Type IX secretion system PorM and gliding machinery GldM form arches spanning the periplasmic space

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Type IX secretion system (T9SS), exclusively present in the Bacteroidetes phylum, has been studied mainly in Flavobacterium johnsoniae and Porphyromonas gingivalis. Among the 18 genes, essential for T9SS function, a group of four, porK-N (P. gingivalis) or gldK-N (F. johnsoniae) belongs to a co-transcribed operon that expresses the T9SS core membrane complex. The central component of this complex, PorM (or GldM), is anchored in the inner membrane by a trans-membrane helix and interacts through the outer membrane PorK-N complex. There is a complete lack of available atomic structures for any component of T9SS, including the PorKLMN complex. Here we report the crystal structure of the GldM and PorM periplasmic domains. Dimeric GldM and PorM, each contain four domains of ~180-Å length that span most of the periplasmic space. These and previously reported results allow us to propose a model of the T9SS core membrane complex as well as its functional behavior.
Bacteria, especially Gram-negative species, have assembled and evolved complex and specific cellular machines, known as secretion systems, to secrete proteins or DNA through the cell envelope into the surrounding medium or inside other cells. In diermed bacteria, protein secretion occurs either as a one-step process, in which substrates are translocated directly from the cytoplasm to the external milieu, or as a two-step process, in which the substrates first cross the inner membrane (IM) into the periplasm using the Sec, Tat, or holins pathways and then cross the outer membrane (OM) through a specialized translocon. After secretion, the substrates might stay attached to the OM surface, be released into the extracellular milieu, or be injected into a target cell. The type IX secretion system (T9SS) uses a two-step process. Depending on the bacterial strain, the T9SS coners very distinct functions. In F. johnsoniae, the T9SS contributes to gliding motility by secreting SprB, a cell-surface adhesin that is required for movement on solid surfaces. P. gingivalis, a non-motile bacterium, is a human oral pathogen and a major causative agent of periodontitis, as its T9SS secretes potent proteolytic enzymes called gingipains that degrade host cell tissues and interfere with innate host defense mechanisms. To date, 18 genes have been identi®ed as essential for T9SS function in P. gingivalis. Among them are a group of five genes, porP–porK–porL–porM–porN, which belong to a co-transcribed operon. The last four genes have orthologues in the F. johnsoniae genome, gldK–gldl–gldM–gldn, together with other extra orthologues of porN. PorK, PorL, PorM, and PorN, assemble as a >1.4 MDa trans-membrane complex. PorK (or GldK) is a lipoprotein anchored to the OM that interacts with the periplasmic protein PorN. PorL and PorM (Gldl and GldM) are IM proteins that interact via their trans-membrane segments. The core of PorL resides in the cytoplasm, whereas PorM, similar to GldM, has a long periplasmic domain. PorM interacts with both PorK and PorN complex, and therefore spans the entire periplasm by being anchored in the IM and interacting with the OM complex. PorM (or GldM) is therefore a central structural component of the T9SS and an interesting target for structure and function studies. Here, we present the atomic structures of the periplasmic domains of both PorM and GldM, that exhibit 22% amino-acid identity, and provide information regarding the contribution of each domain for interaction with PorK and PorN.

Results
Three-dimensional structure of GldM. The GldM (accession number GI: 58531935) and PorM periplasmic domains (GI: 188595218) (GldMp, PorMp) were cloned from residues 36–513 and 36–516, respectively. GldMp crystallized readily, and its structure was solved using the Se edge of a SeMet derivative for phasing (Table 1). One molecule was present in the asymmetric unit, but strong contacts exist with a symmetry-related protein in the crystal. The assembly of GldMp as a dimer was confirmed because domain swapping and tight locking to the symmetry-related dimer were observed, which have been previously demonstrated for GldM in solution, as assessed by size exclusion chromatography (SEC).

The GldMp dimer is elongated and straight with overall dimensions of ∼180 Å × 50 Å × 35 Å (Fig. 1). The dimer structure contains four domains, D1–D4 (Fig. 1) with seven α-helices and 22 β-strands in the sequence (α1–α2–α3–α4–α5) (β1–β2–β3–β4–β5–β6) (β7–β8–β9–β10–β11–β12–β13–β14) (β15–β16–α6–β17–β18–β19–α7–β20–β21–β22).

