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Hu

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A Unique Bacterial Secretion Machinery with Multiple Secretion Centers

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4	Liqiang Song ¹ , John D. Perpich ² , Chenggang Wu ¹ , Thierry Doan ³ , Zuzanna Nowakowska ⁴ , Jan
5	Potempa ^{2,4} , Peter J. Christie ¹ , Eric Cascales ^{3*} , Richard J. Lamont ^{2*} , Bo Hu ^{1*}
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7	
8	
9	¹ Department of Microbiology and Molecular Genetics, McGovern Medical School, 6431 Fannin St,
10	Houston, Texas 77030
11	² Department of Oral Immunology and Infectious Diseases, University of Louisville, 570 S Preston St,
12	Louisville, Kentucky 40292
13	³ Laboratoire d'Ingénierie des Systèmes Macromoléculaires, Institut de Microbiologie, Bioénergies et
14	Biotechnologie, Aix-Marseille Université — CNRS UMR7255, Marseille, France
15	⁴ Department of Microbiology, Faculty of Biochemistry, Biophysics, and Biotechnology, Jagiellonian
16	University, Krakow, Poland
17	
18	
19	
20	*Correspondence to: Bo Hu, Richard J. Lamont or Eric Cascales
21	Hu: Phone: 713-500-589 ; e-mail: Bo.Hu@uth.tmc.edu; Fax: 713-500-5499
22	Lamont: Phone: 502-852-2112 ; e-mail: rich.lamont@louisville.edu; FAX: 502 852 6394
23	Cascales: Phone: (+33) 491-164-462 ; e-mail: <u>cascales@imm.cnrs.fr</u> ; FAX: (+33) 491-712-124
24	
25 26 27 28 29 30	ORCID IDs: Wu (0000-0002-5700-7188); Doan (0000-0002-5909-4289); Christie (0000-0002-9799-7067); Cascales (0000-0003-0611-9179); Lamont (0000-0002-3147-5039); Hu (0000-0002-9117-879X)
31	Keywords: cryoelectron tomography, type IX secretion, protein transport, proton motive force,

32 Abstract

The Porphyromonas gingivalis type IX secretion system (T9SS) promotes periodontal disease by 33 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-Bacterioidetes 48 superphylum. We visualized the spatial organization of the T9SS in its native context in the 49 Porphorymonas 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.

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57

58 Introduction

59 Bacteria deploy at least eleven different translocation systems to export macromolecules across their cell envelopes¹⁻³. Of these, the recently-discovered Type IX secretion systems (T9SSs) carried by many 60 61 species of the Fibrobacteres-Chlorobi-Bacteroidetes (FCB) superphylum play various critical roles in pathogenesis and colonization⁴⁻⁷. Two bacterial models have emerged for detailed mechanistic and 62 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 aiding in colonization and invasion of gingival and other tissues^{8,9}. The Gld T9SS in *Flavobacterium* 65 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 of gliding motility^{10,11}. Studies have shown that these T9SSs are large, cell envelope-spanning 68 nanomachines composed of at least 14 essential subunits (Fig. 1A)^{6,7}. They translocate substrates, 69 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', which covalently links the substrates to anionic lipopolysaccharides (A-LPSs) for surface displav^{5,12}. 72 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 PMFdependent motors^{15,16,17,18}; ii) a large ~50 nm ring-shaped complex composed of periplasmic PorN and 77 outer membrane (OM)-associated lipoprotein PorK^{19,20}; and iii) an OM-spanning translocon, termed Sov 78 or SprA, configured as a 36-strand β -barrel with a central pore of ~70 Å²¹. Despite this progress, it is not 79 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

85

transport systems, but rather as a composite of apparently independent translocation motors and Sov 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 μ m 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 known to consist of T9SS substrates attached at the cell surface (Fig. 1B - D)⁴. In wild-type cells, two 91 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 the previously visualized PorKN rings^{19,20} as shown in tilt- and top-down views (Fig. S1A, B, Movies S1 94 95 and 2). The rods visualized in side view are missing in a strain lacking PorK (Fig. 1E, F), which is an essential T9SS subunit^{7,19}. The $\Delta porK$ mutant strain also lacks the EDSL (Fig. 1E, F) and fails to secrete 96 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 subunits PorM and PorL with large domains respectively in the periplasm and cytoplasm¹³ are also 100 required for T9SS function, but not for assembly of the PorKN ring¹⁹. Accordingly, here we determined 101 102 that the $\Delta porM$ and $\Delta porL$ 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 particle cryoelectron microscopy (cryo-EM)¹⁹ (Fig. 1L-O). The average number of T9SSs visualized by *in* 106 107 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).

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 \sim 57 nm in height and \sim 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

randomly picked regions of the *P. gingivalis* cell envelope established that these four layers are
constituents of the cell envelope and are not parts of the T9SS machine (Fig. S3C,D). Consequently, the
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.

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 complex ¹³ . 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 <i>AporM</i> 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 length ²³
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

to the OM (Fig. 3B). We quantified the PorMKN complexes of each Class among the entire set of 12,492
complexes in the 694 imaged T9SSs (18 complexes per T9SS \times 694 T9SSs). Among the 12,492
complexes, the Class 1 (90°) and Class 3 (50°) complexes were more abundant (35.5 and 37.5 %,
respectively) than the Class 2 (70°) complexes (27 %) (Fig. S6A). Furthermore, among each of the 694
imaged T9SSs, the Class 3 complexes dominated with an average of 10 complexes and a range of 3 to 16
per T9SS (Fig. S6A). The Class 2 structures were least abundant, insofar as ~14 % of the T9SSs lacked
any Class 2 complexes and the bulk of the remaining T9SSs had ≤ 6 . The Class 1 complexes had an
intermediate distribution with an average of 4 per T9SS and a range of 0-10. Importantly, in the absence
of <i>porM</i> , there was little variation from the 60° global average at the PorKN ring - OM junctions (Fig.
S5D), indicating that PorM contributes directly to the distinct PorKN - OM junction architectures in WT
machines.
Recently, it was shown that PorL and PorM and the F. johnsoniae GldL and GldM homologs form
structurally-similar proton-motive-force (PMF)-transducing motors ^{14,24} . Moreover, in the atomic
structures, PorMp and GldMp dimers possess distinct bends in the hinge regions between the D1 - D2 or
D2 - D3 domain interfaces ^{14,21,22} . These finding have led to a model in which PMF-energy transduction
regulates the activation states of the PorLM and GldLM translocation motors ^{14,25} . Upon sensing of the
PMF, PorLM and GldLM transduce PMF energy in the form of conformational changes in the
periplasmic domains of PorM and GldM as a prerequisite for substrate secretion or gliding motility ^{25,26} . In
this context, we propose that the visualized conformational Classes of the PorKMN complexes
correspond to different activation states of the motors.
Evidence for PMF-induced structural changes. To further explore whether transduction of PMF
energy impacts the conformational status specifically at the PorKN - OM junction, we treated P.
gingivalis cells with the protonophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP), which
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 secretion ²⁵ .
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

PorLM complex assembled across the IM²⁴.

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 for the PMF-responsive energy transducer TonB²⁸, trypsin treatments of spheroplasts derived from 223 224 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 periplasmic arms of GldM to activate the T9SS for transport or gliding motility²². In view of the 232 233 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-

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

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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 cytoplasm²⁴. Recently, the 3D structures of the PorLM and GldLM complexes, were shown to consist of 249 250 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

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

261structural Class, or activation state, tend to spatially cluster within each T9SS (Fig. S6A-B). It is262important to note, however, that PorL's cytoplasmic domain is disordered when purified and analyzed by263single-particle cryo-EM¹⁴. If this domain is also too flexible for detection by *in situ* cryo-ET, the264visualized densities might instead correspond to other Por components. One candidate is PorX, the265response regulator of the PorXY two-component system that was shown to interact with PorL²⁹, but266further 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 \sim 3.5 Å by single particle cryo-EM²⁰. This cryo-EM structure docks well on the SS densities (Fig. 5G-H, Fig. 273 274 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 from other components, e.g., the PorV shuttle protein³⁰. In the cryo-EM structure, the location of 277 278 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 nm^{21} . In line with those findings, the SS densities project ~6-7 nm above the OM (Fig. 5E). Interestingly, 280 281 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
- 288

Recently, however, a protein interactome analysis identified PorW as a mutually interacting partner of 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 functioning in bacteria^{1-3,32,33} (Fig. S9). Deciphering the molecular and structural details underlying IM 300 301 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 μ g ml⁻¹ hemin, and 1 μ g ml⁻¹ menadione. When appropriate, erythromycin 308 (10 μ g 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 $\triangle porN$, (all 33277) and $\triangle sov$ (W83) as described previously³⁴. Upstream and downstream fragments of
- 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 pates 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 <i>porK</i> in <i>P. gingivalis</i> ATCC33277, a 500-bp sequence upstream of PGN_1678
319	containing the promoter region was fused to the coding region of <i>porK</i> 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 <i>P. gingivalis</i> $\Delta porK$ via
323	conjugation with <i>E. coli</i> ^{35,36} , and transconjugants were selected with gentamicin (50 μ g ml ⁻¹) and
324	tetracycline.
325	
326	<i>F. johnsoniae</i> CJ1827 (UW101 <i>rpsL</i> , Str ^R) and its $\Delta gldM$ isogenic mutant have been previously
327	described ³⁷ . 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 MgSO ₄ , 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 µM.
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 µL 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

339

on ice, 17 μ L of boiling 4× Laemmli loading buffer were added, and immediately vortexed and boiled for 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 µM CCCP were prepared and subjected to limited proteolysis using trypsin. 343 Proteolysed spheroplasts were resuspended in 1 mL of CelLytic (Sigma-Aldrich) in presence of aprotinin 344 (Sigma-Aldrich), and incubated for 30 min on a wheel at room temperature. Unsolubilized material was discarded by centrifugation for 10 min at $20,000 \times g$, and subjected to immunoprecipitation using anti-345 346 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 CelLytic 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 overnight at 37°C with 10 ng/ µl⁻¹ of Trypsin/Lys-C Mix from *Pseudomonas aeruginosa* (Promega) in 25 352 353 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 × 150 mm, 2 μ m, 100 Å, Dionex) by a 6–40% linear gradient of mobile phase B 359 $(0.1\% \text{ (v/v) formic acid/80\% (v/v) acetonitrile) in mobile phase A (0.1\% (v/v) formic acid) for 52 min.$ 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

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

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

377

369

378 CryoET data collection and 3-D reconstructions. Frozen-hydrated specimens were imaged at -170 °C 379 using a Polara G2 electron microscope (FEI Company) equipped with a field emission gun and a direct 380 detection device (Gatan K2 Summit). The microscope was operated at 300 kV with a magnification of \times 9,400, resulting in an effective pixel size of 4.5 Å at the specimen level. We used SerialEM³⁸ to collect 381 382 low-dose, single-axis tilt series with dose fractionation mode at about 8 µm defocus. Tilt series were 383 collected from -51° to 51° with increment of 3° and a cumulative dose of \sim 70 e⁻/Å² distributed over 35 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 385 386 series, automatic fiducial seed model generation, alignment and contrast transfer function correction of tilt series by IMOD⁴¹, and weighted back projection (WBP) reconstruction of tilt series into tomograms using 387 388 Tomo3D⁴². Each tomographic reconstruction is $3,710 \times 3,838 \times 1,600$ pixels and ~1.30 Gb in size. In 389 total, 4,227 tomographic reconstructions from 6 different strains were generated.

391	Sub-tomogram averaging and correspondence analysis. We used tomographic package I3 for sub-
392	tomogram analysis as described previously ⁴³ . 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

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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 <i>P. gingivalis</i>

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			
445	Correspondence and requests for materials should be addressed to B.H. or R.J.L.		
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544		

545 Figure legends

546 Figure 1. Porphyromonas gingivalis type IX machine revealed by cryo-ET. A, Schematic 547 representation of the structure and function of the T9SS of *P. gingivalis*. The different subunits are 548 indicated, as well as the putative route for substrate transport (dashed red line). B, A tomographic slice of 549 a wild-type P. gingivalis cell. C, Zoomed-in view of (B) showing the outer membrane (OM), inner 550 membrane (IM), Electron Dense Surface Layer (EDSL) and a type IX machine (T9SS) embedded in the 551 cell envelope. **D**, 3D surface view showing multiple T9SSs embedded in the wild-type *P. gingivalis* cell 552 envelope. E, A tomographic slice of a $\Delta porK$ mutant of P. gingivalis and F, zoomed-in view showing the 553 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.
- 560

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.

568

Figure 3. Proton-motive force-dependent conformational change of the *P. gingivalis* PorM protein
 and *F. johnsioniae* 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 \sim 32 nm and a continuous inner ring of \sim 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

Figure 5. Organization of secretion pore (Sov) at the OM. A and B, Central-section and top-down
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 Δsov 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.













 $(PorL_5)_{18}(PorM_2/PorK_2/PorN_2)_{18}(Sov)_8$