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► **To cite this version:**

Liqiang Song, John Perpich, Chenggang Wu, Thierry Doan, Zuzanna Nowakowska, et al.. A Unique Bacterial Secretion Machinery with Multiple Secretion Centers. *Proceedings of the National Academy of Sciences of the United States of America*, 2022, 119 (18), 10.1073/pnas.2119907119 . hal-03655004

HAL Id: hal-03655004

<https://amu.hal.science/hal-03655004>

Submitted on 29 Apr 2022

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1 **A Unique Bacterial Secretion Machinery with Multiple Secretion Centers**

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31 **Keywords:** cryoelectron tomography, type IX secretion, protein transport, proton motive force,

32 **Abstract**

33 The *Porphyromonas gingivalis* type IX secretion system (T9SS) promotes periodontal disease by
34 secreting gingipains and other virulence factors. By *in situ* cryoelectron tomography, we report that the *P.*
35 *gingivalis* T9SS consists of eighteen PorM dimers arranged as a large, caged ring in the periplasm. Near
36 the outer membrane, PorM dimers interact with a PorKN ring complex of ~52 nm in diameter. PorMKN
37 translocation complexes of a given T9SS adopt distinct conformations energized by the proton motive
38 force, suggestive of different activation states. At the inner membrane, PorM associates with a
39 cytoplasmic complex that exhibits 12-fold symmetry and requires both PorM and PorL for assembly.
40 Activated motors deliver substrates across the outer membrane via one of eight Sov translocons arranged
41 in a ring. The T9SSs are unique among known secretion systems in bacteria and eukaryotes in their
42 assembly as supramolecular machines composed of apparently independently-functioning translocation
43 motors and export pores.

44

45 **Significance Statement**

46 The newly described type IX secretion systems (T9SSs) translocate virulence factors and can mediate
47 specialized gliding motility among bacterial pathogens of the *Fibrobacteres-Chlorobi-Bacteroidetes*
48 superphylum. We visualized the spatial organization of the T9SS in its native context in the
49 *Porphyromonas gingivalis* cell envelope by *in situ* cryoelectron tomography. The T9SS exhibits distinct
50 symmetries across the bacterial cell envelope: i) a cytoplasmic complex requiring PorL and PorM for
51 assembly exhibits 12-fold symmetry, ii) a periplasmic complex composed of PorM exhibits 18-fold
52 symmetry and attaches to a continuous PorKN ring near the outer membrane, and iii) eight Sov
53 translocons are arranged with 8-fold symmetry at the cell surface. The T9SS is the largest of the known
54 bacterial secretion systems, and evidently arranges as multiple, independently-functioning translocation
55 motors.

56

57

58 **Introduction**

59 Bacteria deploy at least eleven different translocation systems to export macromolecules across their cell
60 envelopes¹⁻³. Of these, the recently-discovered Type IX secretion systems (T9SSs) carried by many
61 species of the *Fibrobacteres–Chlorobi–Bacteroidetes* (FCB) superphylum play various critical roles in
62 pathogenesis and colonization⁴⁻⁷. Two bacterial models have emerged for detailed mechanistic and
63 structural analyses of the T9SS. The Por T9SS in *Porphyromonas gingivalis*, a key contributor to human
64 periodontitis and an emerging systemic pathogen, transports various heme-binding proteins and toxins
65 aiding in colonization and invasion of gingival and other tissues^{8,9}. The Gld T9SS in *Flavobacterium*
66 *johnsoniae* secretes adhesins SprB and RemA and dozens of other effector proteins, but also functions as
67 a rotary motor to direct the movement of a surface-bound adhesin along helical tracks, resulting in a form
68 of gliding motility^{10,11}. Studies have shown that these T9SSs are large, cell envelope-spanning
69 nanomachines composed of at least 14 essential subunits (Fig. 1A)^{6,7}. They translocate substrates,
70 exported to the periplasm by the general secretory (Sec) pathway, across the outer membrane (OM). At
71 the cell surface, they release their substrates to the milieu or deliver them to an ‘attachment complex’,
72 which covalently links the substrates to anionic lipopolysaccharides (A-LPSs) for surface display^{5,12}.

73
74 Three stable subassemblies have been identified through biochemical or single-particle microscopy
75 approaches: i) an inner membrane (IM) molecular motor composed of the PorL and PorM subunits^{13,14}
76 with structural and functional homologies with the MotAB/PomAB flagellar and ExbBD/TolQR PMF-
77 dependent motors^{15,16,17,18}; ii) a large ~50 nm ring-shaped complex composed of periplasmic PorN and
78 outer membrane (OM)-associated lipoprotein PorK^{19,20}; and iii) an OM-spanning translocon, termed Sov
79 or SprA, configured as a 36-strand β -barrel with a central pore of ~70 Å²¹. Despite this progress, it is not
80 yet known how these substructures interact or coordinate their activities, in part because there is no
81 overarching view of the T9SS in the native context of the bacterial cell envelope. Here, we visualized the
82 intact T9SS of *P. gingivalis* by *in situ* cryoelectron tomography (cryo-ET). Remarkably, the T9SS
83 presents not as a single functional entity, as shown for all known bacterial and eukaryotic protein

84 transport systems, but rather as a composite of apparently independent translocation motors and Sov
85 translocons arranged symmetrically as distinct rings in the cytoplasm, periplasm, and cell surface.

