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Structural and functional analyses of the *Porphyromonas gingivalis* type IX secretion system PorN protein

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Porphyromonas gingivalis, the major human pathogen bacterium associated with periodontal diseases, secretes virulence factors through the *Bacteroidetes*-specific type IX secretion system (T9SS). Effector proteins of the T9SS are recognized by the complex via their conserved C-terminal domains (CTDs). Among the 18 proteins essential for T9SS function in *P. gingivalis*, PorN is a periplasmic protein that forms large ring-shaped structures in association with the PorK outer membrane lipoprotein. PorN also mediates contacts with the PorM subunit of the PorLM energetic module, and with the effector's CTD. However, no information is available on the PorN structure and on the implication of PorN domains for T9SS assembly and effector recognition. Here we present the crystal structure of PorN at 2.0-Å resolution, which represents a novel fold with no significant similarity to any known structure. In agreement with *in silico* analyses, we also found that the N- and C-terminal regions of PorN are intrinsically disordered. Our functional studies showed that the N-terminal disordered region is involved in PorN dimerization while the C-terminal disordered region is involved in the interaction with PorK. Finally, we determined that the folded PorN central domain is involved in the interaction with PorM, as well as with the effector's CTD. Altogether, these results lay the foundations for a more comprehensive model of T9SS architecture and effector transport.

Porphyromonas gingivalis, an anaerobic, nonmotile gram-negative bacterium, is the major human oral pathogen associated with periodontal diseases (1, 2). Chronic *P. gingivalis* infection is also linked to rheumatoid arthritis, heart disease, diabetes, Alzheimer disease, and other systemic diseases (3–9). Virulence factors responsible for *P. gingivalis* pathogenesis are secreted through the *Bacteroidetes*-specific type IX secretion system (T9SS) (10–12). These virulence factors are mainly cysteine proteases called gingipains that degrade host cell tissues and interfere with innate host defense mechanisms (13).

Among other virulence factors secreted by the T9SS are the heme-binding protein Hbp35 involved in extracellular iron uptake (14) and the peptidylarginine deiminase PPAD whose citrullination activity is linked to rheumatoid arthritis (15). T9SS acts as a two-step mechanism: the substrate proteins are first exported into the periplasm by the Sec machinery after cleavage of their N-terminal signal peptide; then, a conserved C-terminal domain (CTD) is specifically recognized by the T9SS that transports them across the outer membrane (OM) (16, 17). Once outside the cell, the substrates are either delivered into the extracellular medium or remain attached to the cell surface through anchoring to anionic lipopolysaccharide (18–21).

Beside *P. gingivalis*, the T9SS is also encoded on the genome of a large subset of *Bacteroidetes* species, including the model bacterium *Flavobacterium johnsoniae* (10). In this nonpathogenic soil bacterium, the T9SS contributes to gliding motility by secreting SprB, a cell surface adhesin organized as filaments and that is required for movement on solid surfaces (22). Studies on *F. johnsoniae* motility led to the model that the T9SS is a new rotary machine driven by proton motive force powering SprB rotation (23).

To date, 18 genes have been identified as essential for T9SS function in *P. gingivalis* (12, 24). Among them, the *porK-L-M-N* genes (*gldK-L-M-N* in *F. johnsoniae*) are cotranscribed (25). The subunits encoded by these genes assemble into three subcomplexes: (i) a transenvelope complex comprising the PorK-L-M-N proteins; (ii) an OM-associated translocon complex through which effectors are transported across the OM and constituted of the Sov translocon, the plug protein, and PorV that is proposed to convey the effectors to (iii) the attachment complex, responsible for effector processing and attachment at the cell surface or release in the medium (12, 20). The PorK-L-M-N assembles as a >1.4-MDa complex that was proposed to constitute the T9SS core complex (26). This complex is proposed to select the effectors to be secreted via contacts between the effector CTDs and the PorM and PorN proteins (27). This core complex can be divided in two subcomplexes: the PorL-M inner membrane complex and the PorK-N OM-associated complex. PorL and PorM are both inner membrane proteins that interact through their

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transmembrane helices (25). PorL has a cytoplasmic domain, whereas PorM harbors an extended periplasmic domain that spans most of the periplasmic space (28, 29). In *F. johnsoniae*, the GldL-M complex was proposed to be the rotary motor associated with gliding motility (30). Very recently, a partial structure of this complex was solved by cryo-EM, revealing a GldL₅-GldM₂ stoichiometry and demonstrating structural and functional homologies with known molecular motors (30).

The PorL-M complex interacts with the PorK-N OM-associated complex, through contacts between the PorM periplasmic domain and both PorN and PorK (25, 28). PorN is a periplasmic protein, whereas PorK is an OM lipoprotein (25). Cryo-EM and cryo-electron tomography observations revealed that the PorK-N complex assembles into large ring-shaped structures of 50 nm diameter (31, 32). These PorK-N rings are located in the periplasm, underneath the OM, and are proposed to be inserted into the OM by the PorK lipid moiety. These ring structures comprise approximately 32 to 36 subunits of each protein with a PorK:PorN molar ratio of 1:1. However, the resolution of the cryo-EM data was not sufficient to provide details on the structure of each partner, their organization into the ring, or their mode of interaction.

In this study, we focused on the PorN protein (PGN_1673; TIGR03523 family, GI: 188595217). *In silico* analyses and protease accessibility experiments suggested that the N- and C-terminal regions of PorN are intrinsically disordered. We further showed that these regions are involved in PorN oligomerization and PorK interaction. We then solved the structure of the PorN central core by X-ray crystallography, revealing a new fold, and showed that it mediates contacts with PorM and effector CTDs.

Results

PorN presents N- and C-terminal intrinsically disordered regions

In a previous study we have shown that the PorN periplasmic protein (PorN_p, residues 23–359, *i.e.*, devoid of the signal peptide) forms dimers and interacts with PorM and PorK (25). In order to get structural information, we overproduced and purified PorN_p and carried out crystallization trials. Despite extensive screens, no crystal was obtained. Bioinformatic analysis of the PorN sequence suggested the presence of intrinsically disordered N- and C-terminal regions, which could prevent crystallization (Fig. 1). As intrinsically disordered regions are usually more prone to proteolysis, the PorN_p protein preparation was subjected to limited proteolysis assay with different proteases, and a stable domain (PorN_p-G) was obtained after overnight incubation with the endoprotease Glu-C at a 1:1000 (*w:w*) ratio (Fig. 2). Edman sequencing of PorN_p-G revealed that the protease-resistant folded domain starts at residue 55, and the molecular mass (24,949 Da) obtained by mass spectrometry suggested that it ends at residue 265.

