Type IX Secretion: the generation of bacterial cell surface coatings involved in virulence, gliding motility and the degradation of complex biopolymers

Paul D. Veith, Michelle D. Glew, Dhana G. Gorasia and Eric C Reynolds*

Oral Health Cooperative Research Centre, Melbourne Dental School, Bio21 Institute, The University of Melbourne, Melbourne, Australia

*For correspondence. E-mail: e.reynolds@unimelb.edu.au

Running title: The Type IX Secretion System

Summary

The Type IX Secretion System (T9SS) is present in over 1000 sequenced species/strains of the *Fibrobacteres-Chlorobi-Bacteroidetes* superphylum. Proteins secreted by the T9SS have an N-terminal signal peptide for translocation across the inner membrane via the SEC translocon and a C-terminal signal for secretion across the outer membrane via the T9SS. Nineteen protein components of the T9SS have been identified including three, SigP, PorX and PorY that are involved in regulation. The inner membrane proteins PorL and PorM and the outer membrane proteins PorK and PorN interact and a complex comprising PorK and PorN forms a large ring structure of 50 nm in diameter. PorU, PorV, PorQ and PorZ form an attachment complex on the cell surface of the oral pathogen, *Porphyromonas gingivalis*. *P. gingivalis* T9SS substrates bind to PorV suggesting that after translocation PorV functions as a shuttle protein to deliver T9SS substrates to the attachment complex. The PorU component of the attachment complex is a novel Gram negative sortase which catalyses the cleavage of the C-terminal signal and conjugation of the protein substrates to lipopolysaccharide, anchoring them to the cell surface. This review presents an overview of the T9SS focusing on the function of T9SS substrates and machinery components.

(I) INTRODUCTION

Overview

Over the last decade, a novel Gram negative bacterial secretion system has emerged in the *Fibrobacteres-Chlorobi-Bacteroidetes* superphylum (CFB group). This is the ninth such protein secretion system to be discovered in bacteria and has therefore been referred to as the Type IX Secretion System (T9SS). In Gram negative bacteria, proteins may be secreted across the outer membrane (OM) by various systems numbered I to IX, excluding VII which is specific to mycobacteria and Gram positive bacteria. Of these, some systems secrete substrates directly from the cytoplasm to the outside of the cell, whereas the other systems are terminal branches of the general secretory pathway (GSP) and are concerned only with the transport of protein across the OM. Secretory proteins that use the GSP contain N-terminal signal peptides that enable them to be transported across the cytoplasmic membrane via the universal Sec translocase into the periplasm (Chatzi *et al.*, 2013). Substrates of the T9SS also exhibit typical N-terminal signal peptides, and in T9SS mutants these substrates accumulate in the periplasm (Chen *et al.*, 2011) demonstrating that the T9SS is another terminal branch of the GSP.

The T9SS was first suggested to be called by this name in 2013 (McBride & Zhu, 2013, Sato *et al.*, 2013) and is now in consistent use. Prior to 2013, the T9SS was known as the PorSS (Sato *et al.*, 2010), and the identified components of this system were given gene/protein names with the prefix “Por”. The first component identified was PorT, in 2005 (Sato *et al.*, 2005). The term “Por” does not refer to *Porphyromonas*, but rather the porphyrin molecule that gives *P. gingivalis* its black pigmentation when grown on blood agar, hence proteins were given the prefix “Por” to indicate they were essential for the production of this black

pigment (Shoji *et al.*, 2002). The black pigment is generated only when the gingipains, especially the Lys-specific gingipain, Kgp, are secreted and attached to the cell surface via their association with anionic lipopolysaccharide (A-LPS) (Shoji *et al.*, 2002). The first non-gingipain mutants found to be apigmented or beige were the PGN_1239 glycosyltransferase (Chen *et al.*, 2000), *vimA* (Abaibou *et al.*, 2001) and *porR* (Shoji *et al.*, 2002) mutants. VimA and PorR are now known to be involved in the biosynthesis of A-LPS, hence in these mutants, gingipains can be secreted but not attached to the cell surface as recently reviewed (Shoji & Nakayama, 2016). All other “Por” proteins are components of the T9SS as they are required for the secretion and or attachment of T9SS substrates but not the biosynthesis or transport of A-LPS.

After considering the novelty of the T9SS, this review focusses on the secreted proteins (T9SS substrates) including their structure, function, secretion signal and means of attachment to the cell surface, and then turns to the components of the secretion system to build a working model of type IX secretion and its relationship to gliding motility. While this review encompasses the *Bacteroidetes* phylum, most of what is known about the T9SS has been learnt from studies of the oral pathogen *Porphyromonas gingivalis*, and the gliding bacterium, *Flavobacterium johnsoniae*.

The T9SS is novel

Several researchers have reported an inability to find homologs of components of other secretion systems in *P. gingivalis* and other T9SS-containing species leading to the conclusion that *P. gingivalis* harbours a novel terminal branch of the GSP (Nguyen *et al.*, 2007, Sato *et al.*, 2010, McBride & Zhu, 2013). Furthermore, the many T9SS components found to be essential for protein secretion do not have homologs outside of the CFB group.

Consistent with the T9SS being a new secretion system, T9SS substrates contain a novel C-terminal domain (CTD) signal comprising approximately 80 amino acid residues which directs their secretion across the OM (Seers *et al.*, 2006). A further novelty of the T9SS is that it typically attaches the T9SS substrates to the cell surface. Although other secretion systems have been shown to secrete surface layer proteins, it is not a general feature of those systems. Only one or two surface layer proteins are typically secreted by other systems such as the T1SS (Ford *et al.*, 2007) or T2SS (Noonan & Trust, 1997).

(II) T9SS SUBSTRATES

T9SS substrates are usually cell-associated proteins that contain a conserved CTD signal.

The discovery of the T9SS was driven by the intense study of the major virulence factors of *P. gingivalis* called gingipains. The gingipains are abundantly expressed cysteine proteinases and adhesins encoded by the genes *rgpA*, *rgpB* and *kgp*. RgpA and Kgp form cell-associated complexes of non-covalently bound proteinases and adhesins designated the RgpA-Kgp proteinase-adhesin complexes (O'Brien-Simpson *et al.*, 2003). The membrane-associated forms of the gingipains were shown to be extensively glycosylated with as much as 30% carbohydrate, however the site(s) of glycosylation were not determined (Curtis *et al.*, 1999). The three gingipains share much sequence similarity over their various domains, and therefore the presence of a conserved C-terminal sequence was not immediately associated with their secretion and attachment to the cell surface, nor was it linked to the extensive glycosylation. A proteomic study of the *P. gingivalis* OM was conducted using two dimensional gel electrophoresis and mass spectrometry (MS) (Veith *et al.*, 2002). In this

study, four proteins (RgpA, RgpB, P27 and P59) were observed to migrate on 2D gels as very diffuse spots at a MW that was between 15-40 kDa higher than that predicted by the protein sequence. The only portion of sequence that was found to be similar between these proteins was within the most C-terminal 50 amino acid residues, and hence a link was established between the identification of highly modified proteins and the presence of conserved C-terminal residues in the encoded sequences. The extensive and heterogeneous modification to these proteins was postulated to arise from covalent bonding to a glycolipid such as LPS, which in turn provides a mechanism for their cell-surface attachment (Rangarajan *et al.*, 1997, Veith *et al.*, 2002, O'Brien-Simpson *et al.*, 2003). In our earlier publications, we referred to this conserved region as the C-terminal domain (CTD), however since the primary function of this domain is now known, it has the more functional name, the T9SS CTD signal or T9SS signal. The T9SS signal is found in 34 *P. gingivalis* W50 proteins (Veith *et al.*, 2013) and thousands of other proteins across the CFB group (see below). These proteins are putative substrates of the T9SS.

Independent of the *P. gingivalis* studies, the T9SS signal has been identified in numerous proteins of several other *Bacteroidetes* species. A xylanase from *Rhodothermus marinus* was shown to be cell-attached according to activity assays, and confirmed to be associated with the cells by immunocytochemical staining (Karlsson *et al.*, 2004). The T9SS signal is located in the 5th domain ("D5") of this xylanase, and as the authors discovered its presence in other putative cell-associated enzymes, they also concluded that this domain is involved in cell-surface attachment. The T9SS signal (D5) was also found to be present in the sequences of cell surface-associated cellulolytic enzymes from *Cytophaga hutchinsonii* (Xie *et al.*, 2007) and in the sequences of several proteins identified from outer membrane preparations of the fish pathogen, *Flavobacterium psychrophilum* (Duchaud *et al.*, 2007, Dumetz *et al.*, 2008).

Hence the cell surface location of proteins that contain the T9SS signal in their encoded sequence has been recognised within several major classes of the *Bacteroidetes*.

Direct evidence for the involvement of the T9SS signal in secretion and attachment was provided in two *P. gingivalis* studies. In the first of these, RgpB was expressed with its C-terminal 72 amino acids deleted (Seers *et al.*, 2006). The truncated RgpB accumulated in the periplasm in an inactive form that included the pro-domain. It was not modified, nor was it found in OM fractions, indicating that the T9SS signal was essential for the secretion and attachment of RgpB. In the second study (Nguyen *et al.*, 2007), deletion of the two most C-terminal residues (VK) of RgpB was also sufficient to abolish secretion, modification and cell-surface attachment. Earlier evidence for the role of the T9SS signal in secretion was provided by the construction of a chimera comprising the N-terminal portion of Kgp fused to the C-terminal region of RgpB. Kgp was found on the surface of whole cells and in culture fluid with an activity equivalent to wild type, suggesting that the T9SS signal of RgpB was able to direct the secretion and maturation of the Kgp proteinase (Sato *et al.*, 2005). Kgp was not extensively modified in this strain, potentially due to the His tag that was inserted between the portions of Kgp and RgpB sequences. Since Kgp is itself a T9SS substrate, this experiment was not definitive in showing that the T9SS CTD signal alone was sufficient for directing the secretion and modification of substrates. Definitive evidence for this was provided in a later communication from the same group. Shoji *et al.* (2011) fused the T9SS CTD signal of HBP35 to a green fluorescent protein (GFP) from an unrelated organism that did not contain the T9SS. Although the GFP-CTD fusion did not fluoresce when expressed in *P. gingivalis* due to the anaerobic environment, GFP was shown to be secreted and extensively modified. Furthermore, N-terminal CTD truncations showed that a minimal length of 22 C-terminal residues of the HBP35 T9SS CTD signal still retained activity

indicating that they were essential for the secretion and modification of fused GFP (Shoji *et al.*, 2011). These 22 residues include motif D & E but lack the well conserved motif B (see below). Together, the T9SS CTD signal has been shown to be both essential and sufficient for the secretion of substrates and to direct their modification and attachment to the cell surface. Recently, the C-terminal 12 residues of the *F. johnsoniae* gliding motility adhesin RemA T9SS signal were found to be essential for its secretion and its T9SS signal was also sufficient to enable the secretion of GFP (Kulkarni *et al.*, 2017).

From the above studies in *P. gingivalis*, the T9SS CTD signal is able to direct both secretion and modification, and it was proposed for some time that the T9SS signal was the site of the modification. Extensive site-directed mutagenesis of potential glycosylation sites however did not support this proposal (Slakeski *et al.*, 2011). Recently, other evidence suggested that the T9SS CTD signal was not the site of modification. Firstly, peptides derived from the T9SS signal have never been recovered from MS analyses of hydrolysed mature substrates in either *P. gingivalis* or other T9SS substrate-producing species (Veith *et al.*, 2002, Sato *et al.*, 2005, Veith *et al.*, 2009, Veith *et al.*, 2013). Initially the absence of these peptides was thought to be due to the extensive modification, potentially at multiple sites which would hamper both the digestion of this domain with trypsin and the recovery and identification of modified peptides. However the cumulative evidence from the large number of identified T9SS substrates is more consistent with cleavage of the T9SS signal. Secondly, the highly modified forms of RgpB and HBP35 are not detected in Western blots employing anti-T9SS signal antibodies that readily recognise the precursor forms (Shoji *et al.*, 2011, Glew *et al.*, 2012). Thirdly, and most conclusively, cleaved T9SS signals have been recovered from the culture supernatants of wild type *P. gingivalis* as well as from other species (Glew *et al.*, 2012, Veith *et al.*, 2013). These cleaved signal domains were full length and unmodified

indicating that they were released into the culture fluid after secretion. Their cleavage sites were determined by MS and the preferred cleavage was determined to be N-terminal to a small residue (Ser, Ala or Gly) at a well conserved distance 27-34 residues N-terminal to motif B (**Fig. 1**). Hence T9SS substrates are secreted first across the IM as directed by an N-terminal signal peptide which is subsequently cleaved by signal peptidase I, and then secreted across the OM as directed by the T9SS CTD signal and subsequently cleaved by a component of the T9SS.

