Supporting Information

Systematic dissection of the *Agrobacterium* type VI secretion system reveals machinery and secreted components for subcomplex formation

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Information S1

Plasmid construction and generation of in-frame deletion mutants

The plasmid pJQ200KS-ΔtssF was created by ligating the SacI/BamHI-digested tssF PCR product 1 (~500 bp DNA fragment upstream of the tssF open reading frame [ORF]) and the BamHI/XmaI-digested tssF PCR product 2 (~500 bp DNA fragment downstream of the tssF ORF) into SacI/XmaI sites of pJQ200KS and used to generate the deletion mutant ΔtssF (EML1090). The rest of pJQ200KS derivatives (Supplementary Table S1) were created by ligating the XbaI/BamHI-digested PCR product 1 (~500 bp DNA fragments upstream of each target gene) and the BamHI/XmaI-digested PCR product 2 (~500 bp DNA fragments downstream of each target gene) into XbaI/XmaI sites of pJQ200KS and used to generate each of the deletion mutants (Supplementary Tables S1 and S2). For each in-frame deletion mutant confirmed by PCR, at least 2 independent colonies were selected to determine its ability in mediating Hcp secretion.

For complementation, the gene of interest containing its ribosomal-binding sequence (RBS) and ORF was cloned to be driven by a lac promoter on the broad host range vector pRL662 [1]. The PCR products of tssK and clpV genes were digested by HindIII/XbaI and cloned into the same sites of pRL662 to create the plasmids pTssK and pClpV. The PCR-amplified fha and tssE genes were digested by BamHI/XbaI and cloned into the same sites of pRL662 to create the plasmids pFha and pTssE. The remaining genes were amplified with primers described in Supplementary Table S2, and the PCR products were digested by XhoI/XbaI and cloned into the same sites of pRL662 to create the plasmids pTssG, pTssF, pTssC40, pTssC41, pTssB, pTssA, pHcp, pAtu4346, pAtu4347, pVgrG-1, and pVgrG-2.

To convert each of Δfha and ΔtssC41 back to the wild type (revertant), pJQ200KS derivatives harboring the fha and tssC41 genes, including their respective upstream and downstream regions, were used for double crossover. The PCR-amplified products were digested by XbaI/XmaI and cloned into the same sites of pJQ200KS to create the plasmids pJQ200KS-fha, and pJQ200KS-tssC41. The resulting revertants were designated as EML2137 & 2138 (fha R-1 and R-2), and EML2141 & 2142 (tssC41 R-1 and R-2), respectively.

The expression vector pET22b(+) was used to overexpress proteins driven by the T7 promoter via IPTG induction in E. coli BL21 (DE3). Each ORF (without stop codon) of ppkA, tssK, fha, tssE, tssC41, tssB, tssA, attu4346, attu4347, attu4349, vgrG-1, rpoA, and aopB was PCR-amplified with primers described in Supplementary Table S2 and cloned into the same sites of pET22b(+) with appropriate enzyme sites. The tssB-tssC41 fragment (without stop codon) was PCR-amplified with primers described in Supplementary Table S2 and cloned into the same sites of pET22b(+) with
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appropriate enzyme sites to create the plasmid pET-TssB-TssC41-His. The *pppA* and *clpV* ORFs (without stop codon) were PCR-amplified, digested by *Hind*III, and cloned into pET22b(+), which was first digested by *Ndel*, followed by Klenow repair, and finally digested by *Hind*III. To construct the pET-N-TssL-His for expressing the N terminus (residues 1 to 255) of TssL, the plasmid pAD-N-TssL [2] was digested by *Ndel/XhoI* and cloned into the same sites of pET22b(+).

To construct the plasmids for expressing proteins without tag, each DNA fragment containing the RBS and ORF (with stop codon) of *tssC41, hcp, atu4347, vgrG-I*, and *exoR-Strep* was PCR-amplified with primers described in Supplementary Table S2 and cloned into pTrc200 with appropriate enzyme sites to create the plasmids pTrc-TssC41, pTrc-Hcp, pTrc-Atu4347, pTrc-VgrG-1, and pTrc-ExoR-Strep.

The plasmid pTssB-Strep used in Strep-Tag pull down assay was created by PCR amplifying *tssB* ORF with primers described in Supplementary Table S2 and cloned into the *XhoI/XbaI* sites of pRL662.

For the constructs used for yeast two-hybrid, the *tssC41* and *tssB* ORFs (without stop codon) were PCR-amplified with primers described in Supplementary Table S2, digested by *Ndel/BamHI*, and cloned into the same sites of pGBK7 or pGADT7 to create the plasmids pGBK7-TssC41, pGBK7-TssB, pGADT7-TssC41, and pGADT7-TssB, respectively.

