruginosa*: the pseudopilus is a multifibrillar
508 and adhesive structure. *J. Bacteriol.* *185*, 2749–2758.
- 509 29. Zoued, A., Durand, E., Brunet, Y.R., Spinelli, S., Douzi, B., Guzzo, M., Flaugnatti, N.,
510 Legrand, P., Journet, L., Fronzes, R., *et al.* (2016). Priming and polymerization of a
511 bacterial contractile tail structure. *Nature* *531*, 59–63.
- 512 30. Zoued, A., Durand, E., Santin, Y.G., Journet, L., Roussel, A., Cambillau, C., and
513 Cascales, E. (2017). TssA: The cap protein of the Type VI secretion system tail.
514 *Bioessays* *39*, 10.
- 515 31. Schneider, J.P., Nazarov, S., Adaixo, R., Liuzzo, M., Ringel, P.D., Stahlberg, H., and
516 Basler, M. (2019). Diverse roles of TssA-like proteins in the assembly of bacterial type
517 VI secretion systems. *EMBO J.* *12*, e100825.
- 518 32. Santin, Y.G., Doan, T., Lebrun, R., Espinosa, L., Journet, L., and Cascales, E. (2018). In
519 vivo TssA proximity labelling during type VI secretion biogenesis reveals TagA as a
520 protein that stops and holds the sheath. *Nat. Microbiol.* *3*, 1304–1313.
- 521 33. Katsura, I. (1987). Determination of bacteriophage lambda tail length by a protein ruler.
522 *Nature* *327*, 73–75.
- 523 34. Boulanger, P., Jacquot, P., Plançon, L., Chami, M., Engel, A., Parquet, C., Herbeuval, C.,
524 and Letellier, L. (2008). Phage T5 straight tail fiber is a multifunctional protein acting as a
525 tape measure and carrying fusogenic and muralytic activities. *J. Biol. Chem.* *283*, 13556–
526 13564.
- 527 35. Jiang, F., Li, N., Wang, X., Cheng, J., Huang, Y., Yang, Y., Yang, J., Cai, B., Wang, Y.-
528 P., Jin, Q., *et al.* (2019). Cryo-EM structure and assembly of an extracellular contractile
529 injection system. *Cell* *177*, 370-383.e15.
- 530 36. Journet, L., Agrain, C., Broz, P., and Cornelis, G.R. (2003). The needle length of bacterial
531 injectisomes is determined by a molecular ruler. *Science* *302*, 1757–1760.
- 532 37. Cornelis, G.R., Agrain, C., and Sorg, I. (2006). Length control of extended protein
533 structures in bacteria and bacteriophages. *Curr. Opin. Microbiol.* *9*, 201–206.
- 534 38. Hirano, T., Yamaguchi, S., Oosawa, K., and Aizawa, S. (1994). Roles of FlhK and FlhB in
535 determination of flagellar hook length in *Salmonella typhimurium*. *J. Bacteriol.* *176*,
536 5439–5449.