Modification of T9SS substrates and relationship to A-LPS

As mentioned above, T9SS substrates appear to be modified by a heterogeneous glycan or glycolipid such as LPS. Two forms of *P. gingivalis* LPS have been described, O-LPS containing O-polysaccharide (OPS) and A-LPS containing anionic polysaccharide (APS) (Shoji & Nakayama, 2016). Both O-LPS and A-LPS consist of the same (or similar) lipid A and core oligosaccharides (Rangarajan *et al.*, 2008, Paramonov *et al.*, 2009, Paramonov *et al.*, 2015) although the detailed structure of the core region has not been elucidated. The structure of OPS has been found to comprise tetrasaccharide units of GalNAc, Rha, Glc and Gal (Paramonov *et al.*, 2001) while each APS unit is a branched phosphomannan inclusive of 8 mannose sugars (Paramonov *et al.*, 2005). The most common evidence used to show that *P. gingivalis* T9SS substrates are modified with LPS, is their detection in Western blots with MAb-1B5. The epitope for this monoclonal antibody has been demonstrated to include the Man α 1-2Man α 1-phosphate portion of APS (Paramonov *et al.*, 2005), strongly suggesting that the T9SS substrates are modified with A-LPS. In some secretion mutants, MAb-1B5-reactive material accumulates to a small degree in the periplasm along with but not attached to the T9SS substrates suggesting a link between the secretion and modification processes and further supporting that A-LPS is the modification (Chen *et al.*, 2011, Glew *et al.*, 2012).

T9SS substrates do not appear to be modified with O-LPS since in A-LPS specific mutants, O-LPS is still produced, yet substrates are not modified or attached to the cell surface (Shoji & Nakayama, 2016). Genes essential for A-LPS production include members of the Wbp pathway (Shoji *et al.*, 2014), *wbaP* (Shoji *et al.*, 2013, Gorasia *et al.*, 2015), and the *vim* genes (Vanterpool *et al.*, 2005a, Vanterpool *et al.*, 2005b). These mutants are apigmented and no products are detected by MAb-1B5 in Western blots. Secreted T9SS substrates have been observed in the cell-free culture fluid in their non-modified form with their T9SS CTD signals cleaved. These data taken together support the proposal that the T9SS substrate cell surface anchor is A-LPS.

In order to directly demonstrate a covalent linkage between T9SS substrates and A-LPS, Gorasia *et al.*, (2015) deglycosylated T9SS substrates with trifluoromethanesulfonic acid, digested the protein with trypsin and analysed the products by MS. The C-terminal peptides of several T9SS substrates were identified exactly according to their elucidated C-terminal cleavage site except that a residual modification of +630 Da was associated with the C-terminal residue. MS/MS fragmentation data suggested a peptide bond between the C-terminal residue and a serine component of the +630 Da modification. This peptide bond was confirmed by the complete release of the modification by proteinase K cleavage. The modification of T9SS substrates therefore occurs at the C-terminus via a peptide bond (**Fig 1**). The linkage of secreted proteins via a peptide bond to the cell surface is reminiscent of the sortases of Gram positive bacteria which catalyse the transpeptidation of secreted proteins to a glycine residue in the cell wall (Navarre & Schneewind, 1999). Interestingly, in *wbaP* mutants which are unable to synthesise A-LPS, the sortase activity remains functional as T9SS substrates are cleaved at their usual sites however instead of being conjugated to A-LPS, they are instead linked to various amino acids and peptides that had been captured from

the growth medium (Gorasia *et al.*, 2015). The identification of a seryl residue at the C-terminus of the modified T9SS substrate together with the complete C-terminal modification reacting with MAb-1B5 suggests that A-LPS may be modified with an amino acid at the core oligosaccharide region which has been described for LPS from other species (Gozdziewicz *et al.*, 2015). Together these results suggest that each T9SS substrate is attached via its C-terminus to the core region of A-LPS (**Fig. 1**).

Bioinformatics and structure of the T9SS CTD signal

The identification of T9SS CTD signal cleavage sites has allowed the T9SS signal to be defined as the region C-terminal to the cleavage site. In *P. gingivalis*, the length of the T9SS CTD signal ranges from 67-85 amino acids and includes a cleavage motif, an N-terminal spacer region and three well-conserved motifs named B, D and E (**Fig. 1**) (Veith *et al.*, 2013). Motifs B, D and E were found and verified in *P. gingivalis* and *Tannerella forsythia* by specialised motif elucidation software and used for the development of a Hidden Markov Model (Veith *et al.*, 2013). The model was then searched against the SwissProt/TrEMBL databases resulting in the identification of 682 putative T9SS substrates from 87 different organisms. All species that were classified were members of the CFB group. When the model was searched with greater sensitivity against 17 fully sequenced T9SS-positive species, 658 T9SS substrates were predicted with close to 40 substrates per species. This T9SS CTD signal has also been modelled by TIGRfam where it has the name Por_Secre_Tail (TIGR04183). A search for this term in the NCBI protein database in Jan 2017 (www.ncbi.nlm.nih.gov/protein) returned 43,299 hits to 1,011 species/strains within CFB group bacteria comprising mostly *Bacteroidetes* but also *Chlorobi* (green sulfur bacteria), *Ignavibacteriales*, and *Fibrobacter succinogenes*. The ease of predicting T9SS substrates contrasts sharply with other secretion systems where more sophisticated algorithms are

required, and even then require experimental validation (Cianciotto, 2009, McDermott *et al.*, 2011). The crystal structure of the RgpB T9SS CTD signal revealed a β -sandwich of two sheets, one comprising three strands and the other four strands (**Fig. 2**). It is most closely related to an Ig-like fold similar to the preceding Ig-like domain of RgpB that remains part of the mature protein (de Diego *et al.*, 2016). The crystal structure of the uncleavable T9SS CTD signal of PorZ has also been solved and found to contain the same overall β -sandwich structure (Lasica *et al.*, 2016).

The motility adhesin SprB from *F. johnsoniae* is secreted by the T9SS and is therefore a T9SS substrate (Shrivastava *et al.*, 2013). SprB however does not have a Por_Secre_Tail domain but instead has a Bac_Flav_CTERM domain (TIGR04131). The presence of both families in completed genomes strongly correlates to the presence of core T9SS proteins (Kulkarni *et al.*, 2017). The TIGR04131 family is very closely related to the CHU_C family (Pfam13585) which derives its name from *C. hutchinsonii* proteins and has been shown to be necessary for the secretion of a cellulase (Wang *et al.*, 2017). While the T9SS signal of SprB is essential for SprB secretion to the cell surface, it was not able to direct the secretion of GFP, suggesting that additional features in the SprB sequence besides the T9SS signal may be important (Kulkarni *et al.*, 2017). Multiple alignment of protein logos derived from each of these families show that they all have similar characteristics with each one exhibiting a distinct but recognisable motif corresponding to motifs B, C and D (**Fig. 3**). Their differences may reflect the post-translocation fate of the T9SS substrates they carry with SprB-like substrates being directed to the motility apparatus, cellulases potentially being directed to a cellulolytic machine and substrates belonging to the TIGR04183 family being directed to PorV and subsequently to PorU for cleavage and cell surface attachment (**Fig. 4**).

Structure and function of T9SS substrates

With so many predicted T9SS substrates, it is beyond the scope of this review to comprehensively address their assorted functions. A survey of the T9SS substrates predicted by Veith *et al.* (2013), indicate that enzymes involved in the breakdown of complex biopolymers, namely proteinases, glycosidases (including cellulases and chitinases), nucleases and lipases are very common. Other common T9SS substrate classes are adhesins, hemagglutinins and leucine-rich proteins. To gain both an overview as well as some detail we will consider (i) all characterised T9SS substrates of *P. gingivalis*, (ii) the surface layer proteins of *T. forsythia* (iii) the cellulases of *C. hutchinsonii*, and (iv) the motility adhesins of *F. johnsoniae*.

The *P. gingivalis* gingipains RgpA, RgpB and Kgp are major virulence factors and have been studied extensively for over two decades. They are essential for virulence in animal models, are involved in the acquisition of peptides and heme from host proteins and hemoproteins for bacterial nutrition and play a major role in disease pathogenesis through their adhesive properties and by dysregulating host defense and inflammatory responses (O'Brien-Simpson *et al.*, 2003, Guo *et al.*, 2010, Grenier & La, 2011, Smalley & Olczak, 2017). The gingipains are Arg-specific (RgpA and RgpB) and Lys-specific (Kgp) cysteine proteinases, with RgpA and Kgp having adhesin domains C-terminal to their proteinase domains. The immature RgpA and Kgp polypeptides are autolytically processed at several Arg and Lys sites enabling the assembly of the RgpA-Kgp proteinase- adhesin complexes (O'Brien-Simpson *et al.*, 2003). The crystal structures of the proteinase domains reveal a “tooth-like” structure with the “crown” corresponding to a catalytic domain of mixed alpha/beta structure and the “root” corresponding to an immunoglobulin superfamily domain (IgSF) comprising a seven-stranded β -barrel (Eichinger *et al.*, 1999, de Diego *et al.*, 2014, Gorman *et al.*, 2015). The

adhesin domains include ‘adhesin binding motifs’ of unknown structure interspersed with ‘cleaved adhesin’ domains. Three of these cleaved adhesin domains (K1, K2 and K3) from Kgp have been expressed in *E.coli*, crystalized, and found to adopt β -sandwich jelly roll topologies (Li *et al.*, 2010, Li *et al.*, 2011, Ganuelas *et al.*, 2013). Interestingly, some of these structural domains overlap with the cleavage sites determined for the native proteins (Li & Collyer, 2011). These cleavage sites occur in large loops in the solved structures (L9 in K1; L8 in K2 and L10 in K3) suggesting that in *P. gingivalis*, once these domains are folded and secreted to the cell surface, these loops may be excised to facilitate multimerisation and surface coat formation.

In addition to the gingipains, four other proteinases have been identified as T9SS substrates namely PepK (Nonaka *et al.*, 2014), CPG70, PrtT and periodontain. PrtT is unrelated to the gingipains but shares 34% sequence identity to periodontain and both are related to streptococcal proteinases (Madden *et al.*, 1995, Nelson *et al.*, 1999). Periodontain selectively inactivates the human serpin, α_1 -proteinase inhibitor which is the sole known inhibitor of neutrophil elastase and therefore was postulated to be responsible for the clinical observation of increased levels of neutrophil elastase in the gingival crevicular fluid of patients with periodontitis (Nelson *et al.*, 1999). CPG70 includes a zinc carboxypeptidase domain specific for Lys and Arg residues and three PKD domains. CPG70 was essential for virulence in a murine lesion model (Chen *et al.*, 2002), however since it has a role in the proteolytic processing of the RgpA and Kgp polyproteins, it is uncertain whether it has a direct role in virulence or facilitates RgpA-Kgp complex formation (Veith *et al.*, 2004).

Animal peptidylarginine deiminase (PAD) catalyses the conversion of peptidylarginine to peptidyl citrulline, which is considered a physiological post-translational modification that

assists in the functional diversity of the proteins (Gyorgy *et al.*, 2006). *P. gingivalis* PAD (PPAD) is a T9SS substrate and is the only known extracellular PAD produced by a bacterium. PPAD only citrullinates C-terminal arginines unlike the unrelated animal PAD which citrullinates non-terminal arginines within the sequence (Potempa *et al.*, 2003). It is believed PPAD provides protection against acidogenic and acidophilic bacteria through ammonia generation during citrullination helping to maintain a neutral pH (Casiano-Colon & Marquis, 1988). In some genetic backgrounds, citrullinated proteins such as fibrinogen and α -enolase generate an autoimmune response generating anti-citrullinated protein antibodies, a hallmark of rheumatoid arthritis (Mallya *et al.*, 1983). *P. gingivalis* expressing PPAD was able to digest human fibrinogen and α -enolase and citrullinate the resultant peptides containing C-terminal arginine (Wegner *et al.*, 2010). Mice infected with wild type *P. gingivalis* developed collagen-induced arthritis earlier and more severely than a *P. gingivalis* mutant unable to express PPAD providing a molecular basis for the epidemiological link between periodontitis and rheumatoid arthritis (Maresz *et al.*, 2013, Gully *et al.*, 2014). Similarly, a mutant lacking PPAD was significantly less virulent than wild type in producing periodontitis in mice (Gully *et al.*, 2014). The crystal structure of PPAD exhibits a catalytic domain comprising five blades of mixed α/β architecture followed by a C-terminal IgSF domain similar to that of RgpB and Kgp (Goulas *et al.*, 2015) (Montgomery *et al.*, 2016).