The PorN N-terminal disordered region mediates dimer formation

The sequence encoding the PorN proteolysis-resistant fragment, PorN_{ΔNΔC} (residues 55–265), was cloned, and PorN_{ΔNΔC} was purified to homogeneity (Fig. 2). Size-exclusion chromatography–multiangle light scattering (SEC-MALS) analysis of PorN_{ΔNΔC} showed that the PorN folded core is monomeric, in contrast to the dimeric form of full-length PorN (Fig. 2). These results suggest that the N- and/or

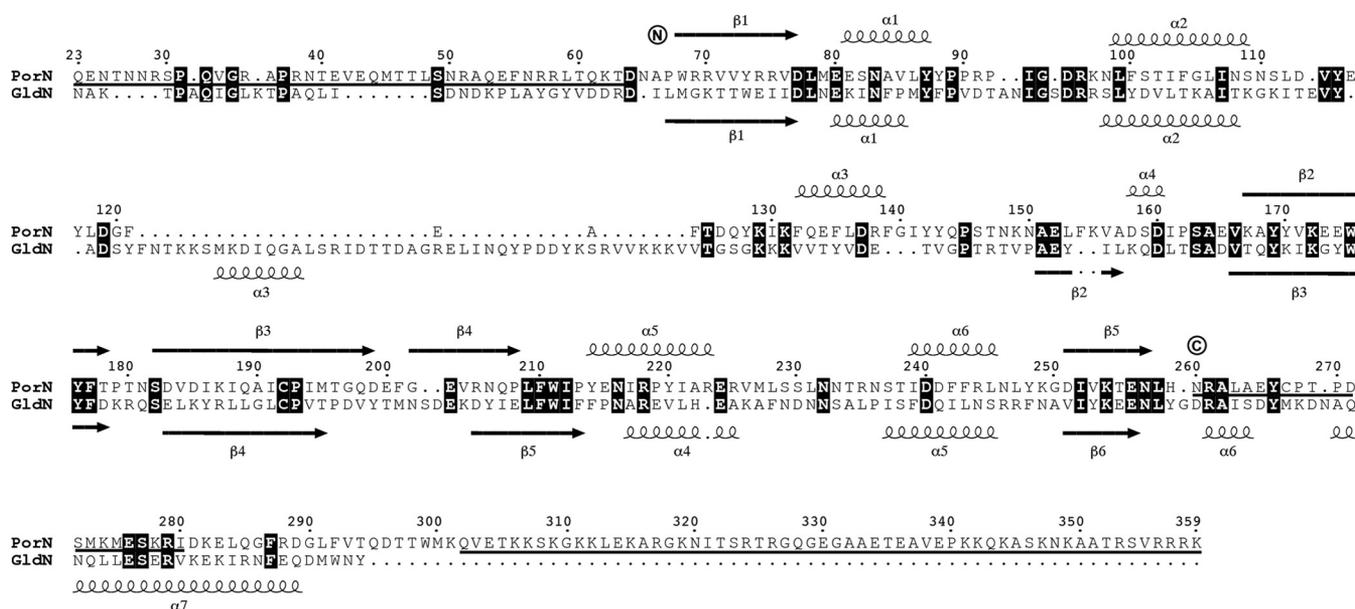


Figure 1. Sequence alignment of *P. gingivalis* PorN and *F. johnsoniae* GldN periplasmic proteins. PorN numbering is indicated on top, and secondary structures as observed in the crystal structure, are shown above the alignment; intrinsically disordered regions predicted by IUPred2A (51) are underlined; N and C termini of the PorN_{ΔNΔC} structure are indicated by the circled N and C, respectively. GldN secondary structures predicted by JPred4 (52) are displayed below the alignment. Amino acid sequences were aligned using T-Coffee (version 11.00.d625267, <http://www.tcoffee.org>) (53), and the figure was prepared using ESPript (version 3.0, <http://esprict.ibcp.fr/>) (54).

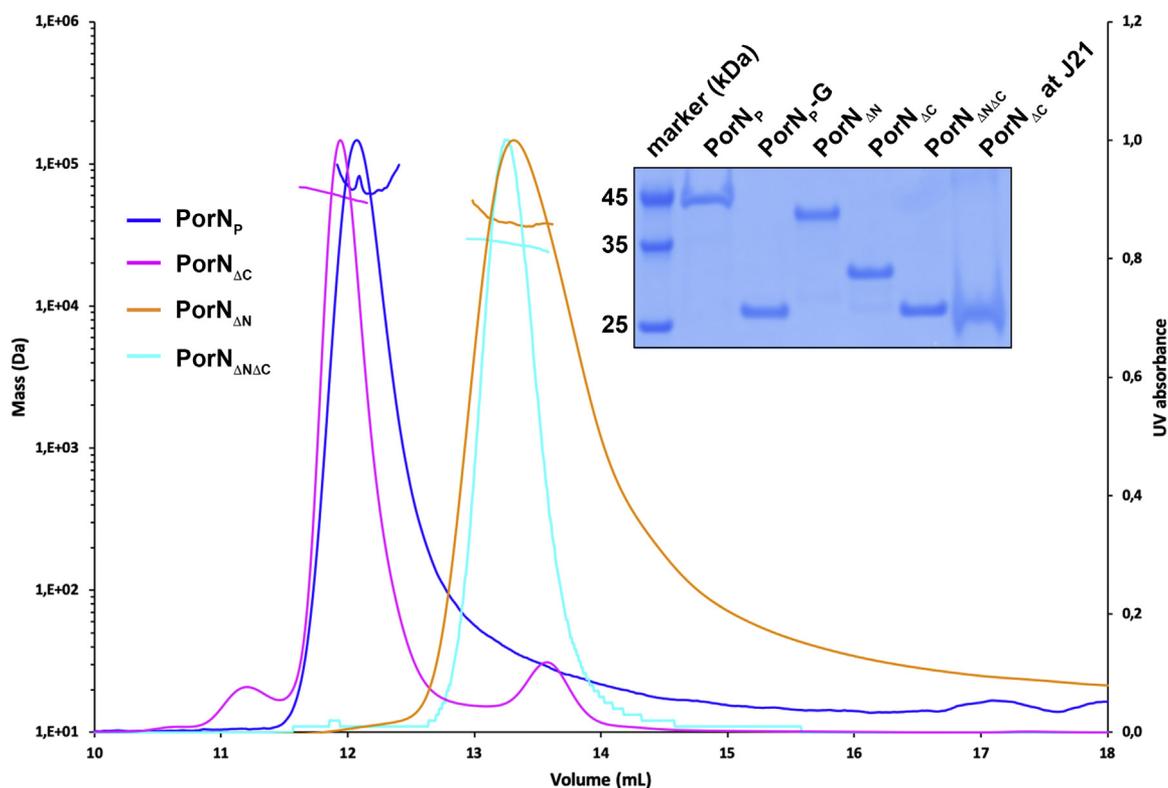


Figure 2. Size-exclusion chromatography–multiangle light scattering analysis of different PorN constructs. Chromatograms of purified PorN_p (blue), PorN_{ΔN} (orange), PorN_{ΔC} (magenta), and PorN_{ΔNΔC} (cyan) are shown. The measured molar masses are ~82.1, ~38.8, ~61.7, and ~28.0 kDa for PorN_p, PorN_{ΔN}, PorN_{ΔC}, and PorN_{ΔNΔC}, respectively. The inset shows the SDS-PAGE/Coomassie blue staining analysis of the different constructs.