**Biochemical fractionation**

Isolation of *A. tumefaciens* cellular fractions was as described [2].
| Table S1. Bacterial strains and plasmids |
|-----------------------------------------|
| **Strain /plasmid**                     |
| **Relevant characteristics**            |
| **Source/ reference**                   |
| **A.tumefaciens**                       |
| C58                                     |
| Wild type virulent strain containing   |
| nopaline-type Ti plasmid pTiC58         |
| Eugene Nester                          |
| EML1213                                 |
| Entire promoter region deletion mutant,|
| C58Δpro                                 |
| This study                              |
| EML1218                                 |
| Entire *imp* operon deletion mutant, C58Δ*imp* |
| This study                              |
| EML1060                                 |
| *ppkA*(atu4330) in frame deletion mutant,|
| C58Δ*ppkA*                              |
| This study                              |
| EML1063                                 |
| *pppA* *(atu4331)* in frame deletion mutant,|
| C58Δ*pppA*                              |
| This study                              |
| EML1068                                 |
| *tssM* *(atu4332)* in frame deletion mutant,|
| C58Δ*icmF*                              |
| [2]                                     |
| EML1073                                 |
| *tssL* *(atu4333)* in-frame deletion mutant,|
| C58Δ*icmH*                              |
| [2]                                     |
| EML1078                                 |
| *tssK* *(atu4334)* in frame deletion mutant,|
| C58Δ*atu4334*                          |
| This study                              |
| EML1521                                 |
| *fha* *(atu4335)* in frame deletion mutant,|
| C58Δ*fha*                               |
| This study                              |
| EML1086                                 |
| *tssG* *(atu4336)* in-frame deletion mutant,|
| C58Δ*atu4336*                          |
| This study                              |
| EML1090                                 |
| *tssF* *(atu4337)* in frame deletion mutant,|
| C58Δ*atu4337*                          |
| This study                              |
| EML1093                                 |
| *tssE* *(atu4338)* in frame deletion mutant,|
| C58Δ*atu4338*                          |
| This study                              |
| EML1097                                 |
| *tagJ* *(atu4339)* in frame deletion mutant,|
| C58Δ*atu4339*                          |
| This study                              |
| EML1100                                 |
| *tssC₄₀* *(atu4340)* in frame deletion mutant,|
| C58Δ*atu4340*                          |
| This study                              |
| EML1105                                 |
| *tssC₄₁* *(atu4341)* in frame deletion mutant,|
| C58Δ*vipB*                              |
| This study                              |
| EML1109                                 |
| *tssB* *(atu4342)* in frame deletion mutant,|
| C58Δ*vipA*                              |
| This study                              |
| EML1113                                 |
| *tssA* *(atu4343)* in-frame deletion mutant,|
| C58Δ*atu4343*                          |
| This study                              |
| EML1117                                 |
| *clpV* *(atu4344)* in frame deletion mutant,|
| C58Δ*clpV*                             |
| This study                              |
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| EML1122 | *hcp (atu4345)* in frame deletion mutant, C58Δhcp | This study |
| EML1127 | *atu4346* in frame deletion mutant, C58Δatu4346 | This study |
| EML1131 | *atu4347* in frame deletion mutant, C58Δatu4347 | This study |
| EML1134 | *vgrG-1 (atu4348)* in frame deletion mutant, C58ΔvgrG-1 | This study |
| EML1137 | *atu4349* in frame deletion mutant, C58Δatu4349 | This study |
| EML1142 | *atu4350* in frame deletion mutant, C58Δatu4350 | This study |
| EML1145 | *atu4352* in frame deletion mutant, C58Δatu4352 | This study |
| EML1166 | *vgrG-2 (atu3642)* in frame deletion mutant, C58ΔvgrG-2 | This study |
| EML1289 | *vgrG-1* and *vgrG-2* double in frame deletion mutant, C58ΔvgrG-1/-2 | This study |
| EML3553 | *atu4346* and *atu4347* double in frame deletion mutant, C58Δatu4346atu4347 | This study |
| EML3700 | *aopB* in frame deletion mutant, C58ΔaopB | This study |
| EML2137 | Complementation of *fha* gene to linear chromosome of Δfha strain, revertant strain of Δfha-1 | This study |
| EML2138 | Complementation of *fha* gene to linear chromosome of Δfha strain, revertant strain of Δfha-2 | This study |
| EML2141 | Complementation of *tssC*41 gene to linear chromosome of ΔtssC*41* strain, revertant strain of ΔtssC*41*-1 | This study |
| EML2142 | Complementation of *tssC*41 gene to linear chromosome of ΔtssC*41* strain, revertant strain of ΔtssC*41*-2 | This study |
| EML829 | ΔactCBA, deletion of actCBA in NT1RE | [4] |

**E. coli**

| DH10B | Host for DNA cloning | Invitrogen |
| BL21(DE3) | Host for overexpressing proteins driven by T7 promoter | [5] |

