8-2016

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Apurva Tadimari Prabhakar
University of Louisville

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By
Apurva Tadimari Prabhakar
B.D.S., M. S. Ramaiah Dental College and Hospital (India), 2012

A Thesis
Submitted to the Faculty of the
School of Dentistry of the University of Louisville
In Partial Fulfillment of the Requirements
For the Degree of

Master of Science
In Oral Biology

Department of Oral Immunology and Infectious Diseases
School of Dentistry, University of Louisville
Louisville, Kentucky

August, 2016

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A Thesis Approved on

July 14, 2016

By the following Thesis Committee:

_____
Jan S. Potempa, Ph.D., D.Sc.
Thesis Director

_____
Dr. Donald Demuth, Ph.D.
Committee Member

_____
David A. Scott, Ph.D.
Committee Member

_____
Mathew. B. Lawrenz, Ph.D.
Committee Member

DEDICATION

I dedicate this thesis to my parents, Mr. Prabhakar Tadimari and Mrs. Sarvamangala Tadimari, who push me to have a good education; whose love, support, encouragement and prayers of day and night make me able to get such success and honor. They made me enjoy learning new things and take new heights in life.

ACKNOWLEDGEMENT

Pursuing my Master's in oral biology student at the University of Louisville School of Dentistry was a magnificent yet challenging experience to me. In all these years, many people were instrumental directly or indirectly in shaping up my academic career. It would not have been possible for me to thrive as a Master's student without the precious support of these personalities. Here is a small tribute to all those people.

First of all, I am thankful to my supervisor Dr. Jan Potempa, who has the attitude and the substance of a genius. It was only due to his valuable guidance, cheerful enthusiasm and ever-friendly nature that I was able to complete my research successfully. His ideas, support, advices, provisions and suggestions made this study possible. I was fortunate enough to have had this chance to work under his direction and be exposed to the various aspects of periodontal pathogen research.

I would like to thank my committee members, Dr. Don Demuth, Dr. David Scott and Dr. Mathew Lawrenz, who took out their precious time and effort to check this manuscript and for providing their valued suggestions. My special thanks to my program director, Dr. Douglas Darling, who has been guiding me since the beginning of the program. The graduate school scholarship that I received, motivated me in my research and was possible only because of him.

Most importantly, I am grateful to Dr. Anna Lasica and Ms. Barbara Potempa, who shaped me as a confident researcher that I am today. Both have been instrumental in teaching me from the very basic lab techniques (read using a pipette) to the most advanced one in the field. Being a fresher to the field, Dr. Anna Lasica and Barbara were very patient, encouraging and ever available to train me, which helped me to learn valuable things.

I also take this opportunity to convey my regards to my lab members and colleagues Apoorv Goel and Lahari Koneru, who were very supportive and by my side on this journey, learning and growing together. I am ever thankful and blessed to have such amazing support of my family and friends, who always motivate me and help me have a positive outlook towards things.

ABSTRACT

CHARACTERIZATION OF PG1604 (PorZ) AS AN ESSENTIAL COMPONENT OF TYPE 9 SECRETION SYSTEM IN *PORPHYROMONAS GINGIVALIS*

Apurva Tadimari Prabhakar

July 14, 2016

Background: The numerous virulence factors of *Porphyromonas gingivalis* are essential for the organism to invade host tissues while escaping immune responses [1, 2]. Some of these factors, like major proteases referred to as gingipains, are secreted by a unique secretion system called PerioGate or Type IX Secretion System (T9SS). T9SS consists of a number of membrane and periplasmic proteins, which provide transportation only for proteins that possess a conserved C-terminal domain (CTD). The CTD functions as a specific recognition signal for the T9SS. PG1604 of *P. gingivalis* W83 is a CTD-containing protein, which has been shown in our initial studies to be essential for secretion of CTD-proteins. However, unlike other CTD proteins, PorZ was also shown by proteomic studies to be present in the wild-type strain as the full-length protein containing the CTD domain (minus a signal peptide) [3, 4].

Objectives: Our objective was to probe structure-function relationship of the PG1604 protein by insertions of an oligohistidine motif or substitutions of six consecutive residues with hexahistidine within the C-terminal domain of PG1604. Effects of these mutations were monitored by various phenotypic tests and allowed to assess their influence on

PG1604 maturation and function. ***Methods:*** 1) Oligohistidine insertions into the PG1604 protein by plasmid-based SLIM mutagenesis. 2) Generation of a *P. gingivalis* mutant strain expressing the modified PG1604 from the chromosome. 3) Phenotype analysis conducted on the whole set of the CTD altered PG1604, including the strain obtained in this study (AT001). Assays included: a) assessment of colony pigmentation on blood agar plates, b) the gingipains activity assay, c) western-blot analysis to detect the presence and subcellular localization of PG1604 (*ant*i-PG1604 and *anti*-HIS tag antibodies) as well as the presence and processing of the Kgp and Rgp proteins. ***Results:*** 1) Some mutants showed the altered secretory phenotype detected by the strains failure to form pigmented colonies on the blood agar plate. 2) The lack of pigmentation correlated with severely decreased gingipains activities and with changes in proKgp and proRgp protein posttranslational processing visualized by western-blot. 4) The PG1604 protein was found to be localized mainly in the outer membrane. 5) HIS-tagged PG1604 may be purified from *P. gingivalis* culture for further analysis. ***Conclusions:*** 1) The CTD of the PG1604 protein is not cleaved off upon its transport through T9SS as full length ~80 kDa protein was shown by western-blot analysis with *anti*-PG1604 serum (migration at) and *anti*-HIS mAb for the PG1604 variant tagged with octahistidine at the C-terminus (*P. gingivalis* strain DM008). 2) The selective modifications of the PG1604 protein, hindered the proper maturation of secreted virulence factors (Kgp and Rgp) due to disrupted function of T9SS in *P. gingivalis*. **Taken together based on our findings we concluded that PG1604 is a novel functional component of T9SS located on the bacterial surface in association with the outer membrane. The**

CTD of PG1604 is not only essential for translocation across the OM but also for this protein function in other CTD-proteins secretion and their posttranslational modifications (A-LPS attachment and proteolytic processing). Therefore, following the nomenclature of the T9SS components we designate the PG1604 protein as PorZ.

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CHAPTER 1

INTRODUCTION

Periodontitis is one of the most prevalent chronic inflammatory diseases that leads to loss of the integrity of periodontium, which is comprised of gingiva, periodontal ligament, and alveolar bone [5]. It is considered as the foremost cause of teeth loss and is said to be involved in the development and also the progression of many systemic problems such as rheumatoid arthritis, atherosclerosis, adverse pregnancy outcomes, aspiration pneumonia and cancer [6]. Recent studies indicate that in US adult population, periodontal disease is quite prevalent with approximately 90% exhibiting at least some form of gingivitis and 47% of the population with periodontitis; with the severe form of periodontitis affecting 18% of those individuals over the age of 65 [7-9]. For individuals and societies, the cost of the treatment is expensive, consuming between 5 and 10% of all healthcare resources [10].

Epidemiologically, studies have shown that poor oral hygiene is associated with more severe disease, but chronic plaque accumulation is not the only predictor of the disease. Other factors such as smoking and systemic problems like diabetes, cardiovascular diseases etc., should be considered as risk factors [11, 12].

Host-microbe interplay in periodontitis and other risk factors

Studies show the human microbiome play an important role in the overall health of the human host. Characterizing the different microbial communities colonizing the human body sites like skin, gut,etc., has been of utmost importance [13, 14]. The oral cavity represents one of the most diverse microbial communities associated with any of the human sites studied. It comprises of a highly complex community with around 700 species identified to be associated with the different environments within the oral cavity. It is probably one of the best characterized communities of the human microbiome [15]. Polymicrobial synergy is seen among the organisms which form these communities in oral cavity maintaining the tissue homeostasis and normal immune responses. In periodontal disease, the shift from healthy tissue to diseased is associated with a drastic change from a symbiotic microbial community (Figure 1), comprising mostly of facultative bacterial genera such as *Streptococci* and *Actinomyces*, to a dysbiotic microbial community structure made up of mainly anaerobic genera from the phyla *Proteobacteria*, *Bacteroidetes*, *Spirochaetes* and *Synergistetes*, leading to a dysregulated host immune response. The dysbiotic oral microbiota encompassing many pathogenic species can thrive in an inflammatory environment so that more damage is actually done to the host tissues than to causing agents [16].

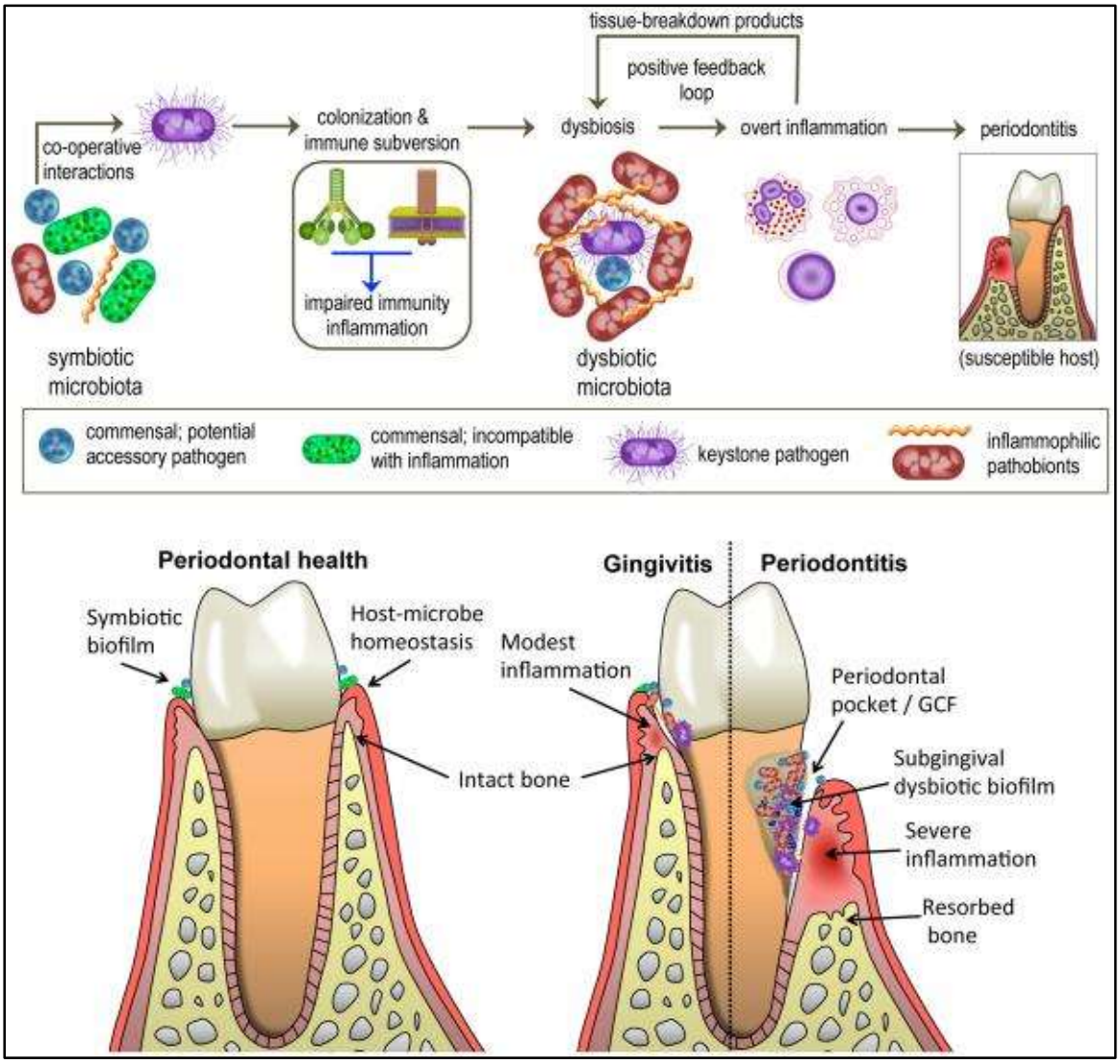

Figure 1. Polymicrobial synergy and dysbiosis in periodontitis
In a predisposed individual, periodontitis is induced by a polymicrobial community which is comprised of distinct organisms playing different roles. Keystone pathogens along with accessory pathogens, destabilize the host immune response causing microbial dysbiosis due to immune system over-activation leading to periodontal tissue destruction. The lower part of the figure depicts the progression of healthy periodontium to gingivitis (periodontal inflammation without bone loss; gingival crevice ≤3 mm) to periodontitis (formation of periodontal pockets ≥4 mm and inflammatory bone loss). Adapted from *Nat Rev Immunol. 2015 Jan; 15(1): 30–44 [6]*.

The etiology of periodontitis is not just limited to the dysbiotic microbial community. It involves other independent but modifiable risk factors which include lifestyle factors, such as smoking and alcohol consumption. It also includes diseases and unhealthy conditions such as diabetes mellitus, obesity, metabolic syndrome, osteoporosis, and low dietary calcium and vitamin D. These diseases might cause a reduction in the number or function of polymorphonuclear leukocytes (PMNs), which in turn can result in an increased rate and severity of periodontal destruction [17]. Genetic factors also play a role in periodontal disease, which can be a target for individualized prevention and early detection [18, 19].

Role of *Porphyromonas gingivalis* in periodontal disease

P. gingivalis, a Gram-negative oral anaerobe which belongs to the phylum Bacteroidetes, is considered to be one of the keystone pathogens of the periodontal disease [20]. *P. gingivalis* not only alters the growth and development of the entire biofilm but disturb the normal homeostatic host-microbe interaction in the periodontium. It was found in 85.75% of subgingival plaque samples from patients with chronic periodontitis. *P. gingivalis* can locally invade periodontal tissues and evade the host defense mechanisms. It utilizes a panel of virulence factors that cause deregulation of the innate immune and inflammatory responses [21] (Figure 2).