Domain D1 (32–232) is formed by helices 1–5 in an up and down fold. The D1 domain dimers are packed together through helices α1. The D2 (233–320) and D3 (321–405) domains are exclusively formed of β-strands. Each D2 domain swaps its β-strands 1 and 2 with the other D2 domain, whereas D3 domains swap β-strand 7. The main plane of domain D3 is perpendicular to that of domain D2 (Fig. 1). The D4 domains (406–513) are not subject to domain swapping but are packed together in the dimer (Supplementary Table 1). The junctions between domains D1–D2 and D3–D4 are compact and thus prevent flexibility (Fig. 1; Supplementary Fig. 1). However, the D2–D3 junctions are less compact and suggest that some bending may occur in solution. Remarkably, with a 180 Å-extended conformation, GldM spans most of the periplasmic space, as the distances between the IM and OM associated with T3SS, T4SS, and T6SS have been found to be ∼260 Å, ∼170 Å, and 180 Å, respectively.

Three-dimensional structure of PorM. The structural determination of PorMp was more tedious than that of GldM. Full-length PorMp resisted all attempted crystallization assays. Trypsin cleavage experiments were therefore performed, and a de®ned fragment (residues 224–516, PorMp224) was puri®ed and crystallized. Phasing was performed using SeMet substituted PorMp224. Domains D2 and D3 could be traced fairly easily, but domain D4 was only partially constructed due to poor electron density map. In an attempt to stabilize this domain, we raised anti-PorMp llama antibodies and selected a nanobody (nb130) from the resulting library that bound to PorMp with high af®nity (KD = 4.5 nM). We cloned a PorMp fragment between residues 224–516, co-crystallized it with nb130, and determined the structure of the complex (Fig. 2b). Surprisingly, the crystallized structure contained only domains D3 and D4, meaning that domain D2 was cleaved by a protease during crystallization; thus, the resulting structure spans residues 315–516 (PorMp315). The D4 domain was easily traced in the electron density map because it was stabilized by nb130 binding, and it was introduced into the PorMp224 structure, generating a complete model. The resulting PorMp224 structure exhibits three domains that resemble GldM domains D2–D4 (Figs. 1, 2b, and 3). Interestingly, PorMp domains D2 and D3 possess a domain-swapping motif identical to that of GldM (Fig. 3; Supplementary Fig. 2). Finally, we cloned, crystallized, and determined the structure of the N-terminal domain of PorMp (residues 30–212) in complex with a nanobody (PorMpN) (Fig. 2c; nanobody not represented). Using the structure of GldM, we could assemble PorMpN and PorMp224 in a realistic model of full-length PorMp (Fig. 2d).

Comparison between GldM and PorM. Taken individually, the four domains of PorMp superimpose well with those of GldM, with root mean square deviation values ranging from 1.6 to 3.5 Å (Supplementary Table 2; Supplementary Fig. 2). Domains D3 and D4 of PorMp224 and PorMp315 share the same straight topology as those of GldM, whereas domain D2 is bent with respect to D3–D4 at an angle of ∼45° because of the convolution of two rotations and a sliding of D3 monomers (Fig. 3; Supplementary Fig. 3).

Surprisingly, PorMp224, the D1 domain of PorMp, is missing the ®rst helix (residues 30–69), which was probably cleaved during crystallization (Fig. 2c; Supplementary Fig. 2). Another difference between the D1 domains of GldMp and PorMp is the organization of dimer packing. In GldMp, both D1 domains are packed side by side using their α1 helices; in PorMp224, the four monomers in the asymmetric unit do not pack together, probably because the α1 helix that forms the D1 domain interface in GldM is absent in this structure (Fig. 1). Finally, a complete domain can be modeled by assembling the various fragments using the GldM scaffold: PorMp315 was structurally aligned with PorMp224, the PorMp224, D2 domain was aligned with the GldMp...
D2 domain, and the PorM_{D1} domain was aligned with the GldMp D1 domain, together with the modeling of the first PorM_{D2} domain, and the PorM_{D1} domain was aligned with the NATURE COMMUNICATIONS | DOI: 10.1038/s41467-017-02784-7