**S. cerevisiae**

| AH109 | Host for yeast two-hybrid analysis | Clontech |

**Plasmids**

| pRL662 | Gm\(^r\), broad-host range vector derived from pBBR1MCS-2 | [1] |
| Vector Name | Description                                                                 | Supplier |
|-------------|-----------------------------------------------------------------------------|----------|
| pET22b(+)   | Ap⁺, *E. coli* overexpression vector to generate C-terminal His-tagged protein | Novagen  |
| pJQ200KS    | Gm⁺, suicide plasmid containing Gm⁺ and sacB gene for selection of double crossover | [6]     |
| pTrc200     | Sp⁺, pVS1 origin *lacI⁰*, *trc* promoter expression vector                   | [7]     |
| pGADT7      | Ap⁺, AD vector used in yeast two-hybrid assay                               | Clontech|
| pGBKT7      | Km⁺, DNA-BD vector used in yeast-two hybrid assay                           | Clontech|
| pTssM       | Gm⁺, pRL662 expressing TssM driven by *lacZp*                               | [3]     |
| pTssL       | Gm⁺, pRL662 expressing TssL driven by *lacZp*                               | [2]     |
| pTssK       | Gm⁺, pRL662 expressing TssK driven by *lacZp*                               | This study|
| pFha        | Gm⁺, pRL662 expressing Fha driven by *lacZp*                                | This study|
| pTssG       | Gm⁺, pRL662 expressing TssG driven by *lacZp*                               | This study|
| pTssF       | Gm⁺, pRL662 expressing TssF driven by *lacZp*                               | This study|
| pTssE       | Gm⁺, pRL662 expressing TssE driven by *lacZp*                               | This study|
| pTssC₄₀     | Gm⁺, pRL662 expressing TssC₄₀ driven by *lacZp*                             | This study|
| pTssC₄₁     | Gm⁺, pRL662 expressing TssC₄₁ driven by *lacZp*                             | This study|
| pTssA       | Gm⁺, pRL662 expressing TssA driven by *lacZp*                               | This study|
| pClpV       | Gm⁺, pRL662 expressing ClpV driven by *lacZp*                               | This study|
| pHcp        | Gm⁺, pRL662 expressing Hcp driven by *lacZp*                                | This study|
| pAtu4346    | Gm⁺, pRL662 expressing Atu4346 driven by *lacZp*                            | This study|
| pAtu4347    | Gm⁺, pRL662 expressing Atu4347 driven by *lacZp*                            | This study|
| pVgrG-1     | Gm⁺, pRL662 expressing VgrG-1 driven by *lacZp*                             | This study|
| pVgrG-2     | Gm⁺, pRL662 expressing VgrG-2 driven by *lacZp*                             | This study|
| pTssB-Strep | Gm⁺, pRL662 expressing TssB-Strep fusion protein driven by *lacZp*          | This study|
| pET-PpkA-His| Ap⁺, pET22b overexpressing His-tagged PpkA in *E. coli*                     | This study|
| pET-PppA-His| Ap⁺, pET22b overexpressing His-tagged PppA in *E. coli*                     | This study|
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| Expression System | Description | Study | Notes |
|------------------|-------------|-------|-------|
| pET-N-TssL-His   | Ap', pET22b overexpressing His-tagged N terminus of TssL (residues 1 to 255) in *E. coli* | This study | |
| pET-TssK-His     | Ap', pET22b overexpressing His-tagged TssK in *E. coli* | This study | |
| pET-Fha-His      | Ap', pET22b overexpressing His-tagged Fha in *E. coli* | This study | |
| pET-TssE-His     | Ap', pET22b overexpressing His-tagged TssE in *E. coli* | This study | |
| pET-TssC41-His   | Ap', pET22b overexpressing His-tagged TssC41 in *E. coli* | This study | |
| pET-TssB-His     | Ap', pET22b overexpressing His-tagged TssB in *E. coli* | This study | |
| pET-TssA-His     | Ap', pET22b overexpressing His-tagged TssA in *E. coli* | This study | |
| pET-ClpV-His     | Ap', pET22b overexpressing His-tagged ClpV in *E. coli* | This study | |
| pET-Hcp-His      | Ap', pET22b overexpressing His-tagged Hcp in *E. coli* | [3] | |
| pET-Atu4346-His  | Ap', pET22b overexpressing His-tagged Atu4346 in *E. coli* | This study | |
| pET-Atu4347-His  | Ap', pET22b overexpressing His-tagged Atu4347 in *E. coli* | This study | |
| pET-VgrG-1-His   | Ap', pET22b overexpressing His-tagged VgrG-1 in *E. coli* | This study | |
| pET-TssB-TssC41-His | Ap', pET22b overexpressing TssB and His-tagged TssC41 in *E. coli* | This study | |
| pET-RpoA-His     | Ap', pET22b overexpressing His-tagged Atu1923 (RpoA) in *E. coli* | This study | |
| pET-AopB-His     | Ap', pET22b overexpressing His-tagged Atu1131 (AopB) in *E. coli* | This study | |
| pJQ200KS-Δpro    | Gm', used in generating entire promoter region deletion mutant of *A. tumefaciens* C58 | This study | |
| pJQ200KS-Δimp    | Gm', used in generating entire *imp* operon deletion mutant of *A. tumefaciens* C58 | This study | |
| pJQ200KS-ΔppkA   | Gm', used in generating *ppkA* in-frame deletion mutant of *A. tumefaciens* C58 | This study | |
| pJQ200KS-ΔppA    | Gm', used in generating *ppA* in-frame deletion mutant of *A. tumefaciens* C58 | This study | |
| Construct              | Description                                                                 | Study     |
|------------------------|-----------------------------------------------------------------------------|-----------|
| pJQ200KS-ΔtssK         | Gm^r, used in generating `tssK` in-frame deletion mutant of *A. tumefaciens* C58 | This study|
| pJQ200KS-Δfha          | Gm^r, used in generating `fha` in-frame deletion mutant of *A. tumefaciens* C58 | This study|
| pJQ200KS-ΔtssG         | Gm^r, used in generating `tssG` in-frame deletion mutant of *A. tumefaciens* C58 | This study|
| pJQ200KS-ΔtssF         | Gm^r, used in generating `tssF` in-frame deletion mutant of *A. tumefaciens* C58 | This study|
| pJQ200KS-ΔtssE         | Gm^r, used in generating `tssE` in-frame deletion mutant of *A. tumefaciens* C58 | This study|
| pJQ200KS-ΔtagJ         | Gm^r, used in generating `tagJ` in-frame deletion mutant of *A. tumefaciens* C58 | This study|