- 537 39. Boyer, F., Fichant, G., Berthod, J., Vandenbrouck, Y., and Attree, I. (2009). Dissecting
538 the bacterial type VI secretion system by a genome wide in silico analysis: what can be
539 learned from available microbial genomic resources? *BMC Genomics* 10, 104.
- 540 40. Vettiger, A., Winter, J., Lin, L., and Basler, M. (2017). The type VI secretion system
541 sheath assembles at the end distal from the membrane anchor. *Nat. Commun.* 8, 16088.
- 542 41. Atmakuri, K., Ding, Z., and Christie, P.J. (2003). VirE2, a type IV secretion substrate,
543 interacts with the VirD4 transfer protein at cell poles of *Agrobacterium tumefaciens*. *Mol.*
544 *Microbiol.* 49, 1699–1713.
- 545 42. Lin, L., Lezan, E., Schmidt, A., and Basler, M. (2019). Abundance of bacterial Type VI
546 secretion system components measured by targeted proteomics. *Nat Commun.* 10, 2584.
- 547 43. Brunet, Y.R., Khodr, A., Logger, L., Aussel, L., Mignot, T., Rimsky, S., and Cascales, E.
548 (2015). H-NS silencing of the *Salmonella* Pathogenicity Island 6-encoded type VI
549 secretion system limits *Salmonella enterica* serovar Typhimurium interbacterial killing.
550 *Infect Immun.* 83, 2738–2750.
- 551 44. Brodmann, M., Dreier, R.F., Broz, P., and Basler, M. (2017). *Francisella* requires
552 dynamic type VI secretion system and ClpB to deliver effectors for phagosomal escape.
553 *Nat Commun.* 8, 15853.
- 554 45. Liebl, D., Robert-Genthon, M., Job, V., Cogoni, V., and Attree, I. (2019). Baseplate
555 Component TssK and spatio-temporal assembly of T6SS in *Pseudomonas aeruginosa*.
556 *Front Microbiol.* 10, 1615.
- 557 46. Zoued, A., Durand, E., Bebeacua, C., Brunet, Y.R., Douzi, B., Cambillau, C., Cascales,
558 E., and Journet, L. (2013). TssK is a trimeric cytoplasmic protein interacting with
559 components of both phage-like and membrane anchoring complexes of the type VI
560 secretion system. *J. Biol. Chem.* 288, 27031–27041.
- 561 47. Iwai, N., Ebata, T., Nagura, H., Kitazume, T., Nagai, K., and Wachi, M. (2004).
562 Structure-activity relationship of S-benzylisothiourea derivatives to induce spherical cells
563 in *Escherichia coli*. *Biosci. Biotechnol. Biochem.* 68, 2265–2269.
- 564 48. Amann, E., Ochs, B., and Abel, K.J. (1988). Tightly regulated tac promoter vectors useful
565 for the expression of unfused and fused proteins in *Escherichia coli*. *Gene* 69, 301–315.
- 566 49. Datsenko, K.A., and Wanner, B.L. (2000). One-step inactivation of chromosomal genes in
567 *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U. S. A.* 97, 6640–6645.
- 568 50. Chaverroche, M.-K., Ghigo, J.-M., and d'Enfert, C. (2000). A rapid method for efficient
569 gene replacement in the filamentous fungus *Aspergillus nidulans*. *Nucleic Acids Res.* 28,
570 e97.
- 571 51. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T.,
572 Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., *et al.* (2012). Fiji: an open-source
573 platform for biological-image analysis. *Nat. Methods* 9, 676–682.

- 574 52. Zoued, A., Duneau, J.-P., Durand, E., España, A.P., Journet, L., Guerlesquin, F., and
575 Cascales, E. (2018). Tryptophan-mediated dimerization of the TssL transmembrane
576 anchor is required for type VI secretion system activity. *J. Mol. Biol.* *430*, 987–1003.
- 577 53. Kawazura, T., Matsumoto, K., Kojima, K., Kato, F., Kanai, T., Niki, H., and Shiomi, D.
578 (2017). Exclusion of assembled MreB by anionic phospholipids at cell poles confers cell
579 polarity for bidirectional growth. *Mol. Microbiol.* *104*, 472–486.
- 580 54. Ducret, A., Quardokus, E.M., and Brun, Y.V. (2016). MicrobeJ, a tool for high
581 throughput bacterial cell detection and quantitative analysis. *Nat. Microbiol.* *1*, 16077.

