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**Quantitative proteomic analysis of the type IX secretion system mutants in
*Porphyromonas gingivalis***

Running title: Proteomic analysis of the T9SS

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of Orbitrap LC– MS/MS data within the Mass Spectrometry and Proteomics Facility at the Bio21 Institute, The University of Melbourne, Australia.

Summary

Porphyromonas gingivalis is an anaerobic, Gram-negative human oral pathogen highly associated with chronic periodontitis. *P. gingivalis* utilises the type IX secretion system (T9SS) to transport many of its virulence factors including the gingipains to the cell surface. The T9SS is comprised of at least 16 proteins and the involvement of these 16 proteins in the T9SS has been verified by creating gene deletion mutants in *P. gingivalis*. These T9SS mutants are regularly utilised to understand how these proteins function together to allow the secretion of the T9SS substrates. We performed label-free quantitative proteomic analysis on the T9SS protein mutants in *P. gingivalis* to understand the relative abundance of each T9SS component in different mutants. The T9SS components were reduced in abundance in the *porK*, *porL*, *porM*, *porN*, *sov* and *porT* mutants while they were increased in the *porE*, *porU*, *porV*, *porZ* and *porQ* mutants. Sov and PorW appear to be the lowest in abundance and PorV the highest amongst all the T9SS components in *P. gingivalis* wild-type strain. These results are consistent with the proposed role of Sov as the translocation pore in the outer membrane and PorV as the shuttle protein that transports the T9SS substrates between sub-complexes. Together, the label-free quantitative proteomics analyses showed that different T9SS mutants have vastly different abundances of the T9SS components. This knowledge will greatly assist in interpreting the phenotype of the T9SS mutants as well as selecting the right mutant for exploring the role of an individual component.

Key words: *Porphyromonas gingivalis*, Type IX secretion system (T9SS), quantitative proteomics, stoichiometry.

Introduction

Porphyromonas gingivalis is a human oral pathogen highly associated with chronic periodontitis, an inflammatory disease that leads to the destruction of the teeth's supporting tissues. Periodontitis has been linked with systemic diseases including cardiovascular disease, rheumatoid arthritis, diabetes, preterm birth and low birth weight and more recently to Alzheimer's disease (Dominy *et al.* 2019; Linden *et al.* 2013; Tonetti *et al.* 2013). This Gram-negative, black-pigmented, anaerobic bacterium causes dysregulation of the local

immune response leading to dysbiosis and disease progression (Darveau *et al.* 2012; Hajishengallis *et al.* 2012; Hajishengallis and Lamont 2014).

P. gingivalis produces a variety of surface-associated virulence factors that have been implicated in the pathogenesis of chronic periodontitis. The major virulence factors of *P. gingivalis* are Lys-specific (Kgp) and Arg-specific (RgpA and RgpB) cysteine proteinases, called the gingipains, and are responsible for ~85% of its total proteolytic activity (Imamura *et al.* 2003; O'Brien-Simpson *et al.* 2001; O'Brien-Simpson *et al.* 2009; Potempa *et al.* 2003). These gingipains and 30 other proteins, including the virulence factors carboxypeptidase G (CPG70), a 35 kDa heme-binding protein (HBP35) and peptidylarginine deiminase are translocated to the cell surface using the type IX secretion system (T9SS) (Lasica *et al.* 2017; Sato *et al.* 2010; Veith *et al.* 2017).