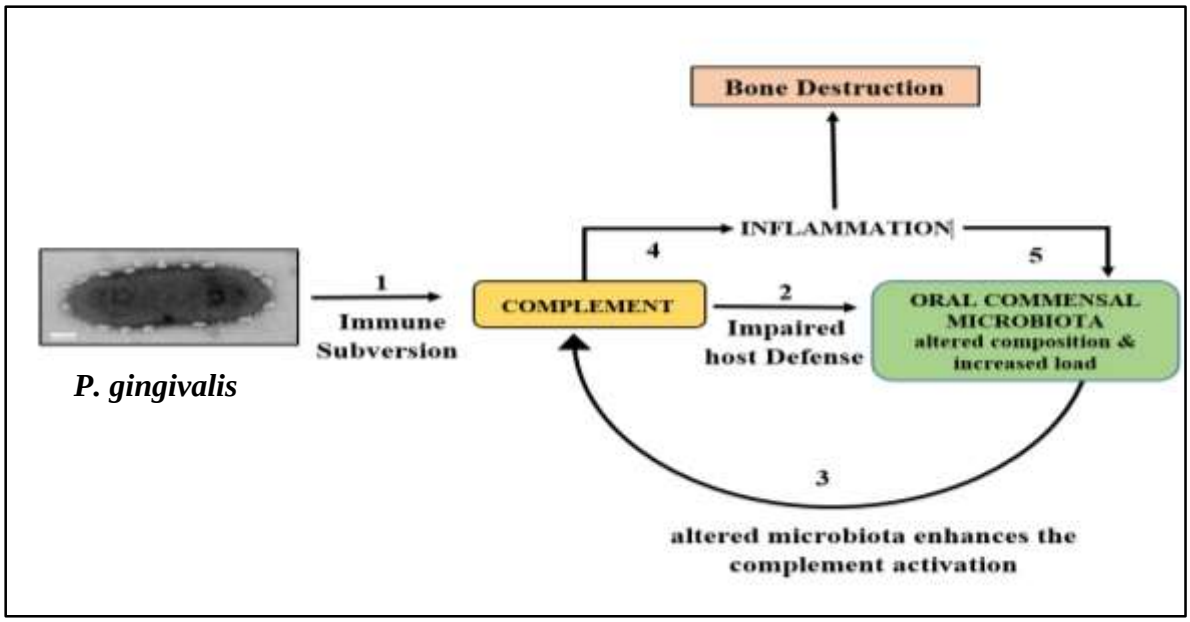

Figure 2. *P. gingivalis*-induced dysbiosis and periodontal disease.
Studies have shown that *P. gingivalis* modulates innate host defense functions that can have a general effect on the oral commensal community. Immune subversion of IL-8 secretion, complement activity, or TLR4 activation can cause an impaired host defense. The host loses its ability to control the oral commensal microbial community leading to an altered oral microbial composition and an increased microbial load. This change from a symbiotic to a dysbiotic microbiota is the cause for pathologic inflammation and bone loss. Adapted from *J Dent Res. 2012 Sep; 91(9): 816–820 [22]*.

P. gingivalis is a non-motile, asaccharolytic bacterium, an obligatory anaerobic rod which forms black-pigmented colonies on blood agar plates due to an absolute requirement for heme, a source of iron, in its growth [23] (Figure 3).

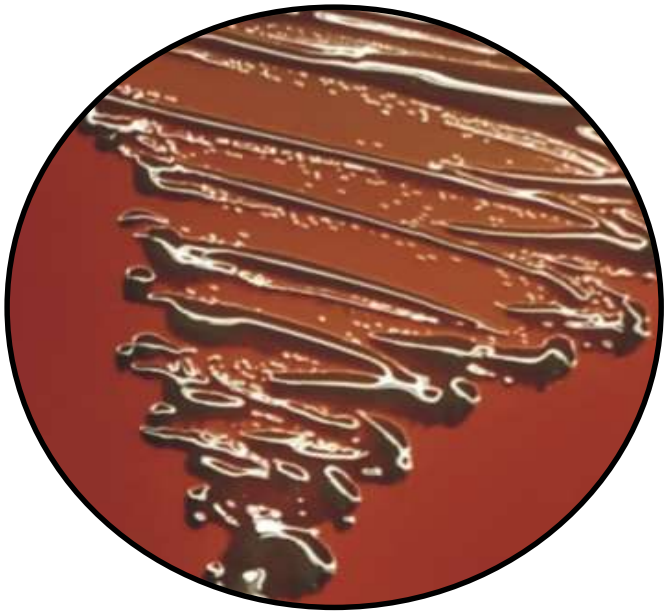

Figure 3. Black-pigmented culture of periodontopathogen *P. gingivalis* on blood agar. The pigmentation is due to accumulation of hemin (oxidized form of heme) on the cell surface when grown on blood agar. Studies show that gingipain are also involved in hemoglobin degradation and acquisition of heme [1]. (Photo by *Tadimari Prabhakar.A*)

The major habitat of *P. gingivalis* is the subgingival sulcus of the human oral cavity. It depends on the fermentation of amino acids for energy production, a property required for its survival in the deep periodontal pocket, where sugar availability is low. Being an obligate anaerobe [24], *P. gingivalis* is the secondary colonizer of dental plaque, often adhering to primary colonizers such as *Streptococcus gordonii* and *P. intermedia* [21].

P. gingivalis is known to produce an array of virulence factors that could penetrate the gingivae and cause tissue destruction directly or indirectly, by induction of inflammation [25]. In order to survive and multiply in a host, *P. gingivalis* has to evade the

host external protective barriers before it could find a suitable ecological niche for colonization. Colonization happens in the presence of virulence factors such as fimbriae, capsules, lipopolysaccharide (LPS), lipoteichoic acids, haemagglutinins, gingipains, outer membrane proteins, and outer membrane vesicles [26] (Figure 4).

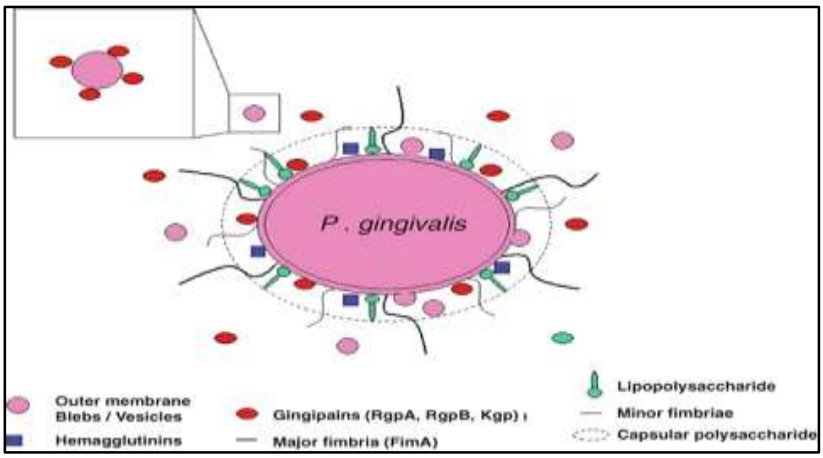

Figure 4. Virulence factors of *P. gingivalis* (Adapted from *The Prokaryotes 2006, pp 428-454*)

Outer membrane vesicles (OMVs) of *P. gingivalis* contain virulence factors such as lipopolysaccharide and gingipains [27]. Lipopolysaccharide circulates systemically in over 50% of periodontal disease patients and is associated with increased matrix metalloproteinase [28]. The gingipain-laden outer membrane vesicles may contribute to tissue destruction in periodontal diseases by serving as a vehicle for the antigens and active proteases [29]. Gingipains are the cysteine proteinases secreted by *P. gingivalis* and are considered the major virulence factors involved in periodontal pathogenesis [30]. Gingipains comprise of Arg-specific cysteine proteinases (Rgp) encoded by *rgpA* and *rgpB*, and a Lys-specific cysteine proteinase (Kgp) encoded by *kgp* [31] (Table 1).

Table 1. The virulence factors and host effectors produced by *P. gingivalis* [25]

Virulence factors	Effect on host evasion
Enzymes (hyaluronidase, chondroitin sulfatase), capsule	Decrease phagocytosis for invasion, chemotaxis inhibitors
Lipopolysaccharide	Bone resorption, Immunoglobulin proteases
Fimbriae, exopolysaccharide, outer membrane proteins	Adhesion or attachment to host outer membrane
Collagenase, trypsin-like protease, gelatinase	Degradation of plasma protease inhibitors, destruction of periodontal tissue
Aminopeptidase	Degradation of iron transport protein

Protein secretion system in *P. gingivalis*:

In Gram-negative bacteria, protein secretion across two lipid bilayers: the cell membrane (the inner membrane; IM) and the outer membrane (OM), is a logistical challenge. To achieve this, Gram-negative bacteria have evolved nine known Types (I to IX) of Secretion Systems (T*x*SS) to translocate proteins synthesized in the cytoplasm to the bacterial surface and beyond [32]. Using specific systems, bacteria are able to assemble cell surface appendages such as pili (T2SS, T4SS, T7SS), curli (T8SS), flagellum (T3SS), secrete proteins extracellularly (T1SS to T6SS) or inject them directly into a host eukaryotic cell (T3SS and T4SS) or into the periplasm of other bacteria (T6SS) [33]. The proteins to be translocated are either secreted directly from the cytoplasm via T1SS, T3SS, T4SS and T6SS or they are first exported to the periplasm across the IM using the

conserved Sec or Tat pathway and then translocated through the OM using T2SS, T5SS, T7SS or T8SS [34].

The most recently discovered system, T9SS, formerly designated the Por secretion system (PorSS), operate in selected bacterial species belonging exclusively to the *Bacteroidetes* phylum, including periodontal pathogens (*P. gingivalis* and *Tannerella forsythia*) and a number of environmental benign microorganisms [35]. T9SS is essential for the secretion of many proteinaceous virulence factors by the periodontal pathogens [36], and thus, has attracted considerable attention as a possible target for pharmaceutical intervention. The system is also required for assembly of the gliding motility machinery of a number of environmental microorganisms such as *Cytophaga hutchinsonii* and *Flavobacterium johnsoniae* [35].

All cargo proteins of T9SS contain a conserved C-terminal domain (CTD) of approximately 70 amino acid residues with an immunoglobulin-like fold [37]. With a few exceptions, these proteins carry a typical cleavable signal peptide and are apparently exported to the periplasm using the Sec system to cross the IM [38]. In the periplasm, T9SS cargo proteins fold and then are directed to an OM translocon by a targeting signal located in the last two β-strand of the CTD [34, 39]. During the translocation, the CTD is cleaved off and the protein is released extracellularly [3] (Figure 5). At least in *P. gingivalis*, the CTD cleavage occurs seemingly simultaneously with the covalent attachment of anionic lipopolysaccharide (A-LPS) to the newly released C-terminal carbonyl group of the truncated protein [40]. The A-LPS attachment serves to anchor the secreted proteins to the OM where they form an electron dense surface layer (EDSL) characteristic for *P. gingivalis* [41] (Figure 6).

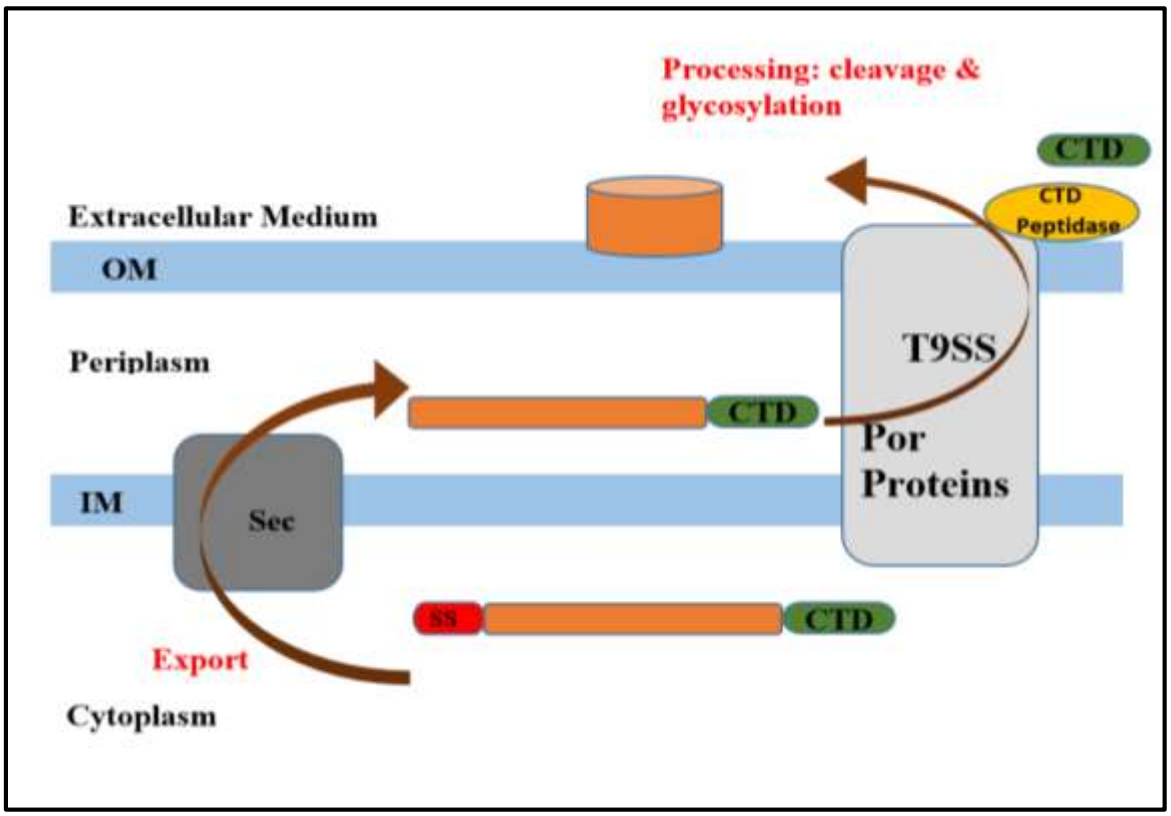

Figure 5. Model of the T9SS (Type IX Secretion System) of *P. gingivalis*. All gingipains are synthesized with an N-terminal signal peptide allowing export to the periplasm via the Sec pathway. Once in the periplasm, they are addressed to the cell surface by a dedicated complex constituted of the products of the *por* genes, the T9SS. The gingipains carry a C-terminal motif, the CTD is required for recruitment to the T9SS. Transport of the gingipain is coupled by cleavage of CTD by peptidase (porU) (Adapted from *Sato* et al., *2010* [42]).