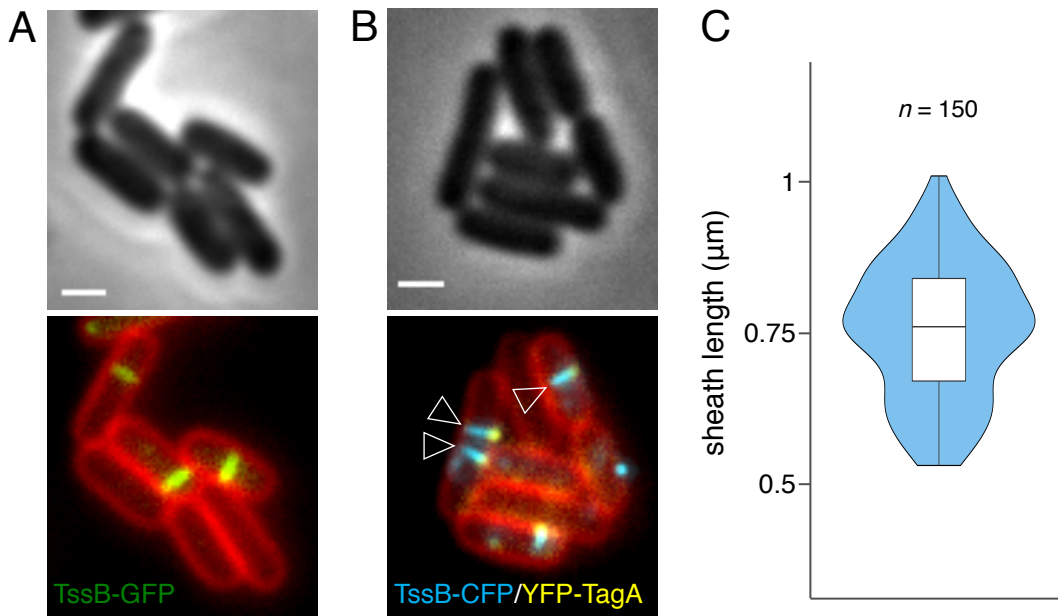


Fig. 1. Statistical measure of the EAEC T6SS sheath length. (A) Representative fluorescence micrograph of EAEC cells producing TssB-sfGFP and labelled with FM4-64 (upper panel, phase channel; lower panel, merge of GFP and FM4-64 channels). Scale bar, 1 μm . (B) Representative fluorescence micrograph of EAEC cells producing TssB-CFP and YFP-TagA, and labeled with FM4-64 (upper panel, phase channel; lower panel, merge of CFP, YFP, and FM4-64 channels). The open arrowheads point T6SS extended sheaths in contact with the TagA stopper. Scale bar, 1 μm . (C) Violin plot representation of the sheath length in wild-type cells. The distribution of the sheath lengths is represented by the outer shape. The bold horizontal bar represents the median value (median = 0.76 μm); the lower and upper boundaries of the internal box plot correspond to the 25th and 75th percentiles respectively; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles. The number of measured sheaths ($n=150$) is indicated above.

