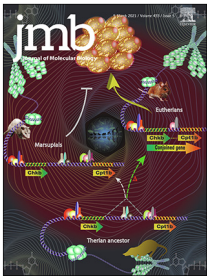

Molecular Strategies Underlying *Porphyromonas gingivalis* Virulence

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Abstract

The anaerobic Gram-negative bacterium *Porphyromonas gingivalis* is considered the keystone of periodontitis diseases, a set of inflammatory conditions that affects the tissues surrounding the teeth. In the recent years, the major virulence factors exploited by *P. gingivalis* have been identified and characterized, including a cocktail of toxins, mainly proteases called gingipains, which promote gingival tissue invasion. These effectors use the Sec pathway to cross the inner membrane and are then recruited and transported across the outer membrane by the type IX secretion system (T9SS). In *P. gingivalis*, most secreted effectors are attached to anionic lipopolysaccharides (A-LPS), and hence form a virulence coat at the cell surface. *P. gingivalis* produces additional virulence factors to evade host immune responses, such as capsular polysaccharide, fimbriae and outer membrane vesicles. In addition to periodontitis, it is proposed that this broad repertoire of virulence factors enable *P. gingivalis* to be involved in diverse human diseases such as rheumatoid arthritis, and neurodegenerative, Alzheimer, and cardiovascular disorders. Here, we review the major virulence determinants of *P. gingivalis* and discuss future directions to better understand their mechanisms of action.

Porphyromonas gingivalis and the Oral Cavity Microbiota

Porphyromonas gingivalis is an anaerobic immotile Gram-negative bacterium, and member of the phylum Bacteroidetes. This bacterium is a natural member of the human oral microbiome and one of the main agents of periodontitis, a set of inflammatory conditions that affect the tissues surrounding the teeth, which eventually causes gum degradation, bone loss and teeth to fall out.[1] Its chronic persistence in the periodontium depends on its ability to evade host immunity without inhibiting the overall inflammatory response.[2,3] *P. gingivalis* is an asaccharolytic bacterium; it generates its metabolic energy by fermenting amino acids, a property decisive for its survival in deep periodontal pockets, where sugars are extremely scarce.[4] To access amino-acids and peptides, *P. gingivalis* secretes a broad variety of proteases that degrade exogenous proteins to generate peptides that enter the periplasm where they are broken into di- or tri-peptides by dipeptidyl peptidases before being transported into the cytoplasm by oligopeptide transporters[5] (Figure 1).

P. gingivalis is unique among pathogenic bacteria in being able to accumulate a cell-surface heme (iron protoporphyrin IX [FePPIX])-containing pigment, μ-oxo-bisheme ($[FePPIX]_2O$), when cultured on blood-containing media, yielding a

black pigmentation. This heme is obtained by degradation of host proteins, mainly hemoglobin, by a cocktail of lysine- and arginine-specific proteinases, called gingipains, and collected by heme-binding proteins such as hemin-binding protein 35 (HBP35) and the HmuY hemophore[6–8] (Table 1 and Figure 1). Gingipains and HBP35 are effectors of a secretion apparatus, the type IX secretion system (T9SS), which is hence the major determinant of *P. gingivalis* pathogenesis.[9] In addition, *P. gingivalis* produces a number of potential virulence factors such as lipopolysaccharides (LPS), capsule and fimbriae, that trigger deleterious effects on host cells, allowing *P. gingivalis* to invade cells and tissues, avoiding the immune surveillance (Table 1 and Figure 1). Once *P. gingivalis* is established within the cell, it secretes an ATP-hydrolase enzyme that prevents ATP-dependent apoptosis, allowing its survival into the host.[10]

P. gingivalis is one of the three components of the so-called "red complex", a bacterial association that also comprises the Gram-negative bacteria *Treponema denticola* and *Tannerella forsythia*. The presence of the red complex is strongly correlated with advanced periodontal lesions such as increased pocket depth and bleeding.[1] Recent studies suggest that, within the red complex, *P. gingivalis* acts as the main pathogen contributing to microbial imbalance and leading to the disease progression, whereas *T. denticola* and *T. forsythia* contribute to the nososymbiocity of the microbial community after homeostasis is disrupted, thereby acting as pathobionts that accelerate disease progression.[11,12] Furthermore, *P. gingivalis* presents synergy biofilm formation with *T. denticola*,[13] which increases the development of gingivitis.[14]

In addition, bacterial commensals or opportunistic pathogens of the human oral flora have been shown to facilitate *P. gingivalis* infection or to increase its

