Porphyromonas gingivalis and related bacteria: from colonial pigmentation to the type IX secretion system and gliding motility

Nakayama K. Porphyromonas gingivalis and related bacteria: from colonial pigmentation to the type IX secretion system and gliding motility. J Periodont Res 2015; 50: 1–8. © 2014 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd

Porphyromonas gingivalis is a gram-negative, non-motile, anaerobic bacterium implicated as a major pathogen in periodontal disease. P. gingivalis grows as black-pigmented colonies on blood agar, and many bacteriologists have shown interest in this property. Studies of colonial pigmentation have revealed a number of important findings, including an association with the highly active extracellular and surface proteinases called gingipains that are found in P. gingivalis. The Por secretion system, a novel type IX secretion system (T9SS), has been implicated in gingipain secretion in studies using non-pigmented mutants. In addition, many potent virulence proteins, including the metallo-carboxypeptidase CPG70, 35 kDa hemin-binding protein HBP35, peptidylarginine deiminase PAD and Lys-specific serine endopeptidase PepK, are secreted through the T9SS. These findings have not been limited to P. gingivalis but have been extended to other bacteria belonging to the phylum Bacteroidetes. Many Bacteroidetes species possess the T9SS, which is associated with gliding motility for some of these bacteria.

Porphyromonas gingivalis is a gram-negative anaerobic bacterium considered a major pathogen in chronic periodontitis (1). Recently, it has been proposed that even in low abundance P. gingivalis is a keystone pathogen with community-wide effects that are critical for the development of dysbiosis in periodontal biofilm (2). In addition, epidemiological and experimental studies have shown that the bacterium may be associated with systemic conditions, such as cardiovascular diseases (3), preterm low birth weight (4), rheumatoid arthritis (5) and non-alcoholic fatty liver disease (6,7).

P. gingivalis requires protoheme for growth. In heme-deprived medium, P. gingivalis cells grow slowly and eventually stop growing after several passages in this medium. On blood agar, these bacterial cells form black-pigmented colonies (Fig 1). The black pigment is derived from the protoheme in erythrocytes. The black pigment phenotype of P. gingivalis has been attributed to the accumulation of the µ-oxo bisheme complex of Fe(III) protoporphyrin IX, [Fe(III)PPIX]2O (8–10). This heme complex, also termed µ-oxo oligomers or dimeric heme, comprises two Fe(III) protoporphyrin IX moieties bridged by an oxygen atom (8). As the optimum pH for P. gingivalis growth is approximately 8 and this bacterium produces an alkaline terminal growth pH because of peptide and amino acid metabolism (11–14), the µ-oxo dimer [Fe(III)PPIX]2O is maintained at an alkaline pH. Interestingly,
pigmented *Prevotella* species, such as *Prev. intermedia* and *Prev. nigrescens*, generate monomeric Fe(III)PPIX.OH from [Fe(III)PPIX]_2O because the terminal growth pH of these bacteria on blood agar for 8 d is approximately 6 (14). In this review, we discuss novel findings, including the type IX secretion system (T9SS), obtained from genetic studies of colonial pigmentation.

**Spontaneous pigment-less mutants**

When *P. gingivalis* cells were grown under hemin excess in a chemostat at pH 7.5 for 2–3 wk (49–73 bacterial generations) and subsequently plated on to blood agar, colonies with atypical morphology were observed (15). One colony variant (W50/BE1) was beige in color, and another colony variant (W50/BR1) was brown. Both colonial variants exhibited decreased virulence (15), and W50/BE1 lacked gelatinase, collagenase and dipeptidyl aminopeptidase activities compared with the parent strain and exhibited reduced hydrophobicity, hemagglutination activity, fimbriation and extracellular matrix proteins, cytokerines, complement proteins, antibodies and protease inhibitors (17–19). Collinson *et al.* (20) showed that BE1 exhibited decreased Rgp activity compared with the wild type, and no Rgp enzyme with large glycan additions, which are associated with the outer membrane, was observed. The gene(s) responsible for the phenotypes of W50/BE1 and W50/BR1 has not been elucidated. However, these early findings suggest that colonial pigmentation is associated with the activity and localization of proteases in *P. gingivalis* cells. Moreover, Rgp was purified from the *P. gingivalis* strain HG66, which secreted soluble Rgp and lacked pigmentation (21,22).

**The isolation of pigment-less mutants using transposon mutagenesis**

Several studies have applied transposon mutagenesis to isolate pigment-less *P. gingivalis* mutants (23–26). Simpson *et al.* (26) reported a non-pigmented mutant with an insertion sequence element (IS1126) at the promoter locus of *kgp*. In addition, Chen *et al.* (25) isolated non-pigmented mutants with transposon Tn4351 insertion in a putative glucosyl (rhamnosyl) transferase-encoding gene in several non-pigmented mutants, and Abaibou *et al.* (28) demonstrated that the *vimA* gene, located downstream of *recA*, is responsible for pigmentation. Using Tn4351 transposon mutagenesis, we isolated and characterized two non-pigmented mutants (*porR* and *porT*) (29,30).

**Pigmentation-related genes**

Pigmentation-related genes encode proteins with three types of functions: gingipain activity, gingipain transport and gingipain attachment (31). Rgp and Kgp proteinases are encoded by *rgpA*, *rgpB* and *kgp*. *rgpA* and *kgp* also encode hemagglutinins (adhesins) and the hemoglobin receptor at the 3’-terminal region of these genes. *kgp* single mutants and *rgpA rgpB kgp* triple mutants form less-pigmented and non-pigmented colonies, respectively, whereas *rgpA rgpB* double mutants form pigmented colonies (27,32). Smalley *et al.* (33) revealed that Rgp activity is crucial for converting oxyhemoglobin into methemoglobin, a form more susceptible to Kgp-mediated degradation, resulting in the release of iron(III) protoporphyrin IX and the production of µ-oxo heme dimers.

The *porR* mutant exhibited a pleiotropic phenotype: Rgp and Kgp proteinases were mainly present in the culture supernatant, mutant cells had no hemagglutinins activity and Rgp-mediated processing of fimbriinn was delayed (29). The *porR* mutant had altered phenol extractable polysaccharides. The monoclonal antibody (mAb) 1B5, which reacts with the sugar portions of *P. gingivalis* cell surface polysaccharides and membrane-type Rgp proteinases (17), did not react with cell lysates from the *porR* mutant, indicating that *porR* is involved in the biosynthesis of cell surface polysaccharides that might function as anchors for Rgp, Kgp, hemagglutinins and the hemoglobin receptor protein. *P. gingivalis* has two different lipopolysaccharide (LPS) molecules, O-LPS and A-LPS. O-LPS...
possesses the conventional O-antigen, whereas A-LPS has a different O-antigen comprising an anionic polysaccharide repeat unit that reacts with mAb 1B5 (34,35). Recently, another mAb (TDC-5-2-1) that recognizes the O-antigen of O-LPS, which is present in almost all wild-type cells, was generated; however, the glycan epitope recognized by this mAb has not been identified (36,37). As the porR mutant reacts with mAb TDC-5-2-1, this identified (36,37). As the porR mutant reacts with mAb TDC-5-2-1, this mutant might possess O-LPS but lacks A-LPS. The porR gene encodes a putative transaminase (29). We recently proposed that the final product synthesized through the Wbp pathway, which involves WbpA (PGN_0613 [UgdA], PGN_1243), WbpB (PGN_0168), WbpE (PGN_1236 [PorR]) and WbpD (PGN_0002), is a sugar substrate required for the biosynthesis of A-LPS (38). The P. gingivalis strain HG66, typically used for gingipain purification, exhibits no pigmentation on blood agar. This strain has a nonsense mutation in the wbpB gene, which is responsible for the pigmentless phenotype of the strain (38).

The porT mutant was also isolated as a non-pigmented mutant using Tn4351 transposon mutagenesis; however, the porT mutant was quite different from the porR mutant (30). The porR mutant exhibited gingipain activity in the culture supernatant, whereas the porT mutant demonstrated no gingipain activity either in the cell extract or in the culture supernatant. Subcellular fractionation and immunoblot analysis revealed that gingipain proteins accumulate in the periplasmic space, indicating that the PorT protein is involved in gingipain transport across the outer membrane. The subcellular localization of the PorT protein is controversial. We treated the membrane fraction of P. gingivalis cells with 1% Triton X-100 to separate the outer and inner membrane fractions, and the PorT protein was detected in the inner membrane fraction (Triton X-100-soluble fraction). However, using fractionation with Sarkosyl treatment, Nguyen et al. (39) reported that the PorT protein is located in the outer membrane.

**Genome sequence of Porphyromonas gingivalis**

In 2003, researchers at TIGR and the Forsyth Institute determined the whole genome sequence of P. gingivalis W83 (40). The P. gingivalis W83 genome comprises 2.3 megabase pairs and encodes a range of pathways and virulence determinants associated with the novel biology of this oral pathogen. This genome size is consistent with previous measurements using pulsed field gel electrophoresis (41). We determined the whole genome sequence of a different strain, ATCC 33277, typically used as a type strain in studies of the pathogenicity and physiology of P. gingivalis (42). Via genomic comparison with strain W83, we identified 461 ATCC 33277-specific and 415 W83-specific CDSs, and extensive genomic rearrangements were observed between the two strains, including 175 regions in which genomic rearrangements occurred. Interestingly, the genomes of P. gingivalis strains did not encode proteins involved in known secretion systems, such as the type II and III secretion systems, suggesting that P. gingivalis possesses a novel secretion system.

**Discovery of a new protein secretion system**

Genes homologous to porT of P. gingivalis have been identified in many members of the large and diverse Bacteroidetes phylum, whereas there are no porT homologs in bacteria belonging to other phyla. In addition, a porT homolog is not present in a bacterium belonging to the genus Bacteroides, B. thetaiotaomicron. Most bacterial protein secretion systems comprise multiple proteins that form a complex in the cell envelope. Thus, a set of proteins, including PorT, required for a protein secretion system must exist in bacteria with the protein secretion system, but not in bacteria lacking the system. Therefore, we used Venn diagram analysis to identify genes involved in these protein secretion systems. We identified 55 genes, including porT, that are present in P. gingivalis and Cytophaga hutchinsonii but absent in B. thetaiotaomicron and constructed deletion mutants of the genes (43). P. gingivalis strains with deletion mutations in 46 of these genes were generated to determine involvement of these genes in a secretion system for gingipains. Among the 46 mutants, 10 mutations in sov (PGN_0832), which was previously implicated in gingipain secretion (44), porK (PGN_1676), porL (PGN_1675), porM (PGN_1674), porP (PGN_1677), porQ (PGN_0645), porU (PGN_0022), porW (PGN_1877), porX (PGN_1019) and porY (PGN_2001) resulted in decreased Rgp or Kgp activity in cells and culture supernatants. We named this novel secretion system the Por secretion system (PorSS), now referred to as the T9SS (43,45,46) (Fig. 2). PorK, PorN and PorP were detected in the outer membrane fraction, whereas PorL and PorM were detected in the inner membrane fraction. Blue native gel analysis revealed that PorK was associated with PorN, and PorL was associated with PorM (43,47). PorY (PGN_0022) on the T9SS of P. gingivalis chromosome. PGN_0023, a mutant deficient in gingipain secretion (48,49), has been implicated in the O-deacetylation of LPS (50). PGN_0022 (porU)-encoding protein is a C-terminal signal peptidase for proteins that possess conserved C-terminal domains (CTDs) and utilize the T9SS for translocation across the outer membrane (49). Veith et al. (51) first identified the CTDs of P. gingivalis proteins. Seers et al. (52) predicted a role for CTDs in export and attachment to the cell surface, and Shoji et al. (53) verified this role. PG27/LptO/PorV has been associated with PorU and several CTD proteins (54).

**Two-component system for the T9SS of Porphyromonas gingivalis**

PorX (PGN_1019) and porY (PGN_2001) encode the response regulatory protein and histidine sensor kinase, respectively, of a two-component signal transduction system and have roles in regulating the expression of the genes in the transport system (43) (Fig. 2). These genes are located separately on the P. gingivalis chromosome, although the cognate...
Fig. 2. Model of the T9SS of Porphyromonas gingivalis. T9SS comprises more than 10 proteins, including PorK, PorL, PorM, PorN, PorP, PorQ, PorT, PorU, PorV, PorW, and Sov. Some of these proteins were expressed using the two-component system PorXY. PorX and PorY are a response regulator and a sensor kinase, respectively. CTD proteins, such as Kgp (K) and Rgp (R), are translocated across the IM via Sec machinery and subsequently secreted across the OM through the T9SS. CTD, C-terminal domains; CP, cytoplasm; IM, inner membrane; OM, outer membrane; PP, periplasm; T9SS, type IX secretion system.

**Extracellular and surface proteins secreted through the T9SS in Porphyromonas gingivalis**

The T9SS was identified in a study examining the secretion of gingipains. PGN_1728 (kgp), PGN_1733 (hag-A), PGN_1970 (rgpA) and PGN_1466 (rgpB) encode gingipain group proteins, with proproteins that possess CTDs at the C-terminus, and the secretion of these proteins is dependent on the T9SS. However, the T9SS is not specific for gingipain secretion. Seers et al. (52) predicted that CTD-containing proteins other than gingipains, including PG1326 (PGN_1115), PG2100 (PGN_0152, tapA), PG2102 (PGN_0900, tprT), PG1374 (PGN_0852), PG0495 (PGN_1476), PG0232 (PGN_0335, cpg70), PG0561 (PGN_0654), PG0654 (PGN_0693), PG1798 (PGN_1767), PG0553 (PGN_1416, pepK), PG2216 (PGN_2080), PG0350 (PGN_1611), PG1795 (PGN_1770), PG0616 (PGN_0659, hbp35), PG1424 (PGN_0898, pad), PG0614 (PGN_0657), PG1030 (PGN_1321) and PG0290 (PGN_1674), are secreted through this secretion system. In addition, Veith et al. (55) reported that as well as the CTD proteins described above, the outer membrane vesicle contains the following CTD proteins: PG0026 (PGN_0022, porU), PG0182 (PGN_0291), PG0182 (PGN_0152), PG0411 (PGN_1556), PG0561 (PGN_0654), PG1548 (PGN_0509), PG1969 (PGN_1770) and PG2172 (PGN_0123). We compared the proteomes of P. gingivalis strains kgpΔ kgpΔ (T9SS-deficient strain) and kgpΔ kgpΔ (T9SS-sufficient strain) using two-dimensional gel electrophoresis and peptide mass fingerprinting to identify other proteins secreted through the T9SS and identified the following 10 proteins: PGN_0152 (PG2100, tapA), PGN_0291 (PG0182), PGN_0335 (PG0232, cpg70), PGN_0654 (PG0611), PGN_0659 (PG0616, hbp35), PGN_0795 (PG0769), PGN_0898 (PG1424, pad), PGN_1416 (PG0553, pepK), PGN_1476 (PG0495) and PGN_1767 (PG1798) (56).

tapA (PGN_0152, PG2100) was associated with tprA (PGN_0876, PG1385). TprA is a tetratricopeptide repeat (TPR) protein that was upregulated in wild-type P. gingivalis (W83) cells placed in a mouse subcutaneous chamber, and the tprA mutant was clearly less virulent in the mouse subcutaneous abscess model (57). When the tprA mutant was placed in a mouse subcutaneous chamber, nine genes, including PG2102 (tapA), PG2101 (tapB) and PG2100 (tapC), were downregulated in the tprA mutant compared with the wild-type bacteria (58). These mutant genes were also downregulated in the culture medium. Yeast two-hybrid system analysis and in vitro protein binding assays with immunoprecipitation and surface plasmon resonance...
detection revealed that the TprA protein, which has three TPR motifs (collectively known as a protein–protein interaction module), binds to the TapA and TapB proteins. The TapA protein is located on the outer membrane, whereas the TprA and TapB proteins are located in the periplasmic space. The tapA mutant is less virulent than the wild type in mouse subcutaneous infection experiments.

cpg70 (PGN_0335, PG0232) encodes a 69.8 kDa metallocarboxypeptidase (CPG70) that cleaves C-terminal Lys and Arg residues from peptides (59). Purified CPG70 is an N- and C-terminally truncated 91.5 kDa proprotein predicted from the cpg70 gene. The cpg70 mutant was less virulent than the wild type in a mouse subcutaneous infection experiment (59). The RgpA and Kgp proteases and adhesins are C-terminally processed by CPG70 (60).

Abiko et al. (61) cloned hbp35 (PGN_0659, PG0616), which encodes a P. gingivalis outer membrane protein that binds hemin and has a calculated molecular mass of 35,313 Da (62). Subcellular fractionation, sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblot analysis using the anti-HBP35 antibody revealed that hbp35 encodes three cytoplasmic proteins with molecular masses of 40, 29 and 27 kDa and a modified form of the 40 kDa protein on the cell surface (63). The 29 and 27 kDa proteins are N-terminal truncated forms of the 40 kDa protein, and the initiation codons for these proteins are located in the middle of the coding sequence of hbp35. HBP35 exhibits thioredoxin activity and is essential for hemin-depleted conditions. The CTD of HBP35 has been extensively characterized (53). The 22 C-terminal amino acid residues of the CTD of HBP35 are required for cell surface translocation and glycosylation. The CTD region functions as a recognition signal for the T9SS, and the glycosylation of CTD proteins occurs after removal of the CTD region, as CTD-containing peptides were not detected in samples of glycosylated HBP35 protein through peptide map fingerprinting analysis, and antibodies against CTD region peptides did not react with glycosylated HBP35 protein (53).

pad (PGN_0898, PG1424) encodes a prokaryotic peptidylarginine deiminase (PAD). McGraw et al. (64) purified and characterized the biochemical and enzymatic properties of the PAD enzyme from P. gingivalis and proposed that PAD, acting in concert with arginine-specific proteases from P. gingivalis, promotes the growth of the pathogen in the periodontal pocket by enhancing the survivability of this bacterium and mediating the circumvention of host humoral defenses (64). Subsequently, research interests were focused on the relationship between P. gingivalis PAD and rheumatoid arthritis (5,65,66). Experimental evidence of a relationship between PAD and rheumatoid arthritis has recently been proposed. Using the chamber model, Marés et al. (67) showed that infection with viable wild-type P. gingivalis exacerbated collagen-induced arthritis in a mouse model, manifested through earlier onset, accelerated progression and enhanced disease severity, including significantly increased bone and cartilage destruction. Additional studies showed that infection with wild-type P. gingivalis significantly increased levels of autoantibodies to collagen type II and citrullinated epitopes, as a PAD null mutant did not elicit similar host responses. Consistently, Gully et al. (68) reported that the development of experimental periodontitis was significantly reduced in PAD-deficient P. gingivalis, and the extent of collagen-induced arthritis was significantly reduced in animals exposed to previous induction of periodontal disease through oral inoculation with a PAD-deficient strain vs. the wild type.

PepK protein, encoded by pepK (PGN_1416, PG0553), is secreted via the T9SS and anchored on to the cell surface through binding to A-LPS (56,69). Enzymatic analysis using outer membrane fractions of wild-type, pepK and gingipain-deficient mutant strains suggests that PepK has Lys-specific serine endopeptidase activity, and the activation of this protein requires processing through Rgp (69).

**T9SSs in other bacteria**

The comparative analysis of 37 Bacteroidetes bacteria genomes revealed T9SS genes in bacteria belonging to the phylum Bacteroidetes (45). Mutant analysis has revealed functional T9SSs in three other bacterial species (C. hutchinsonii, Flavobacterium johnsoniae, Tannerella forsythia) in the phylum Bacteroidetes.

In F. johnsoniae, a gliding bacterium that digests insoluble chitin, a chiA-encoded chitinase (Fjoh_4555) is secreted via the T9SS (43,70). The F. johnsoniae genome encodes proteins with CTDs similar to the P. gingivalis CTD. However, the C-terminal region of ChiA is not similar to that of P. gingivalis CTD, although it is necessary for T9SS-mediated secretion (70).

Wang et al. (71) constructed an orthologous porU mutant in C. hutchinsonii, a widely distributed gram-negative cellularlytic bacterium, and this mutant showed defects in cellulosome degradation and protein secretion. In addition, C. hutchinsonii CHU_0344, a dominant extracellular protein that possesses a C-terminal CTD, is secreted through the T9SS (71).

T. forsythia is one of the three bacteria implicated in the ‘Red Complex’ with P. gingivalis and T. denticola, which are important for chronic periodontitis (72). We constructed porK, porT and sov orthologous T. forsythia mutants and observed that these single mutants lack the surface layer (S-layer) and express less-glycosylated versions of the S-layer glycoproteins TfsA and TfsB (73). Compared with the proteins secreted from the porK and wild-type strains, the secretion of several proteins containing CTD-like sequences is porK gene-dependent. Tomek et al. (74) obtained similar results using porK and porU orthologous mutants, showing that the TfsA and TfsB glycoproteins in these mutants, which are N-terminally processed for Sec-mediated translocation across the cytoplasmic membrane, are
O-glycosylated, revealing that T9SS-mediated translocation across the outer membrane is not associated with O-glycan attachment. In wild-type bacteria, TfsA and TfsB are likely further glycosylated with rough-type LPS on the cell surface (74).

**T9SS and gliding motility**

While the periodontal pathogens *P. gingivalis* and *T. forsythia* are non-motile, the phylum Bacteroidetes includes many gliding bacteria, such as *F. johnsoniae* and *C. hutchinsonii* (75). *F. johnsoniae* cells attach to and move along surfaces at speeds of up to 5 μm/s in a process known as gliding motility (76). Electron microscopic analyses have failed to identify motility machineries such as flagella and type IV pili, implicating in twitching motility (77). Among them, *F. johnsoniae* gliding motility is achieved by another mechanism (77).

Bacteroidetes gliding motility is closely associated with the T9SS (43,45,46). *F. johnsoniae* genes *gldK*, *gldL*, *gldM*, *gldN*, *sprA*, *sprE* and *sprT*, which are essential for gliding motility, are homologous to *P. gingivalis* T9SS-related genes *porK*, *porL*, *porM*, *porN*, *sov*, *porW* and *porT*, respectively (43). This association may not be surprising because bacterial motility is typically associated with secretion systems. For example, flagellar motility and the type III secretion system have the same origin, and type IV pili, implicated in twitching motility, are associated with the type II secretion system.

Recently, we proposed a helical loop track model for the gliding motility of bacteria (78). In *F. johnsoniae*, the filamentous surface protein SprB is propelled along a left-handed helical loop on the bacterial cell surface (Fig. 3). When SprB adheres to a solid surface and can no longer move with respect to that surface, the cell is helically propelled in the opposite direction.

**Coda**

Many oral bacteria associated with periodontal diseases belong to the phylum Bacteroidetes. These bacteria are members of the genera *Porphyromonas*, *Prevotella*, *Tannerella* and *Capnocytophaga* and possess T9SS-related genes. Our unpublished data obtained from mutant studies suggest that *Prev. intermedia* has a functional T9SS. The proteins secreted through T9SSs include many virulence factors, such as gingipains in *P. gingivalis*. More than 10 proteins comprising T9SSs have been identified, but the precise interactions of these proteins remain unknown. Thus, the elucidation of T9SSs is only just commencing.

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**Acknowledgements**

Koji Nakayama would like to thank the members of the Division of Microbiology and Oral Infection, Department of Molecular Microbiology and Immunology, Nagasaki University Graduate School of Biomedical Sciences, for their assistance.

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