An interesting feature of both GldM and PorM is their D2–D3 domain-swapping motifs. To test whether this domain swapping exists in vivo, we first performed bacterial two-hybrid (BACTH) experiments. We found that the D2–D3 construct oligomerizes, whereas the D1 and D4 isolated domains do not interact with themselves (Fig. 4a). On the basis of the structure of PorM, we introduced cysteine residues at different positions within the D2 and D3 domains of the full-length PorM protein (Fig. 4b). SDS-PAGE analyses in absence of reducing agent demonstrated that residues Ala-318 from one monomer is at close distance from residue Ala-391 from the second monomer, whereas the Met-325 residues from two monomers face each other in the dimer (Fig. 4c). These results confirm that the domain swapping occurs in vivo, in the context of the full-length protein.

The most striking difference observed between GldMp and PorM is their overall topology, which results from the PorM kink between D2 and D3 (Fig. 3; Supplementary Fig. 3). This kink is the result of two rotations around a vertical axis and around a horizontal axis, as in a cardan mount (Supplementary Fig. 3). Of note, the PorM D2–D3 bending movement can occur in the left or the right direction, leading to two non-superimposable structures. The observation of bending in a unique direction suggests that the two forms may equilibrate by exchange through a transient straight form resembling GldMp. During crystalization, the equilibrium would be displaced towards the form accommodated in the crystal.

We previously reported that the periplasmic domain of PorM interacts with both PorK and PorN. The contribution of PorM domains for contacting PorK and PorN was tested by BACTH. Our results show that the PorM–D4 domain is sufficient for interacting with PorN. By contrast, PorM interaction with PorK requires the D2–D3 and D4 domains, suggesting that either the three domains are required for interaction or that D2–D3-mediated dimerization of the D4 domain (monomeric in the isolated form) is necessary to properly interact with PorK (Fig. 5a).

### Table 1 Data collection phasing and refinement statistics

|                        | GldMp | GldMp SeMet-SAD | PorM_{D2} | PorM_{D2,SeMetMAD} | PorM_{D15}/nb130 | PorM_{D10}/nb01 |
|------------------------|-------|----------------|-----------|--------------------|------------------|----------------|
| **Data collection**    |       |                |           |                   |                  |                |
| Space group            | P6_{4}22 | P6_{4}22       | P4_{2}2 | P4_{2}2            | P1               | P2_{1}         |
| Cell dimensions        |       |                |           |                   |                  |                |
| a, b, c (Å)            | 71.4, 71.4, 426.9 | 71.40, 71.40, 426.09 | 77.0, 77.0, 228.6 | 77.4, 77.4, 226.9 | 55.2, 77.2, 156.3 | 80.3, 99.9, 80.3 |
| α, β, γ (°)            | 90, 90, 120 | 90, 90, 120 | 90, 90, 90 | 90, 90, 90 | 90.2, 91.7, 97.2 | 90, 93.8, 90 |
| **Wavelength Resolution (Å)** | 0.9677 | 0.97908 | 0.97888 | 0.979109 | 0.979336 | 0.976256 |
| Resolution (Å)         | 46.7-2.0 | 47.3-2.4 | 40.2-2.85 | 30-3.1 | 50-3.1 (3.27-3.1) | 50-3.1 (3.27-3.1) |
| R_{merge}              | 0.120 | 0.146 (3.212) | 0.076 | 0.091 | 0.093 (0.827) | 0.075 |
| Completeness (%)       | 101.1 (1.2) | 13.6 (0.9) | 14.8 (2.2) | 13.2 (2.8) | 13.1 (2.5) | 9.2 (9.8) |
| Redundancy             | 13.6 (14.4) | 20.4 (21.7) | 8.0 (8.0) | 9.1 (9.7) | 9.2 (9.8) | 2.9 (2.9) |
| **Refinement**         |       |                |           |                   |                  |                |
| Resolution (Å)         | 43.4-2.0 | 39.4-2.85 | 39.4-2.85 | 39.4-2.85 | 39.4-2.85 | 39.4-2.85 |
| No. of reflections     | 45,244 | 16,861 | 145,522 | 48,833 | 145,522 | 48,833 |
| R_{work}/R_{free}      | 22.2/25.9 | 21.7/24.9 | 18.0/21.5 | 21.3/24.2 | 18.0/21.5 | 21.3/24.2 |
| No. of atoms           |       |                |           |                   |                  |                |
| Protein                | 3534 | 4469 | 16,861 | 48,833 | 145,522 | 48,833 |
| Ligand/ion             | 24/— | 1/4 | —/— | —/— | —/— | —/— |
| Water                  | 298 | 60 | 60 | 60 | 60 | 60 |
| B-factors              |       |                |           |                   |                  |                |
| Protein                | 69.6 | 109.5 | 53.4 | 109.4 | 53.4 | 109.4 |
| Ligand/ion             | 89.4/— | —/167.1 | —/— | —/— | —/— | —/— |
| Water                  | 68.9 | 78.2 | 50.6 | 90.5 | 50.6 | 90.5 |
| R.m.s deviations       |       |                |           |                   |                  |                |
| Bond lengths (Å)       | 0.009 | 0.01 | 0.01 | 0.009 | 0.01 | 0.009 |
| Bond angles (°)        | 1.16 | 1.23 | 1.13 | 1.12 | 1.13 | 1.12 |