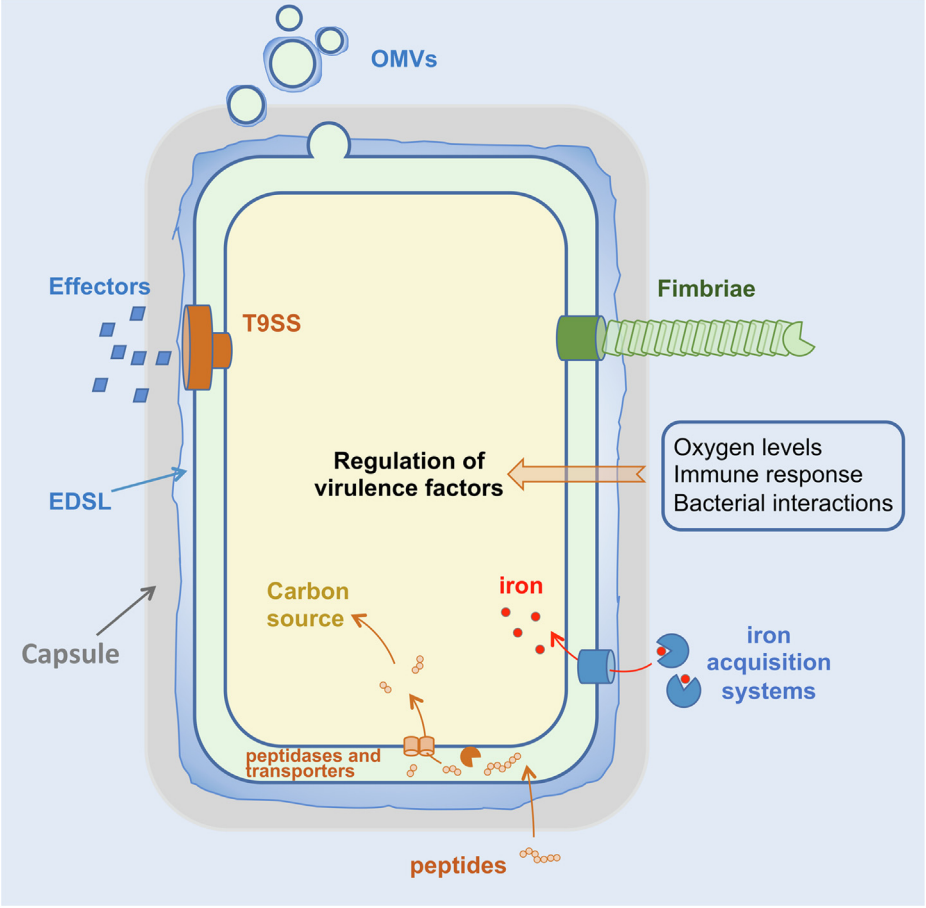

Figure 1. Major virulence factors of *P. gingivalis*. Schematic representation of a *P. gingivalis* cells (yellow, cytoplasm; green, periplasm; blue lines, inner and outer membranes). The cell is surrounded by an electron dense surface layer (EDSL, blue) made of gingipains anchored to the cell surface, and by the capsule (grey). Gingipains and other effectors (blue lozenges) are secreted by the Type IX secretion system (T9SS, orange). Other virulence factors include fimbriae (green), outer membrane vesicles (OMVs, blue). Mechanisms of acquisition of essential elements: iron (red circles) acquisition systems (blue), and di- and tri-peptides (peptidases and oligopeptide transporters, orange) that serve as carbon sources.

Table 1 Major virulence factors of *P. gingivalis.*

Virulence factor	Major role	Actions
T9SS	Secretion system	– Cell surface exposition of gingipains – Secretion of iron-chelating proteins
Gingipains	Proteolytic enzymes	– Degradation of host proteins – Processing of fimbriae subunits
PAD	Peptidylarginine deiminase	– Citrullination of host proteins
Type V pili and Mfa	Fimbriae	– Adhesion to host cells – Bacterial aggregation and biofilms
Lipopolysaccharides	Protection	– Triggers host signaling pathways
Capsule	Protection	– Protection against aggressions – Protection against host complement
OMVs	Extracellular vesicles	– Toxin delivery and transport

survival. *Streptococcus gordonii* enhances accumulation of *P. gingivalis* in dual species communities, helps adhesion to gingival tissue by increasing expression of *P. gingivalis* fimbrial adhesins and promotes its survival in murine oral infection models.[15] Similarly, *Acinetobacter baumannii* increases the abundance of *P. gingivalis* in dual species communities, adapting to each other.[16] Finally, the yeast-like fungus *Candida albicans*, a common species of the oral cavity, depletes oxygen within poly-species biofilms,[17] hence protecting anaerobic bacteria such *as P. gingivalis* in unfavourable, high-oxygen, conditions. In addition, this *C. albicans-P. gingivalis* microbial community influences host immunity by attenuating macrophage and fibroblast responses and reducing cytokine production, promoting tissue invasion and colonization.[18,19]

Proteins Secreted by Porphyromonas gingivalis

The major secreted proteins of *P. gingivalis* participate in proteolytic degradation of host proteins, protein citrullination and heme acquisition (Table 1 and Figure 1). All these secreted factors share a Sec-dependent signal sequence for export into the periplasm, and a Ig-fold C-terminal domain (CTD) that addresses the proteins to the T9SS.