86

87 **Results and Discussion**

88 **The T9SS: A large, caged-ring structure.** *P. gingivalis* cells are small (~0.5 μm in diameter) and well
89 suited for cryo-ET imaging (Fig. 1B, Fig. S1A). A typical three-dimensional (3D) reconstruction of a *P.*
90 *gingivalis* cell revealed the IM and OM, as well as an electron dense surface layer (EDSL), which is
91 known to consist of T9SS substrates attached at the cell surface (Fig. 1B - D)⁴. In wild-type cells, two
92 periplasmic rods attached to the OM and ~50 nm apart were visualized in side views (Fig. 1B-D, Fig.
93 S1A, Movie S1). In fact, the rods constitute the peripheral edges of a large ring similar in size (~50 nm) to
94 the previously visualized PorKN rings^{19,20} as shown in tilt- and top-down views (Fig. S1A, B, Movies S1
95 and 2). The rods visualized in side view are missing in a strain lacking PorK (Fig. 1E, F), which is an
96 essential T9SS subunit^{7,19}. The ΔporK mutant strain also lacks the EDSL (Fig. 1E, F) and fails to secrete
97 T9SS effectors such as the Arg gingipain (Rgp) protease, whereas the *trans*-complemented strain is
98 restored for Rgp secretion (Fig. S2). Similarly, mutants deleted of *porN* are devoid of the EDSL and OM-
99 associated rods, and do not secrete Rgp protease (Figs. S1A and 2). Previously, it was shown that two IM
100 subunits PorM and PorL with large domains respectively in the periplasm and cytoplasm¹³ are also
101 required for T9SS function, but not for assembly of the PorKN ring¹⁹. Accordingly, here we determined
102 that the ΔporM and ΔporL mutants lack the EDSL and do not secrete the Rgp effector, but elaborate the
103 OM-associated rod structures (Figs. S1 and 2). Taken together, these findings strongly indicate that the
104 visualized structures correspond to *in situ* T9SS nanomachines and that the OM-associated rods and rings
105 visualized in side- and top-down views correspond to the PorKN ring complexes characterized by single
106 particle cryoelectron microscopy (cryo-EM)¹⁹ (Fig. 1L-O). The average number of T9SSs visualized by *in*
107 *situ* cryo-ET was ~1.3 per cell with a range of 0 to 4, although this might underestimate the actual number
108 of partially or fully assembled T9SSs per cell (Fig. 1D).

109

110 To determine the 3D structure of the entire T9SS machine, we imaged over 800 wild-type *P. gingivalis*
111 cells, and picked 694 ring-shaped particles from 516 tomograms (Table S1). By sub-tomogram averaging,
112 we generated an overall structure of the T9SS at a resolution of ~ 32 Å (Fig. 1H, Fig. S1C). The T9SS
113 spans the entire cell envelope, with dimensions of ~ 57 nm in height and ~ 52 nm in width, and consists of
114 three distinct substructures: i) a surface structure (SS) on the extracellular side of the OM, ii) a
115 periplasmic structure designated here as the core complex (CC), and iii) a cytoplasmic complex (Fig. 1H-
116 I). The SS is located ~ 6 nm above the OM and below the EDSL, and in top-down view presents as a ring
117 with outer and inner diameters of 48 and 31 nm (Fig. 1H, J). During initial alignment and without
118 symmetry applied, the SS ring appears to be a featureless ring. We applied 18-fold symmetry to smooth
119 the density; however, upon local refinement and classification (see below), we determined that the SS in
120 fact is composed of 8 discontinuous structural units. The CC consists of two parts: i) the PorKN rods/ring
121 structure (side/top-down views) of ~ 8 nm in thickness and 52 nm in diameter (Fig. 1H, K), and ii) strips
122 of densities presenting in side view as arches of ~ 17 nm in height that connect the OM-associated PorKN
123 rods to the IM (Figs. 1H-I, 2A). The cytoplasmic complex consists of a small central knob flanked by two
124 densities when visualized in side-view (Fig. 1H-I). In 3D surface renderings of the intact T9SS shown at
125 45° and 90° (top-down) angles, ring-shaped architectures of the SS, CC, and cytoplasmic complex are
126 readily evident (Fig. L-O).

127

128 Besides these densities, we detected three layers of density in the periplasm and a fourth between the cell
129 surface and the EDSL (Fig. 1H, Fig. S3A,B). A 3D map reconstructed by sub-tomogram averaging of
130 randomly picked regions of the *P. gingivalis* cell envelope established that these four layers are
131 constituents of the cell envelope and are not parts of the T9SS machine (Fig. S3C,D). Consequently, the
132 T9SS presents as a large, caged-ring structure that encompasses a central region of the *P. gingivalis* cell
133 envelope whose IM, periplasmic and OM features are largely intact.

134

135 **The PorMKN core complex (CC).** We next focused on solving refined structures of the CC, with
136 emphasis on the side-view arches. Prior studies have shown that PorM has an N-proximal
137 transmembrane domain (TMD) and a large ~480-residue C-terminal region that extends across the
138 periplasm and interacts with the OM-associated PorKN complex¹³. By X-ray crystallography, the
139 periplasmic regions of PorM (PorMp) and the *F. johnsoniae* homolog GldM (GldMp) presented as
140 elongated dimers with dimensions 180 Å in length and 50 and 35 Å in crosssections^{22,23}. Through local
141 refinements of periplasmic densities associated with T9SSs in WT cells, we determined that the
142 periplasmic arches correspond to 18 distinct knobs arranged in a ring of 52 nm in diameter when
143 visualized in top-down view (Table S1, S3, Fig. 2B, Fig. S4A). The $\Delta porM$ mutant possesses the PorKN
144 ring, but lacks the underlying knobbed ring and the cytoplasmic complex (Fig. 2C, F). In consideration of
145 the PorMp and GldMp X-ray structures^{22,23}, we propose that the side-view arches and top-down-view
146 knobs correspond to 18 PorM dimers, whose N-proximal regions are anchored in the IM and C-terminal
147 regions extend through the periplasm to the OM-associated PorKN ring (Fig. 2D,E).