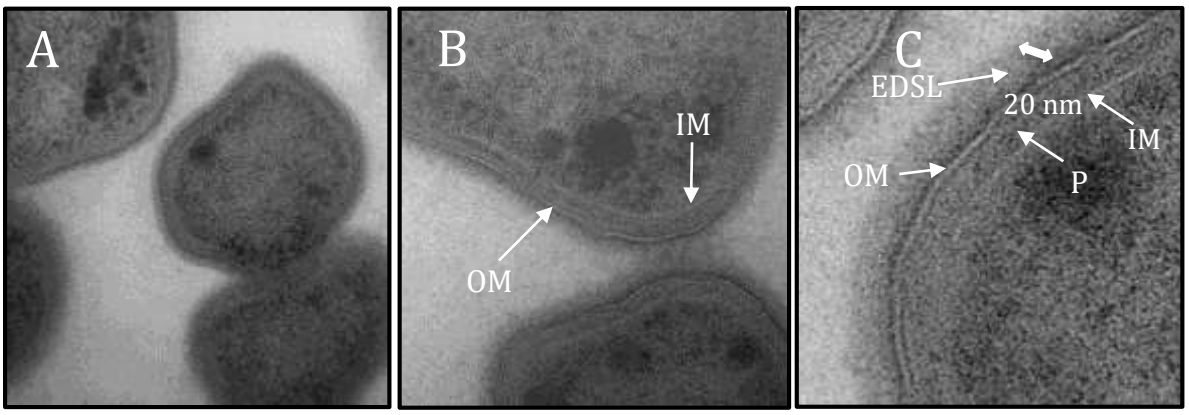

Figure 6: Typical *P. gingivalis* (W83) cells showing EDSL. (A) TEM image of several *P. gingivalis* cells. (B) TEM image of *P. gingivalis* cell showing IM and OM.(C) TEM image of *P. gingivalis* cell showing the presence of a thick EDSL surrounding the membrane of each cell, with dimension shown. Outer Membrane (OM), Inner Membrane (IM), Periplasm (P), Electron Dense Surface Layer (EDSL) (Electron microscope images provided by *Arek Kulczyk, PhD, Harvard University Medical School*).

Seers *et al* in their study demonstrated that secretion of RgpB (member of gingipain family) from the periplasm across the outer membrane is dependent on the conserved CTD [43]. Lack of the CTD leads to the accumulation of gingipains in the periplasm itself [4, 44] and it prevents gingipains from attaching to the outer membrane, which suggests that CTD plays an essential role in the secretion of proteins [45] (Figure 7).

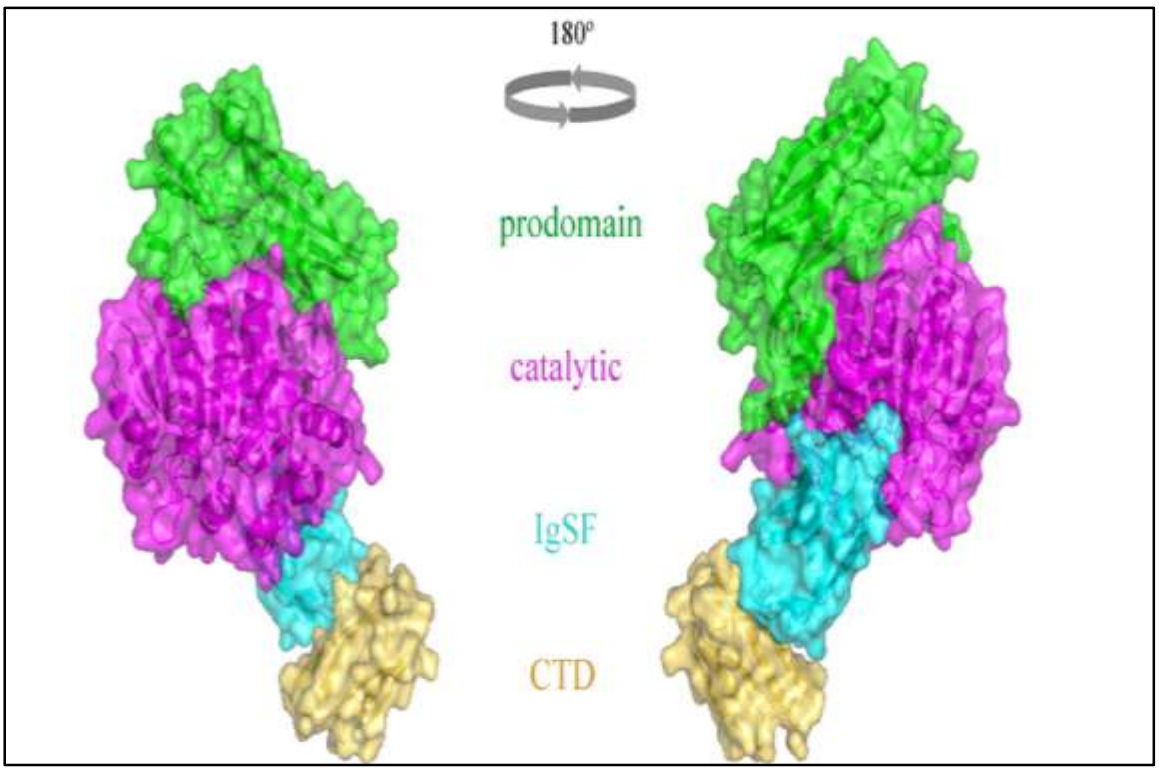

Figure 7. Multi-domain model of the latent full-length pro-RgpB to demonstrate protein domains. Surface representation of the model of the latent full-length proRgpB, transiently present in the periplasm during secretion, including the N-terminal prodomain (green), the catalytic domain (purple), the IgSF domain (cyan) and the CTD (yellow) (Adapted from *Scientific Reports | 6:23123 | DOI: 10.1038/srep23123* [37]).

Presently, 12 unique genes have been found to be indispensable for secretion of CTD-containing proteins in *P. gingivalis* [46]. These genes are conserved in all species bearing genes encoding the CTD-proteins, suggesting they are essential for assembly and/or functioning of T9SS [35]. Among translated products of these genes, six are predicted to be integral β-barrel OM proteins (PorV, PorP, PorQ, PorT, Sov and PG0543) [42], two are inner membrane type I proteins (PorM and PorL), while two are putative lipoproteins (PorK and PorW) probably anchored into the OM facing the periplasm [36, 44]. Finally, PorN is most likely located in the periplasm whereas PorU is found on the bacterial surface. In *P. gingivalis,* inactivation of any of these essential T9SS components leads to the arrest of cargo proteins in the periplasm, with the CTD still intact [47]. Apart from PorU, which

is the surface-located gingipain-like protease that functions as a sortase to cleave off the CTD from secreted proteins [43], little is known about the functional or structural role of other essential T9SS components [42, 47].

Within the periodontal pathogen *P. gingivalis*, about 30 putative proteins bearing the CTD have been detected (Figure 8), including PorU and important virulence factors such as cysteine protease gingipains (RgpA, RgpB, and Kgp) [44], carboxypeptidase D (CPG70) [48], heme-binding protein (HBP35) [39], and peptidyl arginine deiminase (PPAD) [49]. Many of these proteins were found to be A-LPS-modified and migrated higher in SDS-PAGE gels than their predicted molecular mass, thus, confirming their secretion and glycan modification via T9SS [4].

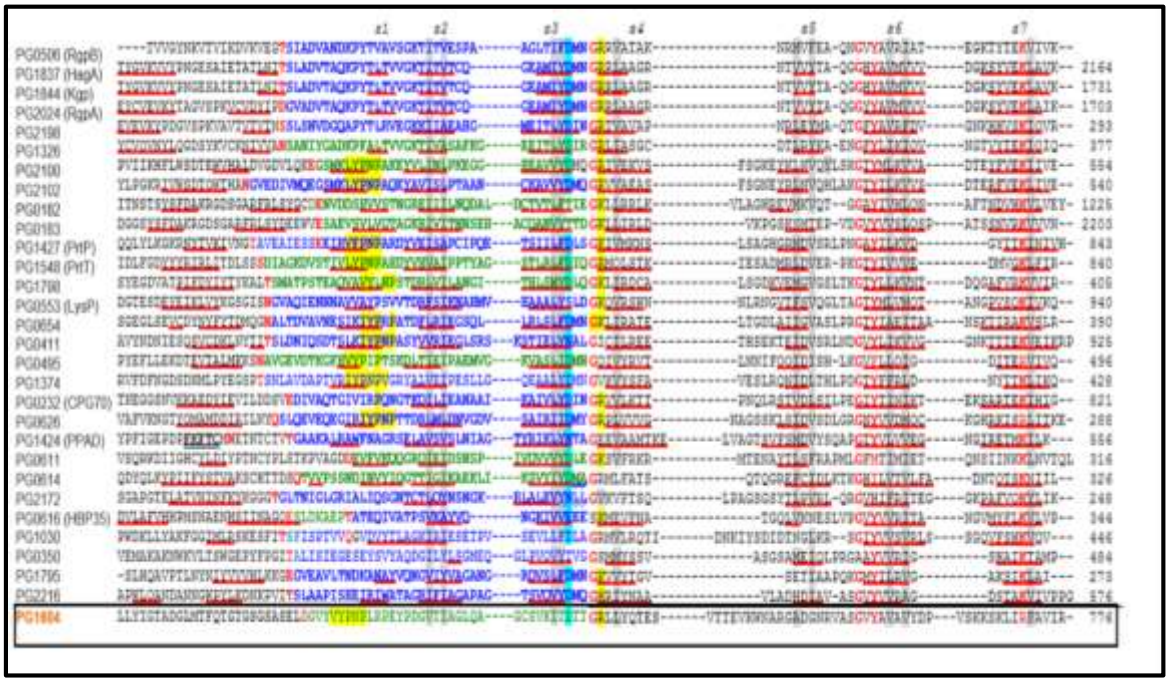

Figure 8. The structure-based alignment of the CTD of proteins secreted by *P. gingivalis* via T9SS (Adapted from Veith., et al, [4]). Conserved hydrophobic residues in β-strands are highlighted.
Bold blue font - CTDs with experimentally determined cleavage sites
Green bold font – cleavage sites predicted from alignment.
L/V-F/Y-P-N-P-A/V-K/R-E/D- a sequential motif conserved in some T9SS secreted proteins. **Thick red** underlining – predicted β-strands
KRVQTIYRNRK – predicted alpha-helixes; S_1- S_7 - β-strands

PG_RS07070 / PG1604 (TIGR DATABASE) / PG1403 (ALAMOS DATABASE) is a unique CTD protein of *P. gingivalis* and is an essential part of the T9SS (unpublished). PG1604 is 776 aa protein containing a signal peptide (1-25 aa) recognized by the Sec system [50] and CTD domain at the C-terminus potentially cleaved by T9SS during further transport. In the W50 strain it is described as immunoreactive 84 kDa antigen as it was detected during *E. coli* library screening with various rat and mouse anti-*P. gingivalis* and/or periodontal patient sera [51]. The authors did not point out this protein as a good vaccine candidate concealing any specific details. The protein was also identified in proteomic analysis of *P. gingivalis* membrane complexes and OMV's [52], but was not further characterized.

Preliminary studies have shown that deletion of PG1604 leads to the absence of the secretory phenotype (white pigmented cells) (Figure 9). Heme accumulation has been shown previously to be dependent on the production of the CTD cargo surface protease Kgp in *P. gingivalis* [53, 54] and non-pigmentation in deletion mutant ΔPG1604 suggests failure of Kgp production or proper processing.

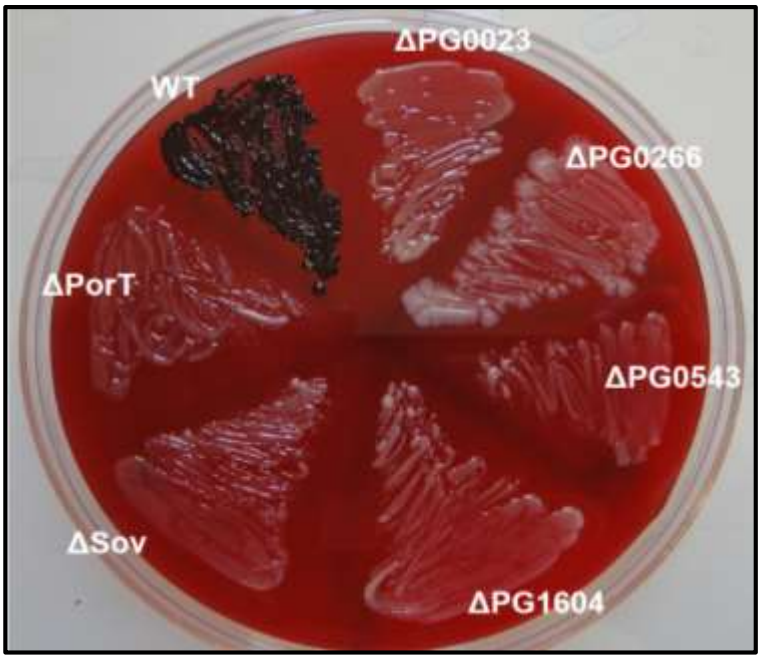

Figure 9. Knockout mutant of PG1604 and a few other T9SS proteins.
The function of PG1604 was probed by targeted mutagenesis of the protein directly in *P. gingivalis* and analyzed effect of the isogenic *PG1604* gene deletion. The mutants yielded non-pigmented colonies indicating a failure to accumulate heme on the cell surface.

Similarity searches of predicted protein tertiary structure performed with a use of RaptorX server revealed a matching tertiary template denoted 4A2L(PDB ID: 4A2L) of the BT3049 protein [55]. TB3049 is a periplasmic domain of a hybrid two- component system (BT4663) of *Bacteroides thetaiotaomicron* [55]. It possesses two β-propeller domains and a C-terminal Ig-like fragment (~120 residues), which is a backbone for homodimerization. This C-terminus is followed by kinase receptor domain anchored in the IM facing the cytoplasm. The 3D model of PG1604 built on the 4A2L template is composed of 2 consecutive β-propeller structures (1β:Q^{26}-L^{311},2β; T^{318}-F^{677}) each consisting of 7 blades followed by the CTD domain of the Ig-like fold encompassing of 6 β- strands (Figure 10), thus revealing structural similarity with BT4663.

β-propellers are known to provide a good platform for protein-protein interactions possessing a variety of functions [56]. Further studies are needed to characterize the structure- function and cell localization of PG1604.

