

1 **A novel widespread bacterial structure related to the flagellar type III**
2 **secretion system**

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27 **Abstract**

28 The flagellar type III secretion system (fT3SS) is a suite of membrane-embedded and cytoplasmic
29 proteins responsible for building the bacterial flagellar motility machinery. Homologous proteins
30 form the injectisome machinery bacteria use to deliver effector proteins into eukaryotic cells, and
31 other family members have recently been reported to be involved in the formation of membrane
32 nanotubes. Here we describe a novel, ubiquitous and evolutionarily widespread hat-shaped
33 structure embedded in the inner membrane of bacteria, of yet-unidentified function, that is related
34 to the fT3SS, adding to the already rich repertoire of this family of nanomachines.

35

36 Type III secretion systems (T3SS) assemble bacterial machinery with diverse functions. In
37 addition to forming the flagellum and the injectisome, they have also been reported recently to be
38 involved in the formation of membrane tubes [1]. The flagellar type III secretion system (fT3SS)
39 consists of a cytoplasmic part containing an ATPase and an inner-membrane (IM)-embedded part
40 known as the core complex (fT3SScc). The fT3SScc consists of five proteins (FliP, FliQ, FliR,
41 FlhB and FlhA), with another protein, FliO, required for assembly but which does not form part
42 of the complex [2,3]. Initially, FliP forms a pentameric platform on which FliQ, FliR and FlhB
43 assemble to create a FliP₅FliQ₄FliR₁FlhB₁ subcomplex upon which an FlhA ring is built [4].

44

45 While using electron cryo-tomography (cryo-ET) to study the process of flagellar assembly in
46 *Helicobacter pylori*, we identified a periplasmic hat-shaped structure embedded in the inner
47 membrane (IM) of the cell (Fig. 1). The structure was abundant and, in contrast to the polar flagella
48 of this species, did not show any preferred spatial localization in the cell (e.g., not exclusively at
49 the cell pole). Carefully reexamining tens of thousands of cryotomograms of other,
50 phylogenetically-diverse bacterial species our lab has imaged over the past 15 years, we found that
51 this hat-like structure is widespread in diverse Gram-negative and Gram-positive bacteria (Fig. 2;
52 see also Supporting Figure S1). In many cases, we observed multiple hat-like structures (up to 10
53 in some cells) distributed around the cell (see Movie S1 for an example from an *E. coli* cell that
54 was partially lysed, enhancing visibility of periplasmic structures). Subtomogram averages of the
55 structure from different species revealed conserved characteristics: a hat-shaped part in the
56 periplasm and two cytoplasmic densities beneath (Fig. 2). In general, the periplasmic hat-like
57 portion had a diameter of ~24-26 nm at its widest point at the outer surface of the IM. The
58 cytoplasmic densities were absent in the averages from three species: *Pseudoalteromonas*

59 *luteoviolacea*, *Hylemonella gracilis* and *Bacillus subtilis*. The absence of these densities in *P.*
60 *luteoviolacea* and *B. subtilis* is likely due to the fact that these were lysed and not intact cells. We
61 also observed that the cytoplasmic density did not resolve into two distinct sections in all species.

62

63 We also identified the same structure in several *H. pylori* flagellar mutants: *fliP*^{*}, $\Delta flgS fliP$ ^{*},
64 $\Delta fliM fliP$ ^{*}, $\Delta fliG fliP$ ^{*}, $\Delta fliO fliP$ ^{*}, and $\Delta fliQ fliP$ ^{*}. The *H. pylori fliP*^{*} strain contains a naturally-
65 occurring point mutation that disrupts the function of FliP [5] and prevents the assembly of the
66 fT3SScc (manuscript in preparation). The other mutants remove additional fT3SScc proteins
67 ($\Delta fliO$ and $\Delta fliQ$), flagellar basal body proteins ($\Delta fliM$ and $\Delta fliG$), or the tyrosine kinase
68 responsible for expression of the class II flagellar genes ($\Delta flgS$) [6]. Curiously, in all of these
69 mutants the diameter of the hat-like density was reduced to only ~20 nm (at its widest part) and
70 the two cytoplasmic densities were missing (or less well resolved) (Fig. 3 A-G). This difference
71 was not due to decreased resolution, since more particles were averaged than from wild-type cells
72 (see Materials and Methods). This observation suggested to us that the hat-like structure is related
73 to the fT3SScc. Indeed the general shape is reminiscent of the MS-ring of the flagellar motor, and
74 we observed the disappearance of two similar cytoplasmic densities in the motor (corresponding
75 to FlhA_C) in the same mutants while studying flagellar assembly (manuscript in preparation). The
76 reduced width of the hat-like structure in *fliP*^{*} cells is also reminiscent of the reduced width of
77 flagellar complexes in the absence of the fT3SScc [7].

78

79 To explore this relationship, we examined *Campylobacter jejuni* mutants of other fTSScc proteins.
80 These included mutants of the C-terminal domains of FlhA ($\Delta flhAc$) and FlhB ($\Delta flhBc$) [8]. In
81 $\Delta flhAc$ cells, compared to wild-type, the periplasmic hat-like part was again smaller in diameter

82 and the two cytoplasmic densities disappeared (Fig. 3 I & J). In contrast, the hat-like structure in
83 $\Delta flhBc$ cells was indistinguishable from the wild-type complex both in diameter and the presence
84 of the associated cytoplasmic densities (Fig. 3 K). This is not too surprising, since, unlike the large
85 pentameric FliP ring or the nonameric FlhA ring, FlhB is a small protein present in a monomeric
86 form in the fT3SScc. Although the absence of the C-terminus of FlhB renders the fT3SS non-
87 functional (no full flagella assemble in $\Delta flhBc$ *C. jejuni* [8]), the fT3SScc can still assemble
88 (manuscript in preparation). To confirm the generality of the relationship between the fT3SScc
89 and the hat-like complex, we imaged an *flhA* mutant in *P. aeruginosa* (*flhA*^{*}, obtained from a
90 transposon insertion mutant library). Here also, the hat-like structure was smaller in size and lacked
91 clear cytoplasmic densities compared to wild-type (Fig. 3 L & M).

92

93 Based on the apparent relationship between the fT3SScc and the hat-like structure, we
94 hypothesized that the novel complex is formed by the flagellar MS-ring protein, FliF, adopting a
95 different, more closed conformation than that seen in the fully-assembled flagellar motor. Hence,
96 we generated and imaged a $\Delta fliF$ mutant in the *H. pylori* *fliP*^{*} background. However, the hat-like
97 complex was still present in this mutant, indicating that it is not formed by FliF (Fig. 3 H). Thus
98 our observations suggest that while the cytoplasmic densities of the complex could be FlhAc, the
99 periplasmic density is not formed by FliF or any of the fT3SScc proteins. Of course, it is also
100 possible that FlhAc does not directly constitute the cytoplasmic densities, but rather that the
101 fT3SScc proteins are regulating the expression (or localization) of another protein(s) that does.

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103 One possibility is that the hat-like structure we discovered here might represent an as-yet
104 unidentified scaffold that helps the fT3SScc assemble. If this structure is some sort of scaffold and

105 the fT3SScc (or part of it) assembles within the hat-like portion and if the cytoplasmic densities
106 are FlhAC, this would explain the disappearance of the cytoplasmic densities and the smaller
107 diameter of the periplasmic portion in *fliP** and *flhA* mutants. The second possibility is that
108 fT3SScc proteins in some way regulate other proteins which themselves form the hat-like complex.
109 Such a regulatory role has been indicated previously for one of the fT3SScc proteins, FliO, which
110 is responsible for the optimal expression of other flagellar genes [9].

111

112 Whatever the function of this hat-like complex, it joins the already-rich repertoire of the (f)T3SS,
113 which has roles in flagellar motility, protein translocation and possibly membrane nanotube
114 formation. Whether the hat-like structure is connected to any of these functions or plays another,
115 yet-unidentified role remains to be elucidated. It is also possible that the apparently ancient
116 structure may have diverged to serve different functions in different species.