148
149 The PorMKN complex exhibits two other noteworthy features. First, in the native T9SS, the PorKN ring
150 is 8 nm thick and the PorM arches are 17 nm in length, whereas in the $\Delta porM$ mutant, the PorKN ring is
151 only 7 nm thick (Fig. 2A,C Fig. S5A, B). In the atomic structure, the PorMp dimers are 18 nm in length²³
152 and composed of 4 domains, D1-D4, with domain D4 directly interacting with PorN^{13,22}. To reconcile
153 these *in situ* and *in vitro* findings, the PorKN ring visualized *in situ* might actually consist of PorK, PorN,
154 and domain D4 of PorM. Alternatively, the interaction between PorM and PorKN might induce a
155 structural change resulting in thickening of the PorKN ring.

156
157 Second, in the global averaged map, the PorKN ring forms a 60° angle with respect to the OM (Fig. 3A).
158 However, when the PorKN rings were locally refined at the junctions with the PorM arches, we detected
159 three distinct structural Classes in which the PorMKN complex forms angles of 90°, 70° and 50° relative

160 to the OM (Fig. 3B). We quantified the PorMKN complexes of each Class among the entire set of 12,492
161 complexes in the 694 imaged T9SSs (18 complexes per T9SS \times 694 T9SSs). Among the 12,492
162 complexes, the Class 1 (90°) and Class 3 (50°) complexes were more abundant (35.5 and 37.5 %,
163 respectively) than the Class 2 (70°) complexes (27 %) (Fig. S6A). Furthermore, among each of the 694
164 imaged T9SSs, the Class 3 complexes dominated with an average of 10 complexes and a range of 3 to 16
165 per T9SS (Fig. S6A). The Class 2 structures were least abundant, insofar as ~14 % of the T9SSs lacked
166 any Class 2 complexes and the bulk of the remaining T9SSs had ≤ 6 . The Class 1 complexes had an
167 intermediate distribution with an average of 4 per T9SS and a range of 0-10. Importantly, in the absence
168 of *porM*, there was little variation from the 60° global average at the PorKN ring - OM junctions (Fig.
169 S5D), indicating that PorM contributes directly to the distinct PorKN - OM junction architectures in WT
170 machines.

171
172 Recently, it was shown that PorL and PorM and the *F. johnsoniae* GldL and GldM homologs form
173 structurally-similar proton-motive-force (PMF)-transducing motors^{14,24}. Moreover, in the atomic
174 structures, PorMp and GldMp dimers possess distinct bends in the hinge regions between the D1 - D2 or
175 D2 - D3 domain interfaces^{14,21,22}. These findings have led to a model in which PMF-energy transduction
176 regulates the activation states of the PorLM and GldLM translocation motors^{14,25}. Upon sensing of the
177 PMF, PorLM and GldLM transduce PMF energy in the form of conformational changes in the
178 periplasmic domains of PorM and GldM as a prerequisite for substrate secretion or gliding motility^{25,26}. In
179 this context, we propose that the visualized conformational Classes of the PorKMN complexes
180 correspond to different activation states of the motors.

181
182 **Evidence for PMF-induced structural changes.** To further explore whether transduction of PMF
183 energy impacts the conformational status specifically at the PorKN - OM junction, we treated *P.*
184 *gingivalis* cells with the protonophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP), which
185 disrupts the PMF and inactivates the T9SS^{14,27}. Approximately 90 % of the visualized T9SSs from CCCP-

186 treated cells possessed all of the structural features of machines from untreated cells, but the remaining 10
187 % lacked PorM arches and the cytoplasmic complex (Fig. 3A,D). Among the 90 % of T9SSs bearing WT
188 features, we identified significant changes in the relative percentages of Classes with distinct PorKN-OM
189 junction angles. Most notably, Class 2 structures (bend-angle 70°) increased significantly, from 27.0 to
190 56.6%, upon CCCP-treatment. Conversely, the Class 1 (bend-angle 90°) and Class 3 (bend-angle 50°)
191 structures decreased from 35.5 to 20.4% and 37.5 to 23%, respectively (Fig. 3E). The significant increase
192 in the percentage of Class 2 structures in PMF-collapsed cells supports a proposal that this Class
193 corresponds to the quiescent or ‘closed’ state of the motor, whereas the Class 1 and 3 structures represent
194 or activated or ‘open’ states. Why activated motors are represented by bend-angles of 90° or 50° could be
195 explained by recent evidence that GldLM in the *F. johnsoniae* T9SS functions as a rotary motor, such that
196 the rotating periplasmic domain of GldM is envisioned to form a large arc as it extends to the PorKN
197 junction^{14,22,23}. Recently, it was also shown that mutations in GldL have different impacts on T9SS
198 activity. Whereas a GldL E49A mutation abolishes both substrate secretion and gliding motility, another
199 mutation, E59A, prevents gliding but does not impact substrate secretion. These findings suggest that
200 GldLM motors exist in a quiescent state and in two activated states, one supporting both gliding motility
201 and substrate secretion and a second only secretion²⁵.

202

203 Whether the *P. gingivalis* Por T9SS rotates is not yet known. If so, a PMF-energized rotating PorM
204 dimer might impose large bend-angles at the PorKN junction, and two such activated (Class 1 and 3)
205 structural states might have been captured by *in situ* cryo-ET imaging. By contrast, in the deenergized
206 state, a nonrotating PorM dimer potentially fixes the PorKN junction at the intermediate bend-angle
207 represented by the Class 2 structures. Even if PorM does not rotate, PMF sensing clearly induces large
208 conformational changes in the PorM dimers that are likely mediated through the recently visualized
209 PorLM complex assembled across the IM²⁴.