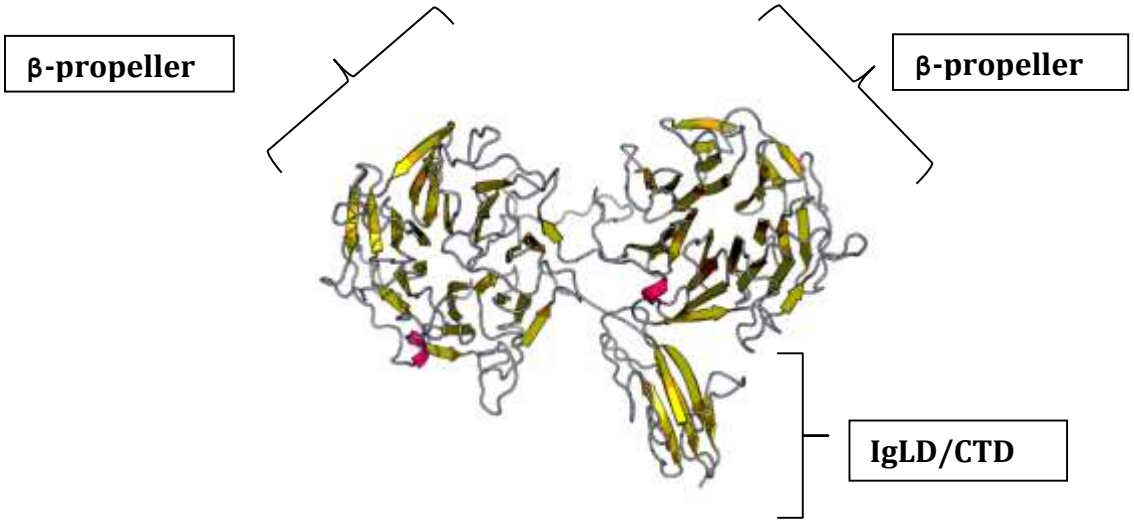

Figure 10. Tertiary structure prediction of PG1604 protein. The model was performed on RaptorX structure prediction server (http://raptorx.uchicago.edu). The most reliable template identified was the putative hybrid two component system BT3049 from *Bacteroides thetaiotaomicron* (PDB ID:4a2lA and 3v9fA) [55] [57]. The quality of a predicted 3D structure model was validated with following measures values: P-value: 3.10e-24; uGDT (GDT): 271 (35); uSeqId (SeqId): 91 (12); Score: 437. Arrows indicate β-strands.

CHAPTER 2

RESEARCH HYPOTHESIS AND SPECIFIC AIMS

Research Hypothesis

We hypothesize that selective modifications of the C - terminal domain of PG1604 by insertion of oligohistidine motives or substitution of 6 consecutive residues with hexahistidine, could affect the secretory phenotype of *P. gingivalis*.

Specific Aims

Aim 1: To probe the structure- function relationship of PG1604 by insertion and substitution with the oligohistidine motif in the CTD and to monitor the changes the in *P. gingiv*alis secretory phenotype caused by mutations (Figure 11).

Aim 2: To determine the post-translational processing and sub-cellular localization of the native and modified PG1604 proteins.

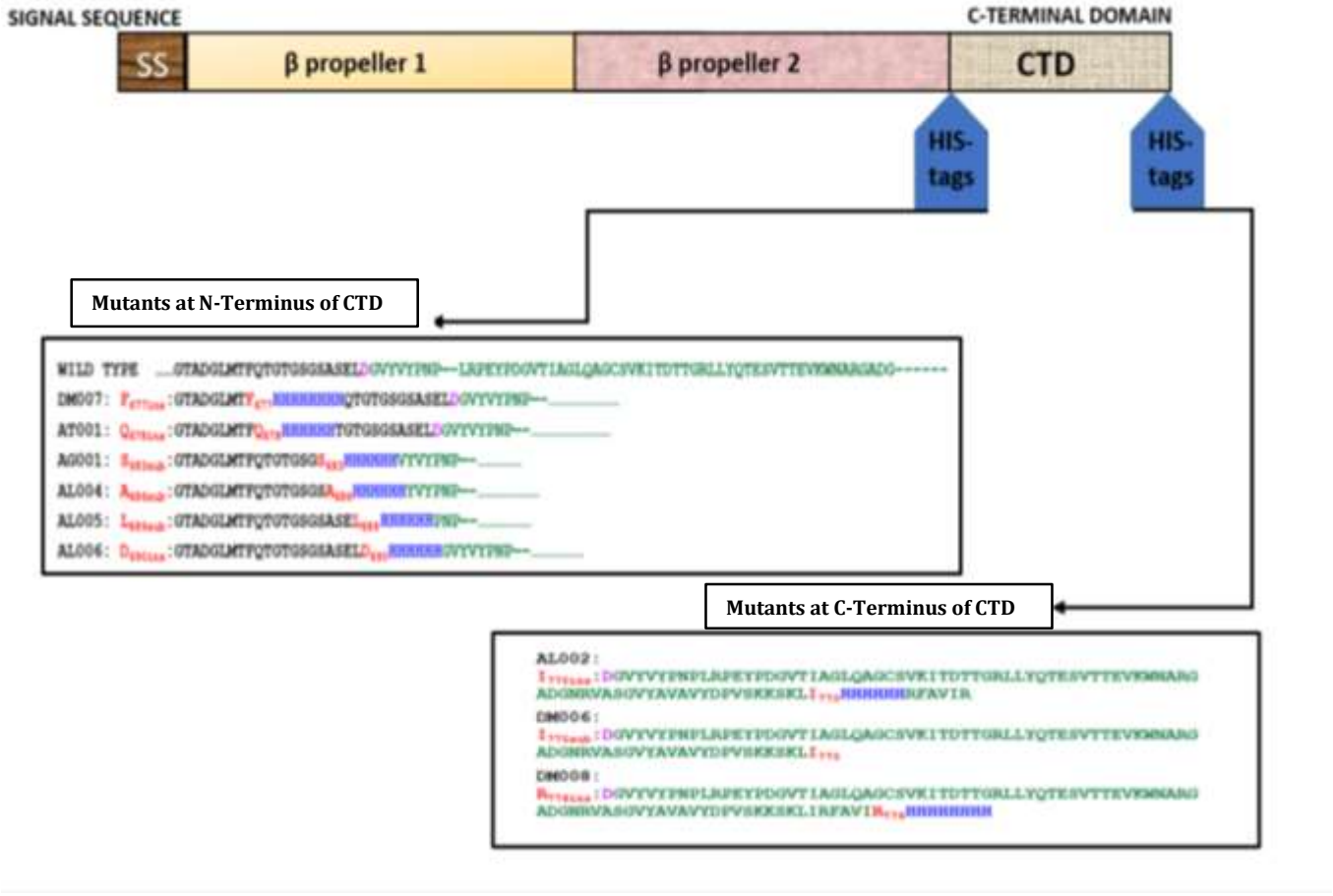

Figure 11. Schematic view of the PG1604 (PorZ) domain structure and details of oligohistinde insertions/substitutions. The names of *P. gingivalis* mutated strains are given on the left of each sequence. **Green** color represents CTDs with their **cleavage site 'D'**. HIS-tag insertions/substitutions are represented by **blue color**, at various sites of CTD indicated by **red** color.

CHAPTER 3

MATERIALS AND METHODS

3.1 Bacterial strains and general bacterial culture conditions

Wild-type strain *P. gingivalis*, W83 and mutant derivatives (Table 2) were cultured in enriched trypticase soy broth medium (eTSB: 30 g trypticase soy broth, 5 g yeast extract, per liter, pH 7.5 and supplemented with 0.5 g L-cysteine, 5 mg hemin and 2 mg menadione) or on blood eTSB agar (eTSB medium + 15 g agar per liter and supplemented with 4% defibrinated sheep's blood) at 37°C in an anaerobic chamber in an atmosphere of 90% N_2, 5% CO_2 and 5% H_2. *Escherichia coli* strain NEB® 5-alpha (New England BioLabs DH5α derivative) was used for all plasmid construction work and was grown in Luria-Bertani (LB) medium and agar. For antibiotic selection in *E. coli*, ampicillin was used at 100 µg/ml and for *P. gingivalis*, erythromycin at a concentration of 5 µg/ml. A schematic summarizing the methods employed in the study is depicted in Figure 14.

Table 2. Bacterial strains used in the study

***P. GINGIVALIS* STRAINS USED IN THIS STUDY**		
STRAIN	**DESCRIPTION**	**SOURCE/REFERENCE**
W83	Wild-type	Reference [58]
AT001	**Insertion** of 6xHIS at site **Q_{678}** in PG1604/ PG_RS07070	This study
DM007	**Insertion** of 6xHIS at site **F_{677}** in PG1604/ PG_RS07070	Laboratory collection
AG001	**Substitution** of 6xHIS at site **S_{683}** in PG1604/ PG_RS07070	Laboratory collection
AL004	**Substitution** of 6xHIS at site **A_{686}** in PG1604/ PG_RS07070	Laboratory collection
AL005	**Insertion** of 6xHIS at site **L_{689}** in PG1604/ PG_RS07070	Laboratory collection
AL006	**Substitution** of 6xHIS at site **D_{690}** in PG1604/ PG_RS07070	Laboratory collection
AL002	**Insertion** of 6xHIS at site **I_{770}** in PG1604/ PG_RS07070	Laboratory collection
DM006	**Substitution** of 6xHIS at site **I_{770}** in PG1604/ PG_RS07070	Laboratory collection
DM008	**Insertion** of 6xHIS at site **R_{776}** in PG1604/ PG_RS07070	Laboratory collection
ΔPG1604	Knockout mutant-PG1604*:: Erm*	Ky-Anh Nguyen, Australia
***E. COLI* STRAINS USED IN THIS STUDY**		
NEB 5-alpha	DH5-alpha derivative; Genotype: *fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	New England BioLabs

Table 3. Plasmids used in the study.

Plasmid	Description	Source/Reference
p1403CeB-H	Master plasmid of PG_RS01060 with erythromycin resistant gene with ampicillin selection in *E. coli* cells and erythromycin selection in *P. gingivalis* cells	Ky-Anh Nguyen, Ph.D. University of Sydney, Australia
pAT1	Mutagenesis plasmid carrying insertion of 6HIS encoding tag at Q678 position of PG1604	This study

3.2 Analysis of growth rates of *P. gingivalis* mutant strains

The growth rates of PG1604 mutants (Table 2) were compared with that of the wild-type strain W83 and ΔPG1604 Dilutions (1:100) of overnight bacterial cultures were inoculated into eTSB to reach starting OD_{600} 0.1 and the bacteria were grown anaerobically without shaking at 37°C. At a time interval of 2 hours, optical density at 600 nm was determined. This was done over a period of 24 hours, through a direct reading in special glass tubes, without exposing the cultures to oxygen. A growth curve was generated by plotting the optical density (y-axis) versus time (x- axis).

3.3 Insertion of hexa-histidine and site-directed mutagenesis of the master plasmid construct

SLIM (Site-directed, Ligase- Independent Mutagenesis) PCR was carried out using Phusion High-Fidelity DNA polymerase (Thermo Scientific) according to 3-step protocol provided by the manufacturer manual (Table 4 and 5). This PCR was used to create insertions of the oligonucleotide motif encoding hexa-histidine into designated sites in the genes of interest in the master plasmids. In essence, for the mutagenesis reaction, four primers were designed (Table 6), adjacent to each other but have opposite orientation. One

pair of primers contained 5' extension encoding for the hexa-histidine peptide. After specific mutation, FD DpnI (Thermo Scientific) was used to remove the non-mutated template.

Table 4. SLIM PCR reaction mixture

COMPONENT	FOR 50µL	FINAL CONCENTRATION	
5X Phusion HF Buffer	10 µL	1X	
10Mm dNTP	2 µL	400 µM	
100% DMSO	1.5 µL	3%	
PRIMERS-4 pcs (10 µM)	4 x 3 µL	250ng	4 pcs primers in one reaction: 2 with insertion/substitution fragment and 2 non-tailed complementary to matrix DNA
Template DNA	X µL	75ng	
Phusion Polymerase	0.5 µL	0.02 U/ µL	
Distilled Water	To fill up to the final reaction volume of 50 µL		

Table 5. PCR 3 step cycling used to for mutagenesis

STEP	TIME	TEMPERATURE	CYCLES
1.Initial	30 seconds	98°C	**1X**
2. Denaturation	5-10 seconds	98°C	**34X**
3. Annealing	10-30 seconds	68°C	**34X**
4. Extension	15-30 s/kb	72°C	**35X***
5. Final Extension	10 minutes	72°C	**1X**
6. Storage	**Hold**	**4°C**	∞

*Repeat from step 2 for 35 times more

Table 6. Primers used to insert 6XHIS tags in the plasmid

Master plasmid/ Gene	Insertion Site	Primer names	Primer Sequence
p1403CeB-H/ PG1604	Q678	PG1604_i_Q678_FwA	**CATCACCATCACCATCAC**ACGG GTACGGGGAGTGGATCAGC
		PG1604_i_Q678_RevB	**GTGATGGTGATGGTGATG**TTGG AACGTCATCAGTCCGTCCG
		PG1604_nt_Q678_FwC	ACGGGTACGGGGAGTGGATCAGC
		PG1604_nt_Q678_RevD	TTGGAACGTCATCAGTCCGTCCG

***Blue font** **indicates nucleotides encoding for hexa-histidine tag.**

3.4 Agarose gel electrophoresis

The DNA of plasmids or PCR products were separated through agarose gel electrophoresis.

3.4a Preparing the gel

1. 0.8% agarose gel was made by mixing 0.8g agarose with 98 ml distilled water and 2 ml 50X TAE buffer (final concentration 1xTAE).

2. The mixture was heated in a microwave oven until all agarose had melted and the solution had started to boil.

3. The gel solution was then left to cool (to approximately 65 °C).

4. Midori Green Advance DNA Stain (4 µl - 6µl; Cat # MG 04) was added after the mixture had cooled, and gently mixed into the agarose

5. The gel was poured slowly into a gel rack, the comb was set on one side of the gel, and any bubbles in the solution removed. The gel was allowed to set (15 to 20 min.).

6. After 20 min., when the gel had solidified, the comb was removed, and the gel, together with the rack, was soaked into a chamber with 1X TAE gel buffer. The gel was placed with the wells facing the electrode that provide the negative current (cathode).

3.4b Loading and running the gel

Loading buffer was added to the samples in order to visualize and sediment it in the gel wells.

1. A DNA ladder (Thermo Scientific GeneRuler 1kb DNA Ladder shown in Figure 12), a mixture of DNA fragments of known size, was loaded along with analyzed samples. This was used to determine the size of the separated DNA molecule by comparing their migration with that of the ladder.