HBP35 is a hemin-binding T9SS substrate that is necessary for efficient growth under limited hemin conditions (Shibata *et al.*, 2003, Shoji *et al.*, 2010). Furthermore, HBP35 was shown to exhibit thioredoxin activity (Shiroza *et al.*, 2008, Shoji *et al.*, 2010). Anti-HBP35 antibodies inhibit *P. gingivalis* hemagglutination, adherence to epithelial cells, hemin binding and cell growth (Hiratsuka *et al.*, 2010, Shibata *et al.*, 2011). Other *P. gingivalis* T9SS substrates that were studied include the TprA-associated proteins TapA and TapC, mutants of which showed

reduced virulence in animal models (Kondo *et al.*, 2010). PG0350 and PG1374, which are Leucine-rich repeat containing proteins that exhibit homology to internalin J from *Listeria monocytogenes*, may be involved in facilitating the invasion of host cells or other intercellular binding activities such as the promotion of biofilms (Zhang *et al.*, 2005, Capestany *et al.*, 2006, Dashper *et al.*, 2009). Recently, Mfa5 was determined to be both a T9SS substrate and a component of Mfa fimbriae, and was essential for proper assembly of the tip fimbrillins providing a novel link between the T9SS and fimbriae (Hasegawa *et al.*, 2016). Hence, in *P. gingivalis*, all characterised T9SS substrates to date appear to have a role in virulence.

As with *P. gingivalis*, *T. forsythia* uses the T9SS to secrete virulence factors including surface layer proteins and BspA as well as predicted proteases, Leucine-rich repeat proteins and an amylase (Sharma, 2010, Narita *et al.*, 2014, Tomek *et al.*, 2014, Veith *et al.*, 2015). In addition, a group of proteinases involved in virulence known as the KLIKK proteases (Koneru *et al.*, 2016) may also be secreted via the T9SS (Veith *et al.*, 2015). Unlike *P. gingivalis* that appears to have an amorphous surface layer composed of T9SS substrates, *T. forsythia* exhibits a true S-layer composed of two homologous T9SS substrates, TfsA and TfsB (Higuchi *et al.*, 2000, Lee *et al.*, 2006) that are important for various virulence traits (Sabet *et al.*, 2003, Sakakibara *et al.*, 2007). This S-layer has been studied by both atomic force microscopy and electron microscopy (EM) techniques and was determined to have a square (P4) lattice structure resulting from coassembly of equimolar amounts of TfsA and TfsB (Sekot *et al.*, 2012, Oh *et al.*, 2013). Homologs of the surface layer proteins are common in *Tannerella* and *Parabacteroides* species suggesting that the generation of T9SS-secreted surface layers may be common in this group of organisms.

C. hutchinsonii digests crystalline cellulose, its primary source of carbon and energy. In mutants lacking the T9SS, *C. hutchinsonii* is defective in its ability to digest cellulose suggesting that the cellulases present on the cell surface of the wild type are secreted by the T9SS (Wang *et al.*, 2014, Zhu & McBride, 2014). One of the most abundant T9SS substrates produced by *C. hutchinsonii* named Cel5A (CHU_1107) was purified and shown to digest cellulose (Veith *et al.*, 2013, Zhu *et al.*, 2013), however its mutation along with individual mutants of other predicted cellulases caused little change in the ability of *C. hutchinsonii* to digest cellulose indicating the presence of redundant cellulases on the cell surface (Zhu *et al.*, 2013, Zhu *et al.*, 2016). However, another predicted T9SS substrate, CHU_3220 (Veith *et al.*, 2013) was found to be essential for the degradation of cellulose and was required for digestion of the crystalline region of cellulose (Wang *et al.*, 2017). The activity and OM-localisation of CHU_3220 was shown to be dependent on the C-terminal domain, “CHU_C”, one of the variant T9SS signals (**Fig. 3**). Hence, CHU_3220 is almost certainly secreted by the T9SS. CHU_3220 was not assumed to be a cellulase itself, but rather an essential component of a cellulolytic machine located on the cell surface (Wang *et al.*, 2017).

F. johnsoniae secretes the motility adhesins SprB and RemA via the T9SS to enable the bacteria to glide over surfaces as recently reviewed (McBride & Nakane, 2015). SprB is a 669 kDa highly repetitive protein that forms long filaments that project outward from the OM and move along the cell surface in a helical track (Nelson *et al.*, 2008, Nakane *et al.*, 2013). SprB is essential for movement in agar but not on glass. SprB has several paralogs and these may be important for motility on different surfaces. RemA is a much smaller protein of 152 kDa, but also was shown to move along a helical track. RemA contains a central lectin domain enabling RemA to bind polysaccharides including polysaccharides secreted by *F. johnsoniae*. This bacterium may therefore be able to provide its own surface on which to

propel itself (Shrivastava *et al.*, 2012). While the attachment mechanism of *P. gingivalis* T9SS substrates to the cell surface is somewhat understood, further studies are required to determine how the motility adhesins of *F. johnsoniae* are attached to the OM in such a way as to allow their movement along a track.

(III) The T9SS

Finding components of the T9SS

Unlike most other multi-component secretion systems identified in bacteria, the genes of the T9SS are widely dispersed in the genome. Indeed the 18 components identified to date in *P. gingivalis* are spread over 13 distinct loci (**Table 1**). Therefore finding all the components of the T9SS represents a significant challenge. The first component of the T9SS was PorT, published in 2005 and identified via transposon mutagenesis (Sato *et al.*, 2005). The second component to be identified was the 280 kDa Sov protein (Saiki & Konishi, 2007). Sov was selected for mutation based on its large size, known detection by proteomics and unknown function. It was noted by multiple research groups that the T9SS CTD signal, PorT and Sov shared a restricted distribution among *Bacteroidetes* species that included *C. hutchinsonii* but excluded *Bacteroides thetaiotaomicron* (Seers *et al.*, 2006, Nguyen *et al.*, 2007, Ishiguro *et al.*, 2009, Sato *et al.*, 2010). Sato *et al.*, (2010) found that *porT* plus 55 other genes were found to be present in both *P. gingivalis* and *C. hutchinsonii* but absent in *B. thetaiotaomicron*. These 55 genes were inactivated resulting in the identification of ten additional components namely PorK, PorL, PorM, PorN, PorP, PorQ, PorU, PorW, PorX and PorY (**Table 1**). The same principle was also used for the identification of PorV (Ishiguro *et al.*, 2009, Chen *et al.*, 2011, Shoji *et al.*, 2011) and PG0534 (Saiki & Konishi, 2010a).

Further comparative genome analyses utilising a larger number of species predicted 29 T9SS components including all 17 known components that have been experimentally verified (**Table 1**) (Heath *et al.*, 2016). PorZ was predicted by both bioinformatic approaches and its involvement in the T9SS was recently confirmed (Lasica *et al.*, 2016). PG0189 was not predicted by either bioinformatic approach but was demonstrated by PSI-BLAST to share the same species distribution as other components of the T9SS and is likely to be a T9SS component based on its association with PorK and PorN (Gorasia *et al.*, 2016), but its involvement needs to be confirmed by mutation. Despite the lack of organisation of the T9SS component genes into operons, the comparative genomics approach has proven to be very powerful in predicting the components of the T9SS. Nevertheless, this approach should be repeated using the more sensitive PSI-BLAST to ensure that weakly conserved candidates such as PG0189 are not missed.

Phenotype of T9SS mutants

The key references pertaining to the characterisation of the T9SS mutants are provided in **Table 1**. The first characteristic of *P. gingivalis* strains lacking an essential component of the T9SS is their lack of black pigmentation when grown on blood agar. This is due to the inability of these mutants to secrete the gingipains, which are required to extract the heme from hemoglobin (Smalley & Olczak, 2017). Mutants that lack A-LPS also produce apigmented colonies due to their inability to attach the secreted gingipains and other T9SS substrates to the cell surface. These mutants are noted for their lack of A-LPS when probed by Western blot using the specific monoclonal antibody MAb-1B5 and by the detection of significant amounts of gingipain activity in vesicle-free supernatants and the absence of cell-associated gingipain activity (Shoji & Nakayama, 2016). T9SS mutants in contrast produce A-LPS but are deficient in both cell-associated and supernatant gingipain activity. Another

property of the gingipains RgpA and Kgp, as well as other T9SS substrates such as HagA, is hemagglutination activity (Sakai *et al.*, 2007), and T9SS mutants are therefore deficient in this activity as well. Furthermore, since secretion is blocked, gingipains and other T9SS substrates accumulate in the periplasm in their non A-LPS-modified form with their T9SS signal intact (Sato *et al.*, 2005). A-LPS-modified forms cannot be detected in T9SS mutants and neither can the cleaved T9SS signal (Glew *et al.*, 2012). The A-LPS-modified T9SS substrates form an extracellular layer referred to as the electron dense surface layer (EDSL) and this is not detectable in T9SS mutants nor in A-LPS mutants (Chen *et al.*, 2011); Slaney, 2006 #293}. The characteristics of T9SS mutants in *F. johnsoniae* include the inability to utilize chitin and defective gliding motility. Interestingly, not all T9SS mutants lack the ability to digest chitin and some mutants retain full gliding motility. These specific phenotypic differences are very helpful to group the T9SS components into functional categories (see below).

Putative OM translocation apparatus

Of the many components of the T9SS identified by Sato *et al.* (2010), only the *porPKLMN* genes were contiguous on the chromosome, making them an attractive candidate for encoding a secretion apparatus. While the *porPKLMN* unit is generally well conserved, in *F. johnsoniae* there is no *porP* homolog immediately upstream of *porK*, and rather, *porKLMN* was experimentally verified to be an operon (Shrivastava *et al.*, 2013). In *P. gingivalis*, the *porPKLMN* genes were shown to be co-transcribed (Vincent *et al.*, 2017) however quantitative proteomics indicates that PorP is more than an order of magnitude less abundant than PorK, PorL, PorM and PorN raising the possibility that there is an additional promoter to drive transcription of the *porKLMN* unit alone (unpublished data). PorK was predicted to be a lipoprotein and was localised to the OM along with PorN by differential detergent solubility

of the IM and OM, while PorL and PorM were localised to the IM (Sato *et al.*, 2010, Shrivastava *et al.*, 2013, Vincent *et al.*, 2017). Blue Native gel electrophoresis (BN-PAGE) and Western blotting revealed that all four of these proteins formed large complexes of over 1 MDa consistent with them forming a secretion apparatus spanning both membranes (Sato *et al.*, 2010). In *F. johnsoniae*, PorK, PorL, PorM and PorN/GldO are absolutely required for gliding motility, chitin utilisation and the secretion of the motility adhesins SprB and RemA (Braun *et al.*, 2005, Rhodes *et al.*, 2010, Shrivastava *et al.*, 2013) whereas some other components such as Sov, PorP, PorT and PorW were previously named with the Spr prefix (**Table 1**) because they exhibited only a partial defect in motility. The PorU, PorV, PorQ and PorZ components are involved in the attachment of T9SS substrates to the cell surface in *P. gingivalis* (Glew *et al.*, 2017) but in *F. johnsoniae*, neither PorU nor PorV are required for full motility (Kharade & McBride, 2015) and it is likely that neither PorQ nor PorZ are required as well since it is thought that all the genes required for gliding motility have already been identified (McBride & Zhu, 2013). This leaves PorK, PorL, PorM and PorN as the most likely candidates to form the essential core secretion apparatus. Indeed, EM examination of purified PorK and PorN complexes isolated from *P. gingivalis* revealed large ring-shaped structures of approximately 32-36 subunits and measuring 50 nm in diameter and ~7 nm in height (Gorasia *et al.*, 2016). Both proteins were resistant to proteinase K treatment of whole cells but were readily degraded by proteinase K after cell lysis suggesting that the ring structure was located on the periplasmic side of the OM (Gorasia *et al.*, 2016). PorL and PorM have two and one predicted transmembrane helices respectively, consistent with their localisation to the IM. Topological predictions suggest that PorL is predominantly cytoplasmic while PorM is suggested to be predominantly periplasmic (Shrivastava *et al.*, 2013). The cytoplasmic localisation of the soluble PorL domain was confirmed by its binding to the cytoplasmic response regulator, PorX (Vincent *et al.*, 2017). Furthermore, cysteine

accessibility assays confirmed the localizations of PorL and PorM as depicted in **Fig. 5** (Vincent *et al.*, 2017). PorL and PorM have been shown to bind to each other (Gorasia *et al.*, 2016, Vincent *et al.*, 2017) and recently, PorM was shown to also interact with PorK and PorN (Vincent *et al.*, 2017). The finding of independent PorK/N and PorL/M complexes by Gorasia *et al.* (2016) suggests that the large complexes observed by Sato *et al.* (2010) by BN-PAGE may also have been two complexes rather than one complex containing all four proteins. However the blue native gels showed clear evidence of two different sized complexes when probed for PorM and PorL, one close to 1 MDa and the other well above the 1.2 MDa standard consistent with the presence of a smaller PorL/M complex and a larger PorK/L/M/N complex. Quantitative proteomics of whole cell WT *P. gingivalis* strains indicate that the abundance of PorK, PorM and PorN is very similar while the abundance of PorL was 3-fold higher (Glew *et al.*, 2017). Taken together, it is likely that PorL and PorM also form large ring structures of a similar number of subunits as PorK and PorN (or greater for PorL) and that together, PorK/L/M/N form a large secretion apparatus that spans the cell envelope (**Fig. 5**). Of greatest difficulty however, is assigning a protein to fulfil the role of forming a pore in the OM. Although there are many known OM-associated components of the T9SS (**Table 1**), none besides PorK and PorN have been shown to be absolutely required for secretion/motility in both *P. gingivalis* and *F. johnsoniae*. Although PorK and PorN appear to be mostly exposed in the periplasm it is possible that one of these proteins contributes just one or two transmembrane segments to the OM pore which given the 32-36 subunit composition of the PorK/N rings could correspond to an OM pore structure composed of up to 72 transmembrane segments. By way of comparison, the curli secretion pore CsgG is formed by 36 transmembrane β -strands with each of the nine subunits contributing four strands (Goyal *et al.*, 2014).