210

211 Among the small fraction (~10 %) of T9SSs from CCCP-treated cells that lacked the PorM arches and
212 cytoplasmic complexes (Fig. 3D), the bend angle of the PorKN - OM junction approximates that observed
213 for $\Delta porM$ mutant machines. CCCP-induced disengagement of the PorM arches therefore phenocopies
214 the $\Delta porM$ mutation with respect to the overall PorKN ring architecture. Importantly, however, this
215 subpopulation of CCCP-deenergized T9SSs retains the surface structures (SS) that are lacking in the
216 $\Delta porM$ or $\Delta porL$ mutant machines (Fig. 2C, 4D). In a small fraction of T9SSs de-energized by collapse
217 of the PMF, PorM and PorL thus appear to disengage from the PorKN ring without destabilization of the
218 previously docked SS.

219
220 We sought to gain further biochemical evidence for PMF-mediated activation of T9SSs, and to this end
221 we capitalized on the availability of antibodies to assay for energy-mediated conformational changes in
222 the PorM and PorN homologs, GldM and GldN, in *F. johnsoniae* cells. Reminiscent of previous findings
223 for the PMF-responsive energy transducer TonB²⁸, trypsin treatments of spheroplasts derived from
224 CCCP-untreated or -treated cells yielded different GldM protease cleavage patterns (Fig. 3F). In the
225 absence of CCCP treatment, GldM remained intact upon exposure to trypsin, whereas CCCP treatment
226 resulted in generation of two GldM cleavage products. By LS-MS/MS spectrometry, we determined that
227 both cleavage sites (downstream residues Lys252 and Arg322, respectively) reside within the linker
228 region between domains D2 and D3 (Fig. 3F). We were unable to assess the effect of PMF energy
229 depletion on the conformational status of GldN, as the GldN trypsin cleavage patterns were unaltered
230 regardless of prior treatment of cells with CCCP (Fig. S7). Overall, these data confirm earlier predictions
231 that GldLM transduces IM energy from the PMF in the form of a conformational change(s) in the
232 periplasmic arms of GldM to activate the T9SS for transport or gliding motility²². In view of the
233 structural homology of GldM and PorM, we suggest that PMF sensing similarly induces conformational
234 changes in the PorM translocation motor. With respect to the PMF-driven conformational changes we
235 observed here for individual PorKMN complexes, it is interesting to note that complexes of a given bend-

236 angle Class tend to spatially cluster within each T9SS (Fig. S6A-B). This clustering could imply that
237 adjacent complexes are coordinately activated in response to PMF energy sensing.

238

239 **Refinement of the cytoplasmic complex.** To better understand the structural basis for energy
240 transduction, we locally refined the cytoplasmic complex (Fig. 4A-C, Fig. S4B). Three distinct densities
241 were evident: i) a central knobbed cylinder projecting ~14 nm from the IM into the cytoplasm, ii) a
242 flanking ring of ~6 nm in thickness and ~20 nm in diameter, and iii) a spoked-ring of ~32 nm in diameter
243 with 12-fold symmetry (Fig. 4B,C, Fig. S4B). PorL and PorM are the only essential T9SSs proteins
244 identified to date with cytoplasmic domains (Fig. 1A). Indeed, the $\Delta porL$ and $\Delta porM$ mutant machines
245 lack the periplasmic arches and cytoplasmic densities, showing a co-dependence on PorL and PorM for
246 assembly of these structures (Figs. 2C, 4D). PorM spans the IM once and its ~9-residue, N-terminal
247 cytoplasmic domain is unlikely to contribute detectable density. By contrast, PorL has two N-terminal
248 transmembrane helices (TMHs) and its large (~230-residue) C-terminal domain resides in the
249 cytoplasm²⁴. Recently, the 3D structures of the PorLM and GldLM complexes, were shown to consist of
250 the 10 TMHs from five PorL subunits arranged in a ring surrounding the two TM helices of the PorM
251 dimer^{14,24}. Thus, the 5 cytoplasmic domains of PorL associated the recently visualized PorLM complex²⁴
252 might contribute to one or more of the cytoplasmic densities detected by *in situ* cryo-ET.

253

254 The PorM dimers are in close juxtaposition with the outer most cytoplasmic knobs, suggesting the
255 possibility that they are composed of PorL pentamers (see Fig. S4B, classes e-h). If so, it is necessary to
256 account for the 18- vs 12-fold symmetry mismatch that exists between the PorM arches and putative PorL
257 knobs. Conceivably, a PorM dimer engages stably with the PorL pentamer only when activated, for
258 example, upon sensing of bound substrate in the periplasm. Such a model of substrate-induced PorLM
259 engagement might allow for one PorL pentamer to interact with and energize more than one spatially-
260 juxtaposed PorM dimer, which would account for our observation that PorKMN complexes of a given

261 structural Class, or activation state, tend to spatially cluster within each T9SS (Fig. S6A-B). It is
262 important to note, however, that PorL's cytoplasmic domain is disordered when purified and analyzed by
263 single-particle cryo-EM¹⁴. If this domain is also too flexible for detection by *in situ* cryo-ET, the
264 visualized densities might instead correspond to other Por components. One candidate is PorX, the
265 response regulator of the PorXY two-component system that was shown to interact with PorL²⁹, but
266 further studies are needed to define subunit contributions to the observed cytoplasmic densities.