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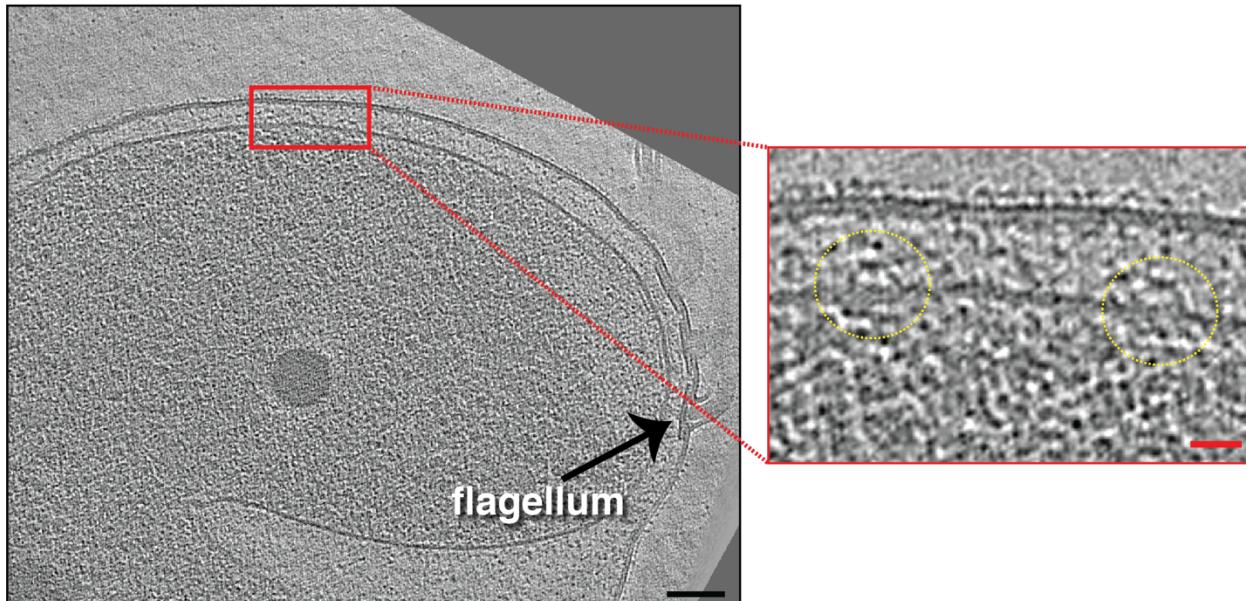
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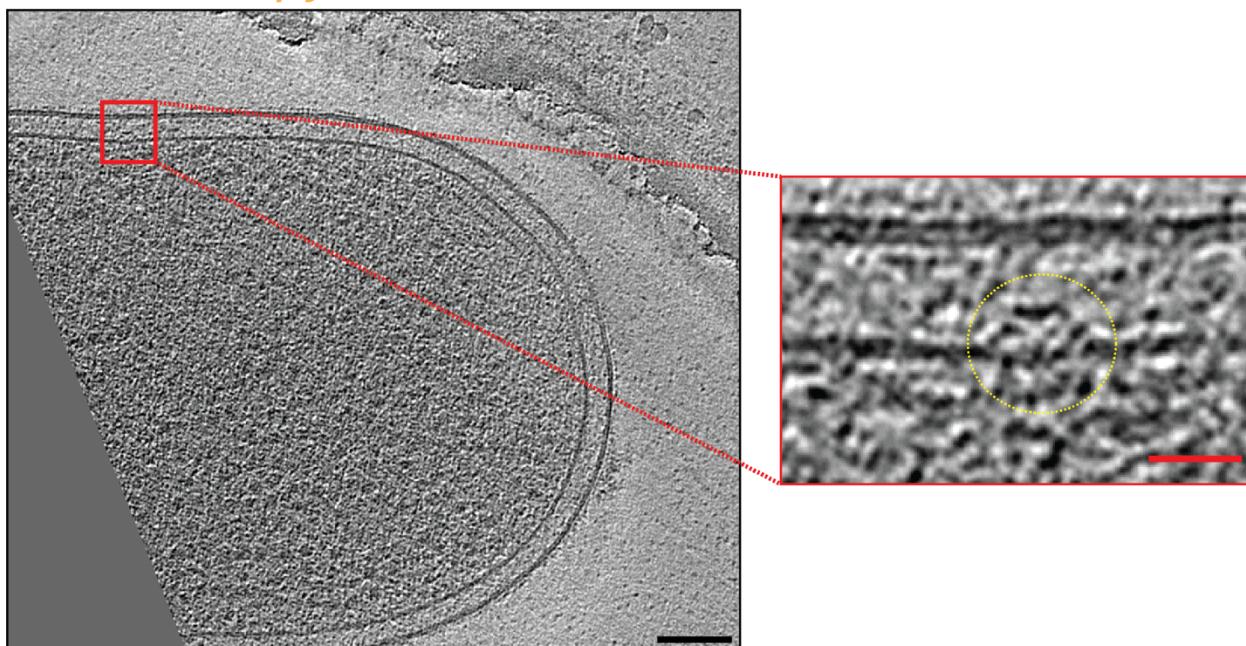
151 **Figures**