2. The samples were loaded into the wells and the lid of the electrophoresis chamber was closed and the current was applied. The gel was run at 90-120 volts (usually 50 min.).

3. The Midori Green Advance DNA stained gel was visualized under UV light and photographed.

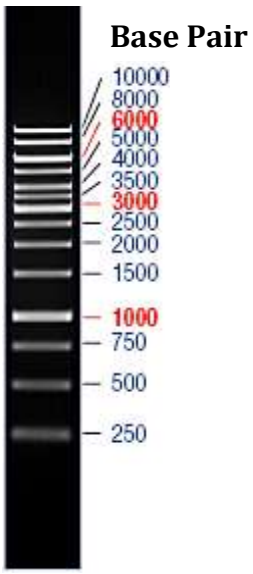

Figure 12. One kb DNA Ladder (Thermo Scientific, Cat # SM0313).

3.5 Transformation of plasmid into competent *E. coli* NEBα cells

Transformation into competent cells was carried out according to the manufacturer's protocol and after that a few (usually 4) antibiotic-resistant colonies were picked and transferred to LB medium (LB agar plate as well as liquid). The plates were kept in incubation at 37°C and liquid media was incubated at 37°C with vigorous shaking at 225 rpm overnight. The plates were stored at 4°C and the plasmid was extracted from the liquid culture using the GeneJET Plasmid Miniprep Kit.

3.6 Extraction of plasmids from bacterial cells

GeneJET Plasmid Miniprep Kit was used for efficient purification of plasmids. Steps were followed according to the manufacturer's protocol. The mutated plasmids (with insertions of the hexa-histidine encoding sequence) were confirmed through sequencing (see 3.8).

3.7 DNA concentration measurements

Nanodrop 2000 spectrophotometer from Thermo Scientific was used to measure the DNA concentration.

3.8 DNA Sequencing

DNA samples (plasmids or PCR products) were sent for sequencing to The Center for Genetics and Molecular Medicine Sequencing Service, University of Louisville.

Sample Preparation

0.4 µg of clean plasmid DNA or 6ng per 100bp of PCR product in total volume of 10.4 µl were mixed with 1.6 µl of appropriate primer (Table 7) of 20µM stock solution.

Table 7. Primers used in the study for sequencing

Primer Name	Target gene	Primer Sequence
PG1604_RevHind	PG1604	GACAAGCTTTGTGTCAGCGAATCACTG
PG1604_seq_rev1	PG1604	TCCGGCTGCATTGTAGAC
PG1604_seq_rev2	PG1604	CGAACAGACCGTTTCC
PG1604_seq_rev3	PG1604	ATAGCTCCGGCTCAAACG
PG1604_seq_rev4	PG1604	TTAGTTGCTCGGAGTAGG
PG1605_seq_fwd	PG1605	TGGGATCAATTGGAGAAATC
ErmF_seq_fwd1	ermF	TGGACGGACAATTAAAGC
ErmF_seq_fwd2	ermF	TTCTGGGAGGTTCCATTG
ErmAM_seq_fwd1	ermAM	TGGCGATGGAGCGGAAAC
ErmAM_seq_fwd2	ermAM	TGGATATTCACCGAACAC

3.9 Generation of isogenic mutants via homologous recombination

Insertions of DNA fragments encompassing modified genes of interest (bearing the 6xHIS encoding motif) into the *P. gingivalis* genome was performed through homologous recombination. Suicide plasmids containing mutated genes of interest were introduced to *P. gingivalis* cells by the electroporation. Briefly, a 40-ml mid-log-phase culture of *P. gingivalis* was harvested by centrifugation at 6,000 × *g* for 15 min., washed three times in electroporation buffer (10% glycerol [vol/vol], 1 mM $MgCl_2$), and re-suspended in 0.5 ml of the same buffer. All steps were carried out at 4°C. Two hundred microliters of the cell suspension was transferred into a pre-chilled 0.2-cm-gap width electroporation cuvette along with 1 μg of plasmid DNA. Electroporation was carried out at 2.5 kV and 10μF capacitance using a micropulser electroporation apparatus (Bio-Rad Laboratories), resulting in time constants of approximately 4.5 msec. After electroporation, the cells were allowed to recover on ice for 2 min. before the addition of 1 ml of eTSB medium to the suspension. Cells were allowed to grow anaerobically at 37°C overnight before being plated onto antibiotic selective media. Obtained mutant was named AT001 and was further sub cultured on selective plates, and genomic integration by a double crossover event was

confirmed by PCR (see section 3.11). DNA sequencing of the appropriate region of the genome, was used to further verify the mutant AT001 using appropriate primers (Refer Table 7).

3.10 Chromosomal DNA isolation

Genomic DNA from *P. gingivalis* AT001 mutated strain was purified using GeneJET Genomic DNA Purification Kit (Thermo Scientific) as described in the protocol provided by the manufacturer.

3.11 PCR reaction to amplify the region of interest in the chromosomal DNA

Chromosomal DNA purified from AT001 served as a template to amplify the region of interest – PG1604 gene, to confirm 6xHIS tag presence. Phusion High-Fidelity DNA polymerase, (Thermo Scientific) was used (Table 8, 9 and 10).

Table 8: Reaction mixture phusion high-fidelity DNA polymerase PCR

COMPONENT	FOR 50µL	FINAL CONCENTRATION
5X Phusion HF Buffer	10 µL	1X
10mM dNTP	1 µL	400 µM
AT001 DNA	1.5 µL	50 ng
Forward Primer (PG1604 SallF)	1 µL	100 µM
Reverse Primer (PG1604 Rev_Hind)	1 µL	100 µM
Phusion Polymerase	0.5 µL	0.02 U/ µL
Distilled Water	To fill up to the final reaction volume of 50 µL	

Table 9. Primers used to amplify the region PG1604 in the chromosomal DNA from clone AT001

Primer Name	Primer Sequence	Purpose
PG1604 SallF	CAGGTCGACATGGAGGAAGGTATT TGGAAATACC	Forward primer in the amplification of PG1604
PG1604_ RevHind	GACAAGCTTTGTGTCAGCGAATCAC TG	Reverse primer in the amplification of PG1604

Table 10. PCR 3 step cycling used for amplification

STEP	TIME	TEMPERATURE	CYCLES
1.Initial	30 seconds	98°C	**1X**
2. Denaturation	5-10 seconds	98°C	**34X**
3. Annealing	10-30 seconds	62°C	**34X**
4. Extension	15-30 s/kb	72°C	**35X***
5. Final Extension	10 minutes	72°C	**1X**
6. Storage	**Hold**	**4°C**	∞

*Repeat from step 2 for 35 times more

3.12 Gingipain activity assay

The Gingipain activity assay was performed to quantify the secreted gingipains in the mutants and later compared with that of the wild type W83 and ΔPG1604. The amidolytic activities of Rgp and Kgp gingipains were assessed by the hydrolysis of the chromogenic substrates: Bz-Arg-pNA and Ac-Lys-pNA, respectively (Bachem). In a 96-well format, samples (in triplicates) were pre-incubated in assay buffer (200 mM Tris-HCl, 100 mM NaCl, 5 mM $CaCl_2$, pH 7.6; freshly supplemented with L-cysteine to 10 mM) for 2 min. prior to the addition of specific substrate to the final concentration of 0.5 mM in a total

volume of 200 µl. The rate of formation of p-nitroanilide (pNA) was measured using absorbance at 405 nm every 20 s over a 10 min. period using a spectrophotometer (SpectraMax M5, Molecular Devices). Enzymatic analysis was performed for whole cell cultures adjusted to OD_{600} - 1.0. Equal volumes of all strains were applied (10 µl for Kgp and 5 µl for Rgp assay) and specific activity was measured as a function of density, and time per ul (mOD//min/µl). Later, total Rgp/Kgp activity in W83 wild-type strain served as positive control and was defined as 100%, allowing cross-comparison between independent experiments and statistical analysis.

3.13 Cellular compartment fractionation for *Porphyromonas gingivalis* W83

Subcellular localization of PG1604 protein in *P. gingivalis* W83 was done using the following protocol [59].

1. 15 ml of culture was grown as grown as per defined conditions (refer section 3.10). OD600 was adjusted to 1.5 prior to fractionation. 0.5 ml of **culture sample** was collected and 15 ml of the adjusted culture was centrifuged at 6000 x g, 15 min. at 4^0 C.
2. The cell pellet was washed once with 15 ml PBS at 4^0 C by re-suspension and re-pelleting, which was re-suspended in 5ml of 0.25 M sucrose, 30mM TRIS, pH 7.6 (solution at RT). Cells were left mixing gently for 10 min. at 4^0 C.
3. Next, the cells were pelleted 12,500 x g for 15 min. at 4^0 C and rapidly re-suspended in 5 ml ice-cold dH_2O. Cells were left mixing gently for 10 min. at 4^0 C.
4. The suspension was pelleted by centrifugation at 12,500 x g for 15 min. at 4^0 C and the supernatant was designated as the **periplasmic/PP sample**.

5. The remaining spheroplasts were washes once with 15 ml PBS, pelleted at 10,000 x g and re-suspended in 5 ml PBS and ultrasonicate for 10 x 5 second pulse (17W per pulse) in ice-water bath with 2 second rest between each pulse. Cellular debris and membranes were pelleted by ultracentrifugation at 150,000 x g for 1 hour at 4^0 C. Supernatant was designated as the **cytoplasmic/CP sample**.
6. The pellet was washed once with 20 ml PBS and re-suspended by first scraping with the sonication tip in 5 ml cold PBS. A minimum number of pulses (usually 2 or 3 x 5 second pulses) were used to disperse the chunks and 0.5 ml of it was collected. Designate it as the **membranes/CE sample**.
7. Cold 0.5 ml of 10% Sarkosyl (lauryl sarcosine; final concentration is 1%) was added and the mixture was left to mix gently for 20 min. at 4^0 C to dissolve the inner membrane or cold 0.5 ml of 10% Triton X-100 with 200 mM $MgCl_2$ (final concentration: 1% Triton X-100 was added), and the mixture was left to mix gently for 30 min. at 4^0 C to dissolve the inner membrane.
8. The Triton X-100- resistant outer membrane was pelleted by ultracentrifugation at 150,000 x g for 1 hour at 4^0 C. The supernatant was designated as the **inner membrane/IM sample** and the remaining pellet was washed once with 20 ml PBS prior to re-suspension in 5 ml PBS by sonication as before and designated as the **outer membrane/OM sample**.

3.14 Purification of the PG1604 from *P. gingivalis* AL005 mutant strain

The growth media of the *P. gingivalis* mutant strain AL005 (L689 substitution 6xHistidine) were clarified by centrifugation at 8,000xg, 30 min. at 4°C. The pellet was re-suspended in cold PBS (approximately 10ml for 1liter pellet). Inhibitor TLCK (100mg -1.36 ml DMSO@ 200mM stock) was added to the mixture to attain final concentration of 1mM. The sample was then sonicated (for 1liter pellet – 1cycle ; 10 pulses ; 3sec ; 30 - 40% power ; 17- 20 watts). The sonicated sample was centrifuged at 150000xg for 1hr at 4°C. The clarified sample was dialyzed against 4 liters of the Binding buffer (20mM sodium phosphate, 500mM sodium chloride, 20mM Imidazole, 0.02% sodium azide; pH – 7.4). The purification column containing Ni Sepharose 6Fast Flow (GE Healthcare) was equilibrated with the Binding buffer before loading a sample. After extensive washing with Binding buffer, protein fraction bound to the matrix was eluted with Elution buffer (20 mM sodium phosphate, 0.5 M sodium chloride, 500 mM imidazole; pH 7.4). Obtained eluate was concentrated by ultrafiltration and the purified sample was further analyzed by western-blot.

3.15 Western- Blot

3.15a Sample preparation:

P. gingivalis W83 and mutant strains (Table 2) were anaerobically grown until OD_{600} 1.5-2.0. 1.5ml of culture subjected to centrifugation at 6,000xg, 15 min. at 4°C and the cells was suspended in 280 µl of PBS (reaching a final volume of approx. 315 µl). Cell were incubated with 35µl TLCK (original concentration 200mM) at RT for 10 min. and sonicated (5 pulses; 3sec; 30 - 40% power; 17- 20 watts). Then 50 µl NuPAGE LDS

loading buffer was added, heated at 100°C for 5 min., finally 50 µl NuPAGE LDS loading buffer with 10% BME was added, heated at 100°C for another 10 min.

3.15b Electrophoresis and transfer of proteins

Total 30-50 µl of *P. gingivalis* whole cell lysates (250 µg of lysate loaded) or purified AL005 concentrate fraction were electrophoresed by using 4-12% Bis-Tris SDS-PAGE (Invitrogen) or Novex® 10-20% Tricine Gel (Invitrogen) at 120 volts for 90 min. Subsequently, proteins were transferred onto nitrocellulose (BioRad) at 100 volts for 60 min. (wet transfer), and then blocked with SuperBlock™ (PBS) Blocking Buffer (Thermo Scientific) for 2h. The membranes were probed with appropriate primary and secondary antibodies (in PBST: 1xPBS with 0.05% Tween 20) against standard (Figure 13).

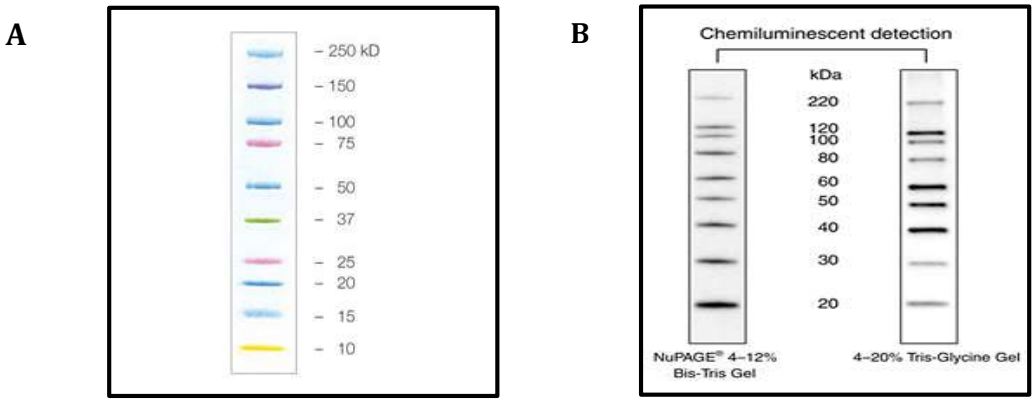

Figure 13. Standard used western-blot analysis: **(A)**Precision Plus Protein™ Kaleidoscope™ Standards #161-0375. **(B)**MagicMark™ XP Western Protein Standard #LC5602.