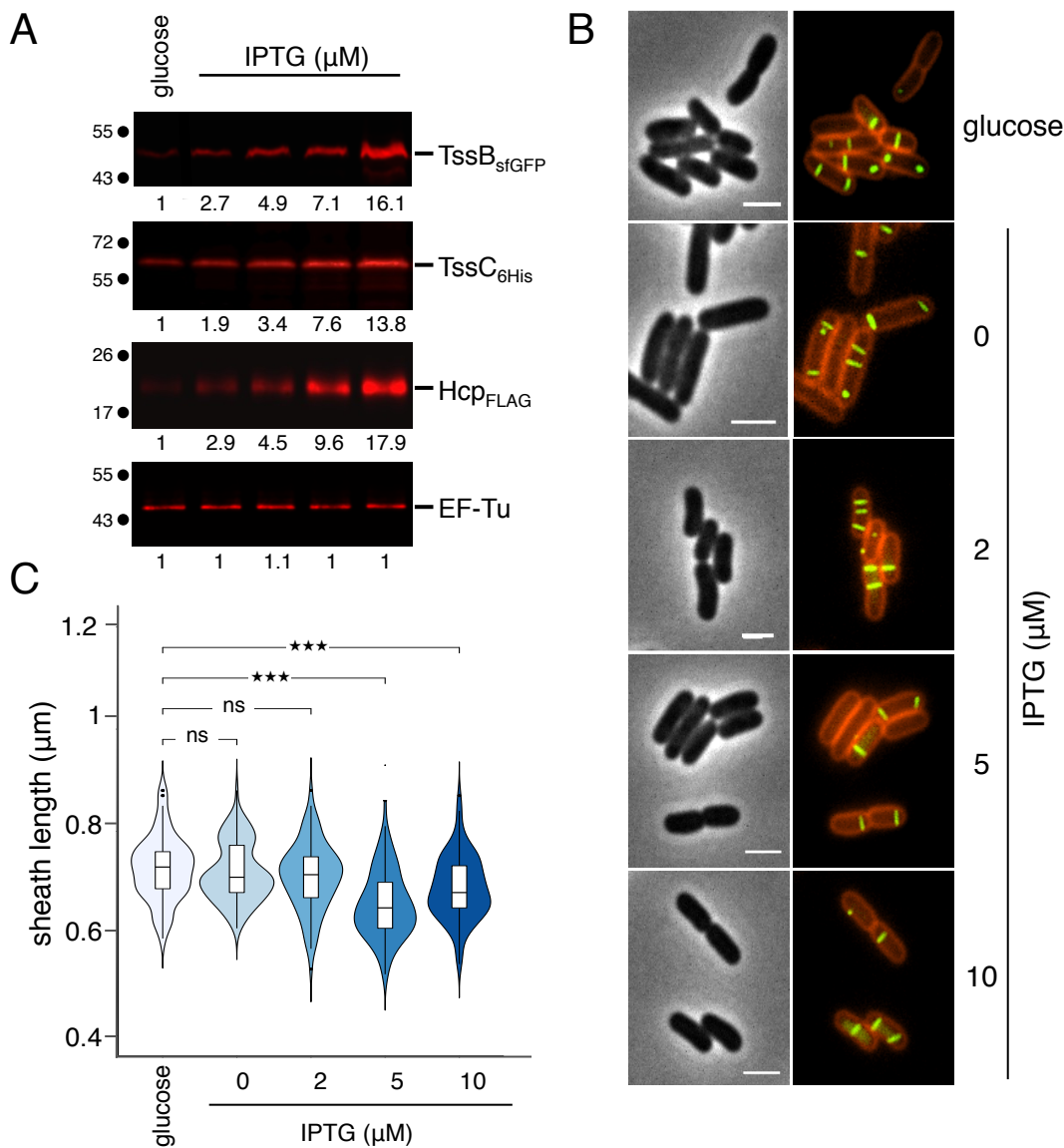
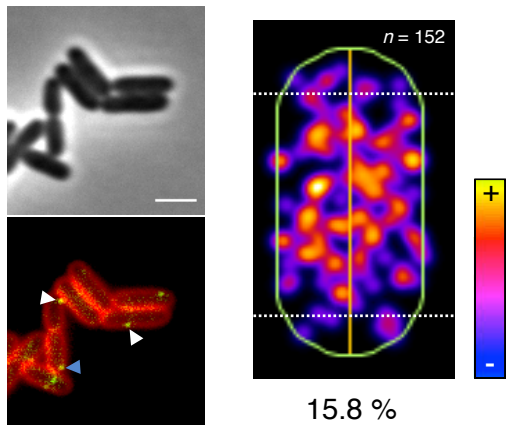
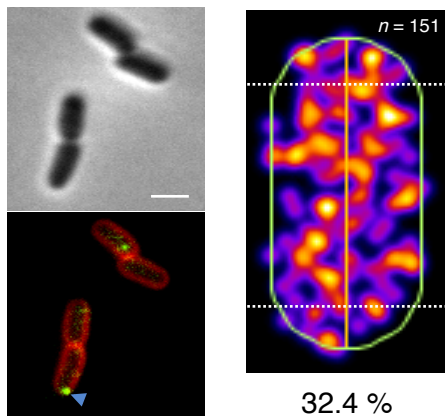


Fig. 2. T6SS tail tube/sheath subunits levels do not determine T6SS sheath length. (A) Western-blot analyses of tail tube/sheath component abundance. 2×10^8 cells of $\Delta tssBC\Delta hcp$ cells producing TssB-sfGFP (TssB_{sfGFP}), 6 \times His-tagged TssC (TssC_{6His}) and FLAG-tagged Hcp (Hcp_{FLAG}) from the pTrc-B_{GFP}-C_{6H}-Hcp_F grown in presence of 0.4% of glucose or of the indicated concentration of IPTG were subjected to 12.5% acrylamide SDS-PAGE and immunodetected with anti-GFP, -His, -FLAG and -EF-Tu monoclonal primary antibodies and secondary antibodies coupled to AlexaFluor[®] 680. The EF-Tu cytoplasmic elongation factor is used as loading control. The increased fold values compared to the glucose condition, measured by densitometric quantification of the band signal intensities and relative to the loading control, are indicated below. Molecular weight markers (in kDa) are indicated on left. (B-C) Representative fluorescence microscopy recordings (B) and statistical analyses of sheath length measurements (C) of $\Delta tssBC\Delta hcp$ cells producing Hcp_{FLAG}, TssB-sfGFP and TssC_{6His} in presence of 0.4% glucose or of the indicated concentration of IPTG, and labelled with FM4-64 (left panels, phase channel; right panels, merge of GFP and FM4-64 channels). Scale bar, 2 μm . In the violin plot representation of the statistical analysis, the distribution of the sheath length is represented by the outer shape. The bold horizontal bar represents the median; the lower and upper boundaries of the internal box plot correspond to the 25th and 75th percentiles respectively; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles. Outliers are shown as black dots. Statistical significance from three independent assays ($n = 100$ for each conditions) relative to the glucose condition is indicated above the plots (ns, non-significant; ***, $p < 0.001$; two-tailed Student's t -test). A comparison of tail tube/sheath subunits levels and sheath lengths is shown in Fig. S1.

