SUPPLEMENTAL INFORMATION

"Type VI secretion and bacteriophage tail tubes share a common assembly pathway"

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SUPPLEMENTAL TEXT

EXTENDED RESULTS AND DISCUSSION

Hcp1 localizes in the cytoplasm.

To determine Hcp1 localization, EAEC cells producing Hcp1 were fractionated. Western blot analysis (Figure S1) showed that Hcp1 was immunodetected in the cytoplasmic fraction, associated with the membrane fraction or released in the culture supernatant. Treatment of the membrane fraction with urea demonstrated that Hcp1 was peripherally-associated and not embedded in the membrane. A faint but reproducible amount of Hcp1 localized in the periplasmic fraction. With the exception of the absence of Hcp1 in the culture supernatant and the periplasmic fraction, the Hcp1 localization pattern was identical in *E. coli* K12 cells (*i.e.*, devoid of T6SS genes). This result is in agreement with fractionation and immunelectron microscopy [1] and with recent findings demonstrating that the T6SS sheath assembles in the cytoplasm [2], but is also in contradiction with several studies reporting cytoplasmic and periplasmic localization of Hcp1 [3-5]. Although we cannot rule out that Hcp1 localization is an artefact due to overproduction, one may hypothesize that the observation reported here depicts the T6SS at rest while upon sheath contraction the tail tube-like structure is propelled through the periplasm. Periplasmic accumulation of Hcp1 might therefore represent Hcp1 proteins upon contraction.

Hcp1 interacts with the sheath-like component TssB1.

The localization of Hcp1 into the cytoplasm is reminiscent of the cytoplasmic TssBC tubular structure recently observed [2]. The TssB and TssC subunits have been postulated to

engulf the putative Hcp conduit. TssB and TssC have been shown to directly interact in *V. cholerae*, *Y. pseudotuberculosis*, *Francisella tularensis* and *Salmonella* Typhimurium. In EAEC, both proteins also interact in a two-hybrid assay (Figure S2A, [6]). While Figure 1B shows that Hcp1 and TssC1 do not interact in a bacterial two-hybrid assay, two of the four combinations to question the Hcp1-TssB1 interaction give a significant positive signal. In these combinations, the N-terminal domain of Hcp1 is freely available, suggesting that the addition of a supplemental domain to the C-terminus of Hcp1 prevents formation of the Hcp1/TssB1 complex. It is worth to note that both N- and C-terminal T25 fusions to Hcp led to a positive signal when Hcp/Hcp interactions were tested (data not shown), suggesting that these additional domains do not affect Hcp hexamer formation.

To further validate interaction between tube and sheath components, we tested the Hcp1-TssB1 and Hcp1-TssC1 interaction by co-immunoprecipitation. Figures S2B and S2C show that TssB1 co-precipitates with Hcp1 while TssC1 does not. Similarly, Hcp2 and TssB2 - the Hcp and TssB proteins encoded within the second EAEC T6SS gene cluster, *sci-2* - also co-immunoprecipitate (Figure S2D, lanes 5 & 6), suggesting that the Hcp-TssB interaction is conserved in T6SS. To test the specificity of the Hcp/TssB complex formation, immunoprecipitation experiments were monitored to assess interaction between the Hcp1-TssB2 and Hcp2-TssB1 non-cognate pairs. As shown in Figures S2C and S2D, Hcp1 does not co-precipitate TssB2 (Figure S2D, lanes 3 & 4) and Hcp2 does not co-precipitate TssB1 (Figure S2C, lanes 5 & 6). All together, results from Figure S2 demonstrate that Hcp-like proteins interact with their cognate TssB protein partner. We further demonstrated that this interaction is specific as no cross-interaction between non-cognate pairs occurs, suggesting that specificity determinants control contacts between Hcp- and TssB-like subunits. These results support a model in which a TssBC sheath surrounds a putative Hcp tail-like structure through interaction between Hcp and TssB. It is worthy to note that the Hcp1-TssB1

interaction was detected upon chemical cross-linking suggesting that the strength of the interaction is not as high as several other structural interactions reported between T6SS components, including the TssB and TssC subunits. This suggests that the Hcp-TssB interaction might be transient which is in agreement with the current model in which the tail-tube slides inside the TssBC sheath upon contraction.

EXTENDED EXPERIMENTAL PROCEDURES

Strains and Plasmids construction. The Δ*sci-1*, Δ*hcp1,* Δ*vgrG1* and Δ*tssBC1* mutant strains were constructed by λ-red recombination engineering using the one-step inactivation procedure developed by Datsenko and Wanner [7] using pKD4-amplified PCR products. λ-red functions were expressed from plasmid pKOBEG [8].

Plasmids used for the bacterial two-hybrid and the co-immunoprecipitation assays, and for complementation of the *vgrG* mutant strain (listed in Supplemental Table S1) were constructed using a ligation- and restriction-free method based on two successive PCR amplifications [9] using oligonucleotides listed in Supplemental Table S1.

Site-directed mutagenesis. Site-directed substitutions were introduced by quick change mutagenesis: complementary pairs of mutagenic oligonucleotides (listed in Supplemental Table S1) were used to amplify the whole plasmid template, and to introduce the mutation at the desired site. The Cys-38 to Ser substitution was introduced into the pUC-HcpFLAG plasmid [10], whereas cysteine substitutions were introduced into the resulting pUC-HcpFLAG C38S plasmid. All constructs were verified by DNA sequencing (GATC).

Homology modeling and Cysteine variants design. An initial triple alignment of template

sequences was performed with COBALT [11]. Pairwise structural alignments using jFatCat_flexible [12] provided anchoring points that were the basis for (minor) corrections to the triple alignment. The target and the three template sequences were then aligned using MAFFT with iterative refinement [13], with the aforementioned triple alignment as a constraint. A separate structure-based quadruple sequence alignment in 3D-Coffee [14] yielded virtually identical results.

Homology models were produced with Modeller, versions 9.8 and 9.10 [15]. Hexameric and dodecameric assemblies of the templates were first constructed within VMD [16] by applying crystallographic symmetry operations. Template 1y12 [17] yielded a dodecamer made of hexamers stacked in the "head-to-tail" configuration. Template 3he1 [18] provided a "tail-to-tail" arrangement, and template 3eaa [5] provided a "head-to-head" structure. Independently, a single hexamer was modeled with restraints from all three hexameric templates at once.

For each of the 3 dodecamer models, residues to be substituted were chosen based on their location in loops that were likely to tolerate substitution and the distance between alpha carbon atoms for Hcp1 subunits in different hexameric rings. Distances less than or close to 6 Å were deemed conducive to disulfide bridge formation. Orientation of the side chain was also taken into account, as well as the estimated loop flexibility given the distance to the β-sheet scaffold.

Fractionation. A total of 8×10^{10} cells were resuspended in 500 µL of 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, sucrose 30% and incubated for 10 min on ice. After addition of 100 $\mu g.mL^{-1}$ of lysozyme and 0.5 mM EDTA and further incubation for 5 min on ice, cells were submitted to an osmotic shock by adding 500 µL of 20 mM Tris-HCl (pH 8.0), 100 mM NaCl. The periplasmic fraction was separated from spheroplasts by centrifugation for 10 min

at 5,000 × *g*. Spheroplasts were lysed by five cycles of freeze and thaw followed by 1 min of sonication (60% duty). Unbroken cells were removed by centrifugation. Soluble and membrane fractions were then separated by ultracentrifugation for 40 min at 100,000 × *g*. Membranes were washed with 20 mM Tris-HCl (pH 8.0), and resuspended in 1 ml of 20 mM Tris-HCl (pH 8.0) or 20 mM Tris-HCl (pH 8.0) 6M urea, incubated on a wheel for 20 min, and then ultracentrifuged for 40 min at 100,000 × *g*. Soluble and urea-solubilized fractions were precipitated with trichloroacetic acid (TCA) 15%, and resuspended in loading buffer prior to analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. The accuracy of the fractionation experiments was verified by immunoblotting using anti-EF-Tu (cytoplasmic marker) and anti-MBP (periplasmic marker) antibodies.

Hcp release assay. Supernatant and cell fractions were separated as previously described with modifications [19]. Briefly, 5×10^9 cells producing FLAG epitope-tagged WT or variant Hcp proteins grown in LB supplemented with ampicillin and Dithiothreitol (DTT) 2 mM were harvested and collected by centrifugation at 2,000 × *g* for 5 min. The supernatant fraction was then subjected to a second low-speed centrifugation and then to a 16,000 × *g* centrifugation for 15 min. The supernatant was filtered on sterile polyester membranes with a pore size of 0.2 μm (membrex 25 PET, membraPure GmbH) before precipitation with trichloroacetic acid (TCA) 15%. Cells and precipitated supernatant were resuspended in loading buffer and analyzed by SDS-PAGE and immunoblotting with the anti-FLAG antibody (Sigma-Aldrich). As control for cell integrity, the membranes were immunodetected with antibodies raised against the periplasmic TolB protein.

Bacterial two-hybrid assay. We used the adenylate cyclase-based two-hybrid technique

using previously published protocols [20,21]. Briefly, pairs of proteins to be tested were fused to the two catalytic domains T18 and T25 of the *Bordetella* adenylate cyclase. After co-transformation of the BTH101 strain with the two plasmids producing the fusion proteins, plates were incubated at 30°C for 2 days. 600 µl of LB medium supplemented with ampicillin, kanamycin and 0.5 mM isopropyl-β-thio-galactoside (IPTG) were inoculated with independent colonies. Cells were grown at 30°C overnight and then spotted on LB agar plates supplemented with ampicillin, kanamycin, IPTG and the chromogenic substrate Bromo-Chloro-Indolyl-β-D-galactopyrannoside (X-Gal). The TolB and Pal proteins, two proteins that interact but are unrelated to the T6SS, were used as negative (when combined with T6SS subunits) or positive (when combined together) controls. The figures show a representative experiment (at least triplicated with identical results).

Co-immunoprecipitation assay. 10^{11} exponentially growing cells were harvested, washed with 20 ml of 10 mM sodium phosphate buffer (NaPi, pH 6.8), and resuspended in NaPi buffer supplemented with *para*-formaldehyde 1%. After incubation at room temperature for 20 minutes, the cross-linking reaction was quenched by the addition of 0.3 M Tris-HCl (pH6.8), and the cells were washed twice in Tris-HCl 20 mM (pH6.8). Cells were resuspended in Tris-HCl 20 mM (pH8.0), NaCl 100 mM and broken by three passages at the French press (1000 psi). The total cell extract was supplemented with CHAPS 10 mM and protease inhibitors (Complete, Roche), incubated for 1 hour with vigorous shaking and ultracentrifuged for 45 min at 20,000 × *g* to discard unsolubilized material. Supernatants were incubated overnight at 4°C with anti-FLAG M2 affinity beads (Sigma Aldrich). Beads were washed twice with Tris-HCl 20 mM (pH8.0), NaCl 100 mM, CHAPS 10 mM, and once in the same buffer without CHAPS. The total extract and immunoprecipitated material were resuspended in Laemmli loading buffer prior to analyses by SDS-PAGE and immunoblotting.

Miscellaneous. Proteins suspended in loading buffer were subjected to SDS- PAGE. For detection by immunostaining, proteins were transferred onto nitrocellulose membranes, and immunoblots were probed with the indicated antibodies, and goat secondary antibodies coupled to alkaline phosphatase, and developed in alkaline buffer in presence of 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium. Anti-TolB antibodies are from our laboratory collection. Anti-EF-Tu (Hycult Biotech), anti-MBP (Sigma-Aldrich), anti-FLAG (Sigma-Aldrich), anti-VSV-G (Sigma-Aldrich) and anti-HA (Roche) monoclonal antibodies and anti-rabbit, -mouse and -rat alkaline phosphatase-conjugated goat secondary antibodies (Beckman coulter) have been purchased as indicated.

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