Values in parentheses are for highest-resolution shell.

**A putative multimer model of a complex.** Using the data from Sato et al.\(^\text{1}\), proposing a mass slightly larger than 1.4 MDa of the (PorKLMN)\(_2\) complex\(^\text{2}\) and from Vincent et al.\(^\text{3}\), together with the structures reported here, we speculated on the possible architecture of the T9SS core machinery. We used Symmdock software\(^\text{18}\) to identify which part of PorM/GldM might accommodate its cargo through the secretins\(^\text{19,20}\).
Discussion

It has been reported by us and others that PorM binds to PorN. The $K_D$ of the PorM–PorN association, $\sim 1 \mu M^3$, is comparable to that of the association between the TssJ lipoprotein with TssM ($K_D = 2–4 \mu M^{21}$), an event that initiates T6SS core complex assembly at the OM$^{15}$. In turn, PorN binds to the PorK lipoprotein; therefore, the T9SS PorK–PorN complex might represent a functional equivalent of the T6SS TssJ lipoprotein, with equivalent binding affinities for PorM or TssM. In the T6SS core complex, TssJ also binds the C-terminal D4 domain of TssM$^{15}$.

Sato et al.$^1$ isolated a core membrane complex of T9SS from *P. gingivalis* that was extracted using DDM. Analysis of this complex by western blot and SDS-PAGE revealed that it contains four components, PorK, PorL, PorM, and PorN, and that its mass is slightly larger than 1.4 MDa$^1$. We recently reported that PorM, PorN, and PorK form homodimers, whereas PorL forms homotrimers$^3$. Hence, the stoichiometry of the assembly is expected to be PorL$_3$/PorM$_2$/PorN$_2$–PorK$_2$, resulting in an overall mass of $\sim 410$ kDa. Therefore, three or four copies of the above-described assembly would be necessary to form the $\sim 1.4$ MDa isolated by Sato et al.$^1$.

In contrast, Gorasia et al.$^{12}$ reported data that differ from those reported above. In their report, a *P. gingivalis* membrane fraction was purified leading to large rings that were analyzed with electron microscopy. These rings, which were attached to the membrane, measure $\sim 50$ nm in diameter (35 nm internally) and are formed of 32–36 1:1 PorK:PorN complexes. They did not observe the presence of PorM or PorL. These authors proposed that the native complex therefore contains 32–36 copies. The same rings were observed on the surface membrane of *P. gingivalis* mutants lacking *porL*, *porM*, and *porP*. Strangely enough, despite the strong interaction measured between PorM and PorN, PorM was not observed in the complex. Furthermore, a pore of 350 Å would be very difficult to occlude during non-secretion periods. We therefore suspect that the gigantic pore reported in Gorasia et al. might be due to the absence of PorM/PorL in the preparations, resulting either from purification or from the use of cells encoding a *porM* mutation. As often observed with protein-forming rings (e.g., phage portals$^{22}$, RAD52, or viral nucleocapsids), ring stoichiometry might vary in the absence of controlling elements.