Gingipains – The gingipain protein family comprises proteases that cleave upstream arginine (RgpA and RgpB) or lysine (Kgp) residues. Gingipains are either secreted in the milieu or anchored to the cell surface and represent the major virulence factors of *P. gingivalis*.[20] Structurally, these proteins share a similar multidomain organization: from N- to C-terminal, the signal peptide is followed by a prodomain, a catalytic domain, an immunoglobulin-superfamily fold (IgSF), and the CTD. Additionally, RgpA and Kgp possess several copies of hemagglutinin/adhesion domains (HA) located between the IgSF and CTD domains.[21] These HA domains, as well as those of the HagA hemagglutinin, have pleiotropic functions as they have been shown to promote co-aggregation with other bacteria[22] and tissue colonization through adhesion to epithelial cells and gingival fibroblasts,[23] and to participate to heme-pigment formation by converting FePPIX. OH monomers into $[FePPIX]_2O$.[24] Expression of gingipain-encoding genes is downregulated once the pathogen has invaded the gingival epithelial cells, suggesting that these proteinases are important for the early stages of *P. gingivalis* infection.[25] Indeed, gingipains proteolyse a broad repertoire of substrates. Gingipains participate to the erosion of periodontal tissues and to the degradation of iron-binding proteins, and target important extracellular matrix components, such as tight-junction associated protein JAM1, hence disrupting the epithelial barrier function and allowing *P. gingivalis* penetration into subepithelial tissues.[26] In addition, by cleaving T-cell surface proteins such as CD4 and CD8,[27] the IL-6, IL-8, IL-12 cytokines[28] and the gamma-interferon (IFN-γ),[29] gingipains interfere with the immune host response and hence facilitate evasion of host defense mechanisms. *P. gingivalis* also secretes Tpr, a cysteine protease of the papain family.[30]

Peptidylarginine deiminase – Members of the *Porphyromonas* genus are the only bacteria known to produce and to secrete a peptidylarginine deiminase (PAD), an enzyme involved in the citrullination of proteins[31] (Table 1). Citrullination is an enzymatic reaction that converts arginine into citrulline, a neutral, non-natural amino acid. By neutralizing a positively-charged residue, this post-translational modification increases the overall hydrophobicity of target proteins and causes protein unfolding and dysfunction.[32] Citrullinated

proteins are targeted by the immune system, and ultimately lead to autoimmune diseases such as rheumatoid arthritis (RA). Indeed, a correlation between the development of RA and severe periodontitis was noticed early[33] and further results demonstrated that *Porphyromonas* PAD (PPAD) is a significant factor in the development of experimental periodontitis and RA in mice.[34] At the structural level, PPAD presents an organization similar to gingipains, with the catalytic domain followed by IgSF and CTD domains.[35]

Hemin-binding protein – The hemin-binding protein 35 (HBP35) is a secreted protein that plays a significant role in heme acquisition.[36] In addition, HBP35 facilitates *P. gingivalis* binding to erythrocytes and host epithelial gingival cells.[37] HBP35 possesses a functional 4-cysteine thioredoxin motif,[36,38] which physiological role is unknown.

The Type IX Secretion System

The T9SS is a secretion apparatus that is restricted to bacteria of the Bacteroidetes phylum.[39] Type IX secretion is a two-step mechanism, in which effectors first cross the inner membrane (IM) by the Sec pathway before being recruited to and transported by the T9SS through the outer membrane (OM)[40] (Figure 2). Deletion of T9SS genes causes effector accumulation in the periplasm.[9,41] Processing and activation of T9SS effectors, though studied since decades, remains unclear. Periplasm-sequestered gingipains have shown partial enzymatic activity,[42,43] but the requirement of an Ig-like domain for T9SS transport and the internal diameter of the OM translocon[44,45] suggest that T9SS effectors achieve proper folding in the periplasm, before translocation through the OM. Effectors are addressed to the secretion apparatus by a conserved ~80-amino acid CTD.[46] In *P. gingivalis*, CTDs are cleaved when effectors reach the cell exterior, and effectors are either released into the extracellular medium or anchored to the surface by covalent attachment to anionic lipopolysaccharides (A-LPS).[47,48] In wild-type *P. gingivalis*, CTD proteins attached to the cell surface yield an additional electron-dense surface layer that can be observed by electron microscopy.[49]

Secretion signal – More than 600 CTD-containing proteins have been bioinformatically identified in at least 20 Bacteroidetes species.[50] Such as T9SS subunits, CTD domains are restricted to the Bac-

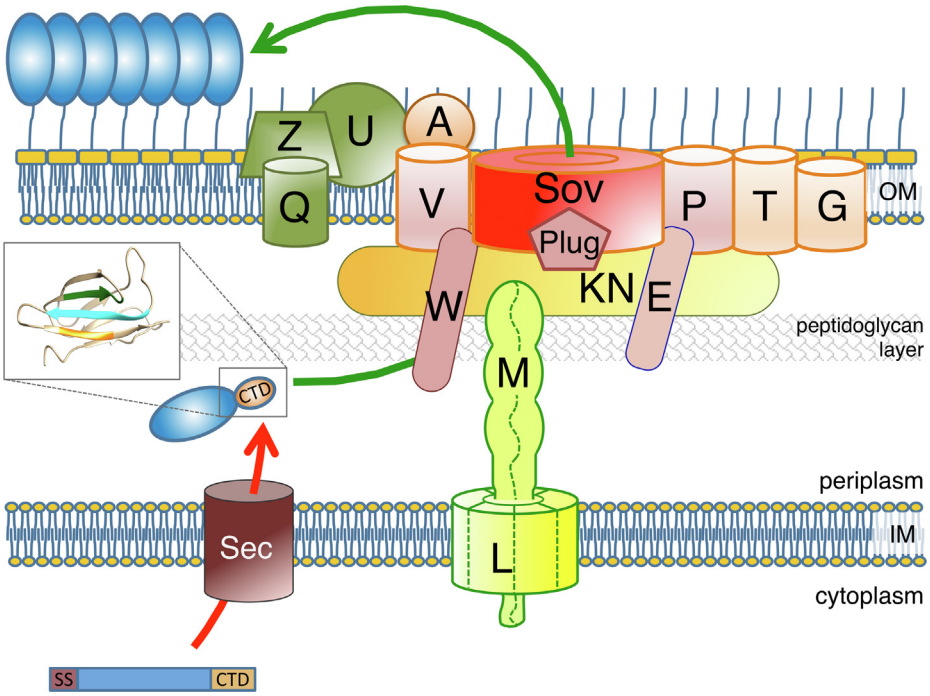

Figure 2. Organization and mechanism of action of the *P. gingivalis* Type IX secretion system. Schematic representation of the T9SS and of the effector secretion pathway. The different subunits of the T9SS are shown, with their localizations. Subunits of the trans-envelope complex (PorK-L-M-N) are coloured yellow; subunits of the translocon complex (Sov, Plug, PorV, PorW, PorP, PorE, and possibly PorA, PorT and PorG) are coloured pink; subunits of the attachment complex (PorQ, PorZ, PorU) are coloured green. The translocation pathway of T9SS effectors (blue; ss, signal sequence, CTD, C-terminal domain) is depicted: export by the Sec machine (red arrow), and then transport to the cell exterior or to the cell surface by the T9SS (green arrow). The insert highlights the structure of the RgpB CTD (PDB: 5HFS). Note that all contacts shown in this figure have not been experimentally established (see text for details).

teroidetes phylum, suggesting co-evolution between CTD substrates and the T9SS apparatus.[50] CTDs belong to two different families: type-A (TIGR04183) and type-B (TIGR0413143).[51] Type-A CTDs are the most abundant and have been studied in different Bacteroidetes species. CTDs are necessary for secretion through the T9SS, as deletion of these domains causes accumulation of the effectors into the periplasm.[52] Type-A CTDs are also sufficient for targeting a T9SS-unrelated protein to the secretion apparatus, as fusion of the type-A CTD of *P. gingivalis* HBP35 to the jellyfish Green Fluorescent Protein (GFP) supports secretion to the cell exterior in a T9SS-dependent manner.[53] Finally, effectors such as *F. johnsoniae* ChiA, are handled by the T9SS, in absence of any identifiable type-A or type-B CTD.[54,55]

Most *P. gingivalis* CTDs belong to type-A. The structures of CTDs from three *P. gingivalis* T9SS substrates (RgpB, PorZ and HBP35) have been reported.[44,56,57] The structures are well conserved: they comprise a β-sandwich with an Ig-like fold conformation organized in two parallel β-sheets that are formed by three and four antiparallel strands respectively[44,56,57] (see insert in Figure 2). How CTD substrates are recruited to the T9SS is not well understood, but a recent non peer-reviewed study showed that CTDs interact with two subunits of the trans-envelope complex, PorM and PorN,[58] suggesting that PorM and PorN could constitute an entry gate for the effectors into the T9SS. Sequence alignment of type-A CTDs showed that five motifs, named A-to-E, are conserved including three (B, D and E) with a high degree of conservation.[46,50] It has been demonstrated that the two last residues of motif E, are necessary for effector secretion.[59] In contrast, the 22-aminoacid C-terminal fragment of HBP35, which comprises only motifs D and E, still supports a significant level of transport, suggesting that motif B improves substrate transport by the T9SS without being fully indispensable.[53] These motifs are likely involved in CTD folding and/or interactions with T9SS components.[46,59] Indeed, a recent study showed that motif D is required for proper interaction with PorM.[58]

Once substrates are translocated across the OM, CTDs are removed by the sortase-like protein PorU, which belongs, with PorV, PorZ and PorQ to the attachment complex.[60] The cell surface-exposed attachment complex is responsible for the covalent anchoring of T9SS substrates to A-LPS.[61] Interestingly, PorU and PorZ, also possess a CTD and are hence transported to the cell surface by the T9SS.[48] In contrast to other T9SS substrates, the PorU and PorZ CTDs are not cleaved,[48,56] suggesting that specific signals or motifs distinguish the PorU/Z and effector CTDs.

T9SS components – While the number of genes required for proper function of the T9SS varies between species, a set of 18 genes is necessary in *P. gingivalis*. Surprisingly, except the *porKLMNP* locus, these genes are widespread within the genome, which is an uncommon situation for other secretion apparatuses in which genes are usually clustered.[39,62]

The T9SS trans-envelope core complex. The trans-envelope core complex comprises 4 subunits, PorK, -L, -M and -N, which localize in the cell envelope (coloured yellow in Figure 2). These genes are, together with *porP*, genetically linked on the chromosome and co-transcribed (Table 1).[63] Blue native gel electrophoresis (BN-PAGE) assays suggested that the PorKLMN proteins assemble large complexes of over 1.2 MDa.[9] PorL and PorM are inner membrane proteins, while PorN is a periplasmic protein and PorK an outer membrane lipoprotein.

PorL presents two TMHs and a cytoplasmic domain.[63] PorM is anchored into the IM by a single TMH, and possesses a large periplasmic C-terminal domain (PorMp). PorM forms homodimers and interacts with PorL via their transmembrane helices (TMHs), and with PorN in the periplasm.[63] The crystal structures of the periplasmic domains PorM and its homologue GldM from *F. johnsoniae* were solved: they both comprise four domains, including a N-terminal all-α-helical domain, and three Ig-like domains.[64,65] Interestingly, the dimer presents swapping elements, were strands from a monomer complete the structure of the second monomer. In addition, kinks exist between the first and second and between the second and third domains,[64,65] as well as between the transmembrane portion and first periplasmic domain.[66] Recently, it has been shown that the PorLM sub-complex serves as energy module by converting electrochemical energy into mechanical energy to power T9SS assembly or effector transport.[63,66] In *F. johnsoniae*, gliding motility is dependent on the proton-motive force and on a functional GldLM module (PMF).[66,67] The cryo-EM structure of a truncated GldLM complex demonstrated that it forms an asymmetric motor of $GldL_5GldM_2$ stoichiometry.[66] The GldL TMHs assemble a pentameric cage that surrounds two copies of the GldM TMH;[66] an organization similar to the PMF-dependent ExbBD or MotAB molecular motors.[68–70] Energy modules using PMF usually carry protonotable glutamate or aspartate residues within their TMHs, as in the case of MotAB, ExbBD or TolQR.[71] Interestingly, sequence analysis revealed that PorM and PorL TMHs bears conserved residues, including a glutamate residue in PorL TMH2.[63,72] In the GldLM complex structure, the Arg-9 residue of one GldM TMH copy forms a salt bridge with residue Glu-49 of GldL, and it is suggested that protonation of GldL Glu-49 breaks this Arg-9/Glu-49 ion pair and induces reorientation of the GldLM TMHs.[66] It is proposed that electrochemical energy is converted to mechanical movement via the rotation of the

two copies of GldM TMH, and subsequent conformation changes within the GldM periplasmic domains.[66]

PorK and PorN form a stable complex of 1:1 molecular ratio, associated to the outer membrane.[41] Electron microscopy analyses showed that PorKN assemble into ~50-nm large ring-like structures that can comprise 32–36 copies of PorKN.[41] Recently, an *in vivo* cryo-electron tomography study confirmed these *in vitro* results and further proposed an organization in double-layered ring.[73]

The T9SS translocon. Until recently, the identity of the T9SS OM pore was a mystery. As a conserved and large (267 kDa) predicted OM component required for T9SS, the Sov protein was an obvious candidate.[39,74–76] In 2018, the *F. johnsoniae* Sov homologue SprA was isolated in complex with a peptidyl-prolyl cis–trans isomerase, and either the PorV OM β-barrel or the Fjoh_1759 plug protein[45] (coloured pink in Figure 2). Cryo-electron microscopy analyses of these complexes showed that SprA is a giant 36-strand β-barrel with a lateral opening and delimiting a 70-Å internal channel that is large enough to allow the transit of folded substrates. In the "PorV" complex, SprA displays a lateral opening that is filled by a loop of PorV. This loop penetrates inside the lumen of the SprA pore,[45] supporting the hypothesis that PorV collects substrates from the translocon and shuttles them to the attachment complex.[77] In the "Plug" complex, PorV is absent and the lateral opening of SprA is unobstructed, but the pore is plugged at the periplasmic entrance by the Fjoh_1759 protein.[45] These two complexes are likely to represent two different states of the mechanism of transport: while the "PorV" complex may correspond to the open complex, prior to substrate engagement, the "Plug" complex may represent the pore after substrate release, occluded to preserve membrane permeability. Recently, a partner of PorV, named PorA, was identified in *P. gingivalis* and proposed to be a component of the translocon complex. However, PorA is absent in most T9SS$^+$ bacteria and might be specific to gingipains.[73] More recently, PorA was suggested to be involved in a signaling pathway from the translocon to the PorXY-SigP transcriptional regulatory system.[78]

The attachment complex. As mentioned above, most of the T9SS substrates are anchored to the cell surface rather than being secreted into the extracellular medium. In *P. gingivalis*, the CTD signal of the substrate is cleaved at the cell surface and the new C terminus is covalently attached to A-LPS by a sortase-like mechanism carried out by PorU.[61,79]

PorU possesses a CTD domain and hence is transported to the cell surface in a T9SS-dependent manner; however, the PorU CTD is not cleaved. Deletion of the *porU* gene leads to partial substrate secretion but prevents CTD cleavage and substrate attachment.[48] PorU has therefore a dual activity: CTD cleavage and A-LPS attachment. Substrate maturation is mediated by the PorU cysteine proteinase activity. Similar to sortases, PorU proteins bear a conserved arginine residue that is likely part of the catalytic site involved in A-LPS attachment.[61] In addition, PorU is involved in the activation of gingipains by removing their inhibitory N-terminal pro-domain.[80]

PorU is a subunit of the T9SS attachment complex, which also comprises PorV, PorZ and PorQ[60] (coloured green in Figure 2). PorV (formerly known as LptO) is a 14-strand β-barrel outer membrane protein and a member of the fatty acid FadL transporters family.[45,49,77] Initially, PorV was shown to be involved in deacetylation of A-LPS and hence in the coordination of LPS and T9SS effector processing.[49,81] PorV binds to multiple CTD proteins;[77] a result that correlates well with the PorV structure in complex with SprA, where a loop outwards the PorV barrel penetrates the interior of SprA through its lateral opening.[45] It is suggested that this loop mediates recognition of T9SS substrates in the translocon and shuttles them to the attachment complex. Indeed, PorV associates tightly with the Sov translocon[45] and with the PorU C-terminal signal peptidase.[82]

The function of the cell surface-exposed PorZ subunit is not well defined but it has been proposed based on its crystal structure that it could bind and recruit A-LPS.[56] Indeed, recent data demonstrated that PorZ specifically binds to A-LPS and feeds the PorU sortase with A-LPS.[83] BN-PAGE analysis showed that PorZ interacts with PorQ,[73] a FadL-like OM β-barrel protein family that improves the efficiency of the type IX secretion apparatus.[9,73,77,79]

Additional T9SS components. Other uncharacterized or poorly characterized T9SS components have been reported, all being associated to the OM. These include the PorP, PorT, and PorG OM β-barrels and the PorE, PorF and PorW outer membrane lipoproteins. The essential PorG subunit interacts with the PorKN ring and has been suggested to facilitate assembly or stability of the ring.[41,84] By contrast, no information is available regarding the accessory PorF protein except that it improves the efficiency of the T9SS.[76] PorW, known as SprE in *F. johnsoniae*, is an OM lipoprotein that interacts with the Sov translocon and a protein of unknown function, PGN_1783.[73] It is proposed that PorW links the PorKN submembrane ring to the Sov translocon.[73] While the localization of PorT has been subject of debate,[43,85,86] it is now admitted that it is an OM β-barrel, but its role in type IX secretion remains unknown.

In *P. gingivalis*, the *porP* gene is co-transcribed with the *porKLMN* genes and its product has been shown to interact with the PorK and PorM proteins.[63] Interestingly, *porP* gene homologues

(*sprF*) are in multiple copies in the *F. johnsoniae* genome, and are genetically linked to genes encoding *porE* homologues and substrates with a type-B CTD, suggesting that SprF-PorE modules are involved to the secretion of specific T9SS type-B substrates.[87] In *P. gingivalis*, only one substrate, PG1035, possesses a type-B CTD. Interestingly, PG1035 was shown to form a complex with PorP and PorE.[73] The PorE lipoprotein (formerly known as PG1058) is composed of four domains: a tetratricopeptide repeats domain, a five-bladed β-propeller domain, a carboxypeptidase regulatory domain-like fold, and an OmpA_C-like domain.[88] Recently, the structure of the OmpA_C-like domain was solved in complex with a peptidoglycan fragment, suggesting that PorE anchors the T9SS to the peptidoglycan layer.[89]

Finally, the housekeeping PGN_0300 Skp/OmpH-like periplasmic chaperone was shown to be necessary for the function of the *P. gingivalis* T9SS, likely by stabilizing the PorU sortase.[90]

Regulation of T9SS genes – Little is known regarding the regulatory mechanism of T9SS genes expression in *P. gingivalis*. Three regulators have been identified based on the downregulation of T9SS genes and reduction of gingipain activity in their absence: the two-component system PorY sensor and PorX response regulator,[9] and the SigP extracytoplasmic sigma factor.[91] By contrast to most response regulators, PorX does not bind to DNA, but interacts with SigP, and this interaction is necessary for SigP to bind to promoter regions of several T9SS genes.[91] In addition, PorX binds to the C-terminal hydrophobic region of the T9SS PorL protein, suggesting that PorX may regulate the activity of the T9SS.[92] How the PorXY two-component system is activated is not known but recent data have shown that the translocon-associated PorA protein participates to the signaling cascade.[78] Finally, other factors have been shown to modulate the expression of *P. gingivalis* genes encoding T9SS subunits and effectors, such as the orphan response regulator RprY,[93] (p)ppGpp levels,[94] deletion of T9SS accessory proteins;[56] or dual-species biofilm formation with *S. gordonii*.[95]

Other Virulence Factors

Fimbriae – Fimbriae are a variety of adhesive pilus structures on the cell surface of bacteria (Figure 1). In pathogenic species, fimbriae are often crucial virulent factors, being involved in attachment and infection of target cells, evasion of the host immune system, or biofilm formation.[96] *P. gingivalis* produces short (Mfa pili) and long (Type V pili) fimbriae (Table 1). By their adhesive properties they participate to polyspecies biofilm formation with synergistic species, cell host colonization, and development of periodontitis.[97,98] Type V pili are composed of the major FimA fimbrillin. Interestingly, FimA are first transported to the cell surface as lipoproteins and are then proteolytically processed by the Arg-protease RgpB gingipain before being polymerized into fimbrial structures by a strand-exchange mechanism.[99–101] In addition to promoting adhesion to host tissues, long fimbriae bind α5β1-integrins[102] and inhibit the Toll-like receptor (TLR)-mediated proinflammatory response[103] allowing *P. gingivalis* to invade host cells. Finally, long fimbriae mediate coaggregation with other oral pathogens such as *Treponema denticola*, *Streptococcus oralis* or *S. gordonii*.[102,104] Similar to Type V pili FimA, the major fimbrillin of the Mfa short fimbriae, Mfa1, is also processed by gingipains before being polymerized.[105] Interestingly, recent data have shown that mimetic peptides are efficient to inhibit polymerization of Mfa pili and hence reduce the ability of *P. gingivalis* to form dual-species biofilms with *S. gordonii*.[106]

Lipopolysaccharide and capsule – Such as other Gram-negative bacteria, the outer leaflet of the *P. gingivalis* OM is mainly composed of lipopolysaccharides (LPS). LPS molecules are recognized by the host and commonly trigger intracellular signaling pathways (Table 1). *P. gingivalis* produces two types of LPS: O-LPS and A-LPS. A-LPS triggers a reduced proinflammatory activity compared to conventional LPS and is required for serum resistance.[3,107] Because A-LPS is necessary for attachment of gingipains and other T9SS effectors at the cell surface, mutations affecting A-LPS production lead to loss of virulence and aberrant protein sorting into the OM.[108] The polysaccharide portion of A-LPS is synthesized by a specific pathway, recently designed as Wbp/Vim.[109–112] In addition to the electron-dense surface layer constituted of T9SS effectors attached to A-LPS, most strains of *P. gingivalis* are covered by a capsule that protects the bacterium from aggressions and from killing by the host complement (Table 1 and Figure 1), and hence encapsulated strains are more virulent in a mouse model of infection.[113,114]

Outer membrane vesicles – Gram-negative bacteria normally produce outer membrane vesicles (OMVs) that are composed of a single membrane derived from their OM.[115] OMVs are virulence factors involved in the release of toxins, defence against other bacteria and bacterial adherence.[116] Due to the additional layer of effectors bound to the A-LPS of the OM, *P. gingivalis* OMVs are enriched in gingipains and other CTD proteins anchored at the cell surface and hence participate to toxin delivery and pathogenicity[86,108,117–119] (Table 1 and Figure 1).

Conclusive Remarks and Research Outlook

P. gingivalis is an important human pathogen. Initially identified as the etiologic agent of oral diseases such as gingivitis and periodontitis, the

presence of *P. gingivalis* was more recently linked to the development of neuroinflammatory disorders. Indeed, early researches correlated periodontitis with dementia and Alzheimer's disease (AD).[120] Later, studies using animal models showed that chronic oral application of *P. gingivalis* caused neuropathologies characteristics of AD.[121] While how *P. gingivalis* induces neuropathologies is not known, mice oral infection with *P. gingivalis* resulted in (i) brain inflammation and increased production of amyloid-β, the main component of amyloid plaques, and (ii) gingipain-dependent cleavage of Tau, a protein necessary for normal neuronal function.[122] Understanding the molecular bases on how *P. gingivalis* induces neurological disorders is of critical importance for therapeutic purposes. Notably, vaccines or treatments that interfere with *Porphyromonas* growth will be of specific interest. Recently, a number of molecules that affect *P. gingivalis* growth or reduce its overall virulence have been identified such as the SSAP or the sialidase DANA inhibitors.[123–125]

Oral diseases provoked by *P. gingivalis* are exacerbated by the presence of other bacterial species, such as those of the "red complex", *T. denticola* and *T. forsythia*, as well as *S. gordonii.* Current researches should now focus to understand how these different bacterial species coordinate their action and how they synergistically cause diseases. Here again, inhibitors or antibodies that target fimbriae biogenesis or adhesins, involved in interspecies interactions have been recently developed.[106,126]

As emphasized in this review, and similarly to what is observed in many pathogens, the virulence of *P. gingivalis* is multifactorial. While the capsule protects the bacterium from external aggressions, LPS and OMVs trigger pro-inflammatory cytokine and chemokine responses, and thus play an important role during the infection process. However the key virulence factor is the type IX secretion system. This multiprotein transport apparatus translocates effectors through the OM and covalently attaches them on the A-LPS at the cell surface, and indirectly participates to fimbriae assembly by secreting proteases that process fimbrial subunits. The major *P. gingivalis* effectors include the gingipain proteases, and the citrullinating PAD toxin. Given the broad range of activities combined with cell surface localization, it is not surprising that gingipains have been considered for a long time as promising targets for designing preventive strategies like inhibitors and vaccines, not only for periodontitis treatment[123,127] but also to prevent brain colonization and neurodegeneration.[122] Several potent inhibitors of gingipains or proteases activity have been described.[127–132] In addition, Cl-amidine, a known inhibitor of citrullinating proteins, is also active on the PAD.[133] However, due to the redundancy of gingipains, a better solution will be to target the secretion apparatus itself. By blocking the T9SS, such inhibitors will prevent cell surface exposure of the gingipains and of PAD, and will indirectly hamper fimbriae biogenesis. While virulence blockers that target other secretion systems have been developed,[134–138] no inhibitor of the T9SS has been described so far. Understanding how the function of the different components of the apparatus, solving the structure of the subunits or of sub-complexes, and defining how effectors are recognized, selected, sorted and transported is of critical importance to rationally design molecules or peptides that will block the biogenesis or mode of action of the T9SS. This will not only provide potential treatments against gingivitis and periodontitis but also might be an alternative strategy to reduce the risk of extra-oral diseases such as cardiovascular and neurological disorders.

CRediT authorship contribution statement

Ignacio Lunar Silva: Writing - original draft. **Eric Cascales:** Writing - review & editing.

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Conflict of Interest Statement

The authors declare no conflicts of interest.

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Abbreviations:
T9SS, Type IX secretion system; CTD, C-terminal domain; PPAD, *Porphyromonas* peptidylarginine deiminase; RA, rheumatoid arthritis; HBP35, hemin-binding protein 35; IM, inner membane; OM, outer membrane; A-LPS, anionic lipopolysaccharide; OMVs, outer membrane vesicles

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