- Antibodies used to detect RgpB and Kgp:

Primary antibodies: anti-Kgp (7B9.F10.F6); 1:250 and anti-RgpB monoclonal (Mouse; 18E6.F7 IgGI); 1:250; Secondary antibodies: anti-mouse (Bio Legend 405306); 1: 5000

- Antibodies used to detect histidine tags and PG1604

Primary antibodies: anti-HIS monoclonal (Mouse; BD552565); 1:2000 and anti-PG1604 polyclonal (Mouse); 2µg; Secondary antibodies: anti-mouse (Bio Legend 405306); 1: 5000

3.15c Visualization of transferred proteins

Membranes were developed using chemiluminescent substrate for HRP – horseradish peroxidase (Thermo Scientific Super Signal West Pico Cat # 34080) at 30 second exposure increments (Imagequant Las 4010).

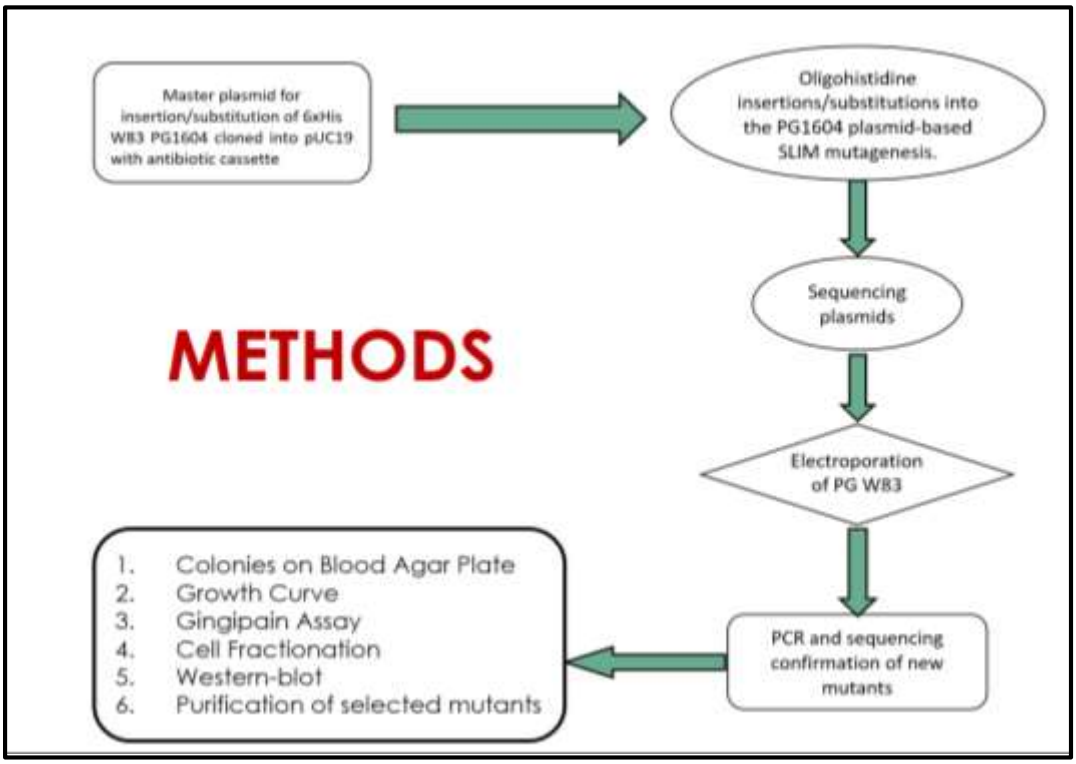

Figure 14. Schematic summary of the experimental approach employed in this study.

CHAPTER 4

RESULTS

The *P. gingivalis* W83 mutant strain expressing the HIS-tag modified PG1604 protein was generated through double crossover recombination with the constructed suicide plasmid. The plasmid was created using the SLIM PCR method using DNA of the master plasmid - p1403CeB-H as a template (see point 4.1 below) the final plasmid was introduced into *P. gingivalis* cells via electroporation. The mutated *P. gingivalis* strain was then used to analyze the phenotype together with other HIS-tagged mutants available as a laboratory collection (created in analogous manner) The wild-type W83 and knock-out (Δ1604) strains were used as positive and negative controls, respectively. Details of all results are presented below.

4.1 Construction of Master Plasmid, p1403CeB-H

The master plasmid p1403CeB-H (7947 bps) was provided by Dr. Ky-Anh (Institute of Dental Research, Westmead Centre for Oral Health and Westmead Millenium Institute, Sydney, Australia). It is pUC19 derivative (*E. coli* vector) which was created by sequential cloning of 3 DNA fragments: a full coding region of the PG1604 gene, followed by the erythromycin resistance cassette (*ermF ermAM* genes) and a fragment of the downstream PG1605 gene. Its construction was confirmed by sequencing. A schematic view of the master plasmid p1403CeB-H is shown in Figure 15.

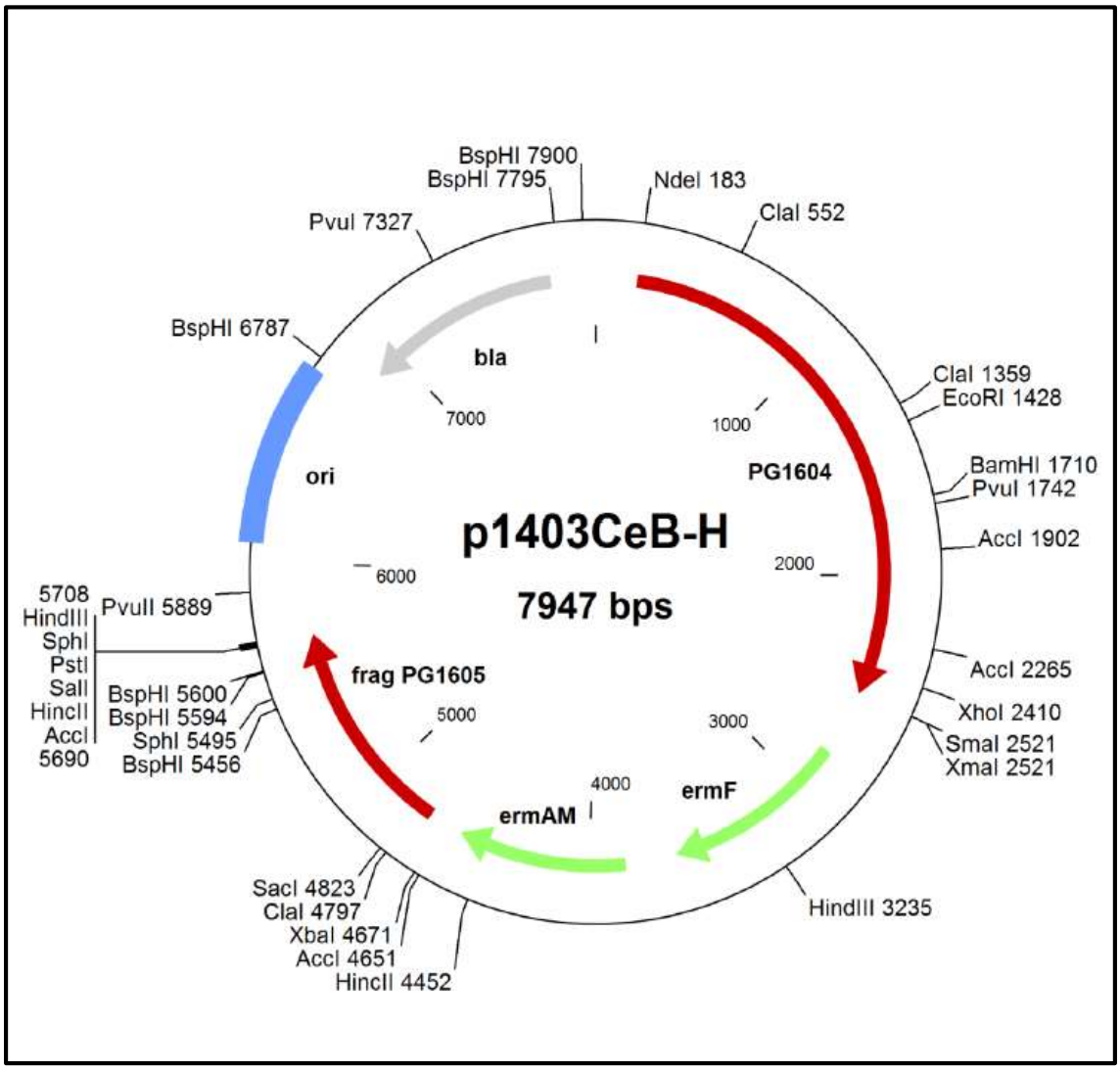

Figure 15. Schematic view of the master plasmid p1403CeB-H.
Blue – *E. coli* origin of replication; **Gray** – *bla* gene, *E. coli* ampicillin selection marker; **Red**- full coding region of PG1604 gene and fragment of downstream PG1605 gene; **Green**- erythromycin resistance cassette (*ermF ermAM* genes). Selected restriction enzymes are marked outside in black.

4.2 Insertion of hexa-histidine and site-directed mutagenesis of the master plasmid p1403CeB-H

Phusion SLIM (Site-directed, Ligase- Independent Mutagenesis) PCR (refer Materials and Methods 3.3) was employed for hexa-histidine insertion at the position Q678 of C-terminal domain of PG1604 (Figures 16 &17)

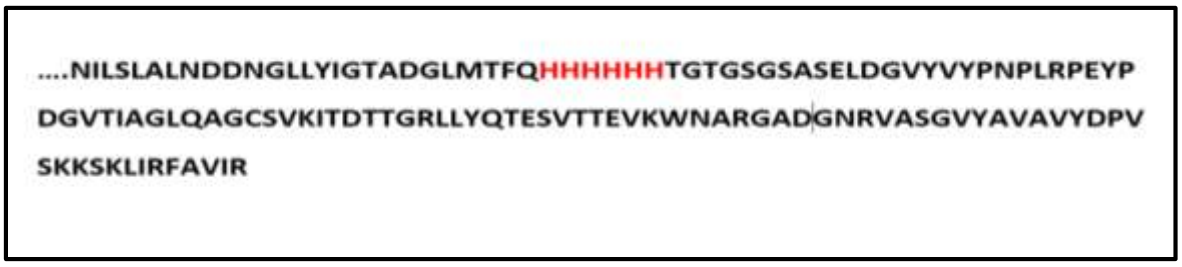

Figure 16. A fragment of PG1604 sequence with 6xHIS insertion at the position Q678 (red font).

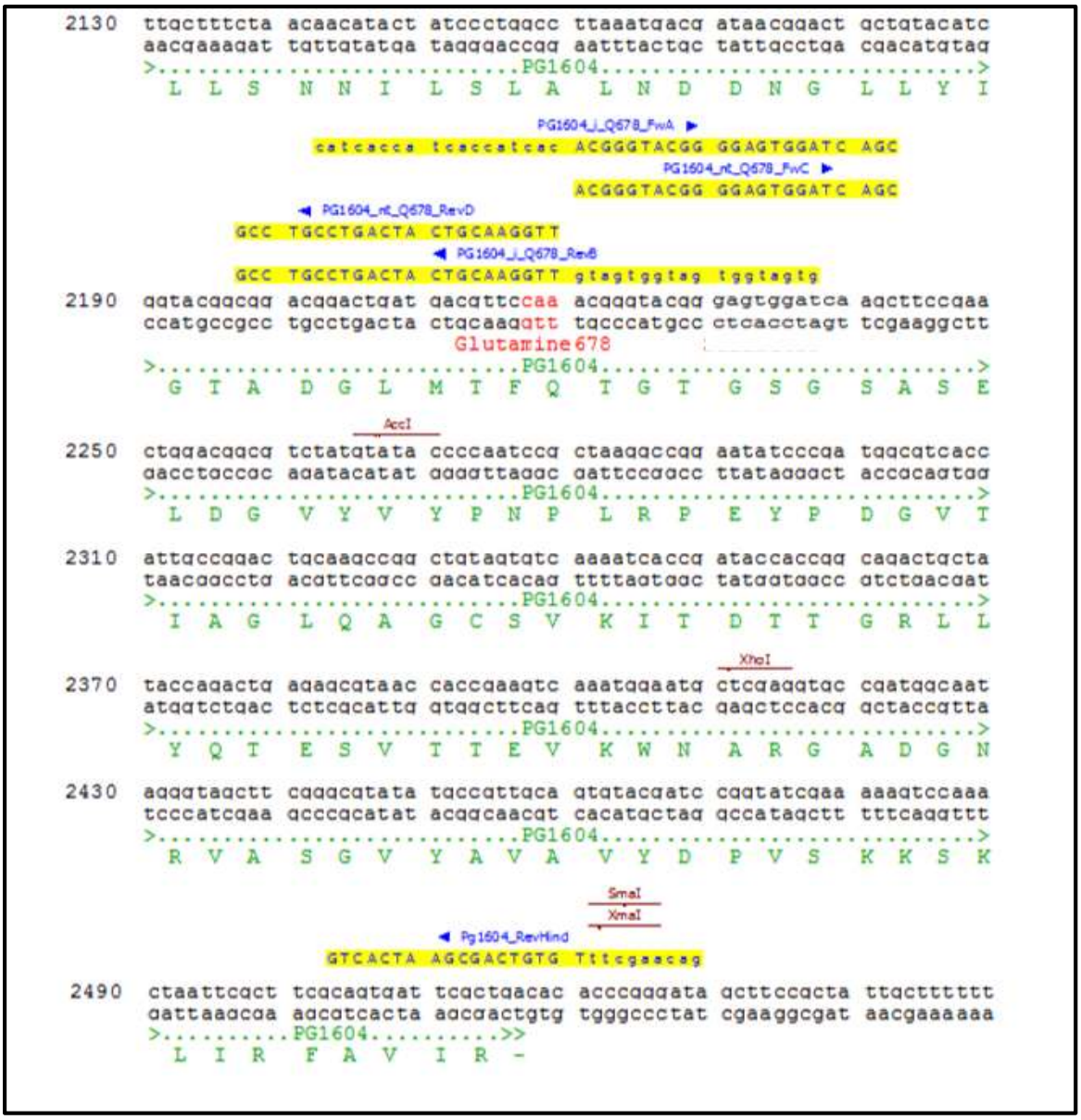

Figure 17. A fragment of the master plasmid p1403CeB-H sequence, representing the region of 6xHIS insertion at the position Q678 (marked with red) in PG1604. Primers used for mutagenesis and sequencing are highlighted in yellow. Amino acid sequence of PG1604 is marked in green.