C-terminal region(s) is/are involved in PorN_p dimer formation. To better understand the contribution of the N- and C-terminal regions to PorN oligomerization, we engineered PorN_p constructs deleted of the N- (PorN_{ΔN}, residues 55–359), C- (PorN_{ΔC}, residues 25–265), or both (PorN_{ΔNΔC}, residues 55–265) terminal regions. These PorN variants were fused to the T18 and T25 domains of the adenylate cyclase, and pairwise interactions were tested by bacterial two-hybrid

(BACTH) analyses. Our results show that PorN_p and PorN_{ΔC} oligomerize but not PorN_{ΔN} or PorN_{ΔNΔC} (Fig. 3A), suggesting that the N-terminal region is required for PorN oligomerization. This result was confirmed *in vitro*. The different PorN constructs, fused to an N-terminal His₆ tag, were purified by metal affinity chromatography and gel filtration and subjected to SEC-MALS experiments. Figure 2 shows that PorN_p and PorN_{ΔC} are dimeric in solution, whereas,

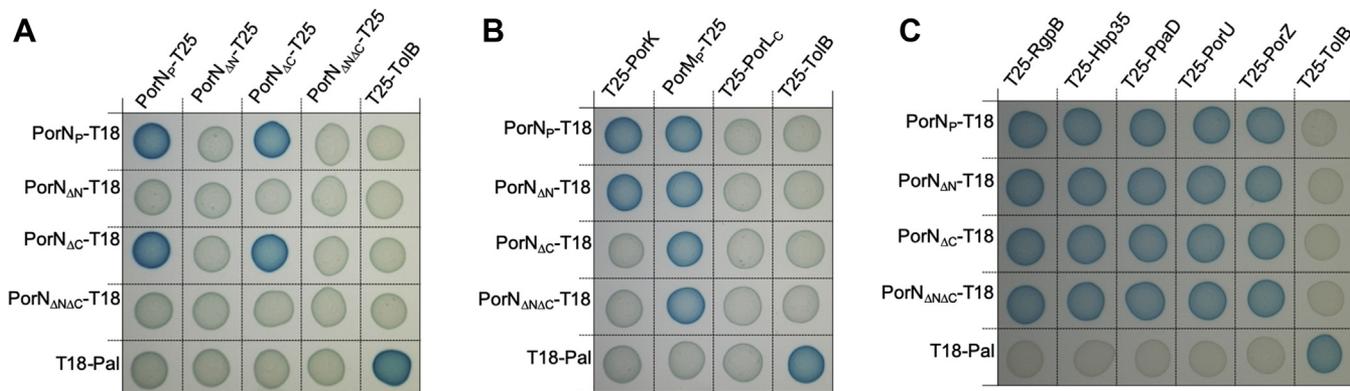


Figure 3. Bacterial two-hybrid analysis. Interaction of PorN variants with (A) themselves, (B) components of the PorKLMN complex, and (C) C-terminal secretion signals of T9SS effectors. BTH101 reporter cells producing the indicated proteins or domains fused to the T18 or T25 domain of the *Bordetella* adenylate cyclase were spotted on X-Gal-IPTG reporter LB agar plates. The blue color of the colony reports interaction between the two partners. Controls include T18 and T25 fusions to TolB and Pal, two proteins that interact but are unrelated to the T9SS. The assay has been performed in triplicate with identical results, and a representative experiment is shown. The different PorN domains are the entire periplasmic domain (PorN_p) and constructs deleted of the N-, or C-, or both terminal domains (PorN_{ΔN}, PorN_{ΔC}, and PorN_{ΔNΔC}, respectively); PorK, soluble, unacylated form of PorK; PorM_p, PorM periplasmic domain; PorL_c, PorL cytosolic domain; RgpB, Hbp35, PPAD, PorU, PorZ, C-terminal domains of *P. gingivalis* gingipain RgpB, hemin-binding protein Hbp35, peptidylarginine deiminase PPAD, and T9SS components PorU and PorZ, respectively.

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similarly to PorN_{ΔNΔC}, PorN_{ΔN} is monomeric (Fig. 2). Taken together, the *in vivo* and *in vitro* assays demonstrate that PorN dimer formation is mediated by the N-terminal intrinsically disordered region.

Contributions of disordered regions and folded core to PorN interactions

PorN was previously shown to interact with the PorK OM lipoprotein, the PorM periplasmic domain, as well as the T9SS effector CTDs (25–27, 31). To gain information on the role of the N- and C-terminal disordered regions and of the central folded core, the PorN variants were assayed for their capacity to interact with PorK, PorM, and effector CTDs by BACTH. The unacylated form of PorK, the PorM periplasmic domain (PorM_p), the PorL cytoplasmic domain (PorL_c), and the CTDs of various T9SS substrates (the gingipain RgpB, the hemin-binding protein Hbp35, the peptidylarginine deiminase PPAD, and the T9SS components PorU and PorZ) were fused to the T25 domain and tested against T18-PorN fusions. Figure 3B shows that PorN_p and PorN_{ΔN} interact with PorK and PorM_p, whereas PorN_{ΔC} interacts only with PorM_p. In addition, all PorN constructs interact with the CTDs of T9SS substrates (Fig. 3C). These results demonstrate that the PorN C-terminal disordered region is required for the interaction with PorK, whereas the PorN central core is sufficient to mediate contacts with PorM and the CTDs.

Crystal structure of the PorN central folded core

As described above, trials to crystallize PorN_p were unsuccessful, probably because of the presence of N- and C-terminal intrinsically disordered regions. Crystallization trials were thus carried out with the PorN_{ΔNΔC} fragment, corresponding to the proteolysis-resistant fragment PorN_p-G. Selenomethionine (SeMet)-substituted PorN_{ΔNΔC} was purified and crystallized, and a single wavelength anomalous dispersion data set was collected at 2.5-Å resolution. Phases were calculated and an initial model comprising residues 67 to 258 was built. Two molecules are present in the asymmetric unit (Fig. S1), but this oligomerization state was likely induced by crystallization as SEC-MALS and BACTH analyses showed that PorN_{ΔNΔC} is

monomeric (Figs. 2 and 3A). However, whether the arrangement of the two PorN molecules in the crystal packing corresponds to a physiological organization, or not, remains to be determined. In parallel, crystallization trials of the PorN_{ΔC} and PorN_{ΔN} fragments were performed. Only crystals of PorN_{ΔC} were obtained; they were isomorphous to those of PorN_{ΔNΔC}, and a native data set was collected at 2.0-Å resolution. The model of PorN_{ΔNΔC} was used to solve the structure of PorN_{ΔC} by molecular replacement. Two molecules are present in the asymmetric unit, with the same orientation as PorN_{ΔNΔC}. The superimposition of PorN_{ΔC} and PorN_{ΔNΔC} shows an r.m.s.d. of 0.94 Å, indicating that the two molecules are almost identical (Fig. S2). As expected, the N-terminal disordered region of PorN_{ΔC} is not visible in the electron density and the refined structure of PorN_{ΔC} comprises residues 66 to 260. Nevertheless, the presence of the missing residues is not compatible with the crystal packing, suggesting that PorN_{ΔC} has undergone proteolysis during crystallization. Indeed, SDS-PAGE analysis of purified PorN_{ΔC} confirmed that the protein was cleaved after 21 days, which corresponds to the crystal growth, and the size of the proteolytic product corresponds to that of PorN_{ΔNΔC} (Fig. 2).