267

268 **The Surface Structure (SS).** Finally, we refined the structure of the SS visualized initially as an
269 extracellular ring (Fig. 1J). Strikingly, the SS presents as eight distinct densities of 8 nm in diameter
270 arranged in a ring of ~48 nm in diameter (Fig. 5A-B, Fig. S4C). Each of the eight densities has a lateral
271 pore near the OM that faces the center of the T9SS supramolecular complex (Fig. 5E-F). The overall size
272 and presence of a lateral pore are highly reminiscent of the 267 kDa SprA/Sov translocon solved at ~3.5
273 Å by single particle cryo-EM²⁰. This cryo-EM structure docks well on the SS densities (Fig. 5G-H, Fig.
274 S8A,B). Moreover, a strain deleted of Sov retained all features of the T9SS except for the SS; this strain
275 also lacked the EDSL and failed to secrete Rgp protease (Fig. 5C,D, Figs. S1, 2). Considering the size of
276 Sov protein, we propose that the SS densities are largely composed of Sov, with potential contributions
277 from other components, e.g., the PorV shuttle protein³⁰. In the cryo-EM structure, the location of
278 detergent associated with the translocon was used to define the likely plane of the OM, giving rise to the
279 proposal that the β -barrel extends ~2 nm above the OM and an extracellular cap projects an additional 5
280 nm²¹. In line with those findings, the SS densities project ~6-7 nm above the OM (Fig. 5E). Interestingly,
281 beneath each of the Sov densities, the OM is missing its inner leaflet (Fig. 5A, Fig. S8A). This gap or hole
282 might be a point of entry for substrates, delivered by the PorLMKN translocation motor, into the Sov
283 translocon. The importance of the translocation motor - Sov contact for a stable association of translocons
284 with the T9SS is underscored by the fact that deletions of any of the translocation motor subunits
285 abolished detectable Sov surface densities (Figs. 2C, 4D). Intriguingly, despite numerous attempts, no
286 studies have yet shown a direct interaction between the PorL/M/K/N proteins and the Sov translocon.

287 Recently, however, a protein interactome analysis identified PorW as a mutually interacting partner of
288 Sov and the PorKN ring. PorW thus appears to constitute a critical link between these subassemblies³¹.

289

290 **Summary.** We have shown that the *P. gingivalis* T9SS assembles as a large supramolecular machine
291 composed of a cytoplasmic complex with 12-fold symmetry, a periplasmic complex with 18-fold
292 symmetry, and a surface structure consisting of 8 symmetrically arranged putative pores (Fig. 5I). Taken
293 together with previous findings, we propose a working model in which the major machine subunits
294 assemble with an overall stoichiometry of (PorL₅/PorM₂/PorK₂/PorN₂)₁₈(Sov)₈ (Fig. 5J). Observed
295 differences in the bend angles at PorMKN - OM junctions (Fig. 3B), coupled with the absence of a
296 discernible central channel (Fig. 2A, Fig. S3A), support our model that the T9SS is a multi-port export
297 apparatus composed of independently - but coordinately - functioning IM energy sensors, periplasmic
298 translocation motors, and OM translocons (Fig. 4J-K, Movie 3). This proposed architecture and
299 mechanism of action strikingly contrast with other known dedicated protein translocation systems
300 functioning in bacteria^{1-3,32,33} (Fig. S9). Deciphering the molecular and structural details underlying IM
301 energy activation, substrate recruitment, and OM export remain as major challenges for future research on
302 this unique family of bacterial transporters.

303

304 **Materials and Methods**

305 ***P. gingivalis* growth and mutant constructions.** *P. gingivalis* ATCC 33277 (33277) and W83 and their
306 isogenic mutants were cultured anaerobically at 37°C in Trypticase soy broth (TSB) supplemented with
307 1 mg ml⁻¹ yeast extract, 5 µg ml⁻¹ hemin, and 1 µg ml⁻¹ menadione. When appropriate, erythromycin
308 (10 µg ml⁻¹) was added to the medium.

309

310 The PCR fusion technique was utilized to generate allelic exchange mutants $\Delta porK$, $\Delta porL$, $\Delta porM$,
311 $\Delta porN$, (all 33277) and Δsov (W83) as described previously³⁴. Upstream and downstream fragments of
312 each gene were amplified by PCR and fused to *ermF* using the primers listed in Table S2. Constructs

313 were confirmed by sequencing and introduced into *P. gingivalis* by electroporation, and mutant strains
314 were selected on TSB plates containing erythromycin. Loss of mRNA from the target gene without
315 disruption of expression of the other genes in the operon (*por* operon) was confirmed by RT-PCR (Fig.
316 S10) using the primers listed in Table S2.

317
318 For complementation of *porK* in *P. gingivalis* ATCC33277, a 500-bp sequence upstream of PGN_1678
319 containing the promoter region was fused to the coding region of *porK* using PCR fusion and the primers
320 listed in Table S2. The resulting PCR product was confirmed by sequencing and cloned into the pT-COW
321 vector to create pT-COW-*porK*. The plasmid was transformed into chemically competent *E. coli* (strain
322 S17-1) and selected using ampicillin. The plasmid was transferred into *P. gingivalis* Δ *porK* via
323 conjugation with *E. coli*^{35,36}, and transconjugants were selected with gentamicin (50 μ g ml⁻¹) and
324 tetracycline.

325
326 *F. johnsoniae* CJ1827 (UW101 *rpsL*, Str^R) and its Δ *gldM* isogenic mutant have been previously
327 described³⁷. *F. johnsoniae* strains were grown in Casitone-Yeast extract (CYE) medium (10 g L⁻¹
328 casitone, 5 g L⁻¹ yeast extract, 8 mM MgSO₄, 10 mM Tris-HCl pH7.6) at 28°C with agitation. The proton-
329 motive force uncoupler carbonyl cyanide-m-chlorophenylhydrazone (CCCP, Sigma Aldrich) was
330 solubilized in ethanol and was added in the culture 30 min prior to cell harvesting, at the final
331 concentration of 10 μ M.