The T9SS is specific to the *Bacteroidetes* phylum (Lasica *et al.* 2017; McBride and Zhu 2013; Sato *et al.* 2010; Veith *et al.* 2017) and is the most recently discovered secretion system in Gram-negative bacteria. To date, 16 potential structural proteins, namely PorK, PorL, PorM, PorN, PorP, Sov, PorT, PorE/PG1058, PorF/PG0534, PorG/PG0189, PorW, PorU, PorV, PorQ, PorZ, Plug/PG2092/PNG_0144, and three regulators PorX, PorY and SigP have been identified as components of the T9SS (Chen *et al.* 2011; Glew *et al.* 2012; Heath *et al.* 2016; Kadowaki *et al.* 2016; Lasica *et al.* 2016; Naito *et al.* 2019; Saiki and Konishi 2010; Sato *et al.* 2010). In *P. gingivalis*, deletion of structural components leads to the accumulation of T9SS substrates in the periplasm (Chen *et al.* 2011; Glew *et al.* 2012; Sato *et al.* 2010). All proteins secreted by the T9SS carry a typical cleavable N-terminal signal peptide for export across the inner membrane via the SEC complex and a conserved C-terminal secretion signal of ~80 residues that directs their secretion across the outer membrane through the Sov pore (Lauber *et al.* 2018; Seers *et al.* 2006; Shoji *et al.* 2011; Veith *et al.* 2013). Substrates are collected by the PorV shuttle protein and delivered to the attachment complex comprising of PorU, PorZ, PorQ and additional PorV (Glew *et al.* 2017). PorU cleaves the C-terminal signal and conjugates the protein substrates to the anionic lipopolysaccharide (A-LPS) anchor using a sortase like reaction (Glew *et al.* 2012; Gorasia *et al.* 2015) producing the electron-dense surface layer, which is characteristic of *P. gingivalis* wild-type morphology (Chen *et al.* 2011; Gorasia *et al.* 2015).

We have shown that PorK, PorN and PorG interact to form a ring-shaped structure of 50 nm in diameter and is anchored on the periplasmic side of the outer membrane (Gorasia *et al.* 2016). PorL and PorM are inner membrane proteins and have been shown to directly interact (Gorasia *et al.* 2016; Vincent *et al.* 2017). The crystal structure of the periplasmic PorM

region showed that it forms a dimer composed of four domains, called D1-D4 (Leone *et al.* 2018). PorM and PorN have been shown to interact with the CTD domain of the T9SS substrates (Vincent 2018). SprA (Sov homolog in *F. johnsoniae*) was recently shown to form an extremely large (36-strand) transmembrane beta-barrel with an internal pore of approximately 70Å (Lauber *et al.* 2018). The SprA pore appeared to be alternately occluded on the periplasmic side by the plug protein (Fjoh_1759) and on the external side by PorV and it was proposed that SprA/Sov functions as the secretion pore (Lauber *et al.* 2018). Apart from these proteins, little is known about the functional or structural roles of the other T9SS components.

The T9SS mutants are highly utilised to study the function of the T9SS proteins. We performed quantitative proteomic analysis on the T9SS *P. gingivalis* mutants to understand the expression of the T9SS proteins in different T9SS mutants and therefore shed some light on the stability of T9SS protein components.

Methods

Bacterial strains and culture conditions

P. gingivalis strains W50 and ATCC 33277 were grown in tryptic soy enriched Brain Heart Infusion broth (TSBHI) (25 g/L Tryptic soy, 30 g/L BHI) supplemented with 0.5 mg/mL cysteine, 5 µg/mL haemin and 5 µg/mL Menadione. For blood agar plates, 5% defibrinated horse blood (Equicell, Bayles, Australia) was added to enriched trypticase soy agar. Mutant strains were grown in the same media as above with the appropriate antibiotic selections. All *P. gingivalis* strains were grown anaerobically (80% N₂, 10% H₂ and 10% CO₂) at 37°C. Mutant *P. gingivalis* strains: *sov*, *porK*, *porL*, *porM*, *porN*, *porP*, *porQ*, *porT* and *porW* were obtained from Professor Koji Nakayama along with their 33277 wild type parental strain (Sato *et al.* 2010). Mutant *P. gingivalis* strains *porU*, *porV*, and *porZ* were created in our laboratory in a 33277 *P. gingivalis* background (Chen *et al.* 2011; Glew *et al.* 2017) (Glew *et al.* 2012). *porE* was also generated in our laboratory in the W50 *P. gingivalis* background (Heath *et al.* 2016).

SDS-PAGE and in-gel digestions

Whole cell cultures of the *P. gingivalis* WT and the mutant strains were lysed in 2% TritonX-100 in the presence of complete protease inhibitors and 5 mM tosyl-L-lysyl-chloromethane hydrochloride (TLCK). The proteins were precipitated using 12% Trichloroacetic acid and the pellet was solubilised in buffer containing 7 M urea, 2 M thiourea, 4% CHAPS and 30

mM Tris-HCL, pH 8. The protein amount was quantified using Bradford reagent and a total of 30 µg of protein from each sample was loaded onto NuPAGE Novex 4-12% Bis-Tris gels and electrophoresed at 126 V for 12 min using MOPS running buffer. The gel was stained overnight using Coomassie Brilliant Blue G-250 and destained in 10% acetic acid and 50% methanol. Each sample was excised as a single band and in-gel digestion was performed after reduction with 10 mM DTT and alkylation with 55 mM iodoacetamide using sequencing-grade-modified trypsin (Promega) overnight at 37°C, as previously published (Mortz *et al.* 2001). Tryptic peptides were extracted from the gel pieces using 50% acetonitrile in 0.1% TFA and sonicated for 10 min in a sonicator bath. The samples were concentrated in a vacuum centrifuge before analysis using LC-MS/MS.

LC-MS/MS

The tryptic peptides were analysed by LC-MS/MS using the Q Exactive Plus Orbitrap mass spectrometer coupled to an ultimate 3000 UHPLC system (Thermo Fisher Scientific). Buffer A was 2% acetonitrile and 0.1% formic acid, buffer B consisted of 0.1% formic acid in acetonitrile. Sample volumes of 1 µL were loaded onto a PepMap C18 trap column (75 µM ID X 2 cm, 100 Å) and desalted at a flow rate of 2 µL/min for 15 min using buffer A. The samples were then separated through a PepMap C18 analytical column (75 µM ID X 15 cm, 100 Å) at a flow rate of 300 nL/min, with the percentage of solvent B in the mobile phase changing from 0 to 3% in 6 min, from 3 to 22% in 90 min, from 22 to 40% in 10 min and from 40-80% in 5 min. The spray voltage was set at 1.8 kV, and the temperature of the ion transfer tube was 250°C. The S-lens was set at 50%. The full MS scans were acquired over a m/z range of 300-2000, with a resolving power of 70000, an automatic gain control (AGC) target value of 3×10^6 , and a maximum injection of 30 ms. Dynamic exclusion was set at 90 s. Higher energy collisional dissociation MS/MS scans were acquired at a resolving power of 17500, AGC target value of 5×10^4 , maximum injection time of 120 ms, isolation window of m/z 1.4, and NCE of 25% for the top 15 most abundant ions in the MS spectra. All spectra were recorded in profile mode.

Relative abundances of proteins were quantified by MaxQuant (Ver 1.5.3.30) (Cox *et al.* 2014) in three analyses according to the background strain. Raw MS/MS files were searched against the W83 (for W50) or 33277 *P. gingivalis* sequence databases with the default recommended parameters except Label Free Quantification (LFQ) min ratio count was set to 1 and the match between runs was selected. MaxQuant normalised the data set as part of data processing. Three biological replicates were used for the analysis. In all strains at least 3 unique peptides were identified for all proteins except for SigP due to its low abundance.

LFQ ratios for each replicate were calculated by dividing the LFQ intensity of each mutant replicate by the average LFQ intensity of the respective wild type strain. The average LFQ intensity of the 33277 wild type strain obtained from Koji Nakayama was from two replicates as one replicate did not work. The average LFQ ratio was calculated as the geometric mean of the three individual ratios.

Results

Quantitative proteomic analysis of the T9SS mutants

P. gingivalis WT strains and T9SS mutants (*sov*, *porE*, *porK*, *porL*, *porM*, *porN*, *porP*, *porQ*, *porT*, *porU*, *porV*, *porW* and *porZ*) were lysed and electrophoresed on a SDS-PAGE gel for a short time so that the whole sample could be subjected to in-gel trypsin digestion. The tryptic fragments were analysed by LC-MS/MS and MaxQuant, resulting in the identification of a total of 1438 proteins including the T9SS proteins (**supplementary table 1**). All the known components of the T9SS (19 proteins) were identified and quantified in each mutant. A plot of the LFQ intensity ratios of the T9SS proteins in each T9SS mutant was plotted relative to the wildtype (**Figure 1**). Two basic trends were observed: (i) mutants in which the majority of the T9SS proteins were decreased in abundance (*porK*, *porL*, *porM*, *porN*, *porT* and *sov*) and (ii) mutants in which the majority of the T9SS proteins were increased in abundance relative to wildtype (*porE*, *porU*, *porV* and *porZ*) (**Figure 1**). In the *porQ*, *porP* and *porW* mutants some T9SS proteins were decreased while others were increased relative to the wildtype (**Figure 1**). In the *porP* mutant specifically PorK, PorL, PorM and PorN increased in abundance while other proteins decreased (**Figure 1**). Collectively, the quantitative proteomic study of the T9SS mutants enabled us to group the T9SS proteins that may function together.

Abundance of T9SS proteins in wildtype *P. gingivalis* strains

To investigate the abundance of the T9SS components in the wild-type *P. gingivalis* strain we used the iBAQ metric of the MaxQuant software which measures the total intensity divided by the number of the identified peptides for one protein (Cox *et al.* 2014). The graph of iBAQ intensities of each T9SS protein in the W50 and ATCC 33277 WT strain was plotted on a log scale (**Figure 2**). Besides the regulators PorY and SigP, PorW was the least abundant protein amongst the T9SS proteins followed by Sov. The next abundant proteins were PorG, PorP, PorE and Plug which all appeared to be of similar levels. PorK, PorL, PorM and PorN were ~10 fold higher in abundance than Sov consistent with the known multimerization of these

proteins (Gorasia *et al.* 2016; Vincent *et al.* 2017) (**Figure 2**). PorU, PorQ and PorZ were of similar abundance to PorK, PorL, PorM and PorN. Finally, PorV appears to be the most abundant T9SS protein which is consistent with its function as a shuttle protein. From the regulatory group of proteins (PorX, PorY and SigP), PorX appears to be nearly 100-fold more than PorY and SigP (**Figure 2**).

Abundance of T9SS substrates in the T9SS mutants

The relative abundance of the selected T9SS substrates were analysed in each T9SS mutant. It appears that each T9SS substrate had its unique pattern and no real trend was observed (**Figure 3**). Kgp and RgpA appeared to be decreased in abundance in the *porE*, *porU*, *porV* and *porZ* mutant whilst increased in abundance in the *porW*, *porK*, *porL*, *porM*, *porN*, *porT* and *sov* mutants. Only PGN_0152 (PG2102), PGN_0693 (PG0654) and PGN_0898 (PG1424) appeared to be decreased in all the mutants (**Figure 3**). Collectively, the T9SS substrates seem quite stable and are not prone to extensive degradation when accumulation in the periplasm occurs.

Discussion

The T9SS was first discovered in *P. gingivalis* nearly a decade ago. It plays a crucial role in the secretion of virulence factors such as the gingipains in this organism. Although many components of the T9SS have been identified, the function of only a few components has been elucidated. It is of a great interest to understand how these proteins interact and work together to recruit and translocate the T9SS substrates across the outer membrane. *P. gingivalis* T9SS mutants are regularly used to explore the mechanism of the T9SS. In this study, we quantitated the abundance of 19 T9SS protein components in 13 *P. gingivalis* T9SS mutants to understand the expression levels of these proteins. Of note, all the T9SS *P. gingivalis* mutants that have been analysed in this study have been complemented and shown to go back to wild-type phenotype and hence there are no polar effects in these mutants (Chen *et al.* 2011; Glew *et al.* 2017; Glew *et al.* 2012; Sato *et al.* 2010).

The analysis showed that *porK*, *porL*, *porM*, *porN*, *porT* and *sov* mutants have a similar profile of T9SS protein components which were decreased in abundance (**Figure 1**).

Previously, we have demonstrated that PorK, PorN and PorG interact to form large ring-shaped structures (Gorasia *et al.* 2016). Although they are known to be interacting partners it appears that their abundances amongst the mutants poorly correlate (**Figure 1**). In most mutants PorN was increased in abundance, however, PorK was not. In contrast to this, the

abundances of PorL and PorM were observed to be highly correlated. These two proteins have been previously shown to interact (Gorasia *et al.* 2016; Vincent *et al.* 2017) and it seems they are dependent on each other for their stability. PorK, PorL, PorM, PorN and PorG have been proposed to be the core components of the T9SS (Gorasia *et al.* 2016; Vincent *et al.* 2017) and Sov was recently shown to be the translocation pore of the T9SS (Lauber *et al.* 2018). Together, it is clear these six proteins are involved in the translocation of the T9SS substrates from the periplasm across the outer membrane. Nothing is known about PorT in terms of its specific function and its interacting partners. Since the *porT* mutant had a similar profile to *porK*, *porL*, *porM*, *porN* and *sov* mutants (**Figure 1**), it is likely that PorT is also a part of the translocation machinery.

The four T9SS protein components (PorQ, PorV, PorU and PorZ) have been described as attachment complex proteins and have been proposed to play a role in attaching the T9SS substrates onto the cell surface of *P. gingivalis* (Glew *et al.* 2017). In these mutants, it appears that the translocation machinery is intact, however, the protein substrates are not anchored to the A-LPS on the cell surface and very little proteolytic activity is generated (Glew *et al.* 2017; Glew *et al.* 2012). In the current study, it was found that the majority of the T9SS proteins were increased in abundance in these mutants. These results support the independence of the attachment system, deletion of one protein component of the attachment system does not greatly reduce any other component. On the contrary, the translocation system appears to be highly interdependent, as the absence of one component adversely affects all the other components. Perhaps, in the attachment mutants the intact translocation machinery is upregulated in an attempt to compensate for the lack of final product on the surface. If the translocation system was not so highly interdependent the quantitative proteomic analysis performed in this study might have provided insights into the assembly order of the T9SS. Due to the high interdependency observed, the assembly order may prove difficult to determine.

Amongst the T9SS protein components, PorW and Sov appear to be the lowest in abundance. This is consistent with Lauber *et al.* study who have shown that Sov homolog, SprA, does not multimerise and have also proposed that there might be one SprA pore per translocation machine (Lauber *et al.* 2018). Since PorW is at a similar abundance as Sov this suggests that PorW may also function as one monomer per machine. PorX, PorY and SigP have a role in regulating the expression of T9SS component encoding genes (Kadowaki *et al.* 2016; Sato *et al.* 2010). PorX has been shown to directly interact with PorY and SigP to regulate the expression of the T9SS proteins (Kadowaki *et al.* 2016) and has also been shown to associate

with PorL (Vincent *et al.* 2016) which is of a similar high abundance (**Figure 2**). The multiple interacting partners of PorX is consistent with it being more abundant than PorY and SigP.

It is a well-known phenomenon that the accumulation of unfolded/misfolded proteins in the periplasm triggers a stress response which in turn leads to the degradation of the accumulated proteins (Miot and Betton 2004; Raivio and Silhavy 2001). The quantification of the relative amount of the T9SS substrates in *P. gingivalis* T9SS mutants did not show any real trend, as the substrates were increased in abundance in some mutants and decreased in others (**Figure 3**). The increase in abundance of the T9SS substrates suggest that they are either resistant to degradation or the stress pathway is not activated in these mutants. In fact, SDS-PAGE gels and immunoblots with antibodies specific to T9SS substrates performed on the periplasm fractions or whole cell lysates from the T9SS mutants have demonstrated that the substrates largely accumulate in their unprocessed forms indicating little degradation (Chen *et al.* 2011; Glew *et al.* 2012; Heath *et al.* 2016). It is speculated that the T9SS substrates are folded in the periplasm and then transported across the outer membrane using the T9SS. The folded state of the T9SS substrates may increase their stability making them resistant to degradation. Additionally, since the T9SS substrates are exposed to the proteolytic environment on the cell surface of *P. gingivalis*, it is likely they are naturally resistant to proteolysis. The T9SS substrates that were reduced in abundance may be down-regulated as part of a feedback mechanism.

In summary, the label-free quantitative proteomics analyses have shown that different T9SS mutants have vastly different abundances of the T9SS components. Deletion of one T9SS protein can have effects on multiple T9SS components. These effects must be understood to fully interpret the phenotype. This knowledge will greatly assist in designing and choosing the right mutant for experiments to understand the role of individual components.

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Figure Legends

Figure 1: Relative abundance of T9SS components in T9SS mutants.

Whole cell lysates of each of the 13 T9SS mutants together with their respective wild type controls were analysed by LC-MS/MS and quantified by MaxQuant in triplicate. The average

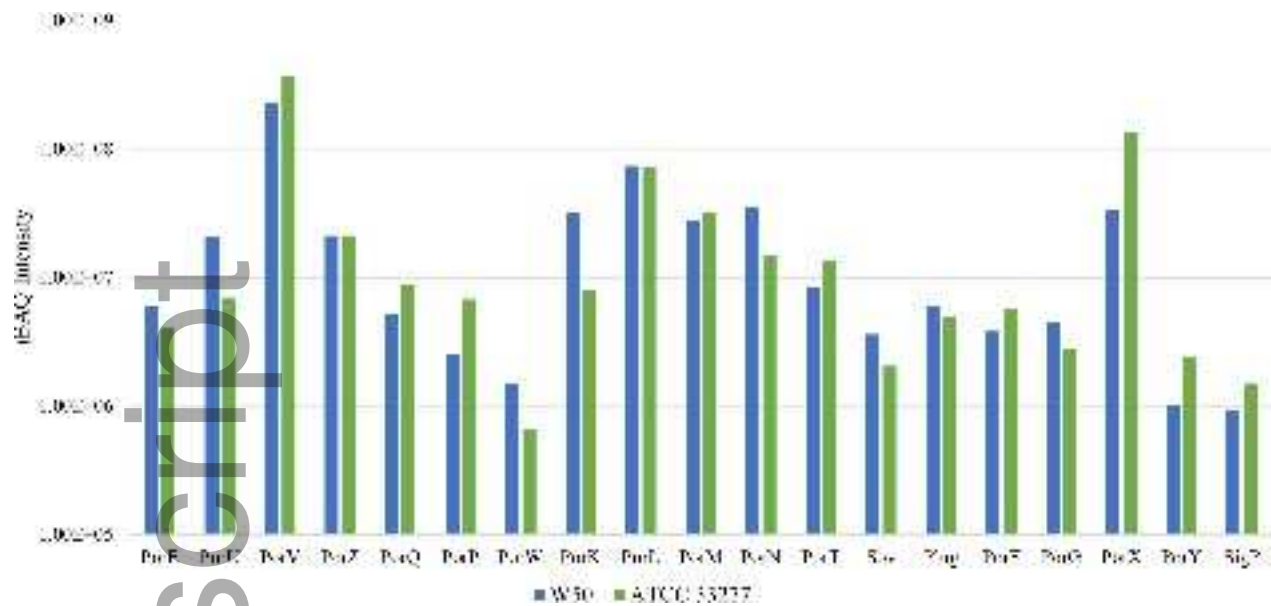
LFQ intensity ratios for 19 T9SS components in each mutant with respect to the wild type is shown on a Log 2 scale.

Figure 2: Stoichiometry of T9SS components in the wild-type W50 and ATCC 33277 *P. gingivalis* strains

The iBAQ metric of the Maxquant software was used as an estimate of the relative molar abundance of each protein. The relative abundances are plotted on a logarithmic scale. The iBAQ intensity of the ATCC 33277 strain was normalised to the overall W50 values.

Figure 3: Abundance of T9SS substrate proteins in T9SS mutants.

Using the same MaxQuant dataset as Figure 1, the average LFQ intensity ratios for 14 T9SS substrates was plotted for each mutant with respect to the wild type on a Log 2 scale.



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