The Attachment complex

In most analysed species, T9SS substrates are generally associated with the OM rather than fully secreted into the growth medium. In *P. gingivalis*, the means of attachment is presumed to be via covalent modification to A-LPS. As discussed above, the T9SS CTD signal is cleaved and the new C-terminus of the T9SS substrate is conjugated to A-LPS via a sortase-like (transpeptidase) mechanism. The protein assigned to this sortase role is PorU. PorU is composed of a central cysteine proteinase of the C25 (gingipain) family domain flanked by large regions of unknown function and an uncleaved T9SS CTD signal (Glew *et al.*, 2012). Of all the T9SS components, PorU is the only predicted proteinase, and since the Gram positive sortases are also classed as cysteine proteinases (C60 family) (Zong *et al.*, 2004), PorU is the best candidate for the sortase role.

PorU is part of an attachment complex comprising the additional proteins PorV, PorZ and PorQ (Glew *et al.*, 2017). In BN-PAGE analyses of wild type *P. gingivalis*, all four proteins co-align, however in specific mutant strains, smaller complexes comprising PorU/V and PorZ/Q were observed suggesting that the primary binding partner of PorU is the PorV outer membrane protein while the primary binding partner of PorZ is the PorQ outer membrane protein. The specific binding of PorU to PorV was suggested from earlier BN-PAGE experiments (Glew *et al.*, 2014) and also from purification of His-tagged PorV complexes (Saiki & Konishi, 2014). PorV and PorQ are strongly localised to the OM by both experimental and bioinformatic approaches (Chen *et al.*, 2011, Veith *et al.*, 2014). PorV is predicted by Pfam to be related to the FadL 14-stranded OM β -barrel protein and this assignment is supported by structural modelling. Structural modelling of PorQ also suggests a FadL fold (unpublished data) and PorV and PorQ are reciprocal best BLAST hits within the

P. gingivalis sequence database sharing 15% sequence identity (31% similarity) over the full length of sequence with an E-value of less than $1e-50$ after three rounds of PSI-BLAST.

PorU and PorZ share similar properties. *P. gingivalis* PorU and PorZ have predicted T9SS CTD signals that were found to remain uncleaved, and consequently the proteins are not modified with A-LPS (Glew *et al.*, 2012, Veith *et al.*, 2013, Lasica *et al.*, 2016). Similarly, the PorU orthologs in *Parabacteroides distasonis*, *C. hutchinsonii* and *T. forsythia* were also found to be full length and lack extensive modification while for PorZ, unprocessed orthologs were identified in *P. intermedia* and *C. hutchinsonii* (Veith *et al.*, 2009, Veith *et al.*, 2013). Despite the lack of T9SS signal cleavage, PorU and PorZ are nevertheless secreted to the cell surface. PorU was weakly localised to the *P. gingivalis* cell surface by whole cell ELISA (Glew *et al.*, 2012), and this localisation was confirmed for PorU by flow cytometry and established for PorZ by multiple techniques (Lasica *et al.*, 2016).

The phenotypes of strains lacking the surface components, PorU and PorZ are distinct from strains lacking their respective outer membrane protein anchors (PorV and PorQ). The *porV* and *porQ* mutants do not appear to have T9SS substrates exposed on the cell surface consistent with a complete T9SS impairment while *porU* and *porZ* mutants exhibit partial secretion (Glew *et al.*, 2012, Glew *et al.*, 2017). The partially secreted T9SS substrates retained their T9SS signal suggesting that PorU and PorZ are involved in signal cleavage and conjugation to A-LPS. Since PorU is the likely sortase, PorZ may be involved in recruiting A-LPS (Glew *et al.*, 2017). Consistent with this proposal, the crystal structure of PorZ and the function of PorZ homologs suggest that PorZ may bind to sugars (Lasica *et al.*, 2016). Since T9SS substrate modification is A-LPS specific, PorZ may recognise the mannose repeat units of APS. If the function of PorZ was limited to the presentation of A-LPS to the

PorU sortase, the *porZ* mutant could be expected to be similar to A-LPS mutants. However, unlike A-LPS mutants, the T9SS substrates produced in the *porZ* mutant are not cleaved (Glew *et al.*, 2017), consistent with the finding that PorU was undetectable on the cell surface on this mutant (Lasica *et al.*, 2016). PorZ therefore also appears to be necessary for the localisation of PorU on the cell surface, as part of the attachment complex.

It is interesting that the phenotypes of mutants lacking PorV and PorQ are different to those lacking PorU or PorZ, and implies that these proteins may have further functionality beyond anchoring PorU and PorZ. Indeed, PorV was shown to bind to multiple T9SS substrates in addition to PorU (Glew *et al.*, 2017). This binding was observed in the *porU* mutant and also in a recombinant RgpB that was engineered to have an uncleavable T9SS CTD signal. Together, the data suggest that the T9SS substrates detected on the cell surface of the *porU* mutant are bound to PorV leading to the proposal that PorV functions as a shuttle protein (Glew *et al.*, 2017). In this proposal, PorV would collect newly secreted T9SS substrates from the secretion channel by binding to their T9SS CTD signals and shuttle them to an attachment complex for permanent anchorage via A-LPS (**Fig. 5**). This shuttle model was supported by quantitative proteomic data which indicated that the PorV shuttle protein (i.e. PorV not incorporated into attachment complexes) was present in a ~100 fold excess over the secretion channels and a 10-fold excess over the attachment complexes. This excess would be required to overcome the relative slowness of the shuttle having to diffuse through the OM to find its target. The proposed activity of PorV therefore is to bind T9SS substrates, both to cleavable T9SS signals as well as to the uncleavable T9SS signal of PorU. The location of motifs B, D and E within the T9SS CTD signal are adjacent in the crystal structures suggesting that together they constitute the binding site for PorV (**Fig. 2**). If that is true, these motifs would face the outer membrane while the cleavage site would be on the outside where

it may have the best exposure to the sortase. While PorU and PorV are essential T9SS components in *P. gingivalis*, in *F. johnsoniae* PorV was found to be essential for the secretion of only a subset of T9SS substrates, while none of the substrates tested required PorU (Kharade & McBride, 2015). This conclusion was based on whether proteins could be secreted into the culture fluid but secretion affected by PorU-dependent CTD cleavage and attachment requires analysis of the ability to localise T9SS substrates to the cell surface which was not done in this study. Nonetheless, this research suggests the presence of multiple terminal branches of the T9SS (**Fig. 4**).

PorV has also been reported to influence the modification status of lipid A. Lipid A analyses of wild type *P. gingivalis* and *porV* mutants in different laboratories gave conflicting results, but suggest that in the absence of PorV, the acylation and phosphorylation states of lipid A are altered (Chen *et al.*, 2011, Rangarajan *et al.*, 2017). The reported intracellular accumulation of A-LPS in the *porV* mutant may explain the higher levels of unmodified (penta-acylated and phosphorylated) lipid A in this mutant (Chen *et al.*, 2011).

Other components of the T9SS

Besides the putative regulatory components (see below), there are seven further proteins with experimental evidence for involvement in the T9SS. In *P. gingivalis*, PorP has been modelled as a 14-stranded OM β -barrel similar to PorV and PorQ and was found associated with PorK and PorM in *E. coli* by immunoprecipitation experiments (Vincent *et al.*, 2017). PorP however was not required for the formation of large complexes comprising PorK, PorL, PorM and PorN (Sato *et al.*, 2010) and moreover the abundance of PorP in wild type cells is approximately 20-fold lower than these other four components (unpublished data). PorP therefore appears to interact with one or more of the secretion channel components but is

neither a core component of it, nor required for its assembly. Intriguingly, *porP* has multiple homologs in *F. johnsoniae* and many other motile species. One of these homologs, *sprF* is adjacent to the *sprB* gene which encodes a motility adhesin. While SprF was found to be essential for the secretion of SprB it was not essential for gliding motility suggesting that the secretion of other motility adhesins may be mediated by other PorP homologs (Rhodes *et al.*, 2011a). SprF was not required for the secretion of chitinase and therefore PorP homologs may be involved in targeting specific T9SS substrates or groups of substrates to a particular extracellular fate. *P. gingivalis* has only the one PorP homolog and this may be related to the observation that all T9SS substrates appear to share the same fate of cell surface attachment via A-LPS.

Recently, PG1058 was identified as an essential component of the T9SS in *P. gingivalis* (Heath *et al.*, 2016). Similar to PorP, PG1058 is unique in *P. gingivalis* whereas there are multiple PG1058 homologs in *F. johnsoniae* and other motile *Bacteroidetes*. PG1058 is a predicted lipoprotein and contains predicted TolB and peptidoglycan-binding domains consistent with a periplasmic location. The existence of multiple PG1058 homologs in species that also contain multiple PorP homologs suggest there may be a functional relationship between these two proteins in recruiting and mediating the secretion of specific T9SS substrates (**Fig. 4**).

Sov, PorT and PorW are essential OM components of the T9SS in *P. gingivalis*, while in *F. johnsoniae* they are essential for chitinase secretion but not absolutely essential for gliding motility (**Table 1**). Their abundance in *P. gingivalis* is lower than the putative components of the secretion channel (unpublished data) and their functions are yet to be described. PG0534 is a predicted TonB-dependent receptor shown to be located in the outer membrane and

required for T9SS function but its specific role has not been determined (Saiki & Konishi, 2010a). A Skp-like protein, PGN_0300 has also been proposed to be involved in the T9SS (Taguchi *et al.*, 2015). Since Skp is known in other species to be involved in the assembly of outer membrane proteins (Noinaj *et al.*, 2017), it remains to be demonstrated that PGN_0300 is specific to the T9SS, rather than simply performing its expected role of assembling certain outer membrane proteins including one or more components of the T9SS.

Regulation of the T9SS

To date, three proteins have been suggested to play a role in regulating the T9SS, SigP, PorX and PorY (**Table 1**). PorX and PorY are homologous to the response regulator and histidine kinase sensor proteins respectively of two-component regulatory systems (Casino *et al.*, 2010), while SigP is an extracytoplasmic function (ECF) sigma factor. Mutation of any of these three components results in the downregulation of T9SS components and a decrease in gingipain activity (Dou *et al.*, 2010, Sato *et al.*, 2010, Kadowaki *et al.*, 2016). PorY was shown to autophosphorylate, as well as interact with and phosphorylate PorX, demonstrating that PorX and PorY function together as a two component system. PorX binds to SigP and SigP in turn was shown to bind to the promoter region of several T9SS component genes (Kadowaki *et al.*, 2016). Together, the data suggest that the PorY sensor responds to a signal transmitted to the periplasm and upregulates components of the T9SS via a cascade of interactions involving PorX and SigP. In addition to binding to SigP, PorX was also shown to bind PorL and the authors speculate that this binding may cause a reversal of the T9SS motor which may be of particular relevance to gliding motility (Vincent *et al.*, 2016). Further study is required to elucidate the signal that activates PorY.

Conclusion and future directions

Recent research has greatly enhanced our understanding of the T9SS. The current emphasis is on understanding the role of each individual component and this is being tackled from several angles including structural biology and interaction analyses. The 3D crystal and cryo-EM structures have been determined for only a small number of components or partial components of the T9SS. Future efforts to determine the 3D structures of more components are essential in order to understand the interconnection of these components and help shed a light on their function. A central question to be resolved is the identity of the OM pore protein(s). Do they consist of PorK and PorN alone or involve other proteins? Do any of the pore proteins interact directly with the T9SS signal and can this interaction be captured or enriched in particular mutants? How are the T9SS substrates physically secreted through the OM? The T9SS is unique in having a large number of OM-associated components, and notwithstanding four such proteins, PorU, PorV, PorQ and PorZ that have been assigned to the cell surface attachment role, there remains at least four further OM-associated components with expected cell surface exposure, Sov, PorT, PG0534 and PorP whose functions are unknown. It is likely that the functions of these proteins relate to the specialization of the T9SS in the cell surface attachment of its substrates and the formation of a surface coat (S-layer, EDSL). They may also provide essential connections, whether physical or regulatory, between the secretion channel and the attachment complex. Acquiring an *in-situ* structure of the T9SS by cryo-EM techniques that shows the connectivity of the PorK/N rings with other components of the system would be particularly helpful in fully elucidating the architecture of the T9SS machinery.

Acknowledgements

This work was supported by the Supported by the Australian Government, Department of Industry, Innovation and Science.

For Peer Review

References

- Abaibou, H., Chen, Z., Olango, G. J., Liu, Y., Edwards, J., and Fletcher, H. M. (2001) vimA gene downstream of recA is involved in virulence modulation in *Porphyromonas gingivalis* W83. *Infect Immun* **69**: 325-335.
- Braun, T. F., Khubbar, M. K., Saffarini, D. A., and McBride, M. J. (2005) *Flavobacterium johnsoniae* gliding motility genes identified by mariner mutagenesis. *J Bacteriol* **187**: 6943-6952.
- Capestany, C. A., Kuboniwa, M., Jung, I. Y., Park, Y., Tribble, G. D., and Lamont, R. J. (2006) Role of the *Porphyromonas gingivalis* InlJ protein in homotypic and heterotypic biofilm development. *Infect Immun* **74**: 3002-3005.
- Casiano-Colon, A., and Marquis, R. E. (1988) Role of the arginine deiminase system in protecting oral bacteria and an enzymatic basis for acid tolerance. *Appl Environ Microbiol* **54**: 1318-1324.
- Casino, P., Rubio, V., and Marina, A. (2010) The mechanism of signal transduction by two-component systems. *Curr Opin Struct Biol* **20**: 763-771.
- Chatzi, K. E., Sardis, M. F., Karamanou, S., and Economou, A. (2013) Breaking on through to the other side: protein export through the bacterial Sec system. *Biochem J* **449**: 25-37.
- Chen, T., Dong, H., Yong, R., and Duncan, M. J. (2000) Pleiotropic pigmentation mutants of *Porphyromonas gingivalis*. *Microb Pathog* **28**: 235-247.
- Chen, Y. Y., Cross, K. J., Paolini, R. A., Fielding, J. E., Slakeski, N., and Reynolds, E. C. (2002) CPG70 is a novel basic metallopeptidase with C-terminal polycystic kidney disease domains from *Porphyromonas gingivalis*. *J Biol Chem* **277**: 23433-23440.

- Chen, Y. Y., Peng, B., Yang, Q., Glew, M. D., Veith, P. D., Cross, K. J., *et al.* (2011) The outer membrane protein LptO is essential for the O-deacylation of LPS and the coordinated secretion and attachment of A-LPS and CTD proteins in *Porphyromonas gingivalis*. *Mol Microbiol* **79**: 1380-1401.
- Cianciotto, N. P. (2009) Many substrates and functions of type II secretion: lessons learned from *Legionella pneumophila*. *Future Microbiol* **4**: 797-805.
- Curtis, M. A., Thickett, A., Slaney, J. M., Rangarajan, M., Aduse-Opoku, J., Shepherd, P., *et al.* (1999) Variable carbohydrate modifications to the catalytic chains of the RgpA and RgpB proteases of *Porphyromonas gingivalis* W50. *Infect Immun* **67**: 3816-3823.
- Dashper, S. G., Ang, C. S., Veith, P. D., Mitchell, H. L., Lo, A. W., Seers, C. A., *et al.* (2009) Response of *Porphyromonas gingivalis* to heme limitation in continuous culture. *J Bacteriol* **191**: 1044-1055.
- de Diego, I., Ksiazek, M., Mizgalska, D., Koneru, L., Golik, P., Szmigielski, B., *et al.* (2016) The outer-membrane export signal of *Porphyromonas gingivalis* type IX secretion system (T9SS) is a conserved C-terminal beta-sandwich domain. *Sci Rep* **6**: 23123.
- de Diego, I., Veillard, F., Sztukowska, M. N., Guevara, T., Potempa, B., Pomowski, A., *et al.* (2014) Structure and mechanism of cysteine peptidase gingipain K (Kgp), a major virulence factor of *Porphyromonas gingivalis* in periodontitis. *J Biol Chem* **289**: 32291-32302.
- Dou, Y., Osbourne, D., McKenzie, R., and Fletcher, H. M. (2010) Involvement of extracytoplasmic function sigma factors in virulence regulation in *Porphyromonas gingivalis* W83. *FEMS Microbiol Lett* **312**: 24-32.
- Duchaud, E., Boussaha, M., Loux, V., Bernardet, J. F., Michel, C., Kerouault, B., *et al.* (2007) Complete genome sequence of the fish pathogen *Flavobacterium psychrophilum*. *Nat Biotechnol* **25**: 763-769.

- Dumetz, F., Duchaud, E., Claverol, S., Orieux, N., Papillon, S., Lapaillerie, D., and Le Henaff, M. (2008) Analysis of the *Flavobacterium psychrophilum* outer-membrane subproteome and identification of new antigenic targets for vaccine by immunomics. *Microbiology* **154**: 1793-1801.
- Eichinger, A., Beisel, H. G., Jacob, U., Huber, R., Medrano, F. J., Banbula, A., *et al.* (1999) Crystal structure of gingipain R: an Arg-specific bacterial cysteine proteinase with a caspase-like fold. *EMBO J* **18**: 5453-5462.
- Ford, M. J., Nomellini, J. F., and Smit, J. (2007) S-layer anchoring and localization of an S-layer-associated protease in *Caulobacter crescentus*. *J Bacteriol* **189**: 2226-2237.
- Ganuelas, L. A., Li, N., Yun, P., Hunter, N., and Collyer, C. A. (2013) The lysine gingipain adhesin domains from *Porphyromonas gingivalis* interact with erythrocytes and albumin: Structures correlate to function. *Eur J Microbiol Immunol (Bp)* **3**: 152-162.
- Glew, M. D., Veith, P. D., Chen, D., Gorasia, D. G., Peng, B., and Reynolds, E. C. (2017) PorV is an Outer Membrane Shuttle Protein for the Type IX Secretion System. *Sci Rep Submitted*.
- Glew, M. D., Veith, P. D., Chen, D., Seers, C. A., Chen, Y. Y., and Reynolds, E. C. (2014) Blue native-PAGE analysis of membrane protein complexes in *Porphyromonas gingivalis*. *J Proteomics* **110**: 72-92.
- Glew, M. D., Veith, P. D., Peng, B., Chen, Y. Y., Gorasia, D. G., Yang, Q., *et al.* (2012) PG0026 is the C-terminal signal peptidase of a novel secretion system of *Porphyromonas gingivalis*. *J Biol Chem* **287**: 24605-24617.
- Gorasia, D. G., Veith, P. D., Chen, D., Seers, C. A., Mitchell, H. A., Chen, Y. Y., *et al.* (2015) *Porphyromonas gingivalis* Type IX secretion substrates are cleaved and modified by a sortase-like mechanism. *PLoS Pathol* **11**: e1005152.

- Gorasia, D. G., Veith, P. D., Hanssen, E. G., Glew, M. D., Sato, K., Yukitake, H., *et al.* (2016) Structural insights into the PorK and PorN components of the *Porphyromonas gingivalis* Type IX secretion system. *PLoS Pathog* **12**: e1005820.
- Gorman, M. A., Seers, C. A., Michell, B. J., Feil, S. C., Huq, N. L., Cross, K. J., *et al.* (2015) Structure of the lysine specific protease Kgp from *Porphyromonas gingivalis*, a target for improved oral health. *Protein Sci* **24**: 162-166.
- Goulas, T., Mizgalska, D., Garcia-Ferrer, I., Kantyka, T., Guevara, T., Szmigielski, B., *et al.* (2015) Structure and mechanism of a bacterial host-protein citrullinating virulence factor, *Porphyromonas gingivalis* peptidylarginine deiminase. *Sci Rep* **5**: 11969.
- Goyal, P., Krasteva, P. V., Van Gerven, N., Gubellini, F., Van den Broeck, I., Troupiotis-Tsailaki, A., *et al.* (2014) Structural and mechanistic insights into the bacterial amyloid secretion channel CsgG. *Nature* **516**: 250-253.
- Gozdziewicz, T. K., Man-Kupisinska, A., Lugowski, C., and Lukasiewicz, J. (2015) Occurrence of glycine in the core oligosaccharides of *Hafnia alvei* lipopolysaccharides--identification of disubstituted glycoform. *Carbohydr Res* **408**: 119-126.
- Grenier, D., and La, V. D. (2011) Proteases of *Porphyromonas gingivalis* as important virulence factors in periodontal disease and potential targets for plant-derived compounds: a review article. *Curr Drug Targets* **12**: 322-331.
- Gully, N., Bright, R., Marino, V., Marchant, C., Cantley, M., Haynes, D., *et al.* (2014) *Porphyromonas gingivalis* peptidylarginine deiminase, a key contributor in the pathogenesis of experimental periodontal disease and experimental arthritis. *PLoS One* **9**: e100838.

- Guo, Y., Nguyen, K. A., and Potempa, J. (2010) Dichotomy of gingipains action as virulence factors: from cleaving substrates with the precision of a surgeon's knife to a meat chopper-like brutal degradation of proteins. *Periodontol 2000* **54**: 15-44.
- Gyorgy, B., Toth, E., Tarcsa, E., Falus, A., and Buzas, E. I. (2006) Citrullination: a posttranslational modification in health and disease. *Int J Biochem Cell Biol* **38**: 1662-1677.
- Hasegawa, Y., Iijima, Y., Persson, K., Nagano, K., Yoshida, Y., Lamont, R. J., *et al.* (2016) Role of Mfa5 in expression of Mfa1 fimbriae in *Porphyromonas gingivalis*. *J Dent Res* **95**: 1291-1297.
- Heath, J. E., Seers, C. A., Veith, P. D., Butler, C. A., Nor Muhammad, N. A., Chen, Y. Y., *et al.* (2016) PG1058 is a novel multidomain protein component of the bacterial Type IX secretion system. *PLoS One* **11**: e0164313.
- Higuchi, N., Murakami, Y., Moriguchi, K., Ohno, N., Nakamura, H., and Yoshimura, F. (2000) Localization of major, high molecular weight proteins in *Bacteroides forsythus*. *Microbiol Immunol* **44**: 777-780.
- Hiratsuka, K., Kiyama-Kishikawa, M., and Abiko, Y. (2010) Hemin-binding protein 35 (HBP35) plays an important role in bacteria-mammalian cells interactions in *Porphyromonas gingivalis*. *Microb Pathog* **48**: 116-123.
- Ishiguro, I., Saiki, K., and Konishi, K. (2009) PG27 is a novel membrane protein essential for a *Porphyromonas gingivalis* protease secretion system. *FEMS Microbiol Lett* **292**: 261-267.
- Kadowaki, T., Yukitake, H., Naito, M., Sato, K., Kikuchi, Y., Kondo, Y., *et al.* (2016) A two-component system regulates gene expression of the type IX secretion component proteins via an ECF sigma factor. *Sci Rep* **6**: 23288.

- Karlsson, E. N., Hachem, M. A., Ramchuran, S., Costa, H., Holst, O., Fex Svenningsen, A., and Hreggvidsson, G. O. (2004) The modular xylanase Xyn10A from *Rhodothermus marinus* is cell-attached, and its C-terminal domain has several putative homologues among cell-attached proteins within the phylum Bacteroidetes. *FEMS Microbiol Lett* **241**: 233-242.
- Kharade, S. S., and McBride, M. J. (2015) *Flavobacterium johnsoniae* PorV is required for secretion of a subset of proteins targeted to the type IX secretion system. *J Bacteriol* **197**: 147-158.
- Kondo, Y., Ohara, N., Sato, K., Yoshimura, M., Yukitake, H., Naito, M., *et al.* (2010) Tetratricopeptide repeat protein-associated proteins contribute to the virulence of *Porphyromonas gingivalis*. *Infect Immun* **78**: 2846-2856.
- Koneru, L., Ksiazek, M., Waligorska, I., Straczek, A., Lukasik, M., Madej, M., *et al.* (2016) Mirolysin, a LysargiNase from *Tannerella forsythia*, proteolytically inactivates the human cathelicidin, LL-37. *Biol Chem* doi: **10.1515/hsz-2016-0267**.
- Kulkarni, S. S., Zhu, Y., Brendel, C. J., and McBride, M. J. (2017) Diverse C-terminal sequences involved in *Flavobacterium johnsoniae* protein secretion. *J Bacteriol* doi: **10.1128/JB.00884-16**.
- Lasica, A. M., Goulas, T., Mizgalska, D., Zhou, X., de Diego, I., Ksiazek, M., *et al.* (2016) Structural and functional probing of PorZ, an essential bacterial surface component of the type-IX secretion system of human oral-microbiomic *Porphyromonas gingivalis*. *Sci Rep* **6**: 37708.
- Lee, S. W., Sabet, M., Um, H. S., Yang, J., Kim, H. C., and Zhu, W. (2006) Identification and characterization of the genes encoding a unique surface (S-) layer of *Tannerella forsythia*. *Gene* **371**: 102-111.