The structure of the PorN folded core consists of an elongated antiparallel β-sheet covered on one side by six short α-helices connected by long loops, with connectivity β1α1α2α3α4β2β3β4α5α6β5 (Fig. 4). The β-sheet is composed of four β-strands on one extremity and two β-strands on the other, with the long 18-residue β-strand β3 being common to the two parts of the β-sheet. The N and C termini of the PorN folded domain are located very close to each other (<10 Å), suggesting that the N- and C-terminal disordered domains are likely to be at close proximity.

A structural similarity search within the Protein Data Bank (PDB) of the PorN folded domain structure using the DALI server (33) did not return any hit with statistically significant similarities. The highest Z-score (4.9) corresponds to the outer membrane enzyme PagL from *Pseudomonas aeruginosa* (PDB entry 2ERV), which folds into an eight-stranded β-barrel. Four β-strands of the PagL β-barrel superimpose with the four-stranded part of the PorN β-sheet with a core r.m.s.d. of 3.3 Å (Fig. S3). Actually, only the PorN β-sheet can be partially

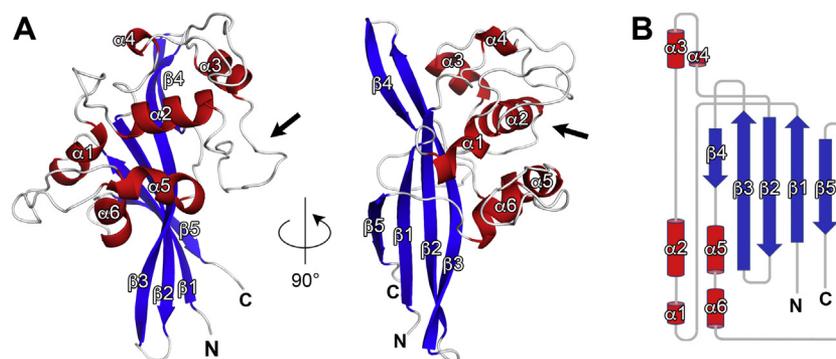


Figure 4. Overall structure of the *P. gingivalis* PorN folded domain. A, the PorN folded domain is shown as a ribbon diagram with red α -helices and blue β -sheets. The positions of the N and C termini are indicated. The figure was prepared using PyMOL (version 1.20, <https://pymol.org>). The position of the insertion (extra loop) in *F. johnsoniae* GldN is indicated by the arrow. B, topology diagram of *P. gingivalis* PorN. The figure was prepared using the TopScan plugin in PyMOL.

superimposed to a part of the structures corresponding to the 10 highest Z-score returned by the DALI server. This absence of similarity with any known structure suggests that the PorN core domain represents a new fold.

Discussion

The T9SS is a large multiprotein complex that delivers substrate proteins into the medium or that anchors them to the cell surface (2, 11, 20, 24). The recent years have started to provide information onto T9SS composition and organization, and the structures of some T9SS subunits have been solved. Here, we pursued this effort and report the structure–function analysis of the *P. gingivalis* PorN periplasmic protein. Together with PorK, PorN assembles large ring-shaped structures located underneath the membrane for which a low-resolution EM structure has been reported (31). Our analyses show that the PorN N- and C-terminal regions are intrinsically disordered, whereas the central domain is folded. The structure of the folded domain was then solved by X-ray crystallography and presents no similarity with any known structure.

It was previously shown that PorN dimerizes (25); our bacterial two-hybrid and SEC-MALS analyses reveal that dimerization is mediated by the N-terminal region. PorN dimerization is likely to be physiologically relevant, as PorN dimers were identified by chemical cross-linking of whole cells (31). Of interest, it was shown that a PorK-N ring is composed of 32 to 36 PorK-N complexes (31), suggesting that 16 to 18 PorN dimers are involved in ring formation. Bacterial two-hybrid assays revealed also the contribution of the other PorN domains for interaction with its partners. The C-terminal domain is involved in the interaction with PorK, while the folded domain is involved in the interaction with the periplasmic domain of PorM. Furthermore, we showed that the PorN folded domain is also involved in the interaction with the CTDs from five T9SS substrates, RgpB, Hbp35, PPAD, PorU, and PorZ. The current model of T9SS substrate transport suggests that substrates are selected in the periplasm by the PorM subunit and then conveyed to the OM-associated Sov translocon for their secretion across the OM. The PorKN ring, located underneath the OM, connects the PorM transenvelope protein to the translocon complex. By interacting with T9SS effectors, our results suggest that, in addition to playing a central role in the T9SS assembly, PorN is also involved in the trafficking of the substrates. We propose that the PorKN ring collects the effectors from the PorLM energetic module and provides them to the translocon.

It is interesting to note that PorN and its homologue in *F. johnsoniae*, GldN, differ significantly. Indeed, a sequence alignment between *P. gingivalis* PorN and *F. johnsoniae* GldN shows that no intrinsically disordered domains are predicted at the GldN C terminus (Fig. 1). Compared with PorN, the GldN folded domain is longer at its C terminus with two extra α -helices. However, an \sim 40-amino acid–long insertion is present after helix α 2 in GldN. Therefore, the GldN and PorN topologies are slightly different and, hence, the mode of binding to GldK might be different as the C-terminal

disordered domain is not present in GldN. This raises the question as whether these potential structural differences between PorN and GldN could reveal differences in the assembly of the T9SS core complex in the two bacterial models *P. gingivalis* and *F. johnsoniae*. However, the GldN extra loop contains a significant proportion of charged residues (15/40), a characteristic shared with the PorN C-terminal region. Interesting, the position of this extra loop in the PorN structure showed that it is spatially located close to the C terminus, suggesting that the GldN extra loop and PorN C-terminal regions may play similar functions, such as interaction with GldK/PorK. Further investigations will be necessary to confirm that GldN interacts with GldK and whether this extra loop of GldN is involved in the interaction.

Experimental procedures

Strains, media growth conditions, and chemicals

Strains used in this study are listed in Table S1. *P. gingivalis* ATCC33277/DSMZ20709 was used as source of DNA for cloning. *P. gingivalis* was grown anaerobically in brain–heart infusion medium supplemented with menadiol (0.5 μ g mL⁻¹) and hemin (5 μ g mL⁻¹). *Escherichia coli* DH5 α (New England Biolabs), BTH101 (34) and Rosetta (DE3) pLysS (Novagen) were used for cloning procedures, bacterial two-hybrid assays, and protein production, respectively. *E. coli* cells were routinely grown aerobically in Lysogeny broth (LB) at 30 °C or 37 °C. Plasmids were maintained by addition of ampicillin (100 μ g mL⁻¹), kanamycin (50 μ g mL⁻¹), or chloramphenicol (40 μ g mL⁻¹).