E. coli cells were transformed with the created plasmid and clones were selected on LB agar plates containing ampicillin. Plasmid DNA was isolated from several colonies and screened for the correct mutation by DNA sequencing of the pertinent region (Figure 18) followed by full plasmid sequencing of the HIS-tagged clone. The correct plasmid was named/denoted pAT1.

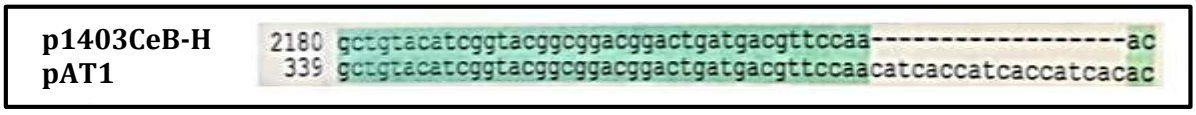

Figure 18. Alignment of the p1403CeB-H master plasmid sequence vs. pAT1 (a fragment).
The results obtained from sequencing being compared to wild-type fragment of PG1604 present in the master plasmid (p1403Che eB-H). Insertion of fragment encoding for 6xHIS (CATCACCATCACCATCAC) in the pAT1 is not highlighted.

4.3 Generation of isogenic mutant via homologous recombination

Double cross-over recombination between the mutated suicide plasmid (pAT1) and the W83 *P. gingivalis* chromosome was achieved by the electroporation method. Clone obtained on selective erythromycin plates was named AT001 and was checked for proper genomic modification by PCR followed by DNA sequencing.

In the similar way as described above, 5 other mutants at a N-terminus of the CTD (DM007, AG001, AL004, AL005, AL006) and 3 mutants at a C-terminus (AL002, DM006, DM008) were created by insertion/substitution of histidine tags at different regions (Figure 19). The deletion mutant ΔPG1604 and the wild-type W83 served as controls.

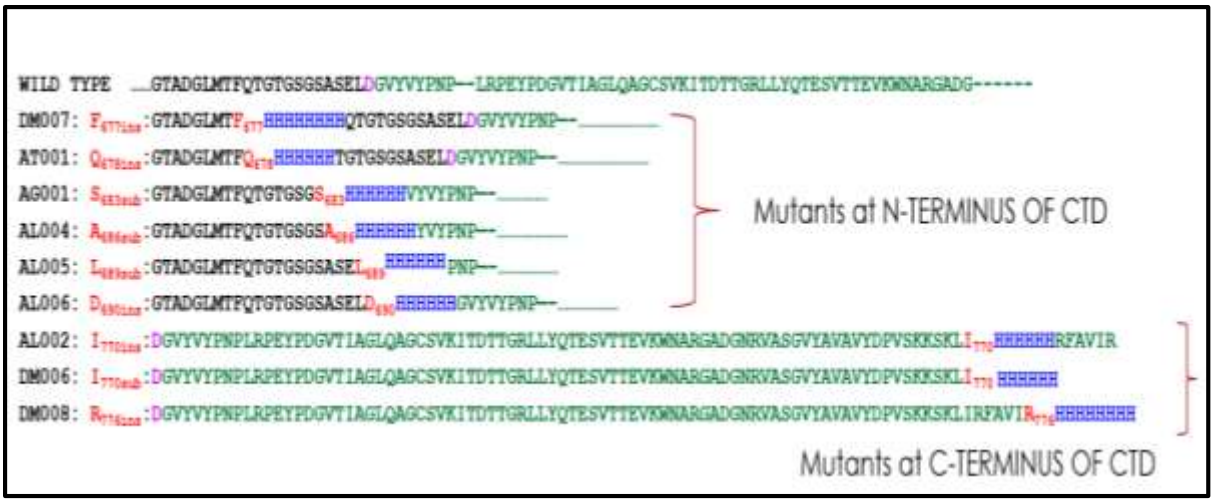

Figure 19. Mutants generated by insertion/substitution of oligohistidines at the C-terminal
domain region of PG1604. The names of *P. gingivalis* mutated strains are given on the left of each sequence. **Green** color represents the CTDs with their **cleavage site 'D'**. HIS-tag insertions/substitutions are represented by **blue color**, at various sites of CTD indicated by **red** color.

4.4 Assessment of the mutant strains on the blood agar plates

P. gingivalis W83, ΔPG1604 and all 9 HIS-tagged mutants were streaked on blood agar plates. Wild-type dark/black pigmentation was observed for most of the strains except for ΔPG1604, DM006 and AL005 which yielded white-pigmented colonies on the blood agar. AL005 turned dark with time, but hemolysis (which accompanies normal growth) was still not pronounced as in the case of other white-pigmented strains (Figure 20).

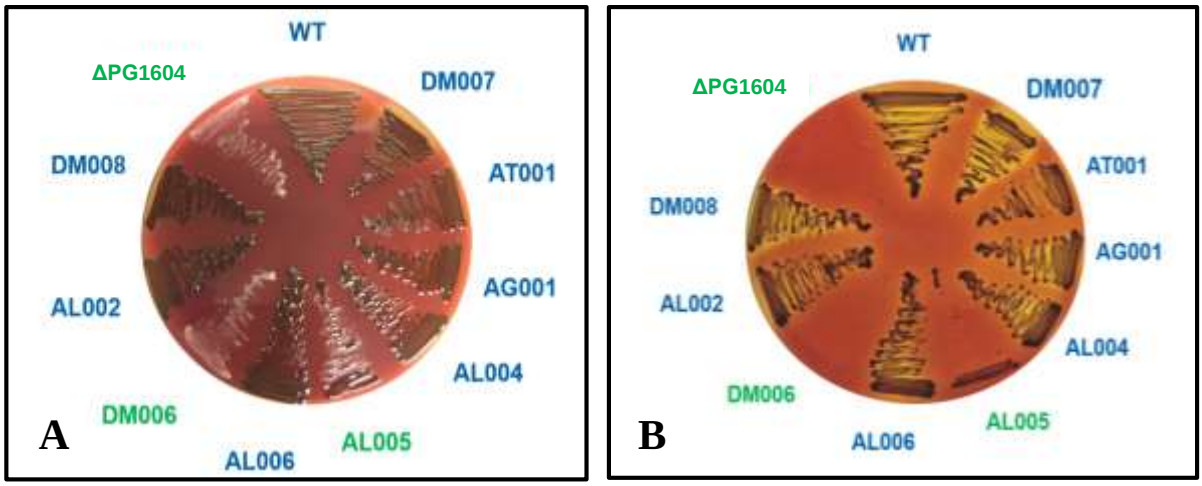

Figure 20. *P. gingivalis* mutants streaked on blood agar plates.
(A) Mutants on the 3rd day of culture with white growth observed for ΔPG1604, DM006 and AL005. (B) The 8th day of culture when all but ΔPG1604, DM006 and AL005 strains are showing hemolysis.

4.5 Analysis of growth rates of *P. gingivalis* mutant strains

The growth rates of 9 HIS-tagged mutants were compared with that of the wild-type W83 and ΔPG1604. A growth curve was generated by plotting the optical density (y-axis) versus time (x- axis) at an interval of 2 hours over 24 hours. The growth pattern of the mutants was not much different than that of the wild-type strain W83 except that a delayed exponential phase seen in ΔPG1604, DM006, DM007 and DM008 (Figure 21). Doubling times were calculated from the growth curves for three density points (OD_{600}: 0.5, 0.55 and 0.6) of the logarithmic growth phase as shown in the appendix.

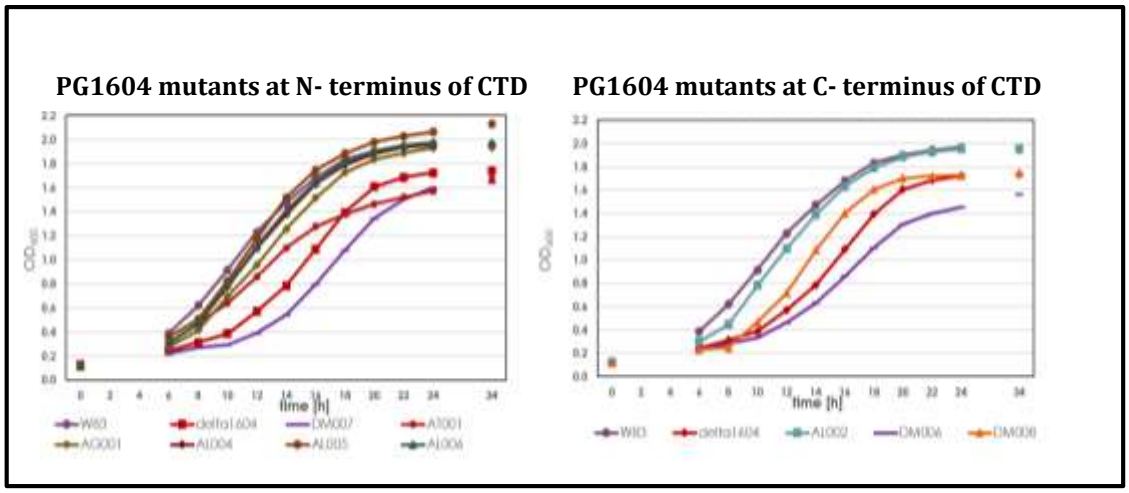

Figure 21. Growth curve of *P. gingivalis* ΔPG1604 and strains producing HIS-tagged PG1604. X-axis represents the time in hours and the Y-axis represents the optical density at 600nm taken at 2 hour interval over 24 hours. Dilutions (1:100) of overnight bacterial cultures were inoculated into eTSB to reach starting OD_{600} 0.1 and the bacteria were grown anaerobically without shaking at 37°C. Wild-type W83 was used as positive control.

4.6 Effect of oligohistidine modification of PG1604 on gingipain activity and protein processing of the mutant strains

The Gingipain activity assay was performed to quantify the secreted gingipains (Kgp and RgpA/B) in the mutants and compared with that of the wild-type W83 and ΔPG1604. t-test was used to assess whether the means of two group are statistically different from each other using the p-value. A varied gingipain activity, both Kgp and Rgp, were observed in the mutants (Figure 22C).

Hexa-histidine insertions after Phe_{677} (mutant DM007) or Arg_{776} (mutant DM008) only slightly decreased the level of gingipains activity, but insertion after Gln_{678} (mutant AT001) caused Rgp gingipain activity to drop to 35% and Kgp to 80% of wild-type. On the other hand, substitution of 6 residues after Ser_{683} (mutant AG001) and Ala_{686} (mutant AL004) with the hexahistidine motif had little effect on gingipain secretion. In contrast,

the substitution of residues after Leu_{689} (mutant AL005) nearly abolished gingipain activity. Even more dramatic effect was exerted by substitution of the 6 C-terminal residues I_{770}RFAVIR with histidines (mutant DM006), which totally obliterated both gingipains activities of the mutant (Figure 22C). In contrast, hexahistidine insertion after Ile_{770} (mutant AL002) did not affect gingipain activity.

Western-blot analysis for the PG1604 mutants was done using specific monoclonal antibodies against of Kgp, Rgp and the results correlated with that of the gingipain activity assay (Figure 22B).

Gingipains are normally expressed with a large N-terminal propeptide, followed by the catalytic protease domain (circa 50 kDa) and hemagglutinin/adhesin domains (except RgpB) and finally the CTD at the C-terminus. During maturation, the nascent polypeptides are proteolytically processed so that only the catalytic and hemagglutinin/adhesin domains remain as a non-covalently associated complex and are secreted onto the cell surface [60]. The released gingipains are additionally modified by the covalent attachment of A-LPS, which in the case of RgpB, is attached directly to the catalytic domain to result in an increase of its molecular mass from 50 kDa to 70-90 kDa. This membrane anchored form of RgpB is referred to as membrane-type RgpB (Mt-RgpB) [60]. mt-RgpB is clearly visible in *P. gingivalis* mutants which showed Rgp activity close to that of the wild-type (DM007, AG001, AL004, AL006, AL002 and DM008). AT001 mutant with lowered Rgp activity (35% of the parental wild-type strain) also possesses significant amounts of mt-RgpB. Conversely, mutants DM006 and AL005 devoid of enzymatic activities showed the full-length pro-RgpB accompanied by forms with partially and fully cleaved N-terminal profragment similar to that observed in ΔPG1604 (Figure 22B). Analogous processing

results were observed for Kgp. Interestingly, minute activity of Kgp in AL005 mutant (13% of the parental wild-type strain) was sufficient for this strain to attain some processed Kgp (50 kDa) although much less pronounced than the unprocessed variant (150 kDa). This is in contrast to ΔPG1604 and DM006, which present only unprocessed Kgp (Figure 22B).

To better understand the effect of substitutions/insertions on PG1604 localization and processing, the mutants were analyzed by western-blot with antibodies specific for PG1604 and the polyhistidine sequential motif. As presented in Figure 22B, a band immunoreactive with anti-PG1604 antibody was clearly visible in all strains except ΔPG1604. Interestingly, two strains (AL004 and AL002) with normal/unaltered gingipain activity, exhibit an extra band at the lower molecular mass of ~70 kDa that is reactive to anti-PG1604 Abs. This band was the only PG1604-immunoreactive band present in the strains devoid of or low in the gingipains activity (mutants AL005 and DM006) (Figure 221B).

Western-blotting with anti-polyhistidine antibodies revealed the presence of the tag in all mutants expressing tagged-PG1604 in the loop preceding the CTD (Figure 22B left panel). However, in the case of the C-terminal mutants, the tag was detected in *P. gingivalis* expressing the C-terminally labeled PG1604 (DM008) (Figure 22B, right panel). The 70 kDa band did not react with anti-polyhistidine antibodies, suggesting that mutated PG1604 was cleaved at the C-terminus losing circa 10 kDa. In the context of an apparent C-terminal truncation of C-terminally modified PG1604, it is important to reiterate that in the AL005 mutant the 70 kDa form of PG1604 still reacted with anti-polyhistidine antibody as the tag lies further upstream (Figure 22B).