152 **Figure 1**

H. pylori



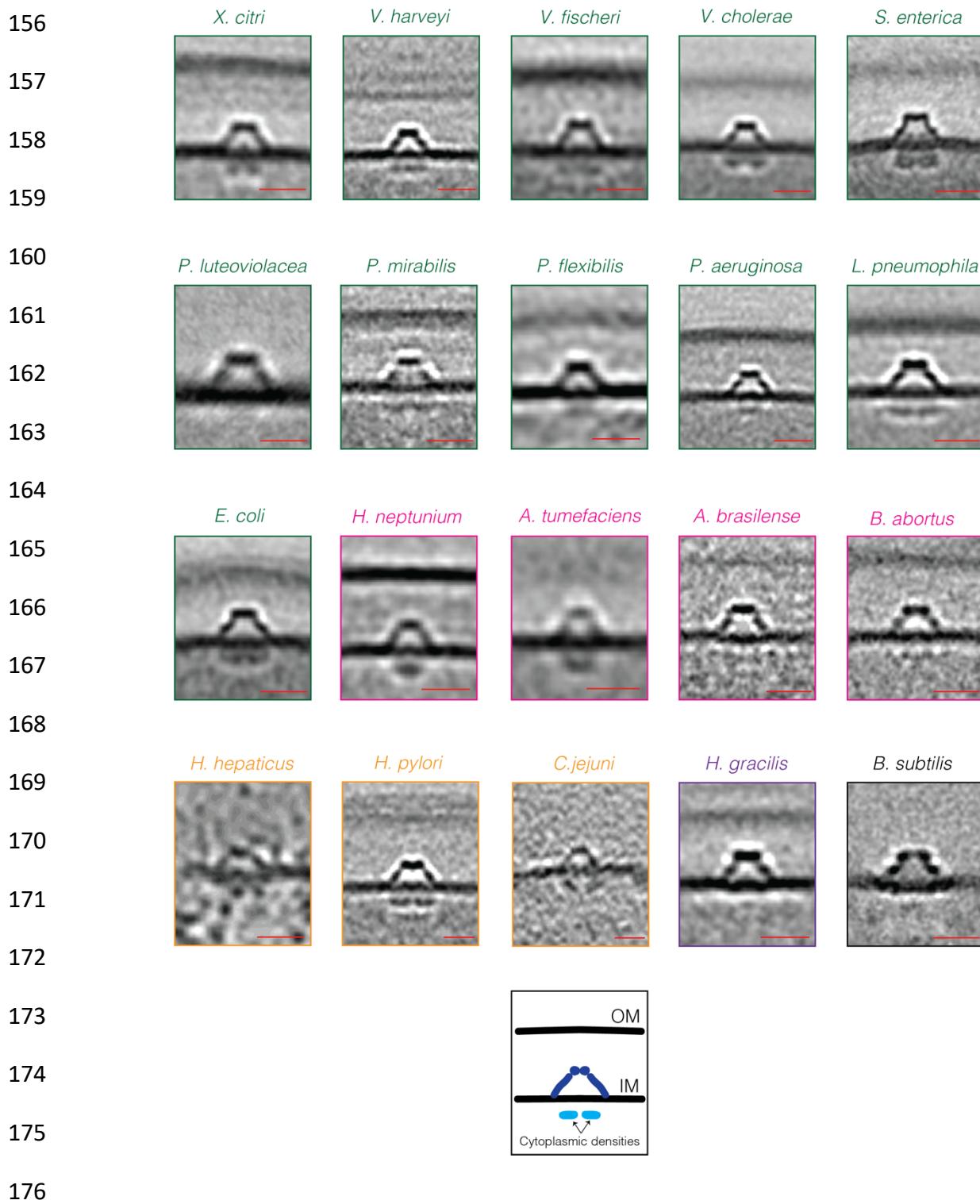
*H. pylori fliP**



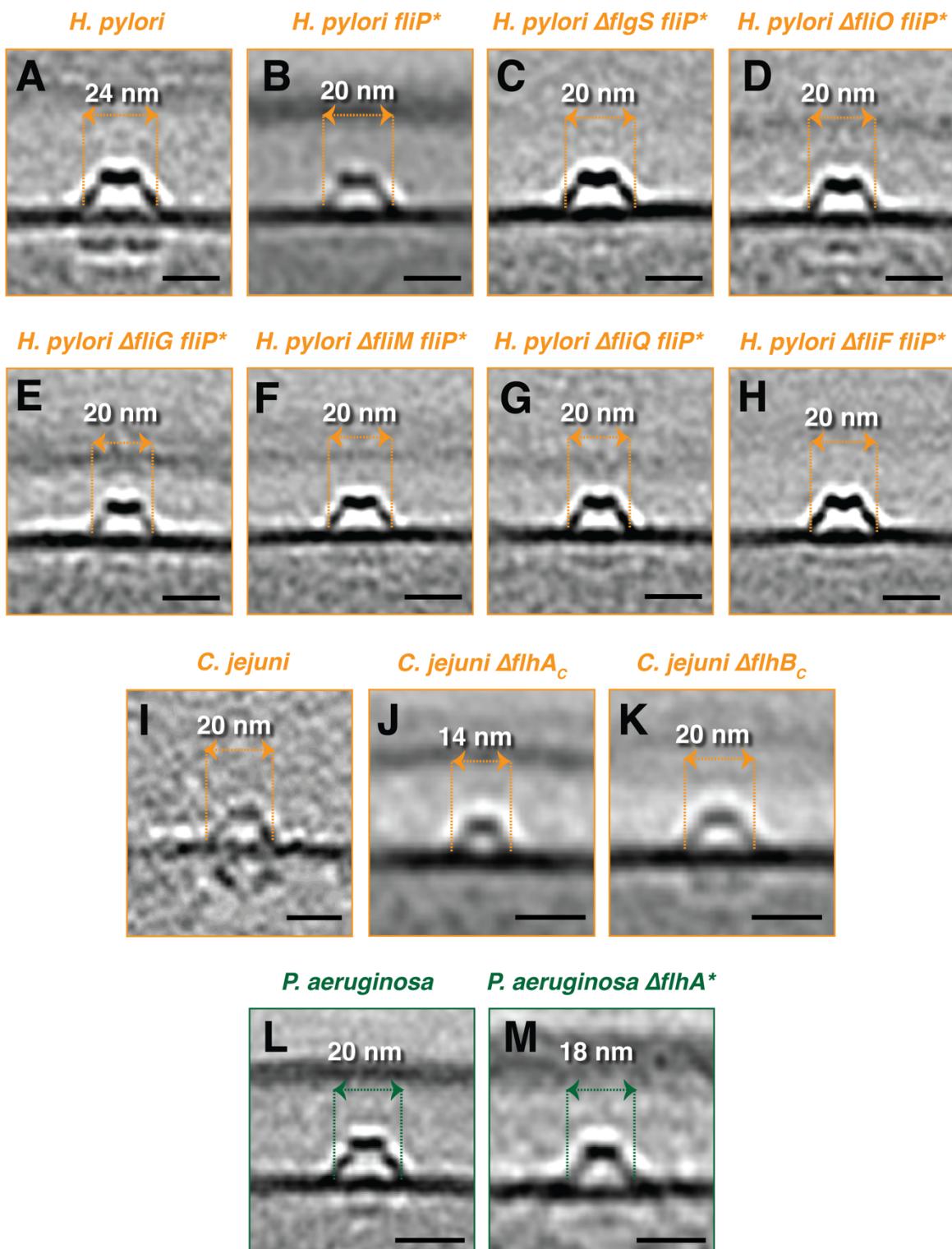
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155 **Figure 2**



177 **Figure 3**



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180 **Figure legends:**

181 **Figure 1: Identification of a novel hat-like complex in *H. pylori*. A & B)** slices through electron
182 cryotomograms of *H. pylori* (A) or *H. pylori fliP** (B) cells showing the presence of hat-like
183 complexes (enlarged in red boxes). Black scale bars 100 nm, red scale bars 25 nm.

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185 **Figure 2: The hat-like complex is a widespread bacterial structure.** A gallery of the hat-like
186 complex in different bacterial species (*Xanthomonas citri*, *Vibrio harveyi*, *V. fischeri*, *V. cholerae*,
187 *Salmonella enterica*, *Pseudoalteromonas luteoviolacea*, *Proteus mirabilis*, *Pseudomonas flexibilis*,
188 *P. aeruginosa*, *Legionella pneumophila*, *Escherichia coli*, *Hyphomonas neptunium*,
189 *Agrobacterium tumefaciens*, *Azospirillum brasilense*, *Brucella abortus*, *Helicobacter hepaticus*,
190 *H. pylori*, *Campylobacter jejuni*, *Hylemonella gracilis* and *Bacillus subtilis*). Sub-tomogram
191 averages are shown, except for *C. jejuni* and *H. hepaticus*, where not enough data was available
192 for averaging so single tomographic slices are shown. Color coding indicates taxonomic class:
193 green, Gammaproteobacteria; pink, Alphaproteobacteria; yellow, Epsilonproteobacteria; purple,
194 Betaproteobacteria; and black, Bacilli. Scale bars are 20 nm.

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196 **Figure 3: The effect of various flagellar-related mutations on the hat-like complex.** Central
197 slices through sub-tomogram averages (except (I), where a single tomographic slice is shown) of
198 the hat-like complex in wild-type cells and the indicated mutants of *H. pylori* (A-H), *C. jejuni* (I-
199 K) and *P. aeruginosa* (L & M). Scale bars are 20 nm.

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Supporting Information for

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A novel widespread bacterial structure related to the flagellar type III 203 secretion system

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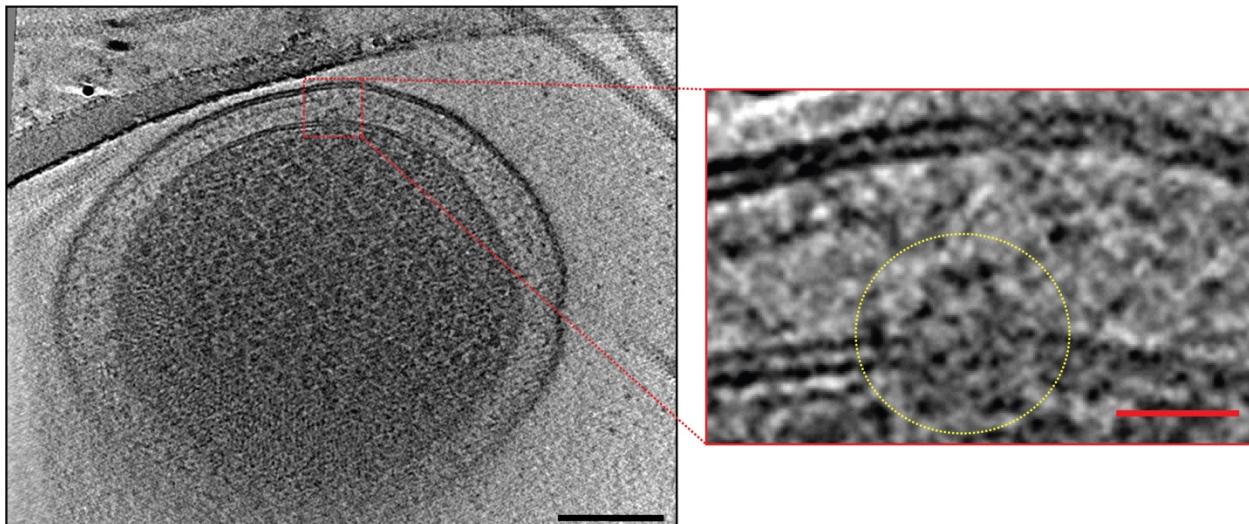
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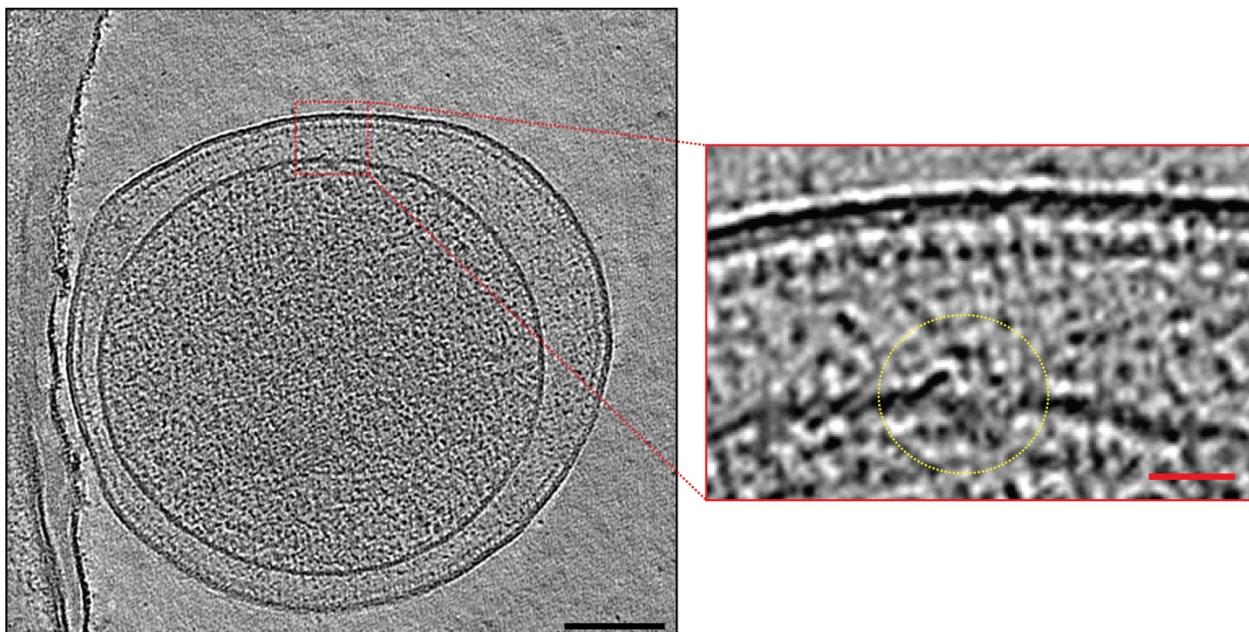
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209 **Supporting Figures:**

S. enterica



E. coli



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V. cholerae

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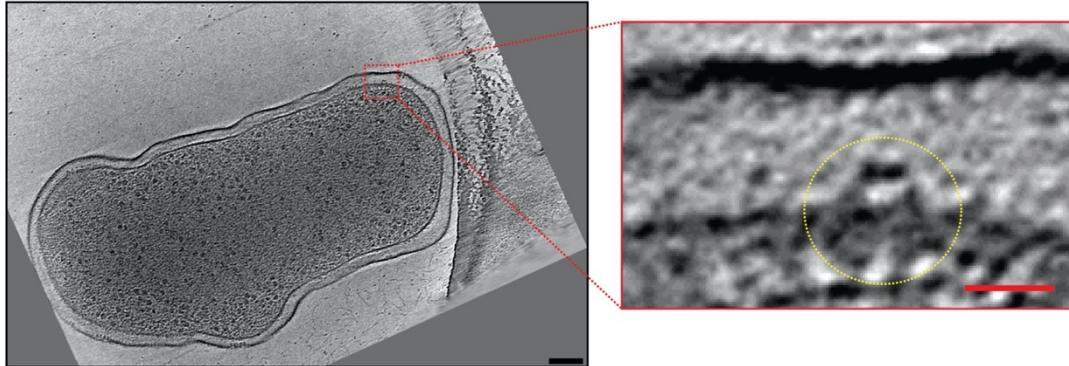
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V. fischeri