By assembling all of the available data, we propose a schematic model of the T9SS core complex and secretion-associated opening.
based on the topology of the PorL3/PorM2/PorN2/PorK2 moieties (Fig. 5b, c). Each PorMp dimer is anchored in the IM by its two helices, which interact with the three helices of the PorL trimer. Close to the OM, the PorM–D4 domain mediates contact with the PorN–PorK complex. The membrane-attached ring of PorN2/PorK2 should be associated with the secretion pore and may control its access by the effector. Interestingly, several possible candidates have been proposed controlling secretion, although no definitive arguments implicating a specific one have been made. To note, Veith et al. proposed that a cascade of several OM components might be associated with the core machinery for the post-treatment of effectors and their eventual association with the OM.

We speculate that the hinge between D2 and D3 may have a role in PorN/PorK opening, as it has been proposed that PorM is energized by the PorL trimer and that the two proteins form an energy transduction system for effector translocation. The putative straight topology of PorM, resembling that of GldM, may therefore be associated with a closed state of the system. This state might be converted to the open form through a conformational change at the D2–D3 interface or through PorL/PorM activation (Fig. 5b, c). Finally, we suggest that due to the structural similarity between PorM and GldM, both classical T9SS and Gld T9SS membrane core complexes might assemble and function in similar ways.

**Methods**

**Protein production.** The sequences corresponding to PorMp (residues 36–516), PorM215 (residues 224–516), and PorMP224 (residues 44–217) were cloned into the pET28a derivative vector pLIC03 (Supplementary Table 3). The sequence corresponding to GldM (residues 36–513) was amplified from Flavobacterium johnsoniae cDNA (ATCC17061, Leibniz Institute DSMZ) and was cloned into pLIC03 with the same protocol as for PorM constructs (Supplementary Table 3). PorMP224, PorMP224, and SeMet GldM were produced in Rosetta E. coli cells and purified by nickel-affinity chromatography followed by SEC [16,17].

**Production of PorM-specific llama nanobodies.** The PorM-specific nanobodies nb01 and nb130 were obtained and purified by standard methods. In brief, a llama (Lama glama) was immunized with purified PorM (Ardèche-lamas France). PorM was injected subcutaneously four times at 1-week intervals using incomplete Freund’s adjuvant, followed by a fifth injection 2 weeks later. Blood samples were collected aseptically 5 days after the last boost. Lymphocytes were isolated from blood samples, and cDNA was synthesized from the acquired RNA using a reverse PCR protocol. A nanobody phage display library of ~10^9 independent transformants was generated using the phagemid vector pHEN4 [24,25]. Phage display selection and screening of specific nanobodies were performed as previously published [25]. After enrichment of antigen-specific clones by rounds of selection on solid-phase-coated antigen, PorM-specific nanobodies were identified, and the inserts of the corresponding pHEN4-derived plasmids were sequenced and cloned into the pHEN6 vector. E. coli Wk6 cells carrying the pHEN6 derivatives were grown at 37 °C in terrific broth supplemented with 0.1% glucose and 100 μg/mL ampicillin to an optical density ~0.6–1.0 and the expression of the nanobodies was induced by the addition of 1 mM IPTG for 16 h at 28 °C. The periplasmatic fraction containing the nanobodies was prepared using mild osmotic shock and the His-tagged nanobodies were immobilized on a 5-mL Ni-NTA column equilibrated in 50 mM Tris-HCl, pH 8, 300 mM NaCl, and 10 mM imidazole. Nanobodies were eluted in 250 mM imidazole and concentrated using the Amicon-technology (10-kDa cut-off) prior to loading on a HiLoad 16/60 Superdex 75 gel filtration column equilibrated in 20 mM Tris–HCl, pH 8, 50 mM NaCl.

**Crystallization and crystallographic processing.** All crystallization trials were performed using the sitting-drop vapor-diffusion method at 293 K in 96-well Greiner plates. Drops were prepared by mixing different volumes (100, 200, and 300 μL) of protein solution and 100 μL of precipitant solution, and were equilibrated at 1.0 and the expression of the nanobodies was induced by the addition of 1 mM IPTG for 16 h at 28 °C. The periplasmatic fraction containing the nanobodies was prepared using mild osmotic shock and the His-tagged nanobodies were immobilized on a 5-mL Ni-NTA column equilibrated in 50 mM Tris–HCl, pH 8, 300 mM NaCl, and 10 mM imidazole. Nanobodies were eluted in 250 mM imidazole and concentrated using the Amicon-technology (10-kDa cut-off) prior to loading on a HiLoad 16/60 Superdex 75 gel filtration column equilibrated in 20 mM Tris–HCl, pH 8, 50 mM NaCl.