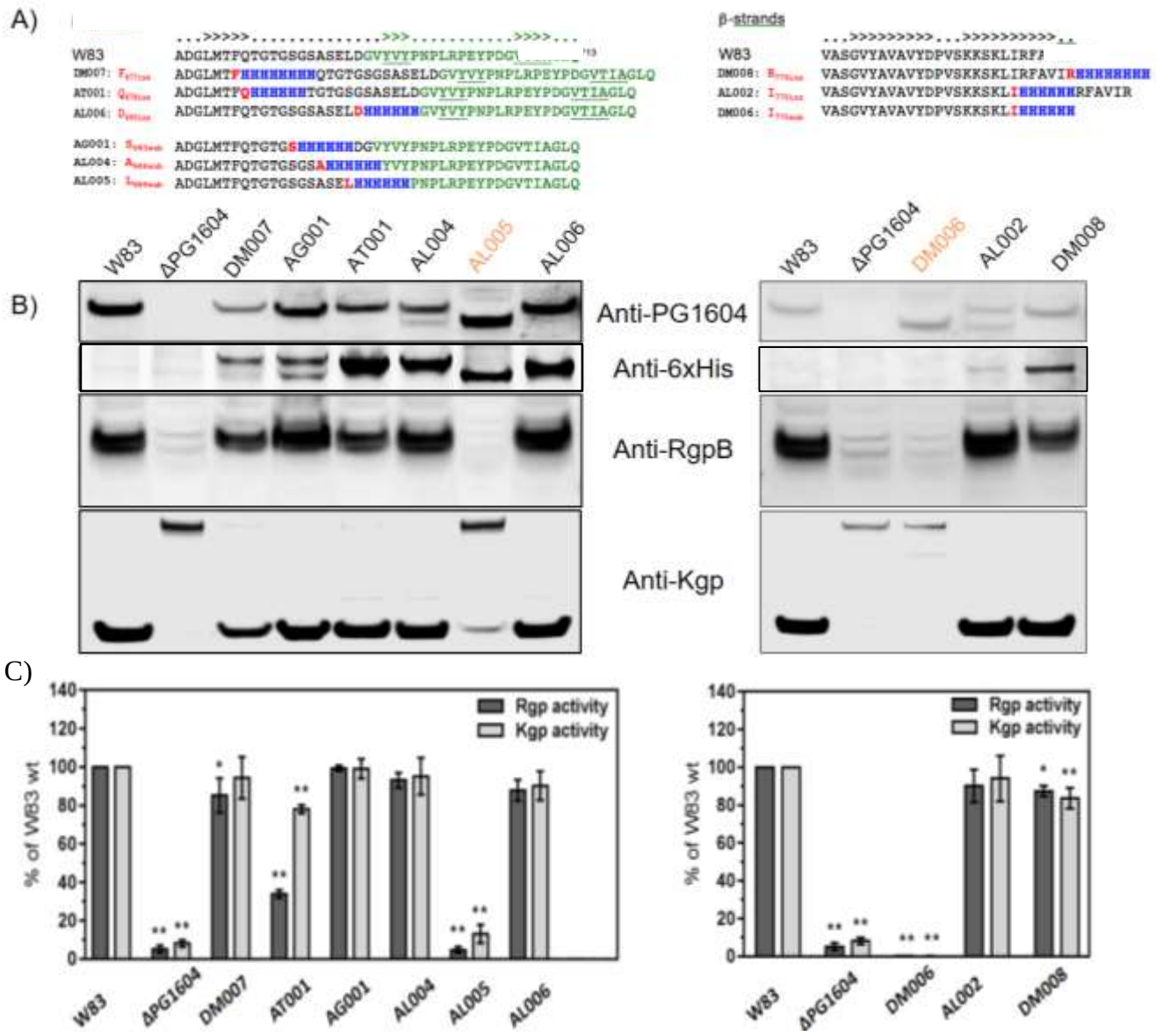

Figure 22. Effect of PG1604 modification at the C-terminus and in a loop preceding the CTD on T9SS function. (A) Location of insertions and substitutions of consecutive residues by hexahistidine in the PG1604 polypeptide chain. Wild-type and mutant strains were grown to stationary phase and diluted equally to OD_{600} = 1.0 then whole cultures were subjected (B) to western-blot analysis for PG1604, oligohistidine, RgpB and Kgp, and (C) were used for gingipain activity assay.

4.7 Subcellular localization of PG1604 protein in the *P. gingivalis* W83

Using the proteomics approach, PG1604 has been identified in the outer membrane [4] and outer membrane vesicles (OMV) [52] of *P. gingivalis*. To confirm the suggested location of the protein, we performed WB on cultures quantitatively separated into whole cells and subcellular fractions, including the cell envelope (IM+OM), IM, OM, OMV, particle-free medium, periplasm and cytoplasm. Majority of PG1604 was found associated with the cell envelope and OM fractions (Figure 23).

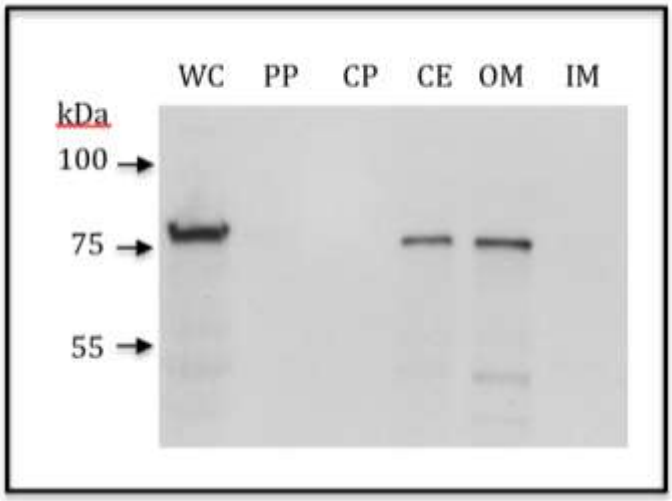

Figure 23. Cellular fractions extracted from W83 reacted to PG1604 specific mouse pAbs. *P. gingivalis* cells were quantitatively fractionated into whole cell extract (WC), periplasm (PP), cytoplasm (CP), cell envelope (CE), outer membrane (OM) and inner membrane (IM). Samples were electrophoresed by using Novex® 10-20% Tricine Gel (Invitrogen) at 125volts for 90 min. Subsequently proteins were transferred onto nitrocellulose (Bio-Rad) at 100 volts for 60 min. PG1604 was detected using PG1604 polyclonal (Mouse), 2µg.

4.8 Purification of PG1604 from the *P. gingivalis* AL005 mutant strain

To obtain larger amounts of truncated PG1604 version (70 kDa) for further analysis, 0.5 liter of the AL005 (L689 substitution 6xHistidine) mutant culture was cultivated to OD_{600} ~ 1.35-1.85 and processed to obtain a sample enriched in our protein of interest (Refer section 3.14) with a use of Ni-Sepharose. HIS-tagged proteins bound to the resin were eluted in a single step with 500 mM imidazole. Thereafter, eluates were concentrated (100x) and total 50 µl of purified, concentrated fraction (0.8 µg/µl concentration) was analyzed by western-blot. Analysis of the purified AL005 mutant revealed a single band at approximately 70kDa immunoreactive to anti-PG1604 antibodies (Figure 24).

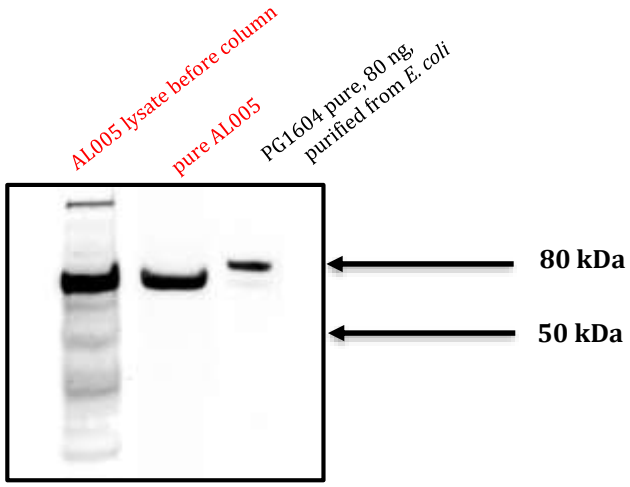

Figure 24. Western-blot analysis of the purified mutant AL005 (L689 substitution 6xHistidine) of PG1604. Total 50 µl of concentrated fraction of AL005 eluted from Ni-sepharose (0.8 µg/µl) and the pure recombinant PG1604 protein (80ng) were electrophoresed by using 4-12% Bis-Tris SDS-PAGE (Invitrogen) at 150 volts for 60 min. Subsequently proteins were transferred onto nitrocellulose (Bio-Rad) at 100 volts for 60 min. PG1604 was detected using PG1604 polyclonal (Mouse), 2µg.

CHAPTER 5

DISCUSSION

The newly discovered T9SS system used by bacterial species belonging to the *Bacteroidete* phylum to secrete proteins or assembly of a gliding-motility apparatus seems to be more complicated than other known secretion systems of Gram-negative bacteria. T9SS is composed of at least 12 essential proteins, including PG1604 a subject of this thesis. As in the case of any other essential components, depletion of *P. gingivalis* of the PG1604 function, either through deletion of the *PG1604* gene or limited site-directed mutations within the sequence preceding the IgSF-like C-terminal domain (AL005 mutated strain) and at the C-terminus of the protein (DM006), disabled the secretion of CTD-bearing cargo proteins (e.g. Kgp and Rgp) which accumulated in the unprocessed (Figure 22B) and inactive forms (Figure 2C) in the periplasm.

The outer membrane (OM) localization of the PG1604 protein shown through cell fractionation (Figure 23) was further supported by cell surface attachment analysis (FACS, unpublished laboratory data), thus confirming that PG1604 is not only a functional component of T9SS, but is also transported outside alike other cargo proteins. However, the unusual feature of PG1604, as the protein indispensable for the T9SS secretory activity and, in the same time the T9SS cargo protein, is its presence in a full-length polypeptide together with the CTD (80 kDa). Clearly, the CTD of PG1604 is not cleaved during the

protein translocation to the surface, as in the case of the other T9SS substrates [44]. This observation is concluded from western-blot analysis performed with anti-PG1604 serum on cell lysates of the wild-type *P. gingivalis* (Figure 22B) as well as by using anti-HIS antibodies and detecting PG1604 at 80 kDa in DM008 strain expressing the protein with octahistidine tag at the C-terminus after the last amino acid, R^{776} (Figure 22B).

Translocation of cargo proteins across the OM by T9SS is associated with CTD cleavage by PorU and simultaneous attachment of A-LPS [36]. Synchronization of these two events apparently depends on the length of a loop between the CTD and a preceding domain of a secreted protein. Insertions at and upstream the cleavage site uncoupled proteolytic cleavage of CTD from A-LPS attachment and proteins modified in this way were released into the medium in non-glycosylated form [61]. In contrast, downstream insertions or amino acid substitutions within the linker region have little effect on the cargo protein cleavage, modification and attachment to the cell surface, at least in the case of RgpB [62]. In the case of PPAD and RgpB, the domain preceding CTD has the immunoglobulin-like fold [45]. Similarly folded domains comprising of 6-8 β-strands preceding the CTD, are predicted with high confidence in 22 out of 29 remaining T9SS cargo proteins. In these proteins, the cleavage sites by the PorU sortase, either experimentally determined or predicted, are located 2-6 residues downstream of the last β-strand. Such consensus cleavage site seems to be missing in PG1604. For now, the mode of further processing after the transport of PG1604 (e.g. attachment of the OM) is unknown.

The tertiary structure of RgpB- and PG1604-derived CTDs is well conserved, but it is strikingly similar when limited to the last two β-strands, which superimpose almost perfectly despite sharing only limited amino acid sequence identity (Potempa *et al.*,

unpublished results). Taking into account that the signal for OM translocation, cleavage of the CTD and A-LPS attachment is located in these two last β-strands [63], this observation strongly supports a contention that their structure, rather than a specific sequential motif is essential for recognition of the signal, apparently in the structural context of the preceding domain. Therefore, mutations of the interdomain linker of PG1604 by the insertion of the oligohistidine or replacement of consecutive residues with this motif, which do not affect the structure of the adjacent domains, do not affect function of PG1604 (DM007, AG001, AT001, AL004, AL006, AL002. DM008) as judged by strain pigmentation (Figure 20), full gingipain processing (Figure 22B) and gingipains activity (Figure 22C). Similarly, several HIS residues can be added to the C-terminus without any effect on *P. gingivalis* secretory phenotype (DM008 strain). In stark contrast, however, substitution of the last 6 residues with hexahistidine (DM006) apparently rendered PG1604 susceptible for limited proteolysis at the C-terminus, leading to the truncated protein devoid of its essential function in T9SS. Interestingly, insertion of 6xHis in the middle of the last β-strand did not affect PG1604 translocation to the surface and its function in the CTD-protein secretion (AL002). Most likely such modification does not destroy the structure of the CTD.

To conclude, the results obtained in this study support that the CTD of PG1604 protein is not cleaved off upon its transport through T9SS, as shown by western-blot with *anti*-PG1604 serum (migration at full length ~80 kDa) and *anti*-HIS mAb for HIS-tagged mutant at the C-terminus (*P. gingivalis* strain DM008). The selective modifications of the PG1604 protein, alter the proper maturation of secreted virulence factors (Kgp and Rgp) and due to disruption of the function of T9SS of *P. gingivalis*. This finding enable us to conclude that PG1604 is a novel functional component of T9SS located mainly in the outer

membrane. Moreover, its CTD is not only essential for its transport through T9SS but also necessary for further physiological functions. Therefore, following the T9SS nomenclature, we designate the PG1604 protein as PorZ. The present study contributes to understanding of the mechanism of recognition and processing of CTD bearing cargo proteins that may provide a significant opportunity to impede maturation of these important virulence factors in the treatment of periodontal disease.

Further studies are needed to determine the functional role of the PG1604 in the T9SS by solving its crystal structure and also, to demonstrate how PG1604 interact with other CTD class proteins of T9SS. This conclusion can be extended on other periodontal pathogens such as *P. intermedia* and *T. forsythia*, which also secrete CTD bearing proteins using T9SS [4, 52].

REFERENCES

Uncategorized References

1. Simpson, W., T. Olczak, and C.A. Genco, *Lysine-specific gingipain K and heme/hemoglobin receptor HmuR are involved in heme utilization in Porphyromonas gingivalis.* Acta Biochim Pol, 2004. **51**(1): p. 253-62.
2. Guo, Y., K.A. Nguyen, and J. Potