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1 **Molecular strategies underlying *Porphyromonas***
2 ***gingivalis* virulence**

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16 **Abstract**

17

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

34 **Highlights**

35

36 - *Porphyromonas gingivalis* is the etiologic agent of gingivitis and periodontitis

37 - *Porphyromonas* pathogenesis relies on a broad repertoire of virulence factors

38 - *Porphyromonas* virulence factors include the T9SS, fimbriae, and OMVs

39 - The T9SS is a multiprotein complex of the cell envelope that transports effectors

40 - T9SS effectors are mainly proteolytic enzymes that degrade the tooth tissues.

41 ***Porphyromonas gingivalis* and the oral cavity microbiota**

42

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

56 *P. gingivalis* is unique among pathogenic bacteria in being able to accumulate a cell-
57 surface heme (iron protoporphyrin IX [FePPIX])-containing pigment, μ -oxo-bisheme
58 ($[\text{FePPIX}]_2\text{O}$), when cultured on blood-containing media, yielding a black pigmentation.
59 This heme is obtained by degradation of host proteins, mainly hemoglobin, by a
60 cocktail of lysine- and arginine-specific proteinases, called gingipains, and collected by
61 heme-binding proteins such as hemin-binding protein 35 (HBP35) and the HmuY
62 hemophore [6-8] (Table 1 and Figure 1). Gingipains and HBP35 are effectors of a
63 secretion apparatus, the type IX secretion system (T9SS), which is hence the major
64 determinant of *P. gingivalis* pathogenesis [9]. In addition, *P. gingivalis* produces a
65 number of potential virulence factors such as lipopolysaccharides (LPS), capsule and
66 fimbriae, that trigger deleterious effects on host cells, allowing *P. gingivalis* to invade
67 cells and tissues, avoiding the immune surveillance (Table 1 and Figure 1). Once *P.*

68 *gingivalis* is established within the cell, it secretes an ATP-hydrolase enzyme that
69 prevents ATP-dependent apoptosis, allowing its survival into the host [10].

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

80 In addition, bacterial commensals or opportunistic pathogens of the human oral flora
81 have been shown to facilitate *P. gingivalis* infection or to increase its survival.
82 *Streptococcus gordonii* enhances accumulation of *P. gingivalis* in dual species
83 communities, helps adhesion to gingival tissue by increasing expression of *P. gingivalis*
84 fimbrial adhesins and promotes its survival in murine oral infection models [15].
85 Similarly, *Acinetobacter baumannii* increases the abundance of *P. gingivalis* in dual
86 species communities, adapting to each other [16]. Finally, the yeast-like fungus
87 *Candida albicans*, a common species of the oral cavity, deplete oxygen within poly-
88 species biofilms [17], hence protecting anaerobic bacteria such as *P. gingivalis* in
89 unfavourable, high-oxygen, conditions. In addition, this *C. albicans*-*P. gingivalis*
90 microbial community influences host immunity by attenuating macrophage and
91 fibroblast responses and reducing cytokine production, promoting tissue invasion and
92 colonization [18,19].

93

94 **Proteins secreted by *Porphyromonas gingivalis***

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

100 *Gingipains* – The gingipain protein family comprises proteases that cleave upstream
101 arginine (RgpA and RgpB) or lysine (Kgp) residues. Gingipains are either secreted in
102 the milieu or anchored to the cell surface and represent the major virulence factors of
103 *P. gingivalis* [20]. Structurally, these proteins share a similar multidomain organization:
104 from N- to C-terminal, the signal peptide is followed by a prodomain, a catalytic
105 domain, an immunoglobulin-superfamily fold (IgSF), and the CTD. Additionally, RgpA
106 and Kgp possess several copies of hemagglutinin/adhesion domains (HA) located
107 between the IgSF and CTD domains [21]. These HA domains, as well as those of the
108 HagA hemagglutinin, have pleiotropic functions as they have been shown to promote
109 co-aggregation with other bacteria [22] and tissue colonization through adhesion to
110 epithelial cells and gingival fibroblasts [23], and to participate to heme-pigment
111 formation by converting FePPIX.OH monomers into [FePPIX]₂O [24]. Expression of
112 gingipain-encoding genes is downregulated once the pathogen has invaded the
113 gingival epithelial cells, suggesting that these proteinases are important for the early
114 stages of *P. gingivalis* infection [25]. Indeed, gingipains proteolyse a broad repertoire of
115 substrates. Gingipains participate to the erosion of periodontal tissues and to the
116 degradation of iron-binding proteins, and target important extracellular matrix
117 components, such as tight-junction associated protein JAM1, hence disrupting the
118 epithelial barrier function and allowing *P. gingivalis* penetration into subepithelial
119 tissues [26]. In addition, by cleaving T-cell surface proteins such as CD4 and CD8 [27],
120 the IL-6, IL-8, IL-12 cytokines [28] and the gamma-interferon (IFN- γ) [29], gingipains
121 interfere with the immune host response and hence facilitate evasion of host defense

122 mechanisms. *P. gingivalis* also secretes Tpr, a cysteine protease of the papain family
123 [30].

124 *Peptidylarginine deiminase* – Members of the *Porphyromonas* genus are the only
125 bacteria known to produce and to secrete a peptidylarginine deiminase (PAD), an
126 enzyme involved in the citrullination of proteins [31] (Table 1). Citrullination is an
127 enzymatic reaction that converts arginine into citrulline, a neutral, non-natural amino
128 acid. By neutralizing a positively-charged residue, this post-translational modification
129 increases the overall hydrophobicity of target proteins and causes protein unfolding
130 and dysfunction [32]. Citrullinated proteins are targeted by the immune system, and
131 ultimately lead to autoimmune diseases such as rheumatoid arthritis (RA). Indeed, a
132 correlation between the development of RA and severe periodontitis was noticed early
133 [33] and further results demonstrated that *Porphyromonas* PAD (PPAD) is a significant
134 factor in the development of experimental periodontitis and RA in mice [34]. At the
135 structural level, PPAD presents an organization similar to gingipains, with the catalytic
136 domain followed by IgSF and CTD domains [35].

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

142

143 **The Type IX secretion system**

144 The T9SS is a secretion apparatus that is restricted to bacteria of the Bacteroidetes
145 phylum [39]. Type IX secretion is a two-step mechanism, in which effectors first cross
146 the inner membrane (IM) by the Sec pathway before being recruited to and transported
147 by the T9SS through the outer membrane (OM) [40] (Figure 2). Deletion of T9SS
148 genes causes effector accumulation in the periplasm [9,41]. Processing and activation
149 of T9SS effectors, though studied since decades, remains unclear. Periplasm-

150 sequestered gingipains have shown partial enzymatic activity [42,43], but the
151 requirement of an Ig-like domain for T9SS transport and the internal diameter of the
152 OM translocon [44,45] suggest that T9SS effectors achieve proper folding in the
153 periplasm, before translocation through the OM. Effectors are addressed to the
154 secretion apparatus by a conserved ~ 80-amino acid CTD [46]. In *P. gingivalis*, CTDs
155 are cleaved when effectors reach the cell exterior, and effectors are either released
156 into the extracellular medium or anchored to the surface by covalent attachment to
157 anionic lipopolysaccharides (A-LPS) [47,48]. In wild-type *P. gingivalis*, CTD proteins
158 attached to the cell surface yield an additional electron-dense surface layer that can be
159 observed by electron microscopy [49].

160

161 *Secretion signal* – More than 600 CTD-containing proteins have been bioinformatically
162 identified in at least 20 Bacteroidetes species [50]. Such as T9SS subunits, CTD
163 domains are restricted to the Bacteroidetes phylum, suggesting co-evolution between
164 CTD substrates and the T9SS apparatus [50]. CTDs belong to two different families:
165 type-A (TIGR04183) and type-B (TIGR0413143) [51]. Type-A CTDs are the most
166 abundant and have been studied in different Bacteroidetes species. CTDs are
167 necessary for secretion through the T9SS, as deletion of these domains causes
168 accumulation of the effectors into the periplasm [52]. Type-A CTDs are also sufficient
169 for targeting a T9SS-unrelated protein to the secretion apparatus, as fusion of the type-
170 A CTD of *P. gingivalis* HBP35 to the jellyfish Green Fluorescent Protein (GFP) supports
171 secretion to the cell exterior in a T9SS-dependent manner [53]. Finally, effectors such
172 as *F. johnsoniae* ChiA, are handled by the T9SS, in absence of any identifiable type-A
173 or type-B CTD [54,55].

174 Most *P. gingivalis* CTDs belong to type-A. The structures of CTDs from three *P.*
175 *gingivalis* T9SS substrates (RgpB, PorZ and HBP35) have been reported [44,56,57].
176 The structures are well conserved: they comprise a β -sandwich with an Ig-like fold

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

191 Once substrates are translocated across the OM, CTDs are removed by the sortase-
192 like protein PorU, which belongs, with PorV, PorZ and PorQ to the attachment complex
193 [60]. The cell surface-exposed attachment complex is responsible for the covalent
194 anchoring of T9SS substrates to A-LPS [61]. Interestingly, PorU and PorZ, also
195 possess a CTD and are hence transported to the cell surface by the T9SS [48]. In
196 contrast to other T9SS substrates, the PorU and PorZ CTDs are not cleaved [48,56],
197 suggesting that specific signals or motifs distinguish the PorU/Z and effector CTDs.

198

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

204

205 The T9SS trans-envelope core complex. The trans-envelope core complex comprises
206 4 subunits, PorK, -L, -M and -N, which localize in the cell envelope (coloured yellow in
207 Figure 2). These genes are, together with *porP*, genetically linked on the chromosome
208 and co-transcribed (Table 1) [63]. Blue native gel electrophoresis (BN- PAGE) assays
209 suggested that the PorKLMN proteins assemble large complexes of over 1.2 MDa [9].
210 PorL and PorM are inner membrane proteins, while PorN is a periplasmic protein and
211 PorK an outer membrane lipoprotein.

212 PorL presents two TMHs and a cytoplasmic domain [63]. PorM is anchored into the IM
213 by a single TMH, and possesses a large periplasmic C-terminal domain (PorMp). PorM
214 forms homodimers and interacts with PorL via their transmembrane helices (TMHs),
215 and with PorN in the periplasm [63]. The crystal structures of the periplasmic domains
216 PorM and its homologue GldM from *F. johnsoniae* were solved: they both comprise
217 four domains, including a N-terminal all- α -helical domain, and three Ig-like domains
218 [64,65]. Interestingly, the dimer presents swapping elements, where strands from a
219 monomer complete the structure of the second monomer. In addition, kinks exist
220 between the first and second and between the second and third domains [64,65], as
221 well as between the transmembrane portion and first periplasmic domain [66].
222 Recently, it has been shown that the PorLM sub-complex serves as energy module by
223 converting electrochemical energy into mechanical energy to power T9SS assembly or
224 effector transport [63,66]. In *F. johnsoniae*, gliding motility is dependent on the proton-
225 motive force and on a functional GldLM module (PMF) [66,67]. The cryo-EM structure
226 of a truncated GldLM complex demonstrated that it forms an asymmetric motor of
227 GldL₅GldM₂ stoichiometry [66]. The GldL TMHs assemble a pentameric cage that
228 surrounds two copies of the GldM TMH [66], an organization similar to the PMF-
229 dependent ExbBD or MotAB molecular motors [68-70]. Energy modules using PMF
230 usually carry protonatable glutamate or aspartate residues within their TMHs, as in the
231 case of MotAB, ExbBD or TolQR [71]. Interestingly, sequence analysis revealed that
232 PorM and PorL TMHs bears conserved residues, including a glutamate residue in PorL

233 TMH2 [63,72]. In the GldLM complex structure, the Arg-9 residue of one GldM TMH
234 copy forms a salt bridge with residue Glu-49 of GldL, and it is suggested that
235 protonation of GldL Glu-49 breaks this Arg-9/Glu-49 ion pair and induces reorientation
236 of the GldLM TMHs [66]. It is proposed that electrochemical energy is converted to
237 mechanical movement via the rotation of the two copies of GldM TMH, and subsequent
238 conformation changes within the GldM periplasmic domains [66].

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

244

245 The T9SS translocon. Until recently, the identity of the T9SS OM pore was a mystery.
246 As a conserved and large (267 kDa) predicted OM component required for T9SS, the
247 Sov protein was an obvious candidate [39,74-76]. In 2018, the *F. johnsoniae* Sov
248 homologue SprA was isolated in complex with a peptidyl-prolyl cis-trans isomerase,
249 and either the PorV OM β -barrel or the Fjoh_1759 plug protein [45] (coloured pink in
250 Figure 2). Cryo-electron microscopy analyses of these complexes showed that SprA is
251 a giant 36-strand β -barrel with a lateral opening and delimiting a 70-Å internal channel
252 that is large enough to allow the transit of folded substrates. In the "PorV" complex,
253 SprA displays a lateral opening that is filled by a loop of PorV. This loop penetrates
254 inside the lumen of the SprA pore [45], supporting the hypothesis that PorV collects
255 substrates from the translocon and shuttles them to the attachment complex [77]. In
256 the "Plug" complex, PorV is absent and the lateral opening of SprA is unobstructed, but
257 the pore is plugged at the periplasmic entrance by the Fjoh_1759 protein [45]. These
258 two complexes are likely to represent two different states of the mechanism of
259 transport: while the "PorV" complex may correspond to the open complex, prior to
260 substrate engagement, the "Plug" complex may represent the pore after substrate

261 release, occluded to preserve membrane permeability. Recently, a partner of PorV,
262 named PorA, was identified in *P. gingivalis* and proposed to be a component of the
263 translocon complex. However, PorA is absent in most T9SS+ bacteria and might be
264 specific to gingipains [73]. More recently, PorA was suggested to be involved in a
265 signaling pathway from the translocon to the PorXY-SigP transcriptional regulatory
266 system [78].

267

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

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

281 PorU is a subunit of the T9SS attachment complex, which also comprises PorV, PorZ
282 and PorQ [60] (coloured green in Figure 2). PorV (formerly known as LptO) is a 14-
283 strand β -barrel outer membrane protein and a member of the fatty acid FadL
284 transporters family [45,49,77]. Initially, PorV was shown to be involved in deacetylation
285 of A-LPS and hence in the coordination of LPS and T9SS effector processing [49,81].
286 PorV binds to multiple CTD proteins [77], a result that correlates well with the PorV
287 structure in complex with SprA, where a loop outwards the PorV barrel penetrates the
288 interior of SprA through its lateral opening [45]. It is suggested that this loop mediates

289 recognition of T9SS substrates in the translocon and shuttles them to the attachment
290 complex. Indeed, PorV associates tightly with the Sov translocon [45] and with the
291 PorU C-terminal signal peptidase [82].

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

298

299 Additional T9SS components. Other uncharacterized or poorly characterized T9SS
300 components have been reported, all being associated to the OM. These include the
301 PorP, PorT, and PorG OM β -barrels and the PorE, PorF and PorW outer membrane
302 lipoproteins. The essential PorG subunit interacts with the PorKN ring and has been
303 suggested to facilitate assembly or stability of the ring [41,84]. By contrast, no
304 information is available regarding the accessory PorF protein except that it improves
305 the efficiency of the T9SS [76]. PorW, known as SprE in *F. johnsoniae*, is an OM
306 lipoprotein that interacts with the Sov translocon and a protein of unknown function,
307 PGN_1783 [73]. It is proposed that PorW links the PorKN submembrane ring to the
308 Sov translocon [73]. While the localization of PorT has been subject of debate
309 [43,85,86], it is now admitted that it is an OM β -barrel, but its role in type IX secretion
310 remains unknown.

311 In *P. gingivalis*, the *porP* gene is co-transcribed with the *porKLMN* genes and its
312 product has been shown to interact with the PorK and PorM proteins [63]. Interestingly,
313 *porP* gene homologues (*sprF*) are in multiple copies in the *F. johnsoniae* genome, and
314 are genetically linked to genes encoding *porE* homologues and substrates with a type-
315 B CTD, suggesting that SprF-PorE modules are involved to the secretion of specific

316 T9SS type-B substrates [87]. In *P. gingivalis*, only one substrate, PG1035, possesses a
317 type-B CTD. Interestingly, PG1035 was shown to form a complex with PorP and PorE
318 [73]. The PorE lipoprotein (formerly known as PG1058) is composed of four domains: a
319 tetratricopeptide repeats domain, a five bladed β -propeller domain, a carboxypeptidase
320 regulatory domain-like fold, and an OmpA_C-like domain [88]. Recently, the structure
321 of the OmpA_C-like domain was solved in complex with a peptidoglycan fragment,
322 suggesting that PorE anchors the T9SS to the peptidoglycan layer [89].
323 Finally, the housekeeping PGN_0300 Skp/OmpH-like periplasmic chaperone was
324 shown to be necessary for the function of the *P. gingivalis* T9SS, likely by stabilizing
325 the PorU sortase [90].

326

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

342

343 **Other virulence factors**

344 *Fimbriae* – Fimbriae are a variety of adhesive pilus structures on the cell surface of
345 bacteria (Figure 1). In pathogenic species, fimbriae are often crucial virulent factors,
346 being involved in attachment and infection of target cells, evasion of the host immune
347 system, or biofilm formation [96]. *P. gingivalis* produces short (Mfa pili) and long (Type
348 V pili) fimbriae (Table 1). By their adhesive properties they participate to polyspecies
349 biofilm formation with synergistic species, cell host colonization, and development of
350 periodontitis [97,98]. Type V pili are composed of the major FimA fimbrillin.
351 Interestingly, FimA are first transported to the cell surface as lipoproteins and are then
352 proteolytically processed by the Arg-protease RgpB gingipain before being polymerized
353 into fimbrial structures by a strand-exchange mechanism [99-101]. In addition to
354 promoting adhesion to host tissues, long fimbriae bind $\alpha 5\beta 1$ -integrin [102] and inhibit
355 the Toll-like receptor (TLR)-mediated proinflammatory response [103] allowing *P.*
356 *gingivalis* to invade host cells. Finally, long fimbriae mediate coaggregation with other
357 oral pathogens such as *Treponema denticola*, *Streptococcus oralis* or *S. gordonii*
358 [102,104]. Similar to Type V pili FimA, the major fimbrillin of the Mfa short fimbriae,
359 Mfa1, is also processed by gingipains before being polymerized [105]. Interestingly,
360 recent data have shown that mimetic peptides are efficient to inhibit polymerization of
361 Mfa pili and hence reduce the ability of *P. gingivalis* to form dual-species biofilms with
362 *S. gordonii* [106].

363

364 *Lipopolysaccharide and capsule* – Such as other Gram-negative bacteria, the outer
365 leaflet of the *P. gingivalis* OM is mainly composed of lipopolysaccharides (LPS). LPS
366 molecules are recognized by the host and commonly trigger intracellular signaling
367 pathways (Table 1). *P. gingivalis* produces two types of LPS: O-LPS and A-LPS. A-
368 LPS triggers a reduced proinflammatory activity compared to conventional LPS and is
369 required for serum resistance [3,107]. Because A-LPS is necessary for attachment of
370 gingipains and other T9SS effectors at the cell surface, mutations affecting A-LPS
371 production lead to loss of virulence and aberrant protein sorting into the OM [108]. The

372 polysaccharide portion of A-LPS is synthesized by a specific pathway, recently
373 designed as Wbp/Vim [109-112]. In addition to the electron-dense surface layer
374 constituted of T9SS effectors attached to A-LPS, most strains of *P. gingivalis* are
375 covered by a capsule that protects the bacterium from aggressions and from killing by
376 the host complement (Table 1 and Figure 1), and hence encapsulated strains are more
377 virulent in a mouse model of infection [113,114].

378

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

386

387 **Conclusive remarks and research outlook**

388 *P. gingivalis* is an important human pathogen. Initially identified as the etiologic agent
389 of oral diseases such as gingivitis and periodontitis, the presence of *P. gingivalis* was
390 more recently linked to the development of neuroinflammatory disorders. Indeed, early
391 researches correlated periodontitis with dementia and Alzheimer's disease (AD) [120].
392 Later, studies using animal models showed that chronic oral application of *P. gingivalis*
393 caused neuropathologies characteristics of AD [121]. While how *P. gingivalis* induces
394 neuropathologies is not known, mice oral infection with *P. gingivalis* resulted in (i) brain
395 inflammation and increased production of amyloid- β , the main component of amyloid
396 plaques, and (ii) gingipain-dependent cleavage of Tau, a protein necessary for normal
397 neuronal function [122]. Understanding the molecular bases on how *P. gingivalis*
398 induces neurological disorders is of critical importance for therapeutic purposes.
399 Notably, vaccines or treatments that interfere with *Porphyromonas* growth will be of

400 specific interest. Recently, a number of molecules that affect *P. gingivalis* growth or
401 reduce its overall virulence have been identified such as the SSAP or the sialidase
402 DANA inhibitors [123-125].

403 Oral diseases provoked by *P. gingivalis* are exacerbated by the presence of other
404 bacterial species, such as those of the "red complex", *T. denticola* and *T. forsythia*, as
405 well as *S. gordonii*. Current researches should now focus to understand how these
406 different bacterial species coordinate their action and how they synergistically cause
407 diseases. Here again, inhibitors or antibodies that target fimbriae biogenesis or
408 adhesins, involved in interspecies interactions have been recently developed
409 [106,126].

410 As emphasized in this review, and similarly to what is observed in many pathogens, the
411 virulence of *P. gingivalis* is multifactorial. While the capsule protects the bacterium from
412 external aggressions, LPS and OMVs trigger pro-inflammatory cytokine and chemokine
413 responses, and thus play an important role during the infection process. However the
414 key virulence factor is the type IX secretion system. This multiprotein transport
415 apparatus translocates effectors through the OM and covalently attaches them on the
416 A-LPS at the cell surface, and indirectly participates to fimbriae assembly by secreting
417 proteases that process fimbrial subunits. The major *P. gingivalis* effectors include the
418 gingipain proteases, and the citrullinating PPAD toxin. Given the broad range of
419 activities combined with cell surface localization, it is not surprising that gingipains have
420 been considered for a long time as promising targets for designing preventive
421 strategies like inhibitors and vaccines not only for periodontitis treatment [123,127] but
422 also to prevent brain colonization and neurodegeneration [122]. Several potent
423 inhibitors of gingipains or proteases activity have been described [127-132]. In addition,
424 CI-amidine, a known inhibitor of citrullinating proteins, is also active on the PPAD [133].
425 However, due to the redundancy of gingipains, a better solution will be to target the
426 secretion apparatus itself. By blocking the T9SS, such inhibitors will prevent cell
427 surface exposure of the gingipains and of PPAD, and will hamper fimbriae biogenesis.

428 While virulence blockers that target other secretion systems have been developed
429 [134-138], no inhibitor of the T9SS has been described so far. Understanding how the
430 function of the different components of the apparatus, solving the structure of the
431 subunits or of sub-complexes, and defining how effectors are recognized, selected,
432 sorted and transported is of critical importance to rationally design molecules or
433 peptides that will block the biogenesis or mode of action of the T9SS. This will not only
434 provide potential treatments against gingivitis and periodontitis but also might be an
435 alternative strategy to reduce the risk of extra-oral diseases such as cardiovascular
436 and neurological disorders.

437

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447

448 **CRedit authorship contribution statement**

449 Ignacio Lunar Silva: Writing – original draft

450 Eric Cascales: Writing – review and editing

451

452 **Keywords**

453 *Porphyromonas gingivalis*; Pathogenesis; Protein transport; Virulence factors; Type IX
454 secretion system

455

456 **Abbreviations used**

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

461

462 **References**

463

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1002

1003 **Legend to Figures**

1004

1005 **Figure 1. Major virulence factors of *P. gingivalis*.** Schematic representation of a *P.*
1006 *gingivalis* cells (yellow, cytoplasm; green, periplasm; blue lines, inner and outer
1007 membranes). The cell is surrounded by an electron dense surface layer (EDSL, blue)
1008 made of gingipains anchored to the cell surface, and by the capsule (grey). Gingipains

1009 and other effectors (blue lozenges) are secreted by the Type IX secretion system
1010 (T9SS, orange). Other virulence factors include fimbriae (green), outer membrane
1011 vesicles (OMVs, blue). Mechanisms of acquisition of essential elements: iron (red
1012 circles) acquisition systems (blue), and di- and tri-peptides (peptidases and
1013 oligopeptide transporters, orange) that serve as carbon sources.

1014

1015 **Figure 2. Organization and mechanism of action of the *P. gingivalis* Type IX**
1016 **secretion system.** Schematic representation of the T9SS and of the effector secretion
1017 pathway. The different subunits of the T9SS are shown, with their localizations.
1018 Subunits of the trans-envelope complex (PorK-L-M-N) are coloured yellow; subunits of
1019 the translocon complex (Sov, Plug, PorV, PorW, PorP, PorE, and possibly PorA, PorT
1020 and PorG) are coloured pink; subunits of the attachment complex (PorQ, PorZ, PorU)
1021 are coloured green. The translocation pathway of T9SS effectors (blue; ss, signal
1022 sequence, CTD, C-terminal domain) is depicted: export by the Sec machine (red
1023 arrow), and then transport to the cell exterior or to the cell surface by the T9SS (green
1024 arrow). The insert highlights the structure of the RgpB CTD (PDB: 5HFS [44]). Note
1025 that all contacts shown in this figure have not been experimentally established (see text
1026 for details).

1027

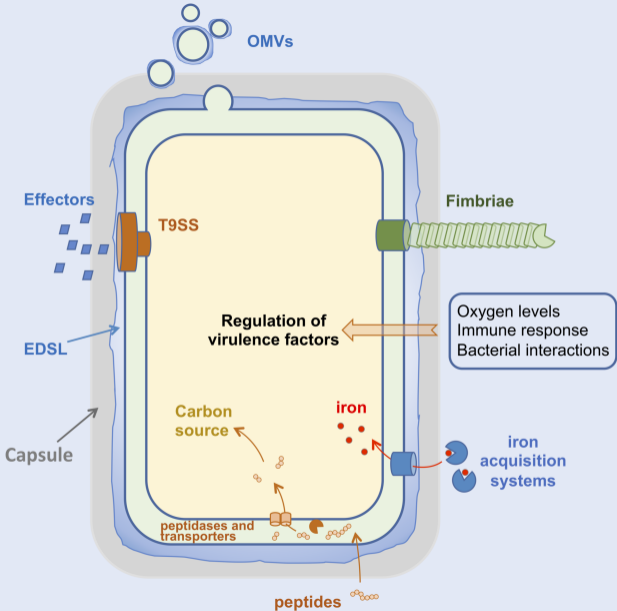
1028 **Table 1. Major virulence factors of *P. gingivalis***

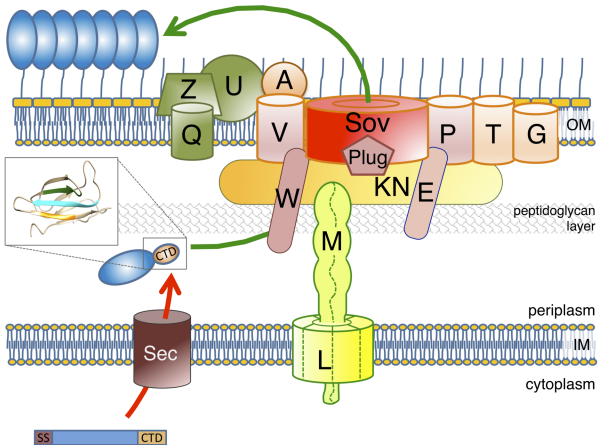
1029

1030	Virulence factor	Major role	Actions
1031 1032	T9SS	Secretion system	- cell surface exposition of gingipains - secretion of iron-chelating proteins
1033 1034	Gingipains	Proteolytic enzymes	- degradation of host proteins - processing of fimbriae subunits
1035	PAD	Peptidylarginine deiminase	- citrullination of host proteins
1036 1037	Type V pili and Mfa	Fimbriae	- adhesion to host cells - bacterial aggregation and biofilms
1038	Lipopolysaccharides	Protection	- triggers host signaling pathways
1039 1040	Capsule	Protection	- protection against aggressions - protection against host complement
1041 1042	OMVs	Extracellular vesicles	- toxin delivery and transport

1043

1044





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

2

3	Virulence factor	Major role	Actions
4	T9SS	Secretion system	- cell surface exposition of gingipains
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13			- protection against host complement
14	OMVs	Extracellular vesicles	- toxin delivery and transport

Healthy gum



Pericoronitis