**Crystallization and crystallographic processing.** All crystallization trials were performed using the sitting-drop vapor-diffusion method at 293 K in 96-well Greiner plates. Drops were prepared by mixing different volumes (100, 200, and 300 μL) of protein solution and 100 μL of precipitant solution, and were equilibrated at 150 μL reservoir volume. Crystallization trials of PorMP224 and PorMP224, SeMet derivatives were performed with the PEGs Suite (Qiagen). After optimization, the final crystallization conditions were 0.02 M sodium acetate pH 4.8–5.8, 0.2 M zinc acetate, 15–25% (w/v) PEG3350, with a protein-to-well ratio of 3:1 (v/v). Crystallization trials of PorMP224, nb130 were performed with the 1:1 complex at 10 mg/mL with 0.2 M Ammonium citrate tribasic pH 7.0 20% (w/v) PEG3350 as precipitant. Crystallization trials of PorMP224, nb01 was performed with the 1:1 complex at 10 mg/mL with 0.1 M Hepes pH 7.5, 0.2 M NaCl, and 25% (w/v) PEG3350 as precipitant. Crystals were briefly soaked in crystallization solution supplemented with 20% (v/v) propylene glycol before being flash-frozen in a nitrogen gas stream at 100 K.

Native PorMP224 diffraction data were collected to 2.85 Å resolution on beamline ID23–1 at the European Synchrotron Research Facilities (ESRF, Grenoble, France), using a Pilatus detector. PorMP224 SeMet MAD data were...
Fig. 4 In vivo validation of the PorMp structure. a Bacterial two-hybrid analysis. BTH101 reporter cells carrying pairs of plasmids producing the indicated PorM fragments fused to T18 or T25 domain of the Bordetella adenylate cyclase were spotted on X-Gal-IPTG reporter LB agar plates. The blue coloration of the colony reports interaction between the two partners. Controls include T18 and T25 fusions to TolB and Pal, two proteins that interact but unrelated to the T9SS. b Ribbon representation of a portion of PorM with the Cys mutants (tested in c) identified by spheres. c Disulfide-bond formation. Total membrane fractions from cells producing the wild-type (WT) FLAG-tagged PorM protein, and the indicated cysteine variants introduced into the cysteine-less C92S PorM, were subjected to 10%-acrylamide SDS-PAGE and immunodetected using the anti-FLAG antibody. The asterisk on right indicates bands corresponding to a PorM dimer. Molecular weight markers are shown on right.