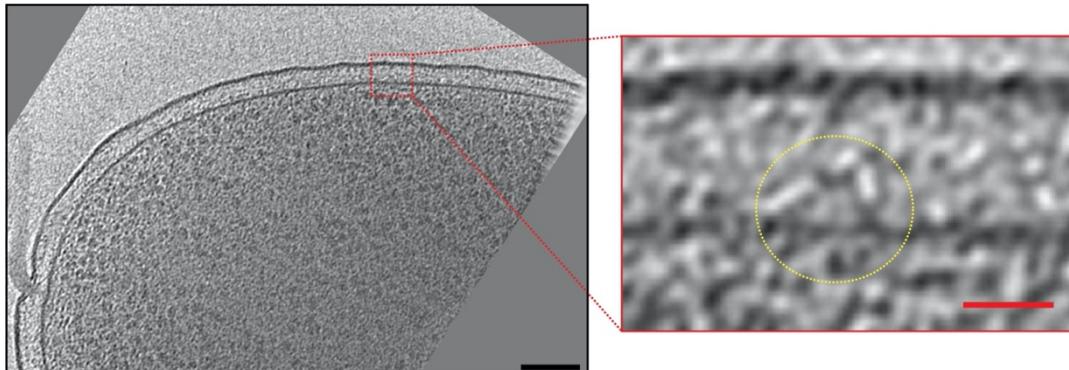
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V. harveyi

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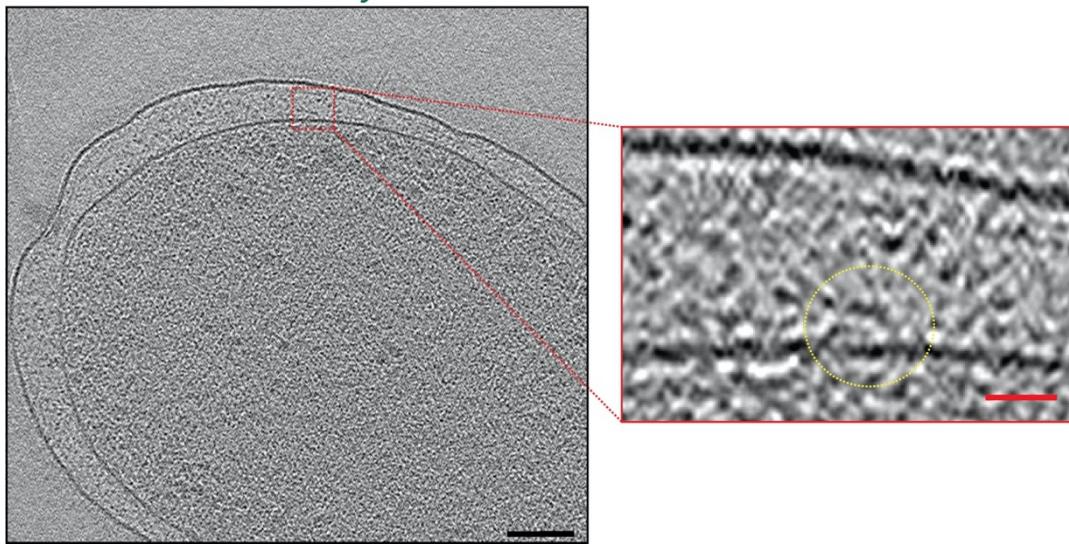
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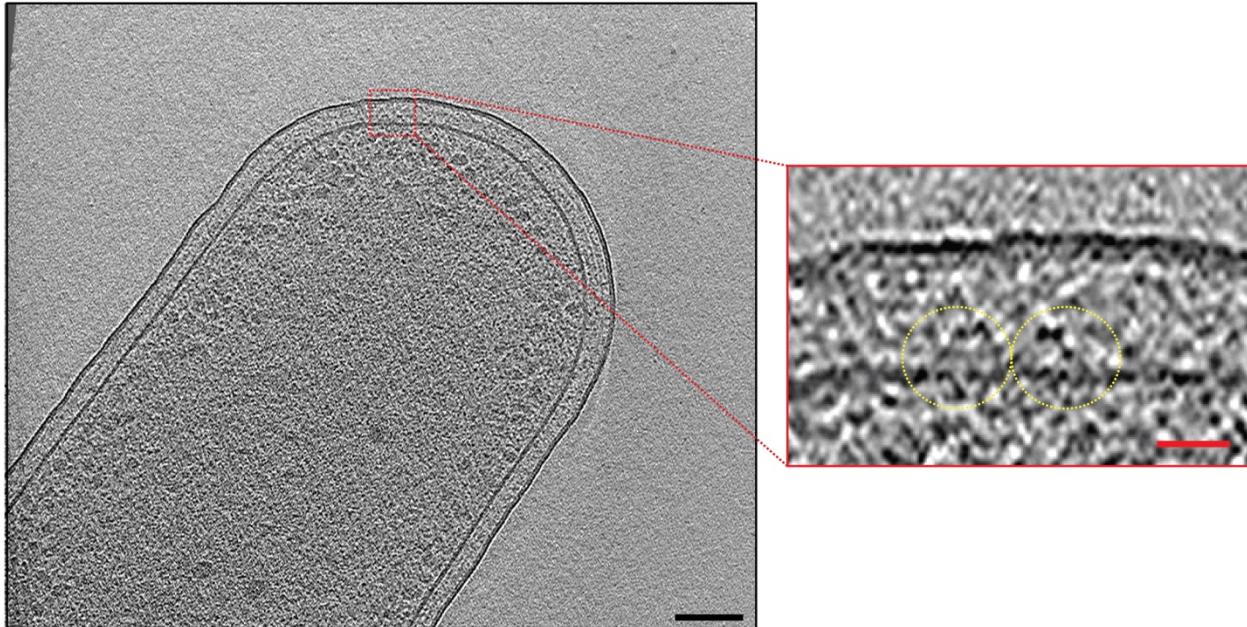
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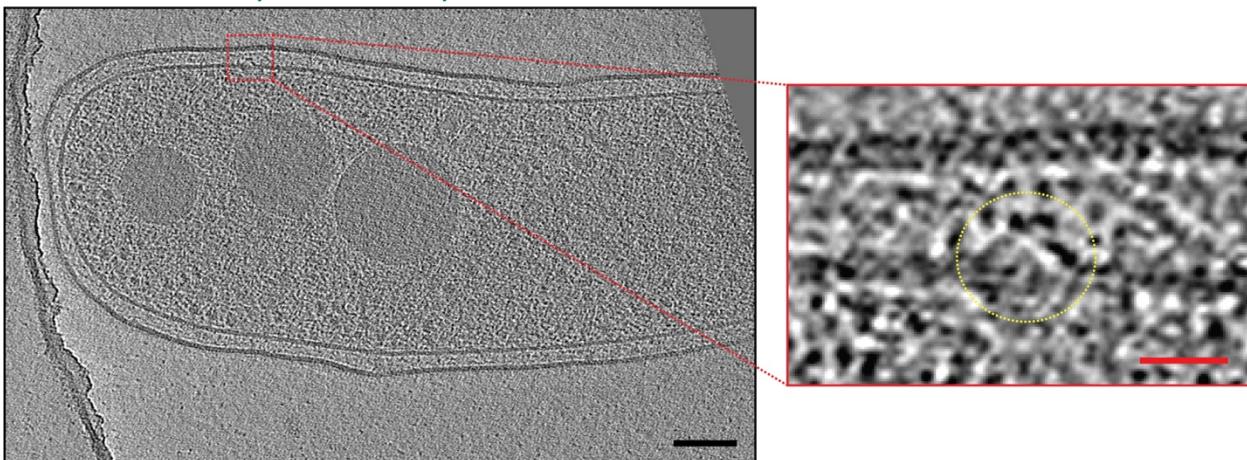
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P. aeruginosa