- Li, N., and Collyer, C. A. (2011) Gingipains from *Porphyromonas gingivalis* - Complex domain structures confer diverse functions. *Eur J Microbiol Immunol (Bp)* **1**: 41-58.
- Li, N., Yun, P., Jeffries, C. M., Langley, D., Gamsjaeger, R., Church, W. B., *et al.* (2011) The modular structure of haemagglutinin/adhesin regions in gingipains of *Porphyromonas gingivalis*. *Mol Microbiol* **81**: 1358-1373.
- Li, N., Yun, P., Nadkarni, M. A., Ghadikolaei, N. B., Nguyen, K. A., Lee, M., *et al.* (2010) Structure determination and analysis of a haemolytic gingipain adhesin domain from *Porphyromonas gingivalis*. *Mol Microbiol* **76**: 861-873.
- Madden, T. E., Clark, V. L., and Kuramitsu, H. K. (1995) Revised sequence of the *Porphyromonas gingivalis* prtT cysteine protease/hemagglutinin gene: homology with streptococcal pyrogenic exotoxin B/streptococcal proteinase. *Infect Immun* **63**: 238-247.
- Mallya, R. K., Young, B. J., Pepys, M. B., Hamblin, T. J., Mace, B. E., and Hamilton, E. B. (1983) Anti-keratin antibodies in rheumatoid arthritis: frequency and correlation with other features of the disease. *Clin Exp Immunol* **51**: 17-20.
- Maresz, K. J., Hellvard, A., Sroka, A., Adamowicz, K., Bielecka, E., Koziel, J., *et al.* (2013) *Porphyromonas gingivalis* facilitates the development and progression of destructive arthritis through its unique bacterial peptidylarginine deiminase (PAD). *PLoS Pathog* **9**: e1003627.
- McBride, M. J., and Nakane, D. (2015) Flavobacterium gliding motility and the type IX secretion system. *Curr Opin Microbiol* **28**: 72-77.
- McBride, M. J., and Zhu, Y. (2013) Gliding motility and Por secretion system genes are widespread among members of the phylum bacteroidetes. *J Bacteriol* **195**: 270-278.

- McDermott, J. E., Corrigan, A., Peterson, E., Oehmen, C., Niemann, G., Cambronne, E. D., *et al.* (2011) Computational prediction of type III and IV secreted effectors in gram-negative bacteria. *Infect Immun* **79**: 23-32.
- Montgomery, A. B., Kopec, J., Shrestha, L., Thezenas, M. L., Burgess-Brown, N. A., Fischer, R., *et al.* (2016) Crystal structure of *Porphyromonas gingivalis* peptidylarginine deiminase: implications for autoimmunity in rheumatoid arthritis. *Ann Rheum Dis* **75**: 1255-1261.
- Nakane, D., Sato, K., Wada, H., McBride, M. J., and Nakayama, K. (2013) Helical flow of surface protein required for bacterial gliding motility. *Proc Natl Acad Sci U S A* **110**: 11145-11150.
- Narita, Y., Sato, K., Yukitake, H., Shoji, M., Nakane, D., Nagano, K., *et al.* (2014) Lack of a surface layer in *Tannerella forsythia* mutants deficient in the type IX secretion system. *Microbiology* **160**: 2295-2303.
- Navarre, W. W., and Schneewind, O. (1999) Surface proteins of gram-positive bacteria and mechanisms of their targeting to the cell wall envelope. *Microbiol Mol Biol Rev* **63**: 174-229.
- Nelson, D., Potempa, J., Kordula, T., and Travis, J. (1999) Purification and characterization of a novel cysteine proteinase (periodontain) from *Porphyromonas gingivalis*. Evidence for a role in the inactivation of human alpha1-proteinase inhibitor. *J Biol Chem* **274**: 12245-12251.
- Nelson, S. S., Bollampalli, S., and McBride, M. J. (2008) SprB is a cell surface component of the *Flavobacterium johnsoniae* gliding motility machinery. *J Bacteriol* **190**: 2851-2857.

- Nelson, S. S., Glocka, P. P., Agarwal, S., Grimm, D. P., and McBride, M. J. (2007)
Flavobacterium johnsoniae SprA is a cell surface protein involved in gliding motility.
J Bacteriol **189**: 7145-7150.
- Nguyen, K. A., Travis, J., and Potempa, J. (2007) Does the importance of the C-terminal residues in the maturation of RgpB from *Porphyromonas gingivalis* reveal a novel mechanism for protein export in a subgroup of Gram-Negative bacteria? *J Bacteriol* **189**: 833-843.
- Nguyen, K. A., Zylicz, J., Szczesny, P., Sroka, A., Hunter, N., and Potempa, J. (2009)
Verification of a topology model of PorT as an integral outer-membrane protein in *Porphyromonas gingivalis*. *Microbiology* **155**: 328-337.
- Noinaj, N., Gumbart, J. C., and Buchanan, S. K. (2017) The beta-barrel assembly machinery in motion. *Nat Rev Microbiol* **15**: 197-204.
- Nonaka, M., Shoji, M., Kadowaki, T., Sato, K., Yukitake, H., Naito, M., and Nakayama, K. (2014) Analysis of a Lys-specific serine endopeptidase secreted via the type IX secretion system in *Porphyromonas gingivalis*. *FEMS Microbiol Lett* **354**: 60-68.
- Noonan, B., and Trust, T. J. (1997) The synthesis, secretion and role in virulence of the paracrystalline surface protein layers of *Aeromonas salmonicida* and *A. hydrophila*. *FEMS Microbiol Lett* **154**: 1-7.
- O'Brien-Simpson, N. M., Veith, P. D., Dashper, S. G., and Reynolds, E. C. (2003)
Porphyromonas gingivalis gingipains: The molecular teeth of a microbial vampire.
Curr Protein Pept Sci **4**: 409-426.
- Oh, Y. J., Sekot, G., Duman, M., Chtcheglova, L., Messner, P., Peterlik, H., *et al.* (2013)
Characterizing the S-layer structure and anti-S-layer antibody recognition on intact *Tannerella forsythia* cells by scanning probe microscopy and small angle X-ray scattering. *J Mol Recognit* **26**: 542-549.

- Paramonov, N., Aduse-Opoku, J., Hashim, A., Rangarajan, M., and Curtis, M. A. (2015) Identification of the linkage between A-polysaccharide and the core in the A-lipopolysaccharide of *Porphyromonas gingivalis* W50. *J Bacteriol* **197**: 1735-1746.
- Paramonov, N., Bailey, D., Rangarajan, M., Hashim, A., Kelly, G., Curtis, M. A., and Hounsell, E. F. (2001) Structural analysis of the polysaccharide from the lipopolysaccharide of *Porphyromonas gingivalis* strain W50. *Eur J Biochem* **268**: 4698-4707.
- Paramonov, N., Rangarajan, M., Hashim, A., Gallagher, A., Aduse-Opoku, J., Slaney, J. M., *et al.* (2005) Structural analysis of a novel anionic polysaccharide from *Porphyromonas gingivalis* strain W50 related to Arg-gingipain glycans. *Mol Microbiol* **58**: 847-863.
- Paramonov, N. A., Aduse-Opoku, J., Hashim, A., Rangarajan, M., and Curtis, M. A. (2009) Structural analysis of the core region of O-lipopolysaccharide of *Porphyromonas gingivalis* from mutants defective in O-antigen ligase and O-antigen polymerase. *J Bacteriol* **191**: 5272-5282.
- Potempa, J., Sroka, A., Imamura, T., and Travis, J. (2003) Gingipains, the major cysteine proteinases and virulence factors of *Porphyromonas gingivalis*: structure, function and assembly of multidomain protein complexes. *Curr Protein Pept Sci* **4**: 397-407.
- Rangarajan, M., Aduse-Opoku, J., Hashim, A., McPhail, G., Luklinska, Z., Haurat, M. F., *et al.* (2017) LptO (PG0027) is required for Lipid A 1-Phosphatase activity in *Porphyromonas gingivalis* W50. *J Bacteriol* **10.1128/JB.00751-16**.
- Rangarajan, M., Aduse-Opoku, J., Paramonov, N., Hashim, A., Bostanci, N., Fraser, O. P., *et al.* (2008) Identification of a second lipopolysaccharide in *Porphyromonas gingivalis* W50. *J Bacteriol* **190**: 2920-2932.

- Rangarajan, M., Aduse-Opoku, J., Slaney, J. M., Young, K. A., and Curtis, M. A. (1997) The prpR1 and prR2 arginine-specific protease genes of *Porphyromonas gingivalis* W50 produce five biochemically distinct enzymes. *Mol Microbiol* **23**: 955-965.
- Rhodes, R. G., Nelson, S. S., Pochiraju, S., and McBride, M. J. (2011a) *Flavobacterium johnsoniae* sprB is part of an operon spanning the additional gliding motility genes sprC, sprD, and sprF. *J Bacteriol* **193**: 599-610.
- Rhodes, R. G., Samarasam, M. N., Shrivastava, A., van Baaren, J. M., Pochiraju, S., Bollampalli, S., and McBride, M. J. (2010) *Flavobacterium johnsoniae* gldN and gldO are partially redundant genes required for gliding motility and surface localization of SprB. *J Bacteriol* **192**: 1201-1211.
- Rhodes, R. G., Samarasam, M. N., Van Groll, E. J., and McBride, M. J. (2011b) Mutations in *Flavobacterium johnsoniae* sprE result in defects in gliding motility and protein secretion. *J Bacteriol* **193**: 5322-5327.
- Sabet, M., Lee, S. W., Nauman, R. K., Sims, T., and Um, H. S. (2003) The surface (S-) layer is a virulence factor of *Bacteroides forsythus*. *Microbiology* **149**: 3617-3627.
- Saiki, K., and Konishi, K. (2007) Identification of a *Porphyromonas gingivalis* novel protein sov required for the secretion of gingipains. *Mol Immunol* **51**: 483-491.
- Saiki, K., and Konishi, K. (2010a) Identification of a novel *Porphyromonas gingivalis* outer membrane protein, PG534, required for the production of active gingipains. *FEMS Microbiol Lett* **310**: 168-174.
- Saiki, K., and Konishi, K. (2010b) The role of Sov protein in the secretion of gingipain protease virulence factors of *Porphyromonas gingivalis*. *FEMS Microbiol Lett* **302**: 166-174.

- Saiki, K., and Konishi, K. (2014) *Porphyromonas gingivalis* C-terminal signal peptidase PG0026 and HagA interact with outer membrane protein PG27/LptO. *Mol Oral Microbiol* **29**: 32-44.
- Sakai, E., Naito, M., Sato, K., Hotokezaka, H., Kadowaki, T., Kamaguchi, A., *et al.* (2007) Construction of recombinant hemagglutinin derived from the gingipain-encoding gene of *Porphyromonas gingivalis*, identification of its target protein on erythrocytes, and inhibition of hemagglutination by an interdomain regional peptide. *J Bacteriol* **189**: 3977-3986.
- Sakakibara, J., Nagano, K., Murakami, Y., Higuchi, N., Nakamura, H., Shimozato, K., and Yoshimura, F. (2007) Loss of adherence ability to human gingival epithelial cells in S-layer protein-deficient mutants of *Tannerella forsythensis*. *Microbiology* **153**: 866-876.
- Sato, K., Naito, M., Yukitake, H., Hirakawa, H., Shoji, M., McBride, M. J., *et al.* (2010) A protein secretion system linked to bacteroidete gliding motility and pathogenesis. *Proc Natl Acad Sci U S A* **107**: 276-281.
- Sato, K., Sakai, E., Veith, P. D., Shoji, M., Kikuchi, Y., Yukitake, H., *et al.* (2005) Identification of a new membrane-associated protein that influences transport/maturation of gingipains and adhesins of *Porphyromonas gingivalis*. *J Biol Chem* **280**: 8668-8677.
- Sato, K., Yukitake, H., Narita, Y., Shoji, M., Naito, M., and Nakayama, K. (2013) Identification of *Porphyromonas gingivalis* proteins secreted by the Por secretion system. *FEMS Microbiol Lett* **338**: 68-76.
- Seers, C. A., Slakeski, N., Veith, P. D., Nikolof, T., Chen, Y. Y., Dashper, S. G., and Reynolds, E. C. (2006) The RgpB C-terminal domain has a role in attachment of

- RgpB to the outer membrane and belongs to a novel C-terminal-domain family found in *Porphyromonas gingivalis*. *J Bacteriol* **188**: 6376-6386.
- Sekot, G., Posch, G., Oh, Y. J., Zayni, S., Mayer, H. F., Pum, D., *et al.* (2012) Analysis of the cell surface layer ultrastructure of the oral pathogen *Tannerella forsythia*. *Arch Microbiol* **194**: 525-539.
- Sharma, A. (2010) Virulence mechanisms of *Tannerella forsythia*. *Periodontol 2000* **54**: 106-116.
- Shibata, Y., Hiratsuka, K., Hayakawa, M., Shiroza, T., Takiguchi, H., Nagatsuka, Y., and Abiko, Y. (2003) A 35-kDa co-aggregation factor is a hemin binding protein in *Porphyromonas gingivalis*. *Biochem Biophys Res Commun* **300**: 351-356.
- Shibata, Y., Okano, S., Shiroza, T., Tahara, T., Nakazawa, K., Kataoka, S., *et al.* (2011) Characterization of human-type monoclonal antibodies against reduced form of hemin binding protein 35 from *Porphyromonas gingivalis*. *J Periodontal Res* **46**: 673-681.
- Shiroza, T., Okano, S., Shibata, Y., Hayakawa, M., Fujita, K., Yamaguchi, K., and Abiko, Y. (2008) Functional analysis of the thioredoxin domain in *Porphyromonas gingivalis* HBP35. *Biosci Biotechnol Biochem* **72**: 1826-1835.
- Shoji, M., and Nakayama, K. (2016) Glycobiology of the oral pathogen *Porphyromonas gingivalis* and related species. *Microb Pathog* **94**: 35-41.
- Shoji, M., Ratnayake, D. B., Shi, Y., Kadowaki, T., Yamamoto, K., Yoshimura, F., *et al.* (2002) Construction and characterization of a nonpigmented mutant of *Porphyromonas gingivalis*: cell surface polysaccharide as an anchorage for gingipains. *Microbiology* **148**: 1183-1191.
- Shoji, M., Sato, K., Yukitake, H., Kondo, Y., Narita, Y., Kadowaki, T., *et al.* (2011) Por secretion system-dependent secretion and glycosylation of *Porphyromonas gingivalis* hemin-binding protein 35. *PLoS One* **6**: e21372.

- Shoji, M., Sato, K., Yukitake, H., Naito, M., and Nakayama, K. (2014) Involvement of the Wbp pathway in the biosynthesis of *Porphyromonas gingivalis* lipopolysaccharide with anionic polysaccharide. *Sci Rep* **4**: 5056.
- Shoji, M., Shibata, Y., Shiroza, T., Yukitake, H., Peng, B., Chen, Y. Y., *et al.* (2010) Characterization of hemin-binding protein 35 (HBP35) in *Porphyromonas gingivalis*: its cellular distribution, thioredoxin activity and role in heme utilization. *BMC Microbiol* **10**: 152.
- Shoji, M., Yukitake, H., Sato, K., Shibata, Y., Naito, M., Aduse-Opoku, J., *et al.* (2013) Identification of an O-antigen chain length regulator, WzzP, in *Porphyromonas gingivalis*. *Microbiologyopen* **2**: 383-401.
- Shrivastava, A., (2013) PhD Thesis. Cell Surface Adhesins, Exopolysaccharides and the Por (Type IX) Secretion System of *Flavobacterium johnsoniae*. In: Biological Sciences. University of Wisconsin-Milwaukee, pp.
- Shrivastava, A., Johnston, J. J., van Baaren, J. M., and McBride, M. J. (2013) *Flavobacterium johnsoniae* GldK, GldL, GldM, and SprA are required for secretion of the cell surface gliding motility adhesins SprB and RemA. *J Bacteriol* **195**: 3201-3212.
- Shrivastava, A., Rhodes, R. G., Pochiraju, S., Nakane, D., and McBride, M. J. (2012) *Flavobacterium johnsoniae* RemA is a mobile cell surface lectin involved in gliding. *J Bacteriol* **194**: 3678-3688.
- Slakeski, N., Seers, C. A., Ng, K., Moore, C., Cleal, S. M., Veith, P. D., *et al.* (2011) C-terminal domain residues important for secretion and attachment of RgpB in *Porphyromonas gingivalis*. *J Bacteriol* **193**: 132-142.
- Smalley, J. W., and Olczak, T. (2017) Heme acquisition mechanisms of *Porphyromonas gingivalis* - strategies used in a polymicrobial community in a heme-limited host environment. *Mol Oral Microbiol* **32**: 1-23.

- Taguchi, Y., Sato, K., Yukitake, H., Inoue, T., Nakayama, M., Naito, M., *et al.* (2015) Involvement of an Skp-like protein, PGN_0300, in the type IX secretion system of *Porphyromonas gingivalis*. *Infect Immun* **84**: 230-240.
- Tomek, M. B., Neumann, L., Nimeth, I., Koerdts, A., Andesner, P., Messner, P., *et al.* (2014) The S-layer proteins of *Tannerella forsythia* are secreted via a type IX secretion system that is decoupled from protein O-glycosylation. *Mol Oral Microbiol* **29**: 307-320.
- Vanterpool, E., Roy, F., and Fletcher, H. M. (2005a) Inactivation of *vimF*, a putative glycosyltransferase gene downstream of *vimE*, alters glycosylation and activation of the gingipains in *Porphyromonas gingivalis* W83. *Infect Immun* **73**: 3971-3982.
- Vanterpool, E., Roy, F., Sandberg, L., and Fletcher, H. M. (2005b) Altered gingipain maturation in *vimA*- and *vimE*-defective isogenic mutants of *Porphyromonas gingivalis*. *Infect Immun* **73**: 1357-1366.
- Veith, P. D., Chen, Y. Y., Chen, D., O'Brien-Simpson, N. M., Cecil, J. D., Holden, J. A., *et al.* (2015) *Tannerella forsythia* outer membrane vesicles are enriched with substrates of the Type IX secretion system and TonB-dependent receptors. *J Proteome Res* **14**: 5355-5366.
- Veith, P. D., Chen, Y. Y., Gorasia, D. G., Chen, D., Glew, M. D., O'Brien-Simpson, N. M., *et al.* (2014) *Porphyromonas gingivalis* outer membrane vesicles exclusively contain outer membrane and periplasmic proteins and carry a cargo enriched with virulence factors. *J Proteome Res* **13**: 2420-2432.
- Veith, P. D., Chen, Y. Y., and Reynolds, E. C. (2004) *Porphyromonas gingivalis* RgpA and Kgp proteinases and adhesins are C terminally processed by the carboxypeptidase CPG70. *Infect Immun* **72**: 3655-3657.

- Veith, P. D., Nor Muhammad, N. A., Dashper, S. G., Likic, V. A., Gorasia, D. G., Chen, D., *et al.* (2013) Protein substrates of a novel secretion system are numerous in the bacteroidetes phylum and have in common a cleavable C-terminal secretion signal, extensive post-translational modification, and cell-surface attachment. *J Proteome Res* **12**: 4449-4461.
- Veith, P. D., O'Brien-Simpson, N. M., Tan, Y., Djatmiko, D. C., Dashper, S. G., and Reynolds, E. C. (2009) Outer membrane proteome and antigens of *Tannerella forsythia*. *J Proteome Res* **8**: 4279-4292.
- Veith, P. D., Talbo, G. H., Slakeski, N., Dashper, S. G., Moore, C., Paolini, R. A., and Reynolds, E. C. (2002) Major outer membrane proteins and proteolytic processing of RgpA and Kgp of *Porphyromonas gingivalis* W50. *Biochem J* **363**: 105-115.
- Vincent, M. S., Canestrari, M. J., Leone, P., Stathopoulos, J., Ize, B., Zoued, A., *et al.* (2017) Characterization of the *Porphyromonas gingivalis* type IX secretion trans-envelope PorKLMNP core complex. *J Biol Chem* **292**: 3252-3261.
- Vincent, M. S., Durand, E., and Cascales, E. (2016) The PorX response regulator of the *Porphyromonas gingivalis* PorXY two-component system does not directly regulate the Type IX secretion genes but binds the PorL subunit. *Front Cell Infect Microbiol* **6**: 96.
- Wang, S., Zhao, D., Bai, X., Zhang, W., and Lu, X. (2017) Identification and characterization of a large protein essential for degradation of the crystalline region of cellulose by *Cytophaga hutchinsonii*. *Appl Environ Microbiol* **83**: e02270-02216.
- Wang, Y., Wang, Z., Cao, J., Guan, Z., and Lu, X. (2014) FLP-FRT-based method to obtain unmarked deletions of CHU_3237 (porU) and large genomic fragments of *Cytophaga hutchinsonii*. *Appl Environ Microbiol* **80**: 6037-6045.

- Wegner, N., Wait, R., Sroka, A., Eick, S., Nguyen, K. A., Lundberg, K., *et al.* (2010) Peptidylarginine deiminase from *Porphyromonas gingivalis* citrullinates human fibrinogen and alpha-enolase: implications for autoimmunity in rheumatoid arthritis. *Arthritis Rheum* **62**: 2662-2672.
- Xie, G., Bruce, D. C., Challacombe, J. F., Chertkov, O., Detter, J. C., Gilna, P., *et al.* (2007) Genome sequence of the cellulolytic gliding bacterium *Cytophaga hutchinsonii*. *Appl Environ Microbiol* **73**: 3536-3546.
- Zhang, Y., Wang, T., Chen, W., Yilmaz, O., Park, Y., Jung, I. Y., *et al.* (2005) Differential protein expression by *Porphyromonas gingivalis* in response to secreted epithelial cell components. *Proteomics* **5**: 198-211.
- Zhu, Y., Han, L., Hefferon, K. L., Silvaggi, N. R., Wilson, D. B., and McBride, M. J. (2016) Periplasmic *Cytophaga hutchinsonii* endoglucanases are required for use of crystalline cellulose as the sole source of carbon and energy. *Appl Environ Microbiol* **82**: 4835-4845.
- Zhu, Y., and McBride, M. J. (2014) Deletion of the *Cytophaga hutchinsonii* type IX secretion system gene sprP results in defects in gliding motility and cellulose utilization. *Appl Microbiol Biotechnol* **98**: 763-775.
- Zhu, Y., Zhou, H., Bi, Y., Zhang, W., Chen, G., and Liu, W. (2013) Characterization of a family 5 glycoside hydrolase isolated from the outer membrane of cellulolytic *Cytophaga hutchinsonii*. *Appl Microbiol Biotechnol* **97**: 3925-3937.
- Zong, Y., Bice, T. W., Ton-That, H., Schneewind, O., and Narayana, S. V. (2004) Crystal structures of *Staphylococcus aureus* sortase A and its substrate complex. *J Biol Chem* **279**: 31383-31389.

Table 1. Components of the T9SS

Names	Description/comments	MW ^a (kDa)	Locus PG ^b	Locus FJ ^c	Pred ^d	Loc ^e	Key References ^g
Sov, SprA	Large OM protein with surface exposure reported in <i>F. johnsoniae</i>	278	PG0809 PGN_0832	Fjoh_1653	Sato Heath	OM	14,19,20,23
-	Predicted 8-stranded OM beta barrel protein associated with PorK and PorN. Involvement in the T9SS needs to be confirmed	23	PG0189 PGN_0297	Fjoh_1692	-	OM	8
-	Putative OM lipoprotein. Multi-domain protein. Multiple copies in some species	75	PG1058 PGN_1296	multiple	Heath	OM P	9
-	Predicted TonB-dependent receptor	88	PG0534 PGN_1437	Fjoh_0118	Heath	OM	20
PorK, GldK	Putative OM lipoprotein, forms large periplasmic rings in association with PorN and interacts with PG0189	53	PG0288 PGN_1676	Fjoh_1853	Sato Heath	OM P	1,8,23,26
PorL, GldL	IM protein with two TMHs. Interacts with PorM and PorX	35	PG0289 PGN_1675	Fjoh_1854	Sato Heath	IM C	1,8,23,26
PorM, GldM	IM protein with periplasmic domains. Interacts with PorL, PorK and PorN	57	PG0290 PGN_1674	Fjoh_1855	Sato Heath	IM P	1,8,23,26
PorN, GldN	Forms large periplasmic rings in association with PorK	39	PG0291 PGN_1673	Fjoh_1856	Heath	OM P	8,17,23,26
GldO	Paralog of PorN; not present in <i>P. gingivalis</i> . Both GldN and GldO need to be disrupted to cause loss of motility in <i>F. johnsoniae</i>	-	-	Fjoh_1857	N/A ^f	OM	16
PorP SprF SprP	Predicted OM beta barrel protein. Multiple copies in some species	33	PG0287 PGN_1677	multiple	Sato Heath	OM	17,23,26,27
PorQ	Predicted 14-stranded OM beta barrel protein (FadL). Binds to PorZ	34	PG0602 PGN_0645	Fjoh_2755	Sato Heath	OM	6,23,24
SigP	ECF sigma factor	22	PG0162 PGN_0274	Fjoh_2436	Heath	C	3,11
PorT, SprT	Predicted 8-stranded OM beta barrel protein	25	PG0751 PGN_0778	Fjoh_1466	Sato Heath	OM	2,15,22,23
PorU	Predicted C25 protease (gingipain). Contains uncleaved T9SS signal. Implicated as the T9SS sortase. Binds to PorV	126	PG0026 PGN_0022	Fjoh_1556	Sato Heath	S	4,5,6,7,12,21,23
PorV, LptO	Predicted 14-stranded OM beta barrel protein (FadL). Binds to T9SS substrates including PorU. Proposed shuttle protein	40	PG0027 PGN_0023	Fjoh_1555	Heath	OM	2,5,6,10,12,21
PorW, SprE	Predicted lipoprotein, contains TPR repeats	132	PG1947 PGN_1877	Fjoh_1051	Sato Heath	-	18,23
PorX	Two-component system response regulator	61	PG0928 PGN_1019	Fjoh_2906	Sato Heath	C	11,23,25
PorY	Two-component system sensor histidine kinase	45	PG0052	Fjoh_1592	Sato	IM	11,23

			PGN_2001		Heath	P	
PorZ	Contains uncleaved T9SS signal. Binds to PorQ	81	PG1604 PGN_0509	Fjoh_0707	Sato Heath	S	6,13

^a Predicted MW after signal peptide cleavage in *P. gingivalis*

^b Locus numbers in *P. gingivalis* W83 (PG) and ATCC33277 (PGN)

^c Locus numbers in *F. johnsoniae* UW101

^d This protein was predicted to be a component of the T9SS by the bioinformatic approach of Sato *et al.* (2010) or Heath *et al.* (2016) as indicated.

^e Subcellular localisation. OM, outer membrane; IM, inner membrane; C, cytoplasm; P, periplasm; S, cell surface. Some proteins have two localisations listed as they are peripheral membrane proteins.

^f GldO is not present in *P. gingivalis*, hence its prediction in *P. gingivalis* is not applicable.

^g References: 1. (Braun *et al.*, 2005) 2. (Chen *et al.*, 2011) 3. (Dou *et al.*, 2010) 4. (Glew *et al.*, 2012) 5. (Glew *et al.*, 2014) 6. (Glew *et al.*, 2017) 7. (Gorasia *et al.*, 2015) 8. (Gorasia *et al.*, 2016) 9. (Heath *et al.*, 2016) 10. (Ishiguro *et al.*, 2009) 11. (Kadowaki *et al.*, 2016) 12. (Kharade & McBride, 2015) 13. (Lasica *et al.*, 2016) 14. (Nelson *et al.*, 2007) 15. (Nguyen *et al.*, 2009) 16. (Rhodes *et al.*, 2010) 17. (Rhodes *et al.*, 2011a) 18. (Rhodes *et al.*, 2011b) 19. (Saiki & Konishi, 2007) 20. (Saiki & Konishi, 2010b) 21. (Saiki & Konishi, 2014) 22. (Sato *et al.*, 2005) 23. (Sato *et al.*, 2010) 24. (Shrivastava, 2013) 25. (Vincent *et al.*, 2016) 26. (Vincent *et al.*, 2017) 27. (Zhu & McBride, 2014)

Figure Legends

Fig 1. Linear architecture and processing of *P. gingivalis* T9SS substrates. T9SS substrates have an N-terminal signal peptide (SP) which is cleaved upon secretion through the SEC translocon and a C-terminal T9SS signal that is cleaved on the cell surface by the PorU sortase. The location of conserved motifs within the T9SS signal are shown together with a protein logo to describe the conserved amino acids within each motif. Motifs B, D & E are significant motifs derived from T9SS substrate sequences found in *P. gingivalis* and *T. forsythia* as reproduced from Veith *et al.* (2013). The cleavage motif is not statistically significant and is derived from the 20 cleavage sites identified in *P. gingivalis* and *T. forsythia* T9SS substrates in Veith *et al.* (2013), and analysed by meme software (<http://meme-suite.org/tools/meme>). The PorU sortase catalyses a transpeptidation reaction replacing the T9SS signal with a modification related to A-LPS via a serine residue.

Fig 2. Crystal structure of the RgpB T9SS CTD signal. The structure is PDB accession 5AG9 as published by De Diego *et al.* (2016) and consists of RgpB (575-736) which includes both the IgSF domain and T9SS CTD signal with a 5 residue substitution mutation in the cleavage site region to aid crystallization. The conserved motifs B (residues FDMNGR shown), D (residues GVVY) & E (residues KVIVK) of the T9SS CTD signal are shown in yellow while the residues close to the C-terminus of the IgSF domain (residues VEG) are shown in pink. The cleavage site is not shown as it is disordered in the crystal but is located adjacent to the residues shown in pink.

Fig 3. Alignment of variant T9SS CTD signals. A) Protein logo representation of TIGR04183. B) Protein logo representation of Pfam13585. C) Protein logo representation of

TIGR04131. The protein logos were created by downloading the aligned sequences for each family from the Conserved Domain Database (<https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>), removing columns that contained mostly gaps and submitting each resultant alignment to the Weblogo tool (<http://weblogo.berkeley.edu/logo.cgi>). The length of the TIGR04131 logo matches the length of the original model (PSSM), and was used as the template for adjusting the position of motifs in TIGR04183 and Pfam13585 to enable alignment. The locations of adjustments can be seen by examining the column numbers below each logo. The need for this adjustment reflects the fact that in the real protein sequences the spacing between each motif is highly variable.

Fig 4. Speculative model for the sorting of T9SS substrates carrying variant T9SS

signals. A) This first model corresponds to Fig 5 and could be seen as the “main terminal branch”. T9SS substrates carrying a ‘Type A’ T9SS signal (which may correlate to the TIGR04183 family) are recognised by Type A PG1058 (C_A) which is bound to Type A PorP (P_A). After translocation through the PorK/N pore, the type A T9SS substrate is collected by PorV and shuttled to the attachment complex comprising PorQ, PorU, PorZ and additional PorV and attached to the cell surface (via A-LPS in *P. gingivalis*). B) In the second model, T9SS substrates carrying a ‘Type B’ T9SS signal (e.g. TIGR04131) are recognised by Type B PG1058 (C_B) which is bound to Type B PorP (P_B). After translocation through the PorK/N pore, the type B T9SS substrate maybe directed to the motility apparatus. Other T9SS signal types may exist for other extracellular fates. Alternatively, variant T9SS signals may combine with specific PG1058 and PorP sorting proteins in order to control the stepwise assembly of extracellular structures like the motility apparatus.

Fig 5. Working Model of Type IX Secretion. T9SS substrates have a conserved T9SS signal (labelled “A”) that directs their secretion. T9SS substrates such as RgpB are transported across the inner membrane via the SEC translocon (Step 1). They are then secreted across the OM via the proposed T9SS translocation apparatus consisting of PorL, PorM, PorK and PorN and delivered to PorV (Step 2). PorK and PorN form a large periplasmic ring structure and potentially also the OM pore. The PorV shuttle protein diffuses through the OM and delivers the T9SS substrate to the attachment complex comprising the PorU sortase, PorZ, PorQ and additional PorV (Step 3). The PorU sortase cleaves the T9SS signal and conjugates the T9SS substrate to the A-LPS anchor to produce the electron dense surface layer (EDSL) or virulence coat (VC) (Step 4). The cleaved T9SS signal is released to allow recycling of PorV (step 5).

Abbreviated Summary (75 word limit – currently 74 words)

The Type IX Secretion System is common in the Bacteroidetes phylum of Gram negative bacteria and is responsible for transporting substrate proteins across the outer membrane via a 'translocation complex' comprised of four core protein components. Some substrates then become part of the gliding machinery allowing locomotion, while other substrates are shuttled to the 'attachment complex' which cause the substrates to be tethered to the outer membrane to form functionally diverse cell surface coatings.

For Peer Review

66x55mm (300 x 300 DPI)

Fig 1. Linear architecture and processing of *P. gingivalis* T9SS substrates. T9SS substrates have an N-terminal signal peptide (SP) which is cleaved upon secretion through the SEC translocon and a C-terminal T9SS signal that is cleaved on the cell surface by the PorU sortase. The location of conserved motifs within the T9SS signal are shown together with a protein logo to describe the conserved amino acids within each motif. Motifs B, D & E are significant motifs derived from T9SS substrate sequences found in *P. gingivalis* and *T. forsythia* as reproduced from Veith et al. (2013). The cleavage motif is not statistically significant and is derived from the 20 cleavage sites identified in *P. gingivalis* and *T. forsythia* T9SS substrates in Veith et al. (2013), and analysed by meme software (<http://meme-suite.org/tools/meme>). The PorU sortase catalyses a transpeptidation reaction replacing the T9SS signal with a modification related to A-LPS via a serine residue.

170x87mm (300 x 300 DPI)

Fig 2. Crystal structure of the RgpB T9SS CTD signal. The structure is PDB accession 5AG9 as published by De Diego et al. (2016) and consists of RgpB (575-736) which includes both the IgSF domain and T9SS CTD signal with a 5 residue substitution mutation in the cleavage site region to aid crystallization. The conserved motifs B (residues FDMNGR shown), D (residues GVV) & E (residues KVIVK) of the T9SS CTD signal are shown in yellow while the residues close to the C-terminus of the IgSF domain (residues VEG) are shown in pink. The cleavage site is not shown as it is disordered in the crystal but is located adjacent to the residues shown in pink.

80x62mm (300 x 300 DPI)

Fig 3. Alignment of variant T9SS CTD signals. A) Protein logo representation of TIGR04183. B) Protein logo representation of Pfam13585. C) Protein logo representation of TIGR04131. The protein logos were created by downloading the aligned sequences for each family from the Conserved Domain Database (<https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>), removing columns that contained mostly gaps and submitting each resultant alignment to the Weblogo tool (<http://weblogo.berkeley.edu/logo.cgi>). The length of the TIGR04131 logo matches the length of the original model (PSSM), and was used as the template for adjusting the position of motifs in TIGR04183 and Pfam13585 to enable alignment. The locations of adjustments can be seen by examining the column numbers below each logo. The need for this adjustment reflects the fact that in the real protein sequences the spacing between each motif is highly variable.

170x91mm (300 x 300 DPI)

Fig 4. Speculative model for the sorting of T9SS substrates carrying variant T9SS signals. A) This first model corresponds to Fig 5 and could be seen as the “main terminal branch”. T9SS substrates carrying a ‘Type A’ T9SS signal (which may correlate to the TIGR04183 family) are recognised by Type A PG1058 (CA) which is bound to Type A PorP (PA). After translocation through the PorK/N pore, the type A T9SS substrate is collected by PorV and shuttled to the attachment complex comprising PorQ, PorU, PorZ and additional PorV and attached to the cell surface (via A-LPS in *P. gingivalis*). B) In the second model, T9SS substrates carrying a ‘Type B’ T9SS signal (e.g. TIGR04131) are recognised by Type B PG1058 (CB) which is bound to Type B PorP (PB). After translocation through the PorK/N pore, the type B T9SS substrate maybe directed to the motility apparatus. Other T9SS signal types may exist for other extracellular fates. Alternatively, variant T9SS signals may combine with specific PG1058 and PorP sorting proteins in order to control the stepwise assembly of extracellular structures like the motility apparatus.

170x84mm (300 x 300 DPI)

Fig 5. Working Model of Type IX Secretion. T9SS substrates have a conserved T9SS signal (labelled "A") that directs their secretion. T9SS substrates such as RgpB are transported across the inner membrane via the SEC translocon (Step 1). They are then secreted across the OM via the proposed T9SS translocation apparatus consisting of PorL, PorM, PorK and PorN and delivered to PorV (Step 2). PorK and PorN form a large periplasmic ring structure and potentially also the OM pore. The PorV shuttle protein diffuses through the OM and delivers the T9SS substrate to the attachment complex comprising the PorU sortase, PorZ, PorQ and additional PorV (Step 3). The PorU sortase cleaves the T9SS signal and conjugates the T9SS substrate to the A-LPS anchor to produce the electron dense surface layer (EDSL) or virulence coat (VC) (Step 4). The cleaved T9SS signal is released to allow recycling of PorV (step 5).

170x79mm (300 x 300 DPI)