332
333 **Spheroplasting and limited proteolysis.** *F. johnsoniae* UW101 cells were grown in 5 mL of CYE
334 medium to an A_{600} =0.8, harvested by centrifugation and resuspended in 100 μ L of 20 mM Tris-HCl pH
335 8.0, 20% sucrose, 1 mM EDTA, and 100 μ g ml⁻¹ of lysozyme. After 30 min incubation at room
336 temperature (20°C), 100 μ L of ice-cold sterile water was added, and the mixture was carefully mixed by
337 three inversions. 50 μ L of the spheroplast suspension were treated with trypsin (100 μ g ml⁻¹). After 5 min

338 on ice, 17 μ L of boiling 4 \times Laemmli loading buffer were added, and immediately vortexed and boiled for
339 5 min prior to SDS-PAGE.

340

341 **Immunoprecipitation and LC-MS/MS analyses.** *F. johnsoniae* spheroplasts obtained from a 50-mL
342 culture treated with 10 μ M CCCP were prepared and subjected to limited proteolysis using trypsin.
343 Proteolysed spheroplasts were resuspended in 1 mL of CellLytic (Sigma-Aldrich) in presence of aprotinin
344 (Sigma-Aldrich), and incubated for 30 min on a wheel at room temperature. Unsolubilized material was
345 discarded by centrifugation for 10 min at 20,000 \times g, and subjected to immunoprecipitation using anti-
346 GldM antibodies bound to Protein A Sepharose CL-4B (GE Healthcare). After 2 hours of incubation at
347 room temperature on a wheel, the beads were washed three times with CellLytic B, resuspended in
348 Laemmli loading buffer, and subjected to SDS-PAGE and InstantBlue staining (Expedeon). Bands
349 corresponding to the proteolytic products were cut, washed with 100 mM acetonitrile/ammonium
350 bicarbonate pH 7.5, reduced by 10 mM dithiothreitol in 100 mM ammonium bicarbonate pH 7.5,
351 alkylated by 55 mM iodoacetamide in 100 mM ammonium bicarbonate pH 7.5 and then digested
352 overnight at 37°C with 10 ng/ μ l⁻¹ of Trypsin/Lys-C Mix from *Pseudomonas aeruginosa* (Promega) in 25
353 mM ammonium bicarbonate pH 7.5, 0.025% (v/v) proteaseMAX surfactant (Promega). Tryptic peptides
354 were extracted from gel bands by 0.1% (v/v) trifluoroacetic acid, 0.01% (v/v) proteaseMAX, 50% (v/v)
355 acetonitrile, and dried by speed vacuum. Samples solubilized in 0.05% (v/v) trifluoroacetic acid, 2% (v/v)
356 acetonitrile were analysed on a ESI-Q-Exactive Plus (ThermoFisher) mass spectrometer coupled to a
357 nanoliquid chromatography (Ultimate 3000, Dionex). Peptides were eluted from a C18 column (Acclaim
358 PepMap RSLC, 75 μ m \times 150 mm, 2 μ m, 100 Å , Dionex) by a 6–40% linear gradient of mobile phase B
359 (0.1% (v/v) formic acid/80% (v/v) acetonitrile) in mobile phase A (0.1% (v/v) formic acid) for 52 min.
360 The peptides were detected in the mass spectrometer in a positive ion mode using a Top 10 Data
361 Dependent workflow. A single full scan acquisition in the Orbitrap at a resolution of 70,000 in a 350–
362 1900 m/z range was followed by a fragmentation MS/MS step, at a resolution of 17,500, of the 10 top
363 ions, in the Higher Energy Collisional Dissociation cell set at 27 eV. The spectra were processed by

364 Proteome Discoverer software (ThermoFisher, version 2.1.0.81) using the Sequest HT algorithm with the
365 search following parameters: *F. johnsoniae* UW101 GldM protein (GeneBank identifier (GI): 58531935);
366 trypsin enzyme (maximum 2 missed cleavages); fixed modification: carbamidomethyl (Cys); variable
367 modification: oxidation (Met); mass values specific for monoisotopic; precursor mass tolerance: ± 10
368 ppm; fragment mass tolerance: ± 0.02 Da.

369 **Preparation of frozen-hydrated specimens.** Strains grown anaerobically at 37°C were harvested in the
370 late log phase by centrifugation at $9000 \times g$. The pellet was resuspended in phosphate-buffered saline
371 (PBS). To prepare CCCP treated wild-type cells, CCCP was added to a final concentration of 20 μM and
372 incubated for 20 min and then mixed with 10 nm diameter colloidal gold beads (used as fiducial markers
373 in image alignment) and deposited onto freshly glow-discharged, holey carbon grids for 1 min. The grids
374 were then blotted with filter paper and rapidly frozen in liquid ethane, using a gravity-driven plunger
375 apparatus as previously described³³.

377 **CryoET data collection and 3-D reconstructions.** Frozen-hydrated specimens were imaged at -170°C
378 using a Polara G2 electron microscope (FEI Company) equipped with a field emission gun and a direct
379 detection device (Gatan K2 Summit). The microscope was operated at 300 kV with a magnification of
380 $\times 9,400$, resulting in an effective pixel size of 4.5 Å at the specimen level. We used SerialEM³⁸ to collect
381 low-dose, single-axis tilt series with dose fractionation mode at about 8 μm defocus. Tilt series were
382 collected from -51° to 51° with increment of 3° and a cumulative dose of $\sim 70 \text{ e}^-/\text{Å}^2$ distributed over 35
383 stacks. Each stack contains ~ 8 images. We used Tomoauto³⁹ to facilitate data processing, which includes
384 drift correction of dose-fractionated data using Motioncorr⁴⁰ and assembly of corrected sums into tilt
385 series, automatic fiducial seed model generation, alignment and contrast transfer function correction of tilt
386 series by IMOD⁴¹, and weighted back projection (WBP) reconstruction of tilt series into tomograms using
387 Tomo3D⁴². Each tomographic reconstruction is $3,710 \times 3,838 \times 1,600$ pixels and ~ 1.30 Gb in size. In
388 total, 4,227 tomographic reconstructions from 6 different strains were generated.

390

391 **Sub-tomogram averaging and correspondence analysis.** We used tomographic package I3 for sub-
392 tomogram analysis as described previously⁴³. A total of 2936 type IX secretion machines ($400 \times 400 \times$
393 400 voxels) were visually identified and then extracted from 1947 tomographic reconstructions. Two of
394 the three Euler angles of each secretion machine were estimated based on the orientation of each particle
395 in the cell envelope. To accelerate image analysis, $4 \times 4 \times 4$ binned sub-tomograms ($100 \times 100 \times 100$ voxels)
396 were used for initial alignment. The alignment proceeds iteratively with each iteration consisting of three
397 parts in which references and classification masks are generated, sub-tomograms are aligned and
398 classified, and finally class averages are aligned to each other. After multiple cycles of alignment and
399 classification for $4 \times 4 \times 4$ binned sub-tomograms, we used $2 \times 2 \times 2$ binned sub-tomograms for refinement.
400 The initial alignments were carried out without symmetry imposed. To determine the symmetry of the SS,
401 CC and cytoplasmic complex, after the initial alignments of the intact machine, the rotation centers of the
402 sub-volumes were moved to the corresponding centers of SS, CC and cytoplasmic complex, respectively,
403 and masks were applied to include the SS, CC or cytoplasmic complex. Once the symmetries were
404 determined through classification, one of the classes was used as reference to align other classes and the
405 corresponding symmetry was applied to the final refinements. Fourier shell correlation (FSC) between the
406 two independent reconstructions was used to estimate the resolution of the averaged structures.

407

408 **Visualization and molecular modeling.** We used IMOD to visualize the maps. IMOD and EMAN2^{41,44}
409 were used to generate 3D surface rendering of *P. gingivalis* cells and UCSF ChimeraX
410 (<http://www.rbvi.ucsf.edu/chimerax>) to visualize sub-tomogram averages in 3D and molecular modelling.
411 Each of 18 PorM dimer structures (PDB 7CMG) was manually fitted onto the each of 18 arches of
412 densities. We docked the Sov homolog SprA (PDB 6H3J) into the 8 pore densities on the cell surface.

413

414 **Quantification and statistical analyses.** Quantification and statistical analyses are integral parts of the
415 algorithms and software used in our high throughput cryoelectron tomography pipeline. In particular,

416 massive data enabled us to use multivariate statistical analysis and classification (which are implemented
417 in tomographic package i3) for processing and interpretation of the sub-tomograms of T9SS extracted
418 from *P. gingivalis* cell reconstructions.

419

420

421 **Acknowledgements.** We are grateful to members of the Hu, Lamont and Cascales labs for critical
422 discussions. We are grateful to Dr. William Margolin for suggestions and critiques of the manuscript,
423 Pascal Mansuelle (IMM proteomic platform, Marseille) for mass spectrometry analyses, and Mark
424 McBride (University of Wisconsin, Milwaukee) for anti-GldM and -GldN antibodies. **Funding:** B.H.
425 was supported in part by McGovern Medical School start-up funds, the Welch Foundation (AU-1953-
426 20180324) and NIH 1R35GM138301. R.J.L. was supported by DE011111, DE012505 and DE023193.
427 E.C. was supported by the Fondation Bettencourt-Schueller, and A*MIDEX grant A-M-AAP-ID-17-33-
428 170301-07.22 and ANR grant ANR-20-CE11-0011. P.J.C. was supported by NIH grants R01GM48476
429 and R35GM131892. C.W. was supported by NIH grant DE030895.

430

431 **Competing interests:** The authors declare no competing interests.

432

433 **Data and materials availability:** Density maps and coordinate data that support the T9SS structures
434 determined by cryo-electron tomography have been deposited in the Electron Microscopy Data Bank
435 (EMDB) as EMD-24227 (Intact machine: PorM arches/ring), EMD-24228 (Cytoplasmic complex) and
436 EMD-24229 (Sov Surface Structure; SS). The authors declare that all other data supporting the findings
437 of this study are available within the paper and its supplementary materials.

438

439 **Author contributions:** B.H., L.S., and R.J.L. designed the research. J.D.P. constructed the *P. gingivalis*
440 mutants and phenotypically characterized mutant strains. B.H. and L.S. collected and processed the data.
441 T.D. and E.C. performed the protease accessibility experiments. B.H. L.S., T.D., E.C., R.J.L. and P.J.C

442 analyzed the data. L.S. and B.H. generated the figures, and P.J.C. wrote the manuscript with
443 contributions from B.H., R.J.L., and E.C.

444

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Figure legends

Figure 1. *Porphyromonas gingivalis* type IX machine revealed by cryo-ET. **A**, Schematic representation of the structure and function of the T9SS of *P. gingivalis*. The different subunits are indicated, as well as the putative route for substrate transport (dashed red line). **B**, A tomographic slice of a wild-type *P. gingivalis* cell. **C**, Zoomed-in view of (B) showing the outer membrane (OM), inner membrane (IM), Electron Dense Surface Layer (EDSL) and a type IX machine (T9SS) embedded in the cell envelope. **D**, 3D surface view showing multiple T9SSs embedded in the wild-type *P. gingivalis* cell envelope. **E**, A tomographic slice of a $\Delta porK$ mutant of *P. gingivalis* and **F**, zoomed-in view showing the absence of T9SSs and EDSL. **G**, 3D surface view of a *P. gingivalis* $\Delta porK$ mutant cell. **H-O**, *In situ* structure of the *P. gingivalis* T9SS. **H**, A central section through longitudinal plane of a global average structure *in situ* showing T9SS densities in the cell envelope. **I**, Outline of major features of the T9SS including the surface structure (SS), periplasmic core complex (CC) and a cytoplasmic (cyto-) complex. **J** and **K**, Cross-section views at the position indicated in (H) (yellow arrows) showing the ring-shaped surface structure and periplasmic PorKN complex. To smooth the map, we imposed an 18-fold symmetry. **L-O**, 3D surface rendering of the intact T9SS in side, cut-away, 45°, and 90° views.

Figure 2. Molecular architecture of PorM. **A**, Central section of the native T9SS (WT) showing that the proposed periplasmic arches of PorM span ~17 nm from the IM to the PorKN ring. **B**, Top-down view of the region denoted in (A) showing 18-fold symmetry of the PorM arches. **C**, A central section of a global average structure of the T9SS visualized in a $\Delta porM$ mutant. **D**, Two PorM dimers (PDB entry 7CMG) docked in the PorM arch density. **E**, An overall view of T9SS with eighteen docked PorM dimers. **F**, 3D surface rendering of the $\Delta porM$ mutant machine showing the presence of the PorKN ring and absence of the PorM arches, cytoplasmic complex, surface structure (SS), and EDSL.

Figure 3. Proton-motive force-dependent conformational change of the *P. gingivalis* PorM protein and *F. johnsoniae* GldM protein. **A**, Central section of the native T9SS (WT) showing that the PorKN

571 ring forms a $\sim 60^\circ$ angle relative to the OM. **B**, 3-D classification reveals three distinct classes of PorKNM
572 complex showing that the PorKN ring forms angles of 90° (Class 1), 70° (Class 2) and 50° (Class 3)
573 relative to the OM. **C**, A proposed model for oscillatory motion of the PorMKN complex. PMF energy-
574 driven conformational changes of PorM drive the oscillation of PorKN yielding bend angles of 90° and
575 50° ; the de-energized PorMKN complex is fixed at an angle of 70° . **D**, 10% of the T9SS machines lack
576 PorM and cytoplasmic complex upon CCCP treatment of WT cells. **E**, The proportion of each bend-angle
577 Class is altered in wild-type cells upon treatment with CCCP. **F**, (i) Limited protease accessibility assay.
578 *F. johnsoniae* wild-type or $\Delta gldM$ mutant cells were treated with CCCP and spheroplasts were treated
579 with trypsin protease. Proteolytic fragments were visualized by immunostaining of western blots with
580 anti-GldM antibodies. GldM, full-length protein, * and **, degradation products. Molecular weight
581 markers are indicated on left. (ii) Identification of the GldM trypsin cleavage sites. The GldM degradation
582 products were enriched by immunoprecipitation using anti-GldM antibodies and subjected to LS-MS/MS
583 identification. The GldM structure (PDB: 6EY4) and magnification are shown, with * and ** denoting
584 cleavage downstream of residues K252 and R322, respectively. For simplification, only a single chain of
585 the GldM dimer is shown.

586

587 **Figure 4. Architecture of the cytoplasmic complex.** **A**, Unrefined structure. **B**, The cytoplasmic
588 complex boxed in (A) was locally refined. A central knobbed cylinder extends ~ 14 nm into the cytoplasm
589 from the IM, and flanking densities extend ~ 6 nm. **C**, Bottom-up view of the region denoted in (B)
590 showing 12-fold symmetry of the outer spoked-ring of ~ 32 nm and a continuous inner ring of ~ 20 nm. **D**,
591 A central section of a global average structure of the T9SS visualized in a $\Delta porL$ mutant showing the
592 presence of the PorKN ring and absence of the PorM periplasmic arches, cytoplasmic complex, SS, and
593 EDSL. **E** and **F**, 3D rendering of the cytoplasmic complex shown in side and bottom-up views.

594

595 **Figure 5. Organization of secretion pore (Sov) at the OM.** **A** and **B**, Central-section and top-down
596 views of the locally refined surface structure showing 8 distinct densities arranged in a ring of 48 nm in

597 diameter. **C**, A central section through longitudinal plane of a global average structure of a Δ_{sov} mutant
598 machine and 3D surface rendering (**D**) showing that the surface densities are absent. **E** and **F**, 3D surface
599 rendering of the putative translocons shown in different views. Arrows in (**F**) denote lateral pores. **G** and
600 **H**, Eight SprA molecules (PDB entry 6H3J) docked into the surface densities. Arrows denote lateral pores
601 facing the center of the T9SS complex. **I**, A side view of the entire of T9SS machine showing
602 cytoplasmic, periplasmic, and surface substructures. **J** and **K**, Schematic of the *P. gingivalis* T9SS multi-
603 port export apparatus, with major machine subunits and postulated routes of substrate translocation
604 shown. Substrates cross the IM through the Sec system, are recruited by the PorLMNK translocation
605 motor, enter a gap in the inner leaflet of OM and then the Sov translocon, and exit Sov through lateral
606 pores for release to the milieu or docking onto the cell surface via the 'attachment complex'. The
607 proposed stoichiometry of the major machine subunits is shown.