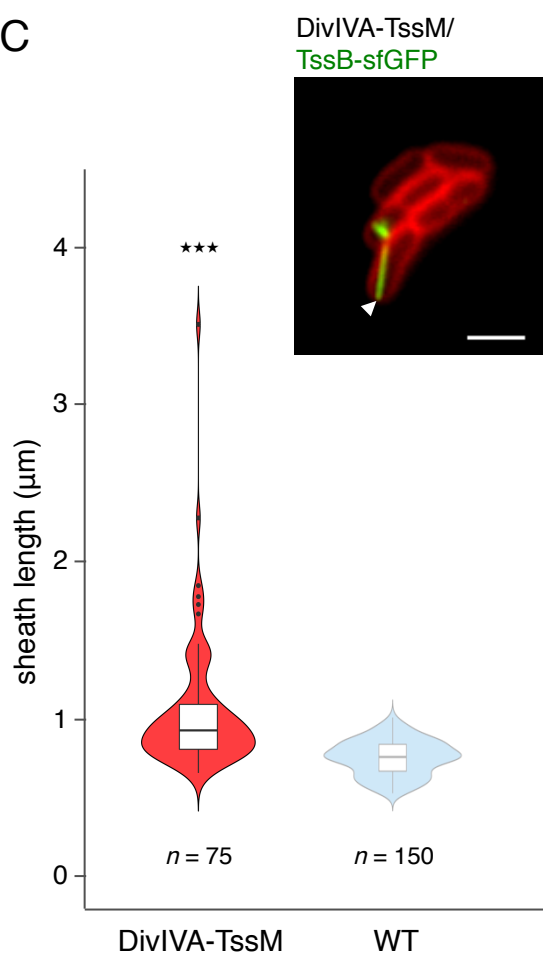
A WT/sfGFP-TssL



B DivIVA-TssM/sfGFP-TssL



C



D DivIVA-TssM/TssB-sfGFP

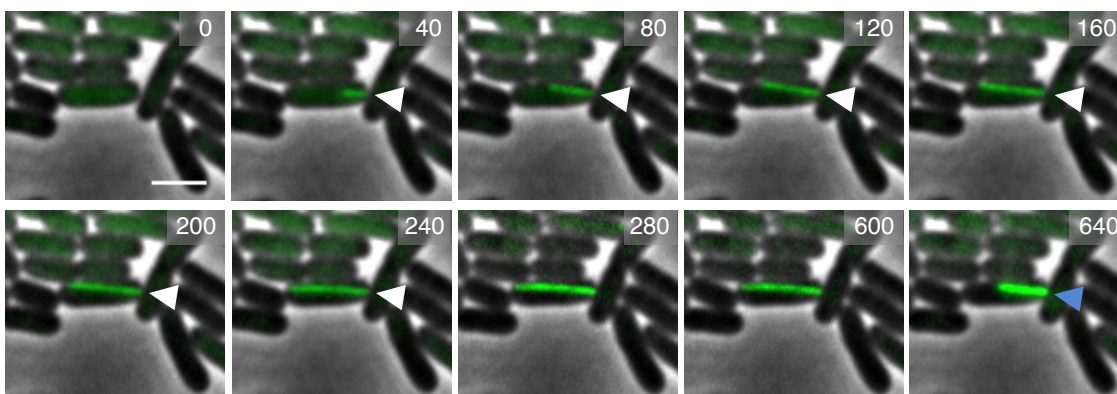


Fig. 3. Sheath length is not controlled by a tape measure protein. Representative fluorescence microscopy recordings of EAEC cells producing sfGFP-TssL in WT (*A*) or DivIV-TssM (*B*) cells, and labelled with FM4-64 (upper panels, phase channel; lower panels, merge of GFP and FM4-64 channels). White and blue arrowheads indicate TssL foci with body or polar localizations, respectively. Scale bar, 2 μ m. The spatial repartition of sfGFP-TssL foci is shown on right, as a projection of foci from $n=152$ and $n=151$ WT and DivIVA-TssM cells, respectively, on a single cell (from blue to yellow, see heatmap color chart on right of panel *A*). The percentage of sfGFP-TssL foci with polar localization is indicated below. (*C*) Violin plot representation of sheath length measurements in DivIVA-TssM cells producing TssB-sfGFP (red). An example of a representative microscopy field is shown in the inset (scale bar, 2 μ m). The distribution of sheath length in WT cells (shown in Fig. 1*B*) is reported in transparency for comparison (blue). The distribution of the sheath length is represented by the outer shape. The bold horizontal bar represents the median value (median = 0.93 μ m and 0.76 μ m for DivIVA-TssM and WT cells, respectively); the lower and upper boundaries of the internal box plot correspond to the 25th and 75th percentiles respectively; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles. Outliers are shown as black dots. The number of measured sheaths ($n=75$) is indicated below. Statistical significance relative to WT cells is indicated above the plots (***, $p < 0.001$; one-tailed Wilcoxon's t -test). (*D*) Fluorescence microscopy time-lapse recordings of DivIVA-TssM cells producing TssB-sfGFP highlighting polar T6SS sheath assembly (white arrowhead) and contraction (blue arrowhead). Scale bar, 2 μ m.