Plasmid constructions

Plasmids and primers used for this study are listed in Table S1. The sequences encoding PorN_p truncated of the N- or C- or both terminal domains (PorN_{ΔN} [residues 55–359], PorN_{ΔC} [residues 25–265], and PorN_{ΔNΔC} [residues 55–265], respectively) were amplified from *P. gingivalis* genomic DNA extracted from 6 \times 10⁹ cells using a DNA purification kit (DNeasy Blood & Tissue, Qiagen). PCRs were performed with a Biometra thermocycler using Q5 DNA polymerase (New England Biolabs) and custom oligonucleotides synthesized by Sigma-Aldrich (listed in Table S1).

For protein production, the sequences were cloned into pLIC03 (kindly provided by the BioXtal company, Marseille). The pLIC03 vector is a derivative of the pET28a+ expression vector (Novagen) in which a cassette coding for a His₆ tag and a tobacco etch virus protease cleavage site followed by the suicide gene *sacB* flanked by BsaI restriction sites was introduced downstream of the ATG start codon. The pLIC03 vector encoding the PorN periplasmic domain (PorN_p) has been described (25). pLIC03 vectors encoding truncated versions of PorN were engineered using the following primers: 5-PorN_{ΔN} and 3-PorN_p (pLIC-PorN_{ΔN}), 5-PorN_p and 3-PorN_{ΔC} (pLIC-PorN_{ΔC}), and 5-PorN_{ΔN} and 3-PorN_{ΔC} (pLIC-PorN_{ΔNΔC}).

For bacterial two-hybrid assays, the sequences were cloned into pUT18C or pKT25 using the following primers:

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T18T25-5-PorN_{ΔN} and T18C-3-PorN_{ΔN} (PorN_{ΔN} into pUT18C), T18T25-5-PorN_{ΔN} and T25C-3-PorN_{ΔN} (PorN_{ΔN} into pKT25), T25T18-5-PorN_{ΔC} and T18C-3-PorN_{ΔC} (PorN_{ΔC} into pUT18C), T25T18-5-PorN_{ΔC} and T25C-3-PorN_{ΔC} (PorN_{ΔC} into pKT25), T18T25-5-PorN_{ΔN} and T18C-3-PorN_{ΔC} (PorN_{ΔNΔC} into pUT18C), and T18T25-5-PorN_{ΔN} and T25C-3-PorN_{ΔC} (PorN_{ΔNΔC} into pKT25). BACTH vectors encoding PorN_p (PorN periplasmic domain, residues 25–359) fused to the T18 domain, and PorK, PorL_C (PorL cytoplasmic domain, amino acids 73–309), PorM_p (PorM periplasmic domain, residues 36–516), and PorN_p fused to the T25 domain have been described (25, 35). BACTH vectors encoding the C-terminal domains of RgpB (residues 661–736), Hbp35 (residues 226–344), PpaD (residues 478–557), PorU (residues 1059–1158), and PorZ (residues 680–776) fused to the T25 domain are described elsewhere (27).

Plasmids for protein production and for bacterial two-hybrid assays were engineered by restriction-free cloning (36), as described (37). Briefly, the DNA fragment of interest was amplified using oligonucleotides introducing extensions annealing to the target vector. The double-stranded product of the first PCR has then been used as oligonucleotides for a second PCR using the target vector (pLIC03, pUT18C, or pKT25) as template. PCR products were then treated with DpnI to eliminate template plasmids and transformed into DH5α-competent cells. All the constructs were verified by colony PCR and DNA sequencing (Eurofins sequencing).

Bacterial two-hybrid assay

Bordetella adenylate cyclase–based bacterial two-hybrid assays (34) were performed as described (38) with modifications. Briefly, the proteins to be tested were fused to the isolated T18 and T25 catalytic domains of the *Bordetella* adenylate cyclase. After introduction of the two plasmids expressing the fusion proteins into the reporter BTH101 strain, plates were incubated at 30 °C for 24 h. Three independent colonies for each transformation were inoculated into 600 μl of LB medium supplemented with ampicillin, kanamycin, and 0.5 mM IPTG. After overnight growth at 30 °C, 15 μl of each culture were dropped onto LB plates supplemented with ampicillin, kanamycin, 0.5 mM IPTG, and 40 μg mL⁻¹ X-Gal and incubated for 6 to 16 h at 30 °C. Controls included interaction assays with TolB/Pal, a protein pair unrelated to the T9SS (38). The experiments were done in triplicate, and a representative result is shown.

Protein production and purification

PorN_p, PorN_{ΔN}, PorN_{ΔC}, and PorN_{ΔNΔC} were produced as described (25) with modifications. Rosetta (DE3) pLysS *E. coli* cells were grown in Turbo Broth medium at 37 °C until reaching optical density at λ = 600 nm (*A*_{600nm}) of 0.6 to 0.8. Expression of the constructs was induced with 1 mM IPTG, and cells were grown for an additional 18 h at 17 °C. Cells were harvested by centrifugation at 4000g for 10 min, resuspended in lysis buffer (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 10 mM

imidazole, 250 μg mL⁻¹ lysozyme, 1 mM PMSF), and frozen overnight at –20 °C. After thawing, 20 μg mL⁻¹ DNase and 1 mM MgSO₄ were added, and cells were lysed by sonication. The pellet and soluble fractions were separated by centrifugation at 16,000g for 30 min, and the proteins were purified from the soluble fraction by immobilized metal ion affinity chromatography using a 5-ml HisTrap crude (GE Healthcare) Ni²⁺-chelating column equilibrated in buffer A (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 10 mM imidazole). The proteins were eluted with buffer A supplemented with 250 mM imidazole and further purified by size exclusion chromatography (HiLoad 16/60 Superdex 75 prep grade, GE Healthcare) equilibrated in 10 mM Hepes pH 7.5, 150 mM NaCl. SeMet-labeled PorN_{ΔNΔC} was produced and purified with the same protocol as the native proteins, except that the cells were grown in SeMet minimal medium (39).

For limited proteolysis assay, 15 μg of purified PorN_p was incubated with 1:1000 (*w:w*) endoproteinase Glu-C (Thermo Fischer) for 16 h at 23 °C. After SDS-PAGE, the band corresponding to the stable Glu-C resistant fragment (PorN_p-G) was excised and analyzed by mass spectrometry and N-terminal Edman sequencing.

For SEC-MALS analysis, the purified PorN_p, (0.75 mg mL⁻¹), PorN_{ΔN} (0.7 mg mL⁻¹), PorN_{ΔC} (0.7 mg mL⁻¹), and PorN_{ΔNΔC} (1.4 mg mL⁻¹) proteins were loaded on a Superdex 200 Increase 10/300 GL column (GE Healthcare) equilibrated in 10 mM Hepes pH 7.4, 150 mM NaCl at a flow rate of 0.6 mL·min⁻¹, using an Ultimate 3000 HPLC system (Fischer Scientific). Detection was performed using an eight-angle light-scattering detector (DAWN8, Wyatt Technology) and a differential refractometer (Optilab, Wyatt Technology).