| pJQ200KS-ΔtssC_{40}    | Gm^r, used in generating `tssC_{40}` in-frame deletion mutant of *A. tumefaciens* C58 | This study|
| pJQ200KS-ΔtssC_{41}    | Gm^r, used in generating `tssC_{41}` in-frame deletion mutant of *A. tumefaciens* C58 | This study|
| pJQ200KS-ΔtssB         | Gm^r, used in generating `tssB` in-frame deletion mutant of *A. tumefaciens* C58 | This study|
| pJQ200KS-ΔtssA         | Gm^r, used in generating `tssA` in-frame deletion mutant of *A. tumefaciens* C58 | This study|
| pJQ200KS-ΔclpV         | Gm^r, used in generating `clpV` in-frame deletion mutant of *A. tumefaciens* C58 | This study|
| pJQ200KS-Δhcp          | Gm^r, used in generating `hcp` in-frame deletion mutant of *A. tumefaciens* C58 | This study|
| pJQ200KS-Δatu4346      | Gm^r, used in generating `atu4346` in-frame deletion mutant of *A. tumefaciens* C58 | This study|
| pJQ200KS-Δatu4347      | Gm^r, used in generating `atu4347` in-frame deletion mutant of *A. tumefaciens* C58 | This study|
| pJQ200KS-ΔvgrG-1       | Gm^r, used in generating `vgrG-1` in-frame deletion mutant of *A. tumefaciens* C58 | This study|
| pJQ200KS-Δatu4349      | Gm^r, used in generating `atu4349` in-frame deletion mutant of *A. tumefaciens* C58 | This study|
| pJQ200KS-Δatu4350      | Gm^r, used in generating `atu4350` in-frame deletion mutant of *A. tumefaciens* C58 | This study|
| pJQ200KS-Δatu4352      | Gm^r, used in generating `atu4352` in-frame deletion mutant of *A. tumefaciens* C58 | This study|
| pJQ200KS-ΔvgrG-2       | Gm^r, used in generating `vgrG-2` in-frame deletion mutant of *A. tumefaciens* C58 | This study|
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| Vector | Description | Source |
|--------|-------------|--------|
| pJQ200KS-Δ46Δ47 | Gm<sup>+</sup>, used in generating *atu4346* and *atu4347* double in-frame deletion mutant of *A. tumefaciens* C58 | This study |
| pJQ200KS-ΔaopB | Gm<sup>+</sup>, used in generating *aopB* in-frame deletion mutant of *A. tumefaciens* C58 | This study |
| pJQ200KS-Δfha | Gm<sup>+</sup>, used in generating revertant strain of Δ*fha* | This study |
| pJQ200KS-ΔtssC41 | Gm<sup>+</sup>, used in generating revertant strain of Δ*tssC*41 | This study |
| pTrc-TssC41 | Sp<sup>R</sup>, pTrc200 expressing TssC<sub>41</sub> without tag | This study |
| pTrc-Hcp | Sp<sup>R</sup>, pTrc200 expressing Hcp without tag | This study |
| pTrc-Atu4347 | Sp<sup>R</sup>, pTrc200 expressing Atu4347 without tag | This study |
| pTrc-VgrG-1 | Sp<sup>R</sup>, pTrc200 expressing VgrG-1 without tag | This study |
| pTrc-ExoR-Strep | Sp<sup>R</sup>, pTrc200 expressing ExoR-Strep fusion protein | This study |
| pGBK7-TssC41 | Km<sup>+</sup>, DNA-BD vector expressing TssC<sub>41</sub> | This study |
| pGBK7-TssB | Km<sup>+</sup>, DNA-BD vector expressing TssB | This study |
| pGBK7-53 | Km<sup>+</sup>, DNA-BD vector expressing murine p53 | Clontech |
| pGAD7-TssC41 | AP<sup>+</sup>, AD vector expressing TssC<sub>41</sub> | This study |
| pGAD7-TssB | AP<sup>+</sup>, AD vector expressing TssB | This study |
| pGAD7-T | AP<sup>+</sup>, AD vector expressing SV40 large T-antigen | Clontech |
| Primer | Plasmids         | Sequence (5' -3') | Source / reference |
|--------|------------------|-------------------|--------------------|
| 1. Promoter deletion 1F-XbaI | pJQ200KS-Agpa | 5'-GC TCTAGA GCCTCT CGAATTCGAGC-3' | This study |
| 2. Promoter deletion 1R-BamHI |  | 5'-CGGATCCATGGTCATGCTCGAATTCGAGC-3' | This study |
| 3. Promoter deletion 2F-BamHI |  | 5'-CGGATCCATGGTCATGCTCGAATTCGAGC-3' | This study |
| 4. Promoter deletion 2R-Xmnal |  | 5'-TCCCCCAGGGGCATCTCGTGAGGTTGCAG-3' | This study |
| 5. Imp deletion 1F-XbaI | pJQ200KS-Δimp | 5'-GC TCTAGA GTCCGTCGAGGTTGTTGCAG-3' | This study |
| 6. Imp deletion 1R-BamHI |  | 5'-CGGATCC ATGGTCATGCTCGAATTCGAGC-3' | This study |
| 7. Imp deletion 2F-BamHI |  | 5'-CGGATCC ATGGTCATGCTCGAATTCGAGC-3' | This study |
| 8. Imp deletion 2R-Xmnal |  | 5'-TCCCCCAGGGGCATCTCGTGAGGTTGCAG-3' | This study |
| 9. PpkA 1F-XbaI | pJQ200KS-ΔppkA | 5'-GCTCTAGA GGAGATGCTACGACAGAC-3' | This study |
| 10. PpkA 1R-BamHI |  | 5'-GCTCTAGA GGAGATGCTACGACAGAC-3' | This study |
| 11. PpkA 2F-BamHI |  | 5'-GCTCTAGA GGAGATGCTACGACAGAC-3' | This study |
| 12. PpkA 2R-Xmnal |  | 5'-TCCCCCAGGGGCATCTCGTGAGGTTGCAG-3' | This study |
| 13. PppA 1F-XbaI | pJQ200KS-ΔpppA | 5'-GCTCTAGA GGAGATGCTACGACAGAC-3' | This study |
| 14. PppA 1R-BamHI |  | 5'-GCTCTAGA GGAGATGCTACGACAGAC-3' | This study |
| 15. PppA 2F-BamHI |  | 5'-GCTCTAGA GGAGATGCTACGACAGAC-3' | This study |
| 16. PppA 2R-Xmnal |  | 5'-TCCCCCAGGGGCATCTCGTGAGGTTGCAG-3' | This study |
| 17. Ts| 5'-GCTCTAGA GGAGATGCTACGACAGAC-3' | This study |
| 18. TsK 1R-BamHI |  | 5'-GCTCTAGA GGAGATGCTACGACAGAC-3' | This study |
| 19. TsK 2F-BamHI |  | 5'-GCTCTAGA GGAGATGCTACGACAGAC-3' | This study |
| 20. TsK 2R-Xmnal |  | 5'-TCCCCCAGGGGCATCTCGTGAGGTTGCAG-3' | This study |
| 21. Fha 1F-XbaI | pJQ200KS-Δfha | 5'-GCTCTAGA GGAGATGCTACGACAGAC-3' | This study |
| 22. Fha 1R-BamHI |  | 5'-GCTCTAGA GGAGATGCTACGACAGAC-3' | This study |
| 23. Fha 2F-BamHI |  | 5'-GCTCTAGA GGAGATGCTACGACAGAC-3' | This study |
| 24. Fha 2R-Xmnal |  | 5'-TCCCCCAGGGGCATCTCGTGAGGTTGCAG-3' | This study |
| 25. Tsq 1F-XbaI | pJQ200KS-ΔtsqG | 5'-GCTCTAGA GGAGATGCTACGACAGAC-3' | This study |
| 26. Tsq 1R-BamHI |  | 5'-GCTCTAGA GGAGATGCTACGACAGAC-3' | This study |
| 27. Tsq 2F-BamHI |  | 5'-GCTCTAGA GGAGATGCTACGACAGAC-3' | This study |
| 28. Tsq 2R-Xmnal |  | 5'-TCCCCCAGGGGCATCTCGTGAGGTTGCAG-3' | This study |
| 29. Tsf 1F-Sacl | pJQ200KS-ΔtsfE | 5'-GCTCTAGA GGAGATGCTACGACAGAC-3' | This study |
| 30. Tsf 1R-BamHI |  | 5'-GCTCTAGA GGAGATGCTACGACAGAC-3' | This study |
| 31. Tsf 2F-BamHI |  | 5'-GCTCTAGA GGAGATGCTACGACAGAC-3' | This study |
| 32. Tsf 2R-Xmnal |  | 5'-TCCCCCAGGGGCATCTCGTGAGGTTGCAG-3' | This study |
| 33. Tsse 1F-XbaI | pJQ200KS-ΔsseE | 5'-GCTCTAGA GGAGATGCTACGACAGAC-3' | This study |
| 34. Tsse 1R-BamHI |  | 5'-GCTCTAGA GGAGATGCTACGACAGAC-3' | This study |
| 35. Tsse 2F-BamHI |  | 5'-GCTCTAGA GGAGATGCTACGACAGAC-3' | This study |
| 36. Tsse 2R-Xmnal |  | 5'-TCCCCCAGGGGCATCTCGTGAGGTTGCAG-3' | This study |
| 37. Tag 1F-XbaI | pJQ200KS-ΔtagA | 5'-GCTCTAGA GGAGATGCTACGACAGAC-3' | This study |
| 38. Tag 1R-BamHI |  | 5'-GCTCTAGA GGAGATGCTACGACAGAC-3' | This study |
| 39. Tag 2F-BamHI |  | 5'-GCTCTAGA GGAGATGCTACGACAGAC-3' | This study |
| 40. Tag 2R-Xmnal |  | 5'-TCCCCCAGGGGCATCTCGTGAGGTTGCAG-3' | This study |
| 41. TssC 1F-XbaI | pJQ200KS-ΔsscC | 5'-GCTCTAGA GGAGATGCTACGACAGAC-3' | This study |
| 42. TssC 1R-BamHI |  | 5'-GCTCTAGA GGAGATGCTACGACAGAC-3' | This study |
| 43. TssC 2F-BamHI |  | 5'-GCTCTAGA GGAGATGCTACGACAGAC-3' | This study |
| 44. TssC 2R-Xmnal |  | 5'-TCCCCCAGGGGCATCTCGTGAGGTTGCAG-3' | This study |
| 45. TssC 1F-XbaI | pJQ200KS-ΔsscC | 5'-GCTCTAGA GGAGATGCTACGACAGAC-3' | This study |
| 46. TssC 1R-BamHI |  | 5'-GCTCTAGA GGAGATGCTACGACAGAC-3' | This study |
| 47. TssC 2F-BamHI |  | 5'-GCTCTAGA GGAGATGCTACGACAGAC-3' | This study |
| 48. TssC 2R-Xmnal |  | 5'-TCCCCCAGGGGCATCTCGTGAGGTTGCAG-3' | This study |
| 49. TssB 1F-XbaI | pJQ200KS-ΔsbsB | 5'-GCTCTAGA GGAGATGCTACGACAGAC-3' | This study |
| 50. TssB 1R-BamHI |  | 5'-GCTCTAGA GGAGATGCTACGACAGAC-3' | This study |
| 51. TssB 2F-BamHI |  | 5'-GCTCTAGA GGAGATGCTACGACAGAC-3' | This study |
| 52. TssB 2R-Xmnal |  | 5'-TCCCCCAGGGGCATCTCGTGAGGTTGCAG-3' | This study |
| 53. TssA 1F-XbaI | pJQ200KS-ΔsasA | 5'-GCTCTAGA GGAGATGCTACGACAGAC-3' | This study |
| 54. TssA 1R-BamHI |  | 5'-GCTCTAGA GGAGATGCTACGACAGAC-3' | This study |
| 55. TssA 2F-BamHI |  | 5'-GCTCTAGA GGAGATGCTACGACAGAC-3' | This study |
| 56. TssA 2R-Xmnal |  | 5'-TCCCCCAGGGGCATCTCGTGAGGTTGCAG-3' | This study |
| 57. ClpV 1F-XbaI | pJQ200KS-ΔclpV | 5'-GCTCTAGA GGAGATGCTACGACAGAC-3' | This study |
| 58. ClpV 1R-BamHI |  | 5'-GCTCTAGA GGAGATGCTACGACAGAC-3' | This study |
| 59. ClpV 2F-BamHI |  | 5'-GCTCTAGA GGAGATGCTACGACAGAC-3' | This study |
| 60. ClpV 2R-Xmnal |  | 5'-TCCCCCAGGGGCATCTCGTGAGGTTGCAG-3' | This study |
| 61. Hcp 1F-XbaI | pJQ200KS-Ahcp | 5'-GCTCTAGA GGAGATGCTACGACAGAC-3' | This study |
| 62. Hcp 1R-BamHI |  | 5'-GCTCTAGA GGAGATGCTACGACAGAC-3' | This study |
| 63. Hcp 2F-BamHI |  | 5'-GCTCTAGA GGAGATGCTACGACAGAC-3' | This study |
| 64. Hcp 2R-Xmnal |  | 5'-TCCCCCAGGGGCATCTCGTGAGGTTGCAG-3' | This study |
| Entry | Description | Sequence/Comment |
|-------|-------------|-----------------|
| 65.   | Atu4346 1F-XbaI | pJQ200KS-Δatu434 5'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-3' This study |
| 66.   | Atu4346 1R-BamHI | 6'-CCGCAGCTTGGATATGAAAGCCGATAAGG-5' This study |
| 67.   | Atu4346 2F-BamHI | 5'-CCGCAGCTTGGATATGAAAGCCGATAAGG-3' This study |
| 68.   | Atu4346 2R-XmaI | 6'-CCGCAGCTTGGATATGAAAGCCGATAAGG-5' This study |
| 69.   | Atu4347 1F-XbaI | pJQ200KS-Δatu434 5'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-3' This study |
| 70.   | Atu4347 1R-BamHI | 6'-CCGCAGCTTGGATATGAAAGCCGATAAGG-5' This study |
| 71.   | Atu4347 2F-BamHI | 5'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-3' This study |
| 72.   | Atu4347 2R-XmaI | 6'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-5' This study |
| 73.   | VgrG-1 1F-XbaI | pJQ200KS-ΔvgrG-1 5'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-3' This study |
| 74.   | VgrG-1 1R-BamHI | 6'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-5' This study |
| 75.   | VgrG-1 2F-BamHI | 5'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-3' This study |
| 76.   | VgrG-1 2R-XmaI | 6'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-5' This study |
| 77.   | Atu4349 1F-XbaI | pJQ200KS-Δatu434 5'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-3' This study |
| 78.   | Atu4349 1R-BamHI | 6'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-5' This study |
| 79.   | Atu4349 2F-BamHI | 5'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-3' This study |
| 80.   | Atu4349 2R-XmaI | 6'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-5' This study |
| 81.   | Atu4350 1F-XbaI | pJQ200KS-Δatu435 5'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-3' This study |
| 82.   | Atu4350 1R-BamHI | 6'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-5' This study |
| 83.   | Atu4350 2F-BamHI | 5'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-3' This study |
| 84.   | Atu4350 2R-XmaI | 6'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-5' This study |
| 85.   | Atu4352 1F-XbaI | pJQ200KS-Δatu435 5'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-3' This study |
| 86.   | Atu4352 1R-BamHI | 6'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-5' This study |
| 87.   | Atu4352 2F-BamHI | 5'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-3' This study |
| 88.   | Atu4352 2R-XmaI | 6'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-5' This study |
| 89.   | VgrG-2 1F-XbaI | pJQ200KS-ΔvgrG-2 5'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-3' This study |
| 90.   | VgrG-2 1R-BamHI | 6'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-5' This study |
| 91.   | VgrG-2 2F-BamHI | 5'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-3' This study |
| 92.   | VgrG-2 2R-XmaI | 6'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-5' This study |
| 93.   | AopB 1F-XbaI | pJQ200KS-ΔaopB 5'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-3' This study |
| 94.   | AopB 1R-BamHI | 6'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-5' This study |
| 95.   | AopB 2F-BamHI | 5'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-3' This study |
| 96.   | AopB 2R-XmaI | 6'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-5' This study |
| 97.   | TssK comN-HindIII | pTssK 5'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-3' This study |
| 98.   | TssK comC-HindIII | 6'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-5' This study |
| 99.   | Fha comN-BamHI | pFha 5'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-3' This study |
| 100.  | Fha comC-BamHI | 6'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-5' This study |
| 101.  | TssG comN-Xhol | pTssG 5'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-3' This study |
| 102.  | TssG comC-Xhol | 6'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-5' This study |
| 103.  | TssF comN-XhoI | pTssF 5'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-3' This study |
| 104.  | TssF comC-XhoI | 6'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-5' This study |
| 105.  | TssE | pTssE 5'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-3' This study |
| 106.  | TssE comN-XbaI | 6'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-5' This study |
| 107.  | TssC6 comN-XhoI | pTssC6 5'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-3' This study |
| 108.  | TssC6X comN-XbaI | 6'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-5' This study |
| 109.  | TssC6 comN-XhoI | pTssC6 5'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-3' This study |
| 110.  | TssC6X comN-XbaI | 6'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-5' This study |
| 111.  | TssC6 comN-XhoI | pTssC6 5'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-3' This study |
| 112.  | TssC6X comN-XbaI | 6'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-5' This study |
| 113.  | TssC6 comN-XhoI | pTssC6 5'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-3' This study |
| 114.  | TssC6X comN-XbaI | 6'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-5' This study |
| 115.  | TssA comN-XhoI | pTssA 5'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-3' This study |
| 116.  | TssA comC-XbaI | 6'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-5' This study |
| 117.  | ClpV | pClpV 5'-GCCTCAAGGTGTTATTGGAGCTACAGGACG-3' This study |
| 130. VgrG-2 | pVgrG-2 | 5'-CCGCTCGAGAGGATTGATTTGACGCTGATAT-3' | This study |
| 131. VgrG-2 | pVgrG-2 | 5'-CTGTCAGAGTTCTGAAAGTTCCAAAGCT-3' | This study |
| 132. PpaA F-Ndel | pET-PpaA-His | 5'-GAACATATTCGGAGAAGAAAGCCGATCAG-3' | This study |
| 133. PpaA F-Nhol | 5'-CCGCTCGAGAGGATTGATTTGACGCTGATAT-3' | This study |
| 134. PpaA F | pET-PpaA-His | 5'-TGGCGATTACCGGATCAAGG-3' | This study |
| 135. PpaA R-HindIII | 5'-CCGCTCGAGAGGATTGATTTGACGCTGATAT-3' | This study |
| 136. TssK F-Ndel | pET-TssK-His | 5'-GAACATATTCGGAGAAGAAAGCCGATCAG-3' | This study |
| 137. TssK R-HindIII | 5'-CCGCTCGAGAGGATTGATTTGACGCTGATAT-3' | This study |
| 138. Fha F-Ndel | pET-Fha-His | 5'-TGGCGATTACCGGATCAAGG-3' | This study |
| 139. Fha R-Sall | 5'-CCGCTCGAGAGGATTGATTTGACGCTGATAT-3' | This study |
| 140. TssE F-Ndel | pET-TssE-His | 5'-GAACATATTCGGAGAAGAAAGCCGATCAG-3' | This study |
| 141. TssE R-Sall | 5'-CCGCTCGAGAGGATTGATTTGACGCTGATAT-3' | This study |
| 142. TssC1 F-Ndel | pET-TssC1-His | 5'-GAACATATTCGGAGAAGAAAGCCGATCAG-3' | This study |
| 143. TssC1 R-Xhol-a | pET-TssB-TssC1-a-H is (144&143) | 5'-CCGCTCGAGCGATTTCCTGGAGAAATACG-3' | This study |
| 144. TssB F-Ndel | pET-TssB-His | 5'-GAACATATTCGGAGAAGAAAGCCGATCAG-3' | This study |
| 145. TssB R-Xhol | pET-TssB-TssC1-a-H is (144&143) | 5'-CCGCTCGAGCGATTTCCTGGAGAAATACG-3' | This study |
| 146. TssA F-Ndel | pET-TssA-His | 5'-GAACATATTCGGAGAAGAAAGCCGATCAG-3' | This study |
| 147. TssA R-Xhol | 5'-CCGCTCGAGAGGATTGATTTGACGCTGATAT-3' | This study |
| 148. ClpV F | pET-ClpV-His | 5'-TGGCGATTACCGGATCAAGG-3' | This study |
| 149. ClpV R-HindIII | 5'-CCGCTCGAGAGGATTGATTTGACGCTGATAT-3' | This study |
| 150. Ato4346 F-Ndel | pET-Ato4346-His | 5'-GAACATATTCGGAGAAGAAAGCCGATCAG-3' | This study |
| 151. Ato4346 R-Xhol | 5'-CCGCTCGAGAGGATTGATTTGACGCTGATAT-3' | This study |
| 152. Ato4347 F-Ndel | pET-Ato4347-His | 5'-GAACATATTCGGAGAAGAAAGCCGATCAG-3' | This study |
| 153. Ato4347 R-Xhol | 5'-CCGCTCGAGAGGATTGATTTGACGCTGATAT-3' | This study |
| 154. Ato4349 F-Ndel | pET-Ato4349-His | 5'-GAACATATTCGGAGAAGAAAGCCGATCAG-3' | This study |
| 155. Ato4349 R-Xhol | 5'-CCGCTCGAGAGGATTGATTTGACGCTGATAT-3' | This study |
| 156. VgrG-1 F-Ndel | pET-VgrG-1-His | 5'-GAACATATTCGGAGAAGAAAGCCGATCAG-3' | This study |
| 157. VgrG-1 R-Xhol | 5'-CCGCTCGAGAGGATTGATTTGACGCTGATAT-3' | This study |
| 158. RpoA F-Ndel | pET-RpoA-His | 5'-GAACATATTCGGAGAAGAAAGCCGATCAG-3' | This study |
| 159. RpoA R-Xhol | 5'-CCGCTCGAGAGGATTGATTTGACGCTGATAT-3' | This study |
| 160. AopB F-Ndel | pET-AopB-His | 5'-GAACATATTCGGAGAAGAAAGCCGATCAG-3' | This study |
| 161. AopB R-Xhol | 5'-CCGCTCGAGAGGATTGATTTGACGCTGATAT-3' | This study |
| 162. ExoR F (Xmal) | pTrC-ExoR-Strep | 5'-CCGCTCGAGAGGATTGATTTGACGCTGATAT-3' | This study |
| 163. ExoR C-Strep | 5'-CCGCTCGAGAGGATTGATTTGACGCTGATAT-3' | This study |
| 164. TssC1 R-BamHI | pGBK7-TssC1 (142&164) | 5'-CGGATCCCGGCTCGGCGCTCTCTTT-3' | This study |
| 165. TssB-BamHI | pGBK7-TssB (144&165) | 5'-CGGATCCCGGCTCGGCGCTCTCTTT-3' | This study |
| 166. Ato4329 RT1 | 5'-GAAGACCGCTGAACTACACTACAC-3' | This study |
| 167. Ato4329 | 5'-CGGATCCGACGATGATAGATCACCGGC-3' | This study |
| 168. PpaA RT1 | 5'-AGGGATCCGATCTCTGATCTCTTT-3' | This study |
| 169. PpaA RT2-BamHI | 5'-CCGCTCGAGCAGCTATCCTTTCTTT-3' | This study |
| 170. TssG RT1 | 5'-CCGCTCGAGCAGCTATCCTTTCTTT-3' | This study |
| 171. TssG-R2-BamHI | 5'-CGGATCCGCGATCCTTTCTTT-3' | This study |
| 172. TssA RT1 | 5'-CGGATCCGCGATCCTTTCTTT-3' | This study |
| 173. TssA-R2-BamHI | 5'-CGGATCCGCGATCCTTTCTTT-3' | This study |
| 174. ClpV RT1 | 5'-CGGATCCGCGATCCTTTCTTT-3' | This study |
| 175. ClpV RT2-BamHI | 5'-CGGATCCGCGATCCTTTCTTT-3' | This study |
| 176. VgrG-1 RT1 | 5'-CGGATCCGCGATCCTTTCTTT-3' | This study |
| 177. VgrG-1 RT2-BamHI | 5'-CGGATCCGCGATCCTTTCTTT-3' | This study |
| 178. Ato4352 RT1 | 5'-TGAGCTGAAAGGCTCATAGTC-3' | This study |
| 179. Ato4352 | 5'-CGGATCCGCGATCCTTTCTTT-3' | This study |
| 180. Ato4353 RT1 | 5'-TGAGCTGAAAGGCTCATAGTC-3' | This study |
| 181. Ato4353 | 5'-CGGATCCGCGATCCTTTCTTT-3' | This study |
| 182. RT2-BamHI | 5'-TGAGCTGAAAGGCTCATAGTC-3' | This study |
| 183. RT2-BamHI | 5'-TGAGCTGAAAGGCTCATAGTC-3' | This study |
| 184. 46/47 1F-Xmal | pQ200KS-Δ66Δ47 | 5'-TGCTAGAGCTATTTCCGTCATAGGAG-3' | This study |
| 185. 46/47 1R-BamHI | 5'-TGAGCTGAAAGGCTCATAGTC-3' | This study |
| 186. 46/47 2F-BamHI | 5'-TGAGCTGAAAGGCTCATAGTC-3' | This study |
| 187. 46/47 2R-Xmal | 5'-TGAGCTGAAAGGCTCATAGTC-3' | This study |

*Restriction enzyme sites are underlined in bold face.*
Table S3. Characteristics of proteins encoded by the imp cluster.

| Gene name | Protein name | Conserved Ortholog<sup>a</sup> | Molecular weight<sup>b</sup> (Da) / pl | Essential for Hcp secretion<sup>c</sup> | Predicted cellular localization<sup>d,e</sup> | Predicted signal peptide<sup>f,g</sup> | Predicted non-classically secreted protein<sup>h</sup> |
|-----------|-------------|-------------------------------|--------------------------------------|------------------------------------------|-----------------------------------------------|-----------------------------------|-----------------------------------------------|
| attu4330  | PpkA       | V                             | 270 a.a.                             | −/+                                      | 1.<sup>i</sup> Unknown                        | 1.<sup>i</sup> No SP                  | No                                             |
|           | TagE       |                               | 29,472 / 6.71                        |                                          | 2.<sup>i</sup> C (cytoplasmic)                | 2.<sup>i</sup> No SP                  | (0.08)                                        |
| attu4331  | PppA       | V                             | 471 a.a.                             | −                                        | 1. Cytoplasmic membrane                       | 1. No SP                          | No                                             |
|           | TagF       |                               | 51,714 / 6.39                        |                                          | 2. C (cytoplasmic)                            | 2. No SP                          | (0.14)                                        |
| attu4332  | TssM       | V                             | 1159 a.a.                            | +                                       | 1. Cytoplasmic membrane                       | 1. No SP                          | No                                             |
|           |            |                                | 128,315 / 6.27                       |                                          | 2. IM (inner membrane protein with 3 TM) (26-48, 57-79, 438-460)<sup>i</sup> | 2. No SP                          | (0.12)                                        |
| attu4333  | TssL       | V                             | 501 a.a.                             | +                                       | 1. Cytoplasmic membrane                       | 1. No SP                          | No                                             |
|           |            |                                | 55,188 / 5.85                        |                                          | 2. IM (inner membrane protein with 1 TM) (254-276)<sup>i</sup> | 2. No SP                          | (0.19)                                        |
| attu4334  | TssK       | V                             | 446 a. a.                            | +                                       | 1. Cytoplasmic                                | 1. No SP                          | No                                             |
|           |            |                                | 49,659 / 5.51                        |                                          | 2. C (cytoplasmic)                            | 2. No SP                          | (0.10)                                        |
| attu4335  | Fha        | V                             | 399 a.a.                             | +                                       | 1. Cytoplasmic                                | 1. No SP                          | No                                             |
|           | TagH       |                               | 43,399 / 4.85                        |                                          | 2. C (cytoplasmic)                            | 2. No SP                          | (0.19)                                        |
| attu4336  | TssG       | V                             | 334 a.a.                             | +                                       | 1. Unknown                                     | 1. No SP                          | No                                             |
|           |            |                                | 36,123 / 9.35                        |                                          | 2. C (cytoplasmic)                            | 2. No SP                          | (0.20)                                        |
| attu4337  | TssF       | V                             | 593 a.a.                             | +                                       | 1. Unknown                                     | 1. No SP                          | No                                             |
|           |            |                                | 65,507 / 6.26                        |                                          | 2. C (cytoplasmic)                            | 2. No SP                          | (0.11)                                        |
| attu4338  | TssE       | V                             | 169 a.a.                             | +                                       | 1. Cytoplasmic                                | 1. No SP                          | No                                             |
|           |            |                                | 19,002 / 5.93                        |                                          | 2. C (cytoplasmic)                            | 2. No SP                          | (0.29)                                        |
| attu4339  | TagJ       | V                             | 274 a.a.                             | −                                       | 1. Cytoplasmic                                | 1. No SP                          | No                                             |
|           |            |                                | 29,813 / 4.88                        |                                          | 2. C (cytoplasmic)                            | 2. No SP                          | (0.09)                                        |
| attu4340  | TssC<sub>41</sub> | V                             | 464 a.a.                             | +                                       | 1. Cytoplasmic                                | 1. No SP                          | No                                             |
|           |            |                                | 50,949 / 5.51                        |                                          | 2. C (cytoplasmic)                            | 2. No SP                          | (0.16)                                        |
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| Gene   | Description | a.a. | Mol. Wt. / pI | Cytoplasmic | Periplasmic | Outer Membrane | Essential for Hcp secretion |
|--------|-------------|------|---------------|-------------|------------|----------------|----------------------------|
| attu4341 | V           | 493  | 55,032 / 5.44 | +           | 1. Cytoplasmic | 2. C (cytoplasmic) | No                          |
| TssC   |             |      |               |             | 1. No SP   | 2. No SP       | (0.39)                     |
| attu4342 | V           | 169  | 19,098 / 5    | +           | 1. Cytoplasmic | 2. C (cytoplasmic) | No                          |
| TssB   |             |      |               |             | 1. No SP   | 2. No SP       | (0.10)                     |
| attu4343 | V           | 351  | 38,491 / 5.14 | +           | 1. Unknown   | 2. C (cytoplasmic) | No                          |
| TssA   |             |      |               |             | 1. No SP   | 2. No SP       | (0.08)                     |
| attu4344 | V           | 892  | 96,506 / 5.09 | +           | 1. Cytoplasmic | 2. C (cytoplasmic) | No                          |
| ClpV   |             |      |               |             | 1. No SP   | 2. No SP       | (0.09)                     |
| TssH   |             |      |               |             |            |                |                            |
| attu4345 | V           | 158  | 17,316 / 5.12 | +           | 1. Unknown   | 2. EC (extracellular) | Yes                        |
| Hcp    |             |      |               |             | 1. No SP   | 2. No SP       | (Score 0.947)              |
| TssD   |             |      |               |             |            |                |                            |
| attu4346 | V           | 129  | 13,737 / 4.93 | −           | 1. Unknown   | 2. P (periplasm) | Yes                        |
| Atu4346|             |      |               |             | 1. Yes(1-25)| 2. Yes(1-25)    | (Score 0.539)              |
| attu4347 | V           | 166  | 18,271 / 9.05 | −           | 1. Unknown   | 2. OM (outer membrane) | Yes                        |
| Atu4347|             |      |               |             | 1. No SP   | 2. No SP       | (Score 0.941)              |
| attu4348 | V           | 816  | 88,523 / 5.98 | (+)         | 1. Cytoplasmic | 2. C (cytoplasmic) | Yes                        |
| VgrG-1 |             |      |               |             | 1. No SP   | 2. No SP       | (Score 0.624)              |
| TssI-1 |             |      |               |             |            |                |                            |
| attu4349 | V           | 318  | 34,604 / 5.24 | −           | 1. Unknown   | 2. C (cytoplasmic) | Yes                        |
| Atu4349|             |      |               |             | 1. No SP   | 2. No SP       | (Score 0.733)              |
| attu4350 | V           | 278  | 29,511 / 5.17 | −           | 1. Periplasmic | 2. OM (outer membrane) | Yes                        |
| Atu4350|             |      |               |             | 1. No SP   | 2. Yes(1-37)   | (Score 0.923)              |
| attu4351 | V           | 224  | 24,945 / 7.74 | NA          | 1. Unknown   | 2. C (cytoplasmic) | No                         |
| Atu4351|             |      |               |             | 1. No SP   | 2. No SP       | (0.11)                     |
| attu4352 | V           | 101  | 10,371 / 7.79 | −           | 1. Unknown   | 2. EC (extracellular) | Yes                        |
| Atu4352|             |      |               |             | 1. No SP   | 2. No SP       | (Score 0.878)              |
| attu3642 | V           | 754  | 81,764 / 5.8  | (+)         | 1. Cytoplasmic | 2. C (cytoplasmic) | Yes                        |
| VgrG-2 |             |      |               |             | 1. No SP   | 2. No SP       | (Score 0.598)              |

a.a.: amino acid.
a: Conserved orthologs: These data have been reported elsewhere [10-12].
b: The molecular weight and isoelectric point (pI) are based on prediction by the software ExPASy (http://www.expasy.ch/tools/pi_tool.html).
c: Essential for Hcp secretion is based on experimental data presented in Fig. 2.
d: The cellular localization is based on prediction by the software PSORTb (http://www.psort.org/psortb/index.html).
e: The cellular localization is based on prediction by the software SOSUIGramN (http://bp.nuap.nagoya-u.ac.jp/sosui/sosuigramn/sosuigramn_submit.html).
f: The prediction of signal peptides was by use of the software SignalP (http://www.cbs.dtu.dk/services/SignalP/).
g: The prediction of signal peptides was by use of the software SOSUIsignal (http://bp.nuap.nagoya-u.ac.jp/sosui/sosuisignal/sosuisignal_submit.html).
h: The prediction was by use of the software SecretomeP (http://www.cbs.dtu.dk/services/SecretomeP/). Non-classically secreted proteins should obtain an NN-score exceeding the threshold of 0.5.

References
1. Vergunst AC, Schrammeijer B, den Dulk-Ras A, de Vlaam CM, Regensburg-Tuink TJ, et al. (2000) VirB/D4-dependent protein translocation from Agrobacterium into plant cells. Science 290: 979-982.
2. Ma LS, Lin JS, Lai EM (2009) An IcmF family protein, ImpLM, is an integral inner membrane protein interacting with ImpKL, and its walker a motif is required for type VI secretion system-mediated Hcp secretion in Agrobacterium tumefaciens. J Bacteriol 191: 4316-4329.
3. Wu HY, Chung PC, Shih HW, Wen SR, Lai EM (2008) Secretome analysis uncovers an Hcp-family protein secreted via a type VI secretion system in Agrobacterium tumefaciens. J Bacteriol 190: 2841-2850.
4. Liu AC, Shih HW, Hsu T, Lai EM (2008) A citrate-inducible gene, encoding a putative tricarboxylate transporter, is downregulated by the organic solvent DMSO in Agrobacterium tumefaciens. J Appl Microbiol 105: 1372-1383.
5. Studier FW, Rosenberg AH, Dunn JJ, Dubendorff JW (1990) Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol 185: 60-89.
6. Quandt J, Hynes MF (1993) Versatile suicide vectors which allow direct selection for gene replacement in gram-negative bacteria. Gene 127: 15-21.
7. Schmidt-Eisenlohr H, Domke N, Baron C (1999) TraC of IncN plasmid pKM101 associates with membranes and extracellular high-molecular-weight structures in Escherichia coli. J Bacteriol 181: 5563-5571.
8. Wu CF, Lin JS, Shaw GC, Lai EM (2012) Acid-induced type VI secretion system is regulated by ExoR-ChvG/ChvI signaling cascade in Agrobacterium
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tumefaciens. PLoS Pathog 8: e1002938.
9. Lai EM, Shih HW, Wen SR, Cheng MW, Hwang HH, et al. (2006) Proteomic analysis of Agrobacterium tumefaciens response to the vir gene inducer acetosyringone. Proteomics 6: 4130-4136.
10. Cascales E (2008) The type VI secretion toolkit. EMBO Rep 9: 735-741.
11. Zheng J, Leung KY (2007) Dissection of a type VI secretion system in Edwardsiella tarda. Mol Microbiol 66: 1192-1206.
12. Zheng J, Ho B, Mekalanos JJ (2011) Genetic analysis of anti-amoebae and anti-bacterial activities of the type VI secretion system in Vibrio cholerae. PLoS One 6: e23876.
Fig. S2

(A) 

![Graph showing OD₄₅₀ nm values for ActC, TssC₄₁, and TssB.]

(B) 

![Graph showing OD₄₅₀ nm values for AopB, Hcp, and ActC.]

(C) 

![Western blot images for AopB, Hcp, and ActC.]

Legend:
- C58 (None)
- C58 (Lysozyme)
- ΔactCBA (None)
- ΔactCBA (Lysozyme)
Fig. S3

| Protein   | T | P | Sp | S | IS |
|------------|---|---|----|---|----|
| TssC_{41}  |   |   |    |   |    |
| TssB       |   |   |    |   |    |
| Hcp        |   |   |    |   |    |
| 47         |   |   |    |   |    |
| VgrGs      |   |   |    |   |    |
| TssM       |   |   |    |   |    |
| Fha        |   |   |    |   |    |
| ActC       |   |   |    |   |    |

* Image of Western blot showing protein expression levels.
Fig. S4
Fig. S5

(A) Vector + Hcp
TssC<sub>41</sub>-His + Hcp
TssB-His + Hcp
TssC<sub>41</sub>-His + TssB + Hcp

(B) Vector + 47
TssC<sub>41</sub>-His + 47
TssB-His + 47
TssC<sub>41</sub>-His + TssB + 47

(C) Vector + VgrG
TssC<sub>41</sub>-His + VgrG
TssB-His + VgrG
TssC<sub>41</sub>-His + TssB + VgrG

(D) Vector + ExoR-Strep
TssC<sub>41</sub>-His + ExoR-Strep
TssB-His + ExoR-Strep
TssC<sub>41</sub>-His + TssB + ExoR-Strep