L. pneumophila



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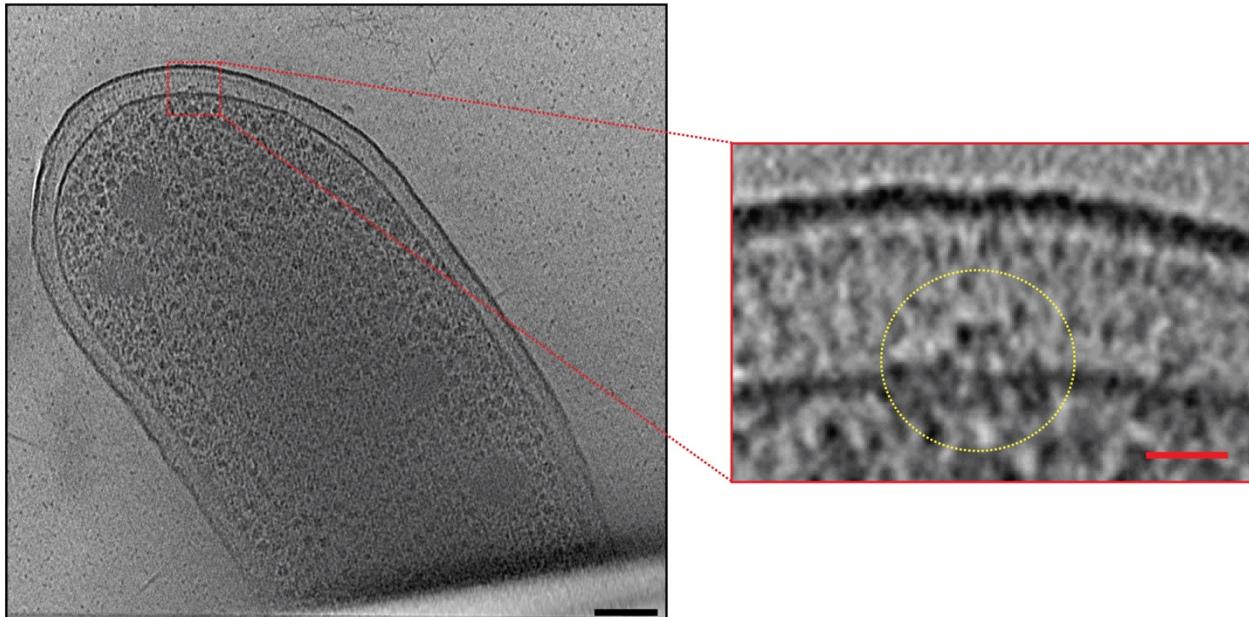
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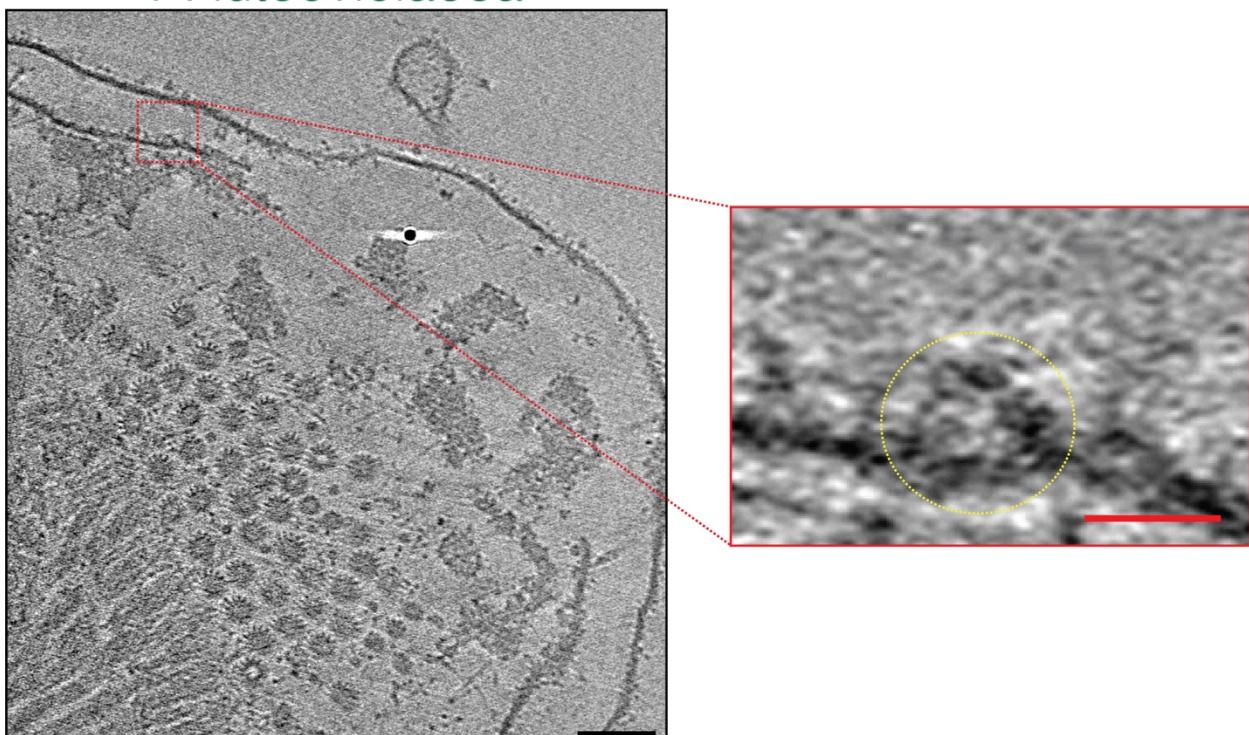
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X. citri



P. luteoviolacea

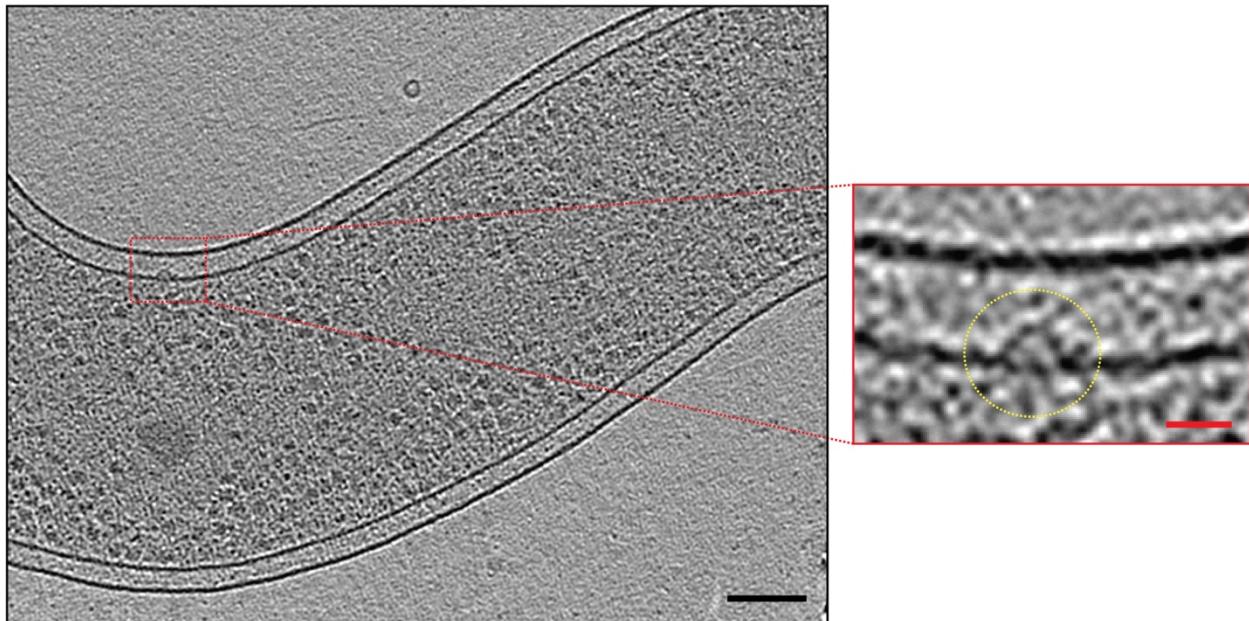


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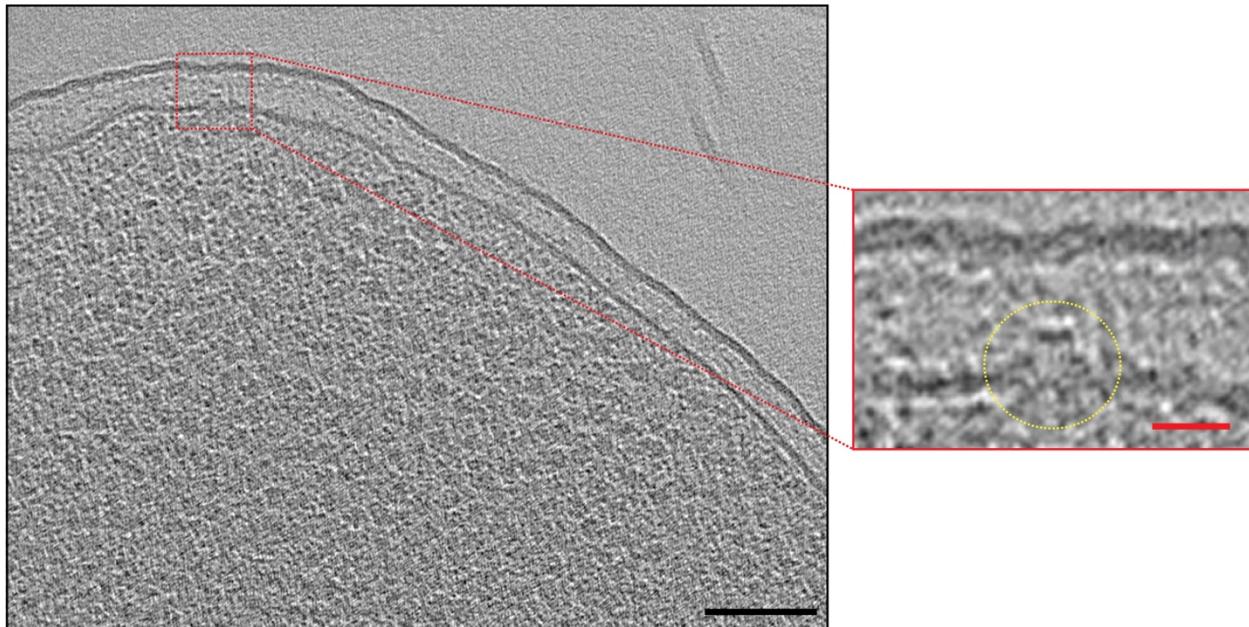
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P. flexibilis



P. mirabilis



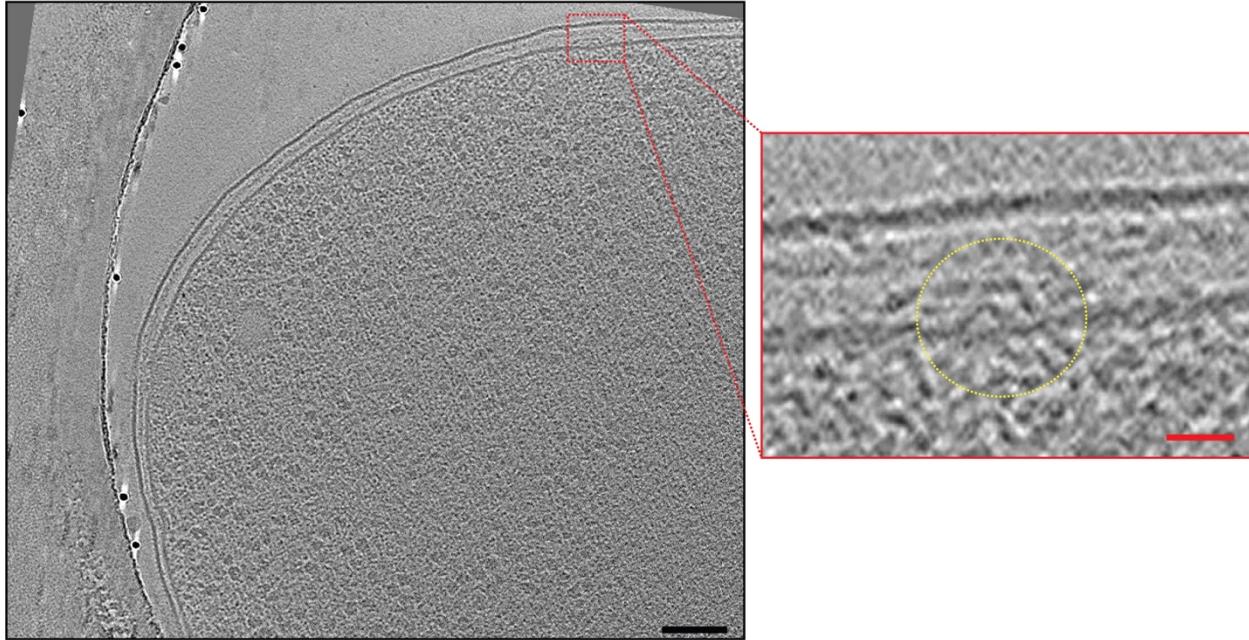
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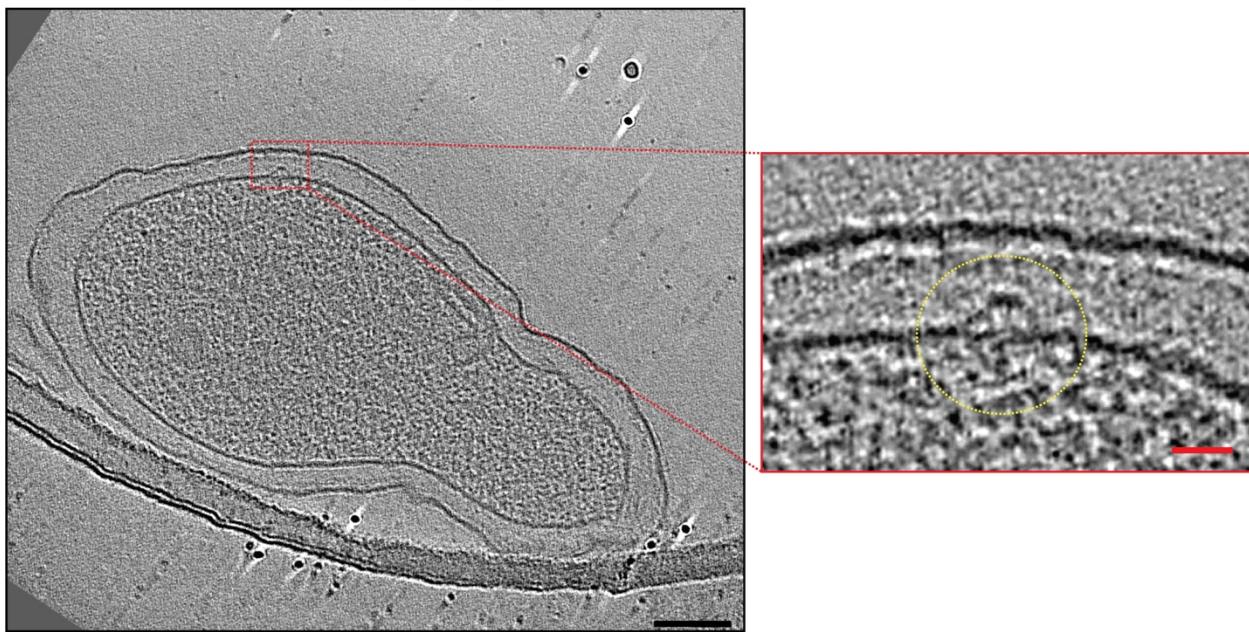
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A. brasiliense



B. abortus

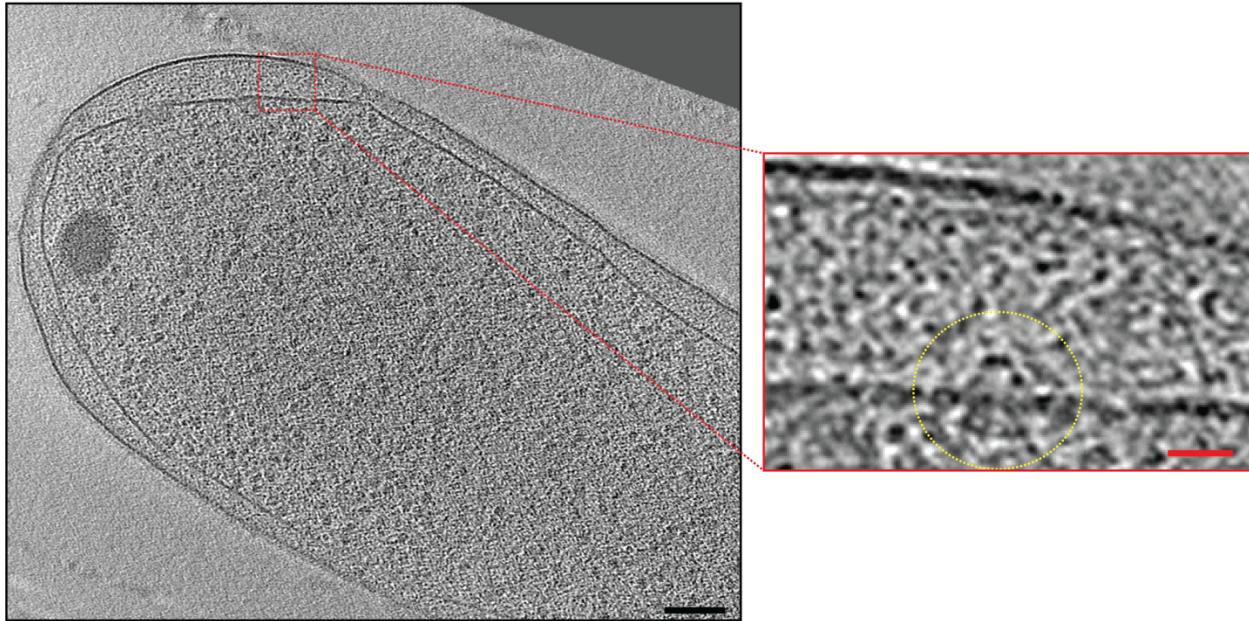


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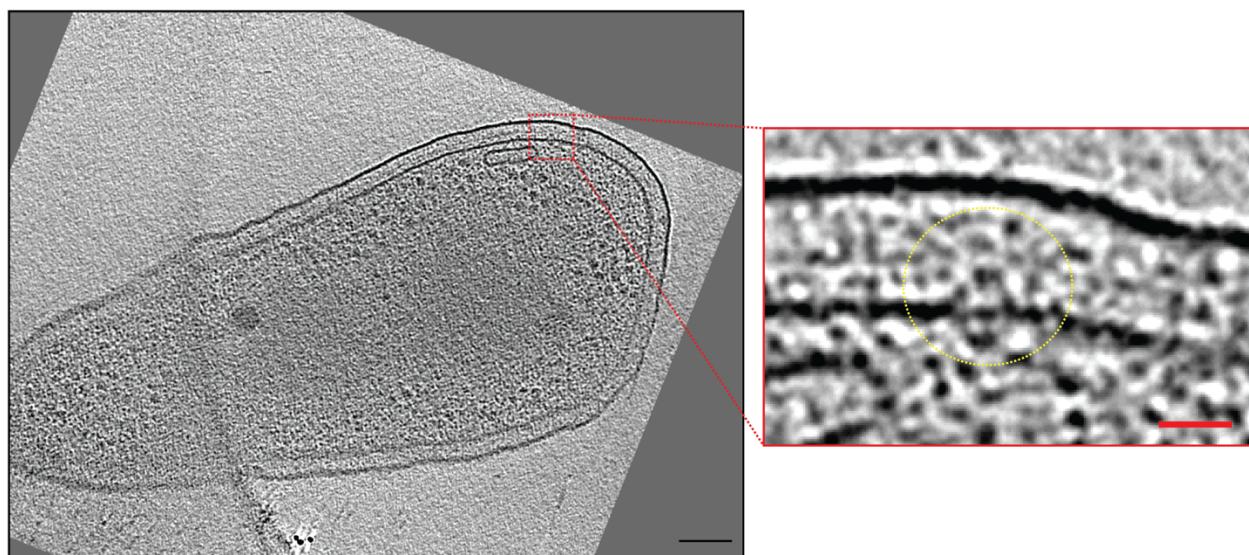
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A. tumefaciens



H. neptunium



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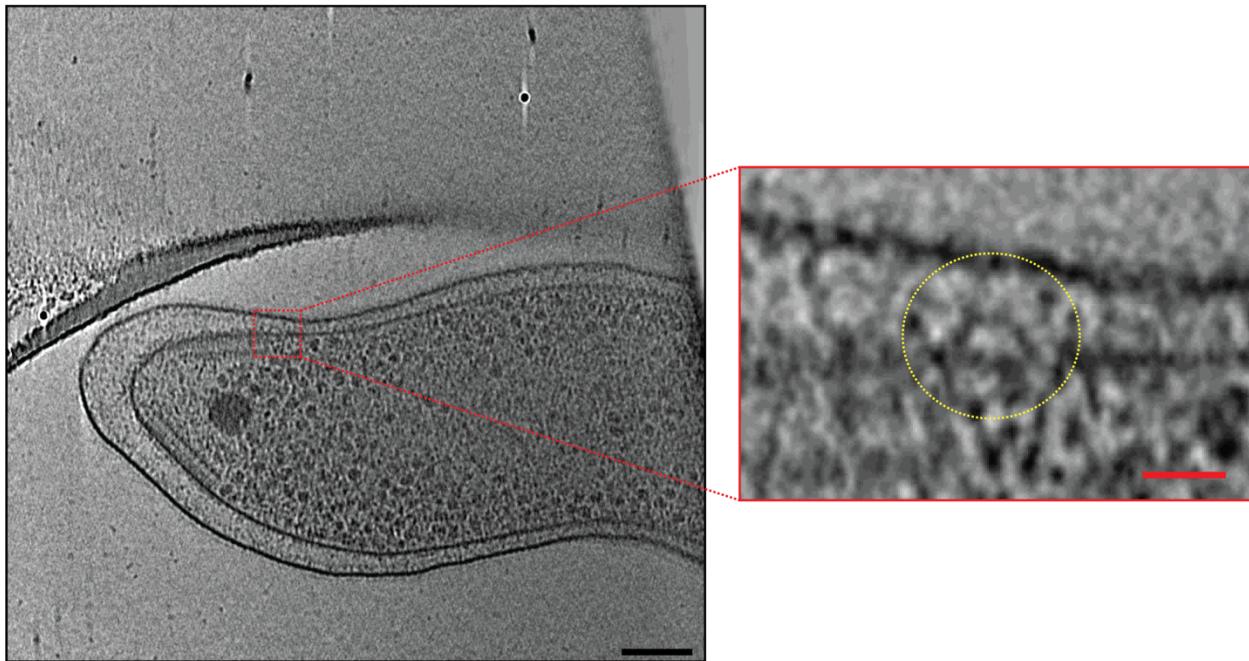
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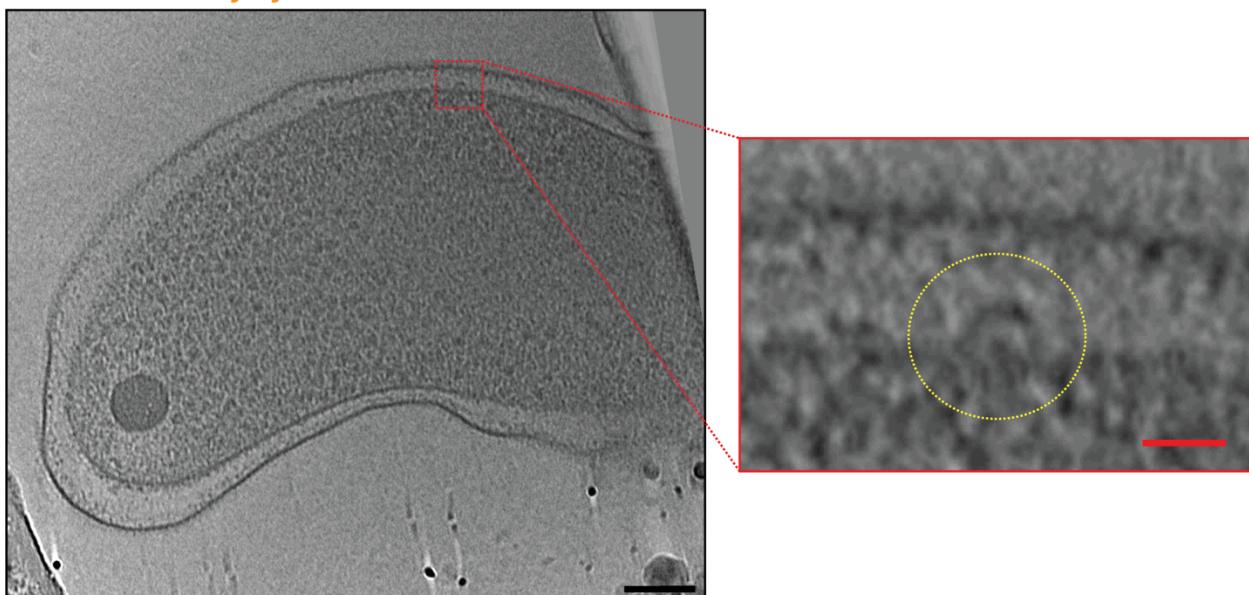
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C. jejuni *dflhAc*



C. jejuni *dflhBc*



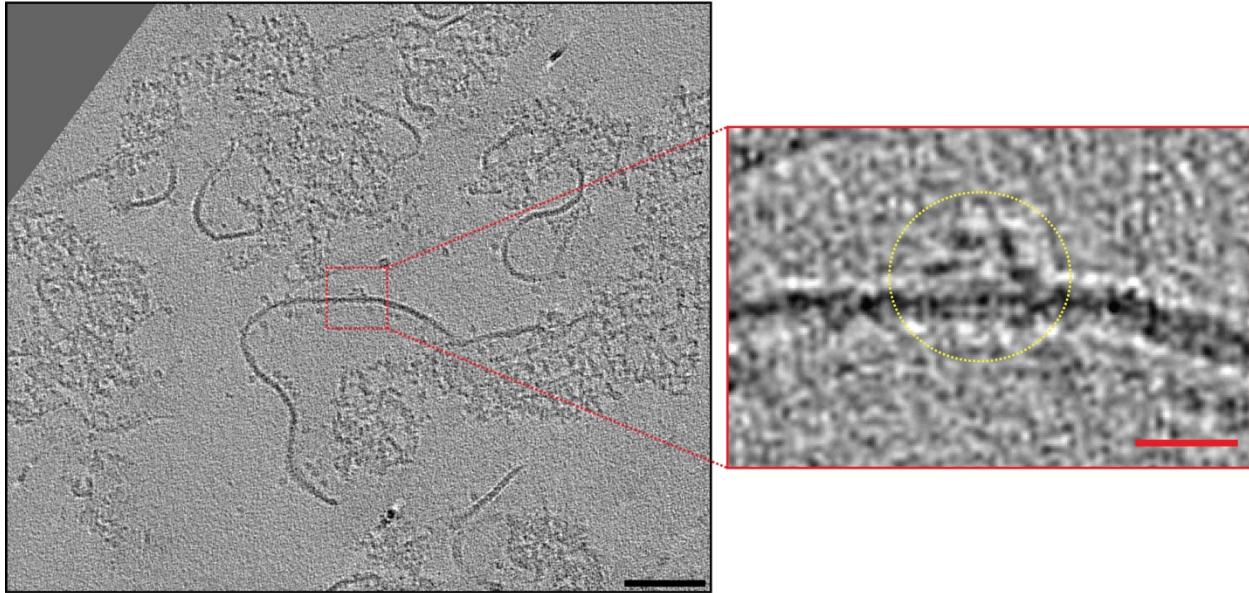
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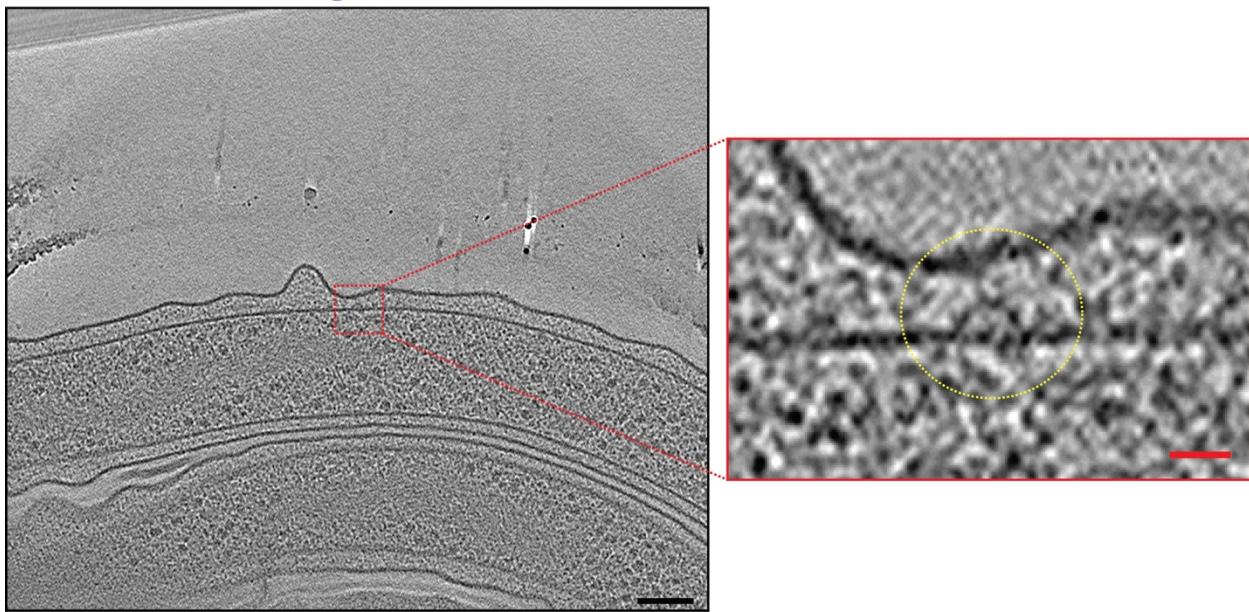
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B. subtilis lysate



H. gracilis



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266 **Figure S1:** Slices through electron crytomograms of various bacterial species highlighting the
267 presence of hat-like complexes (dotted yellow circles in the enlarged views). Black scale bars 100
268 nm, red scale bars 20 nm.

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289 **Movie S1:**

290 An electron crytomogram of a partially-lysed *E. coli* cell highlighting the presence of multiple
291 hat-like complexes in the inner membrane (indicated by red circles).

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312 **Extended Materials and Methods:**

313 **Strains and growth conditions:**

314 *E. coli* cells were grown as described in ref. [1]. *X. citri* cells were grown in 2xTY medium for 14
315 hours to stationary phase. *V. cholerae*, *V. harveyi* and *V. fischeri* were grown as previously
316 described [2]. *P. luteoviolacea* were grown as described in ref. [3]. *P. mirabilis* were grown as
317 described in ref. [4]. *P. aeruginosa* were grown in LB medium at 37° C overnight. The *P.*
318 *aeruginosa flhA** mutant was obtained from a transposon library (mutant number 3296 from the
319 non-redundant library <http://pa14.mgh.harvard.edu/cgi-bin/pa14/downloads.cgi>) from Dianne
320 Newman's lab at Caltech. *L. pneumophila* were grown as described in ref. [5]. *S. enterica* were
321 grown as in ref. [6]. *P. flexibilis* were grown in Lactose growth medium. *H. neptunium* were grown
322 to exponential phase in PYE medium. *A. tumefaciens* wild-type cells with plasmid-borne VirC1-
323 GFP translational fusion under control of the VirB promoter were grown in AB medium with 150-
324 300 ug/ml of kanamycin. *A. brasiliense* and *B. abortus* were grown as described in ref. [7]. *H.*
325 *hepaticus* ATCC 51449 and *H. gracilis* were grown as described in ref. [1,8] *C. jejuni* and its
326 mutants were grown as described in ref. [6,9,10]. *B. subtilis* protoplasts were prepared using
327 lysozyme using a protocol based on ref. [11]. A motile revertant *H. pylori* 26695 isolate was
328 selected by serial passage in Brucella broth supplemented with 10% heat inactivated fetal bovine
329 serum at 37° C, 5% CO₂ for 4 days until cultures reached an OD₆₀₀ ~ 0.4. Non-motile *H. pylori*
330 *fliP** mutants were propagated on TSAII blood agar plates (BD Biosciences) at 37 °C, 5% CO₂ for
331 either 24 or 48 h prior to collection with a sterile cotton swab for grid preparation. *Helicobacter*
332 *pylori* mutants ($\Delta fliM fliP^*$, $\Delta fliO fliP^*$, $\Delta fliS fliP^*$, $\Delta fliG fliP^*$, $\Delta fliQ fliP^*$) were grown directly
333 from glycerol stocks on sheep blood agar at 37 °C with 5% CO₂ for 48 hours. Then, the cells were
334 either collected from the plate using a cotton swab and dissolved in PBS and spun down and

335 plunge-frozen directly, or the cells were spread on a new plate and allowed to grow for 24 hours
336 under the same conditions before plunge-freezing. No difference could be discerned between the
337 two samples by cryo-ET.

338

339 ***H. pylori* mutagenesis:**

340 Flagellar mutants were generated in the non-motile *H. pylori* 26695 background as previously
341 described [12]. Briefly, constructs were generated to replace the coding region of the gene of
342 interest with an in-frame, non-polar kanamycin resistance cassette. The target gene and
343 approximately 500 base pairs (bp) upstream and downstream of flanking regions were amplified
344 and cloned into pGEM T-Easy (Promega). This construct was used as a template for inverse PCR
345 to remove the majority of the target gene coding region and to introduce incompatible restriction
346 sites for directional cloning. A kanamycin resistance cassette driven by a promoter transcribed in
347 the same direction as the endogenous operon was cloned into the ligated inverse PCR plasmid. *H.*
348 *pylori* 26695 was transformed via natural competence, and single colonies resistant to kanamycin
349 (12.5 µg/ml) were selected. PCR was used to verify that the kanamycin resistance cassette had
350 inserted into the target locus in the same orientation as operon transcription.

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352 **Electron cryo-tomography sample preparation and imaging:**

353 Sample preparation for cryo-ET imaging was done as described in references [2,13,14]. Total
354 cumulative electron dose used for each tilt-series in each species was:

Species name	Class	Cumulative electron dose (e ⁻ / Å ²)
<i>Xanthomonas citri</i>	Gammaproteobacteria	120

<i>Vibrio harveyi</i>	Gammaproteobacteria	160
<i>Vibrio fischeri</i>	Gammaproteobacteria	150
<i>Vibrio cholerae</i>	Gammaproteobacteria	160
<i>Salmonella enterica</i>	Gammaproteobacteria	200
<i>Pseudoalteromonas luteoviolacea</i>	Gammaproteobacteria	180
<i>Proteus mirabilis</i>	Gammaproteobacteria	160
<i>Pseudomonas flexibilis</i>	Gammaproteobacteria	100
<i>Pseudomonas fluorescens</i>	Gammaproteobacteria	200
<i>Pseudomonas aeruginosa</i>	Gammaproteobacteria	170
<i>Legionella pneumophila</i>	Gammaproteobacteria	100
<i>Escherichia coli</i>	Gammaproteobacteria	130
<i>Shewanella oneidensis</i> MR1	Gammaproteobacteria	150
<i>Hyphomonas neptunium</i>	Alphaproteobacteria	180
<i>Agrobacterium tumefaciens</i>	Alphaproteobacteria	200
<i>Azospirillum brasilense</i>	Alphaproteobacteria	200
<i>Brucella abortus</i>	Alphaproteobacteria	160
<i>Helicobacter hepaticus</i>	Epsilonproteobacteria	200
<i>Helicobacter pylori</i>	Epsilonproteobacteria	120-130
<i>Campylobacter jejuni</i>	Epsilonproteobacteria	200
<i>Hylemonella gracilis</i>	Betaproteobacteria	75
<i>Bacillus subtilis</i>	Bacilli	160

356 **Image processing and subtomogram averaging:**

357 Three-dimensional reconstructions of tilt-series were performed either automatically through the
358 RAPTOR pipeline used in the Jensen lab [15] or with the IMOD software package [16].
359 Subtomogram averaging was done using the PEET program [17] with a 2-fold symmetrization
360 applied along the particle Y-axis. The number of particles that were averaged are:

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Figure	Number of particles
Figure 2 <i>X. citri</i>	21
Figure 2 <i>V. harveyi</i>	46
Figure 2 <i>V. fischeri</i>	19
Figure 2 <i>V. cholerae</i>	58
Figure 2 <i>S. enterica</i>	38
Figure 2 <i>P. luteoviolacea</i>	50
Figure 2 <i>P. mirabilis</i>	18
Figure 2 <i>P. flexibilis</i>	23
Figure 2 <i>P. aeruginosa</i>	78
Figure 2 <i>L. pneumophila</i>	149
Figure 2 <i>E. coli</i>	31
Figure 2 <i>H. neptunium</i>	23
Figure 2 <i>A. tumefaciens</i>	29
Figure 2 <i>A. brasiliense</i>	20
Figure 2 <i>B. abortus</i>	41
Figure 2 <i>H. pylori</i>	26

Figure 2 <i>H. gracilis</i>	30
Figure 2 <i>B. subtilis</i>	30
Figure 3A	26
Figure 3B	118
Figure 3C	60
Figure 3D	29
Figure 3E	27
Figure 3F	50
Figure 3G	42
Figure 3H	146
Figure 3J	36
Figure 3K	37
Figure 3L	78
Figure 3M	17

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