Crystallization, data collection, and processing

The purified PorN_{ΔC} and SeMet PorN_{ΔNΔC} were concentrated at 10.7 and 10.8 mg mL⁻¹, respectively. Crystallization trials were performed using the sitting-drop vapor-diffusion method at 293 K in 96-well Swissci-3 plates, with Stura Footprint (Molecular Dimensions), Wizard I and II (Rigaku), and Index (Hampton Research) screens and using Tecan and Mosquito (TTP Labtech) robots to fill in the plates and dispense the drops, respectively. Crystals were obtained in several conditions, and after optimization (40), the final crystallization conditions for PorN_{ΔC} and SeMet PorN_{ΔNΔC} were 33 mM Tris pH 8.5, 6.66% PEG 1000, 15.27% PEG 6000, 0.66 M citric acid pH 4.14, and 0.66 M Hepes pH 7.28, 3.2% PEG 3350, 10% glycerol, 3.3 mM NiCl₂ hexahydrate, respectively. Crystals were mounted in cryoloops (Hampton CrystalCap Magnetic) and were briefly soaked in crystallization solution supplemented with 24.7% PEG 6000, 6.66% PEG 8000 for PorN_{ΔC} and 31.3% PEG 3350, 6.6% glycerol for SeMet PorN_{ΔNΔC}. The crystals were flash cooled in a nitrogen-gas stream at 100 K using a home cryocooling device (Oxford Cryosystems).

PorN_{ΔC} diffraction data were collected to 2-Å resolution on beamline ID30 at the European Synchrotron Research Facility (ESRF, Grenoble, France), and single wavelength anomalous

Table 1
Data collection and refinement statistics

	PorN _Δ AC	SeMet PorN _Δ AC
Data collection		
Space group	C222 ₁	C222 ₁
a, b, c (Å)	47.7, 132.5, 133.7	47.9, 132.6, 136.4
α, β, γ (°)	90, 90, 90	90, 90, 90
Resolution (Å) ^a	47.0–2.0 (2.11–2.0)	40.0–2.5 (2.63–2.5)
Unique reflections ^a	28,600 (4169)	15,557 (2194)
Redundancy ^a	3.9 (4.0)	13.4 (13.6)
Completeness (%) ^a	98.3 (99.8)	99.7 (98.3)
I/σ ^a	11.1 (1.5)	16.5 (3.3)
R _{meas} (%) ^a	7.3 (102.6)	9.2 (74.2)
CC1/2	0.998 (0.610)	0.999 (0.761)
Mosaicity	0.09	0.12
Refinement and model quality		
Resolution (Å) ^a	42.5–2.0	
Reflections	28,568	
R _{fac} /R _{free} (%)	19.6/22.8	
Number of atoms		
Protein	1610 (2)/216	
(chains in ASU)/water		
B-factors (Å ²)		
Protein/water	57.9/56.0	
Rmsd		
Bond (Å)	0.009	
Angle (°)	0.94	
Ramachandran plot (%)		
Most favored	96.8	
Allowed regions	1.8	
Outliers	1.4	
PDB accession code	7PVH	

^a Values in parentheses are for the highest-resolution shell.

dispersion data of SeMet PorN_ΔAC were collected to 2.5-Å resolution on beamline Proxima-2 at the Soleil synchrotron. The datasets were integrated with XDS (<https://xds.mr.mpg.de/>) (41) and scaled with SCALA (42) from the CCP4 suite (<https://www.ccp4.ac.uk/>) (43). Heavyatom substructure determination of SeMet PorN_ΔAC, positional refinement, phase calculations, and solvent flattening were performed using autoSHARP (44), SHARP (45) and SOLOMON (46). The initial PorN_ΔAC model was built manually in COOT (47) and was subsequently used as model for molecular replacement with MOLREP (48) to solve the structure of native PorN_ΔAC. The building of PorN_ΔAC was then completed manually with COOT. Refinement, correction, and validation of the structure were performed with autoBUSTER (49), COOT, and Molprobit (50), respectively. Data collection and refinement statistics are reported in Table 1.

Data availability

All details regarding PorN crystal structure (raw data from crystallography experiments, atomic coordinates, and structure factors) have been deposited in the PDB under accession code 7PVH.

Supporting information—This article contains supporting information (25, 27, 34, 38).

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E. C., and P. L. formal analysis; O. F., I. L. S., E. C., and P. L. investigation; P. L. writing – original draft; E. C. writing – review & editing; E. C., A. R., and P. L. supervision; E. C. and A. R. project administration; E. C. and A. R. funding acquisition.

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Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: BACTH, bacterial two-hybrid; CTD, C-terminal domain; OM, outer membrane; PDB, protein data bank; SEC-MALS, size-exclusion chromatography–multiangle light scattering; SeMet, selenomethionine; T9SS, type IX secretion system.

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Structural and functional analyses of the *Porphyromonas gingivalis* type IX secretion system PorN protein

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Running title : Structure-function study of PorN

Supporting Information:

Figure S1 : Organization of the PorN_{ΔNΔC} proteins in the crystal packing.

Figure S2 : Superimposition of the PorN_{ΔNΔC} and PorN_{ΔC} structures.

Figure S3 : Superimposition of the PorN folded domain and PagL.

Table S1 : Strains, plasmids and oligonucleotides used in this study.

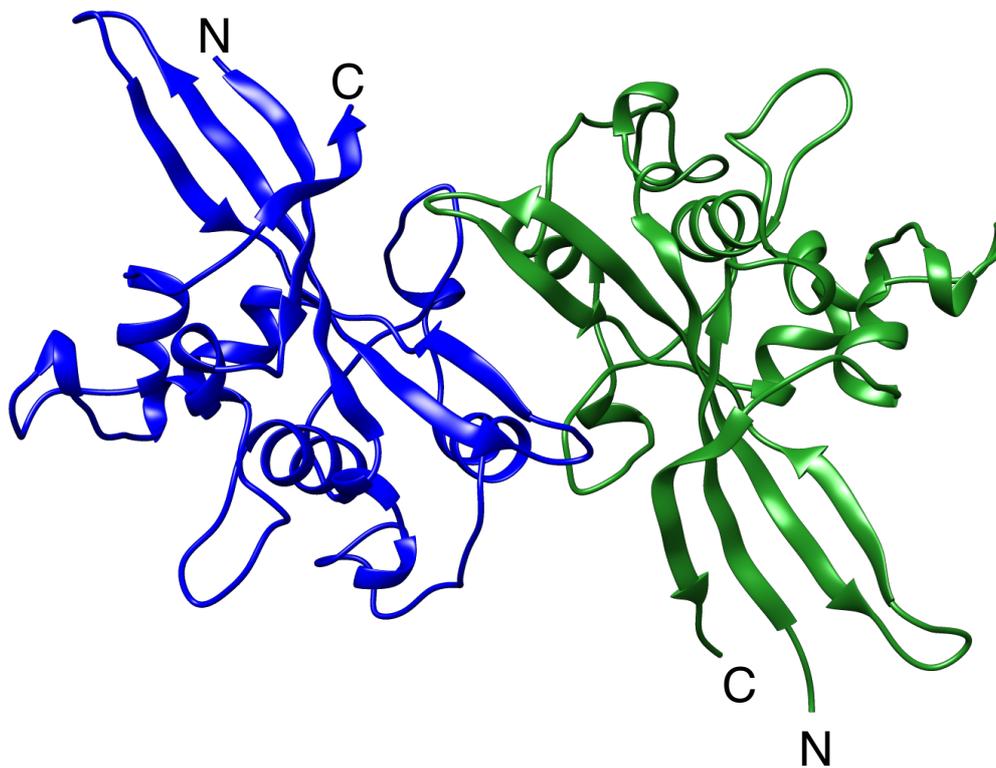


Figure S1 : Organization of the $\text{PorN}_{\Delta\text{N}\Delta\text{C}}$ proteins in the crystal packing. The two monomers are shown in blue and green. The positions of the N- and C-termini are indicated.

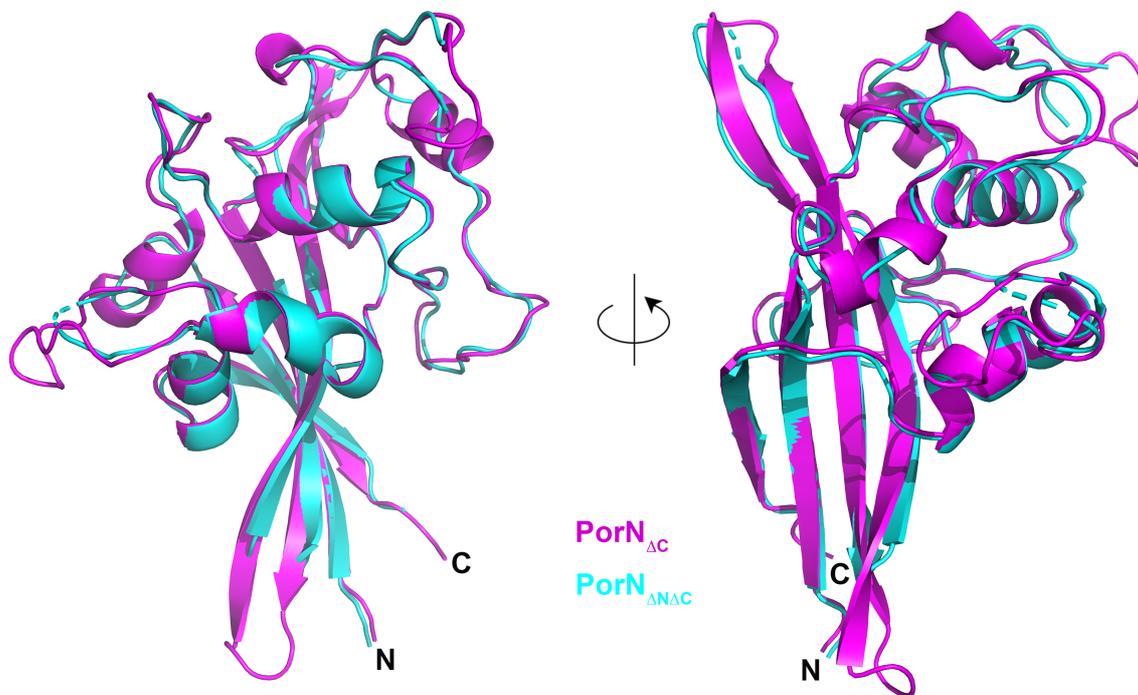


Figure S2 : Superimposition of the PorN_{ΔΔC} (cyan) and PorN_{ΔC} (purple) structures. The positions of the N- and C-termini are indicated.

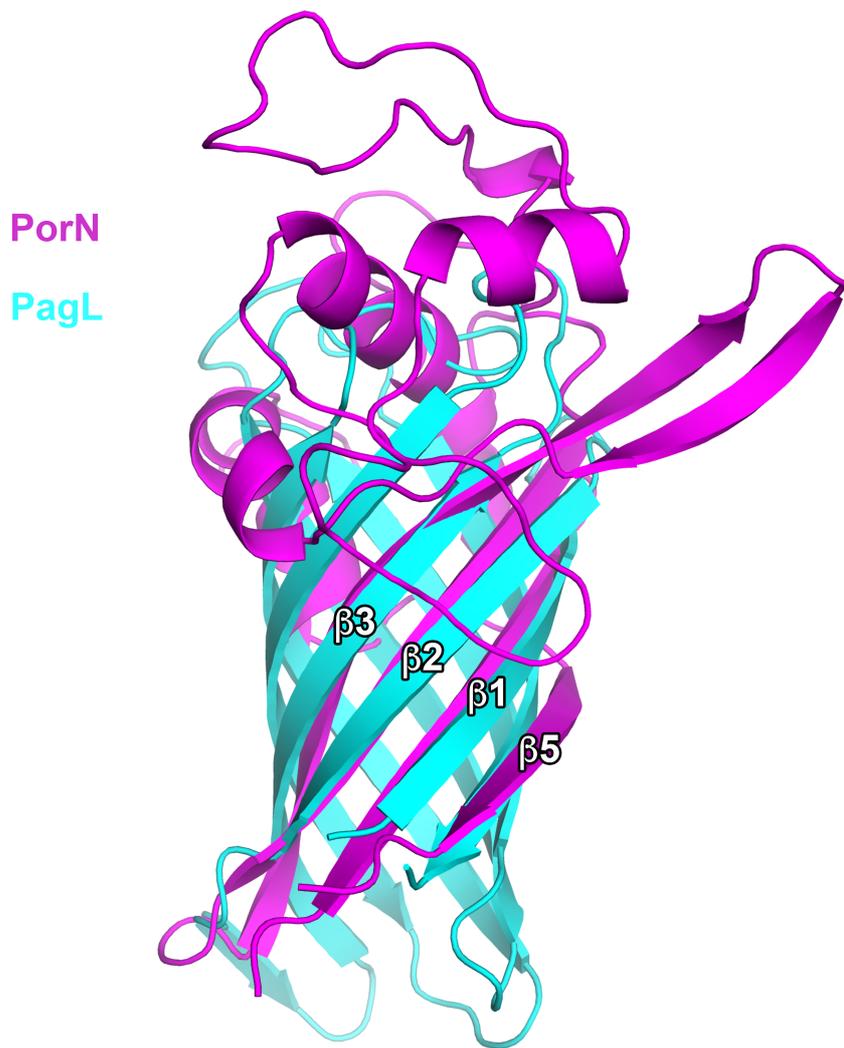


Figure S3 : Superimposition of the PorN folded domain and PagL.
The PorN β -strands that can be superimposed to four β -strands of the PagL β -barrel are labelled.

TABLE S1. Strains, plasmids and oligonucleotides used in this study.

STRAINS

Strains	Description and genotype	Source
<i>Escherichia coli</i>		
DH5 α	F ⁻ , Δ (<i>argF-lac</i>)U169 <i>phoA supE44</i> Δ (<i>lacZ</i>)M15 <i>recA relA endA thi hsdR gyr</i>	Laboratory collection
BTH101	<i>cya-99 araD139 galE15 galK16 rpsL1</i> (Str ^R) <i>hsdR mcrA mcrB</i>	(34)
Rosetta(DE3) pLysS	F ⁻ <i>ompT hsdS_B(r_B⁻ m_B⁻) gal dcm</i> (DE3) pLysSRARE2 (Cam ^R)	Novagen
<i>Porphyromonas gingivalis</i>		
DSM20709	Wild-type <i>Porphyromonas gingivalis</i> (ATCC33277/DSM20709)	DSMZ collection

PLASMIDS

Plasmid	Description and main characteristics	Source
<i>Protein production vectors</i>		
pLIC03	<i>pETG28+</i> derivative, <i>ColE1</i> origin, <i>PT7</i> promoter, <i>N-terminal 6\times<i>His-TEV, Kan</i>^R</i>	Laboratory collection
pLIC-PorN _P	<i>P. gingivalis porN</i> periplasmic domain (residues 25-359), cloned into pLIC03	(25)
pLIC-PorN _{ΔN}	<i>P. gingivalis porN</i> truncated of N-terminal region (residues 55-359), cloned into pLIC03	This study
pLIC-PorN _{ΔC}	<i>P. gingivalis porN</i> truncated of C-terminal region (residues 25-265), cloned into pLIC03	This study
pLIC-PorN _{ΔNΔC}	<i>P. gingivalis porN</i> truncated of N- and C-terminal regions (residues 55-265), cloned into pLIC03	This study
<i>Bacterial two-hybrid vectors</i>		
pUT18C	BACTH vector, <i>ColE1</i> origin, <i>Plac</i> , T18 domain of <i>Bordetella</i> adenylate cyclase, Amp ^R ,	(34)
pKT25	BACTH vector, P15A origin, <i>Plac</i> , T25 domain of <i>Bordetella</i> adenylate cyclase, Kan ^R	(34)
pPorN _P -T18	<i>P. gingivalis porN</i> periplasmic domain (residues 25-359), cloned upstream T18 into pUT18C	(25)
pPorN _{ΔN} -T18	<i>P. gingivalis porN</i> truncated of N-terminal region (residues 55-359), cloned upstream T18 into pUT18C	This study
pPorN _{ΔC} -T18	<i>P. gingivalis porN</i> truncated of C-terminal region (residues 25-265), cloned upstream T18 into pUT18C	This study

pPorN _{ΔNΔC} -T18	<i>P. gingivalis porN</i> truncated of N- and C-terminal regions (residues 55-265), cloned upstream T18 into pUT18C	This study
pT18-Pal	<i>E. coli pal</i> without signal sequence, cloned downstream T18 into pUT18C	(38)
pPorN _P -T25	<i>P. gingivalis porN</i> periplasmic domain (residues 25-359), cloned upstream T25 into pKT25	(25)
pPorN _{ΔN} -T25	<i>P. gingivalis porN</i> truncated of N-terminal region (residues 55-359), cloned upstream T25 into pKT25	This study
pPorN _{ΔC} -T25	<i>P. gingivalis porN</i> truncated of C-terminal region (residues 25-265), cloned upstream T18 into pKT25	This study
pPorN _{ΔNΔC} -T25	<i>P. gingivalis porN</i> truncated of N- and C-terminal regions (residues 55-265), cloned upstream T18 into pKT25	This study
pT25-PorK	<i>P. gingivalis porK</i> without signal sequence, cloned downstream T25 into pKT25	(25)
pPorM _P -T25	<i>P. gingivalis porM</i> periplasmic domain (residues 36-516), cloned upstream T25 into pKT25	(25)
pT25-PorL _C	<i>P. gingivalis porL</i> cytoplasmic domain (residues 73-309), cloned downstream T25 into pKT25	(25)
pT25-RgpB	<i>P. gingivalis rgpB</i> CTD (residues 661-736) cloned downstream T25 into pKTN25	(27)
pT25-Hbp35	<i>P. gingivalis hbp35</i> CTD (residues 226-344) cloned downstream T25 into pKTN25	(27)
pT25-PpaD	<i>P. gingivalis ppaD</i> CTD (residues 478-557) cloned downstream T25 into pKTN25	(27)
pT25-PorU	<i>P. gingivalis porU</i> CTD (residues 1059-1158) cloned downstream T25 into pKTN25	(27)
pT25-PorZ	<i>P. gingivalis porZ</i> CTD (residues 680-776) cloned downstream T25 into pKTN25	(27)
pTolB-T25	<i>E. coli tolB</i> without signal sequence, cloned upstream T25 into pKT25	(38)

OLIGONUCLEOTIDES

Oligonucleotide	Sequence (5' to 3')
<i>Protein production vectors construction</i> ^a	
5-PorN _P	<u>CCGAGAACCTGTACTTCCAATCACAGGAAAATACGAACAACCGCTC</u>
3-PorN _P	<u>CGGAGCTCGAATTCGGATCCTTATTACTTGC GGCGACGAACCG</u>
5-PorN _{ΔN}	<u>CCGAGAACCTGTACTTCCAATCATTCAACAGACGTCTGACACAGAAGACCG</u>
3-PorN _{ΔC}	<u>CGGAGCTCGAATTCGGATCCTTATTATTCGGCCAAAGCTCGGTTGTGC</u>
<i>Bacterial two-hybrid vectors construction</i> ^a	
T18T25-5-PorN _{ΔN}	<u>CGGATAACAATTTACACAGGAAACAGCTATGACCATGTTCAACAGACGTCTGACACAGAAGACC</u>
T18C-3-PorN _{ΔN}	<u>CCTCGCTGGCGGCTAAGCTTGGCGTAATCTTGC GGCGACGAACCGAGC</u>
T25C-3-PorN _{ΔN}	<u>GTTTGC GTAACCAGCCTGATGCGATTGCTGCTTGC GGCGACGAACCGAGC</u>
T25T18-5-PorN _{ΔC}	<u>CGGATAACAATTTACACAGGAAACAGCTATGACCATGGCGCAGGAAAATACGAACAACCGC</u>
T18C-3-PorN _{ΔC}	<u>CCTCGCTGGCGGCTAAGCTTGGCGTAATTCGGCCAAAGCTCGGTTGTGC</u>
T25C-3-PorN _{ΔC}	<u>GTTTGC GTAACCAGCCTGATGCGATTGCTGTTTCGGCCAAAGCTCGGTTGTGC</u>

^a sequence annealing on target vector underlined.