Fig. 5 Topological and functional models of PorM and GldM core membrane complexes. a Bacterial two-hybrid analysis. BTH101 reporter cells carrying pairs of plasmids producing the indicated PorM fragments fused to T18, and PorK or PorN fused to T25 were spotted on X-Gal-IPTG reporter LB agar plates. The blue coloration of the colony reports interaction between the two partners. Controls include T18 and T25 fusions to TolB and Pal, two proteins that interact but unrelated to the T9SS. b Ribbon representation of a portion of PorM with the Cys mutants (tested in c) identified by spheres. c Disulfide-bond formation. The rest of the model collects data from previous reports13,17. K, L, M, and N schematically represent PorK, L, M, and N or GldK, L, M, and N collected to 3.1 Å on the same beamline. The data sets were integrated with XDS27 and were scaled with SCALA from CCP4 Suite v6.3.028. Complete data sets of PorMp224/nb130 and PorMp315/nb01 were collected at beamline PROXIMA 1 at SOLEIL, Paris, France. A Superseded C98 A data set collected at 2.85 Å resolution on beamline ID30A-3 at the European Synchrotron Research Facility (ESRF), Grenoble, France. SeMet GldMp single-wavelength anomalous diffraction (MAD) method using the SeMet PorMp224 data set at 3.1 Å resolution. Heavy-atom substructure determination, positional refinement, phase calculations, and solvent flattening were performed using autoSHARP29, SHARP30, and SOLOMON31. The partial model of SeMet PorMp224 was built using Turbo-Frodo32, and was subsequently used as model for molecular replacement with MOLREP33 to solve the structure of native PorMp224 at 2.85 Å. The structure of GldMp was solved by the SAD method using the SeMet GldMp data set collected at 2.4 Å resolution. Heavy-atom substructure determination, positional refinement, phase calculations, and solvent flattening were performed using autoSHARP29, SHARP30, and SOLOMON31. The partial model of SeMet PorMp224 was built using Turbo-Frodo32, and was subsequently used as model for molecular replacement with MOLREP33 to solve the structure of native GldMp at 2 Å. The building of GldMp was then completed manually with COOT34. The structure of the complex PorMp224/nb130 was solved by molecular replacement with MOLREP33 using the partial model of domains D3 and D4 of PorMp224, and the structure of nb1307 as models. The building of domain D4 of PorMp224 and PorMp315 was then completed manually with COOT34. The structure of the complex PorMp315/nb01 was solved by combining molecular replacement with MOLREP33 using the structure of nb0117 as starting model, and several cycles of automatic building of PorMp315 in the extra density with Buccaneer35 followed by refinement with autoBUSTER36. The building of PorMp315 was then completed manually with COOT34. The structure of GldMp was solved by the SAD method using the SeMet GldMp data set collected at 2.4 Å. Heavy-atom substructure determination, positional refinement, phase calculations, and solvent flattening were performed using autoSHARP29, SHARP30, and SOLOMON31. The partial model of SeMet GldMp was automatically built with Buccaneer35, and was subsequently used as model for molecular replacement with MOLREP33 to solve the structure of native GldMp at 2 Å. The building of GldMp was then completed manually with COOT34.
Refinement, correction, and validation of the different structures were performed with autoBUSTER36, COOT34, and Molprobity37, respectively. More technical details are provided elsewhere16,17. Refinement statistics are reported in Table 1.

SymmDock modeling. SymmDock software35 complex modeling was performed using the straight GlcMp structure as input, with both threefold and fourfold symmetry. The two best solutions were found to be close together resulting in a tight-packed N-terminal domain. To note, SymmDock works by maximizing the contact surface between monomers and minimizing the steric clashes.

Bacterial two-hybrid. PorM–D1, PorM–D2–D3–D4, PorM–D2–D3, and PorM–D4 domains fused to the T18 and T25 domains of the Bordetella adenylate cyclase have been engineered by restriction-free ligation. BACTH experiments have been performed as previously3. After introduction of the two plasmids producing the fusion proteins into the reporter BTH101 strain, plates were incubated at 30 °C for 48 h. Three independent colonies for each transformation were inoculated into 600 μL of LB medium supplemented with ampicillin, kanamycin, and IPTG (0.5 mM). After overnight growth at 30 °C, 106 μL of each culture were dropped onto LB plates supplemented with ampicillin, kanamycin, IPTG, and X-Gal, and incubated for 16 h at 30 °C. Controls included negative assays with TolB/Pal, or Maf/MafG, two protein pairs unrelated to the T9SS. The experiments were done at least in triplicate and a representative result is shown.

In vivo disulfide-bond formation. Cysteine codons were introduced by Quick change site-directed mutagenesis into the plasmid encoding the C92S variant of FLAG-tagged PorM. After gene induction, cells were lysed, and the total membrane fractions obtained after ultracentrifugation were subjected to 10% acrylamide SDS-PAGE, transfer to nitrocellulose, and immunodetection using monoclonal anti-FLAG antibody. Figure 4c uncropped gel is provided as Supplementary Fig. 5.

Data availability. Structures of PorM22, complex PorM22–nboI30 and PorM22–nboI30, and GlcMp were deposited in the Protein Data Bank (PDB) under accession numbers 6EY5, 6EY0, 6EY6, and 6EY4, respectively. Other data are available from the corresponding authors upon reasonable request.

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Author contributions
E.C., A.R. and C.C. designed the study. P.L., J.R., M.S.V., Q.H.T., E.C., C.K., C.C. and A. R. contributed to the analysis of data and preparation of this manuscript. P.L., J.R., Q.H.T. and C.K. performed the proteins production, characterization, crystallization, and
crystallographic experiments. A.D. performed the nanobodies selection, M.S.V. and E.C. performed the BACTH and in vivo experiments.

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