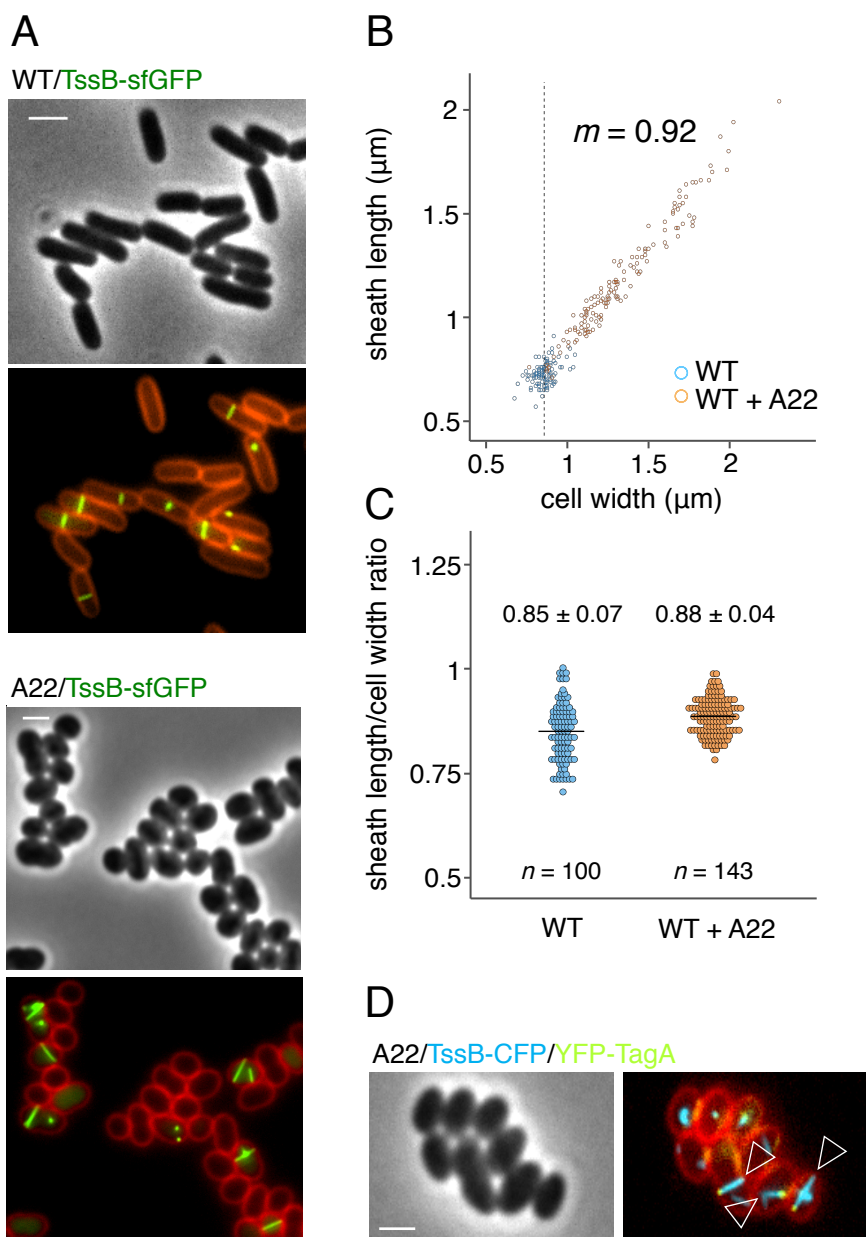


Fig. 4. Cell width dictates T6SS sheath length. (A) Representative fluorescence micrographs of EAEC cells producing TssB-sfGFP in absence (upper panel) or in presence of the MreB inhibitor A22 (lower panel), and labelled with FM4-64 (upper panel, phase channel; lower panel, merge of GFP and FM4-64 channels). Scale bar, 2 μm . (B) Plot representation showing the relationship between sheath length and cell width in absence (blue) and presence of A22 (orange). The dashed line represents the mean of the WT cell width (mean = 0.87 μm). Value of the slope (m) is indicated on right. Kendall's $\tau = 0.82$. (C) Dotplot of the ratios between sheath length and the width of the corresponding cell from cells grown in absence (blue) or presence of A22 (orange). Horizontal bars represent the mean in each condition. The values of the means and standard deviations are indicated above the plots. The number of analyzed cells (n) is indicated below. (D) Representative fluorescence micrograph of EAEC cells producing TssB-CFP and YFP-TagA in presence of the MreB inhibitor A22, and labelled with FM4-64 (left panel, phase channel; right panel, merge of CFP, YFP, and FM4-64 channels). The open arrowheads point T6SS extended sheaths in contact with the TagA stopper. Scale bar, 2 μm .

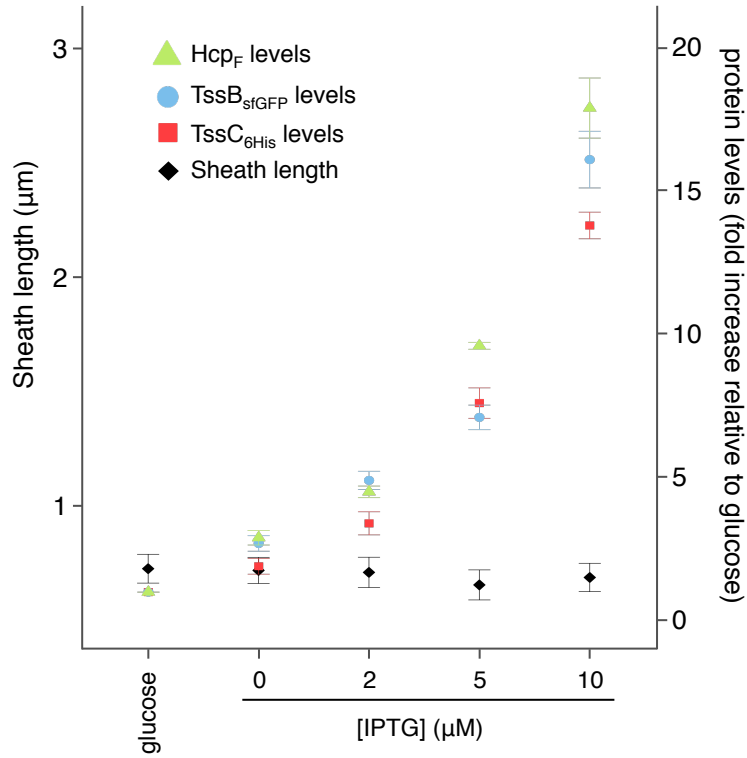


Fig. S1. T6SS tail tube/sheath subunits levels do not determine T6SS sheath length. Graph representation of the fold increase of Hcp_{FLAG} (green triangles), TssB-sfGFP (blue circles), and TssC_{6His} (red squares) cellular levels as a function of the IPTG concentration (relative to the glucose condition) from $\Delta tssBC\Delta hcp$ cells bearing plasmid pTrc-B_{GFP}-C_{6H}-Hcp_F. The corresponding sheath lengths are indicated with black diamonds. The mean and standard deviations from three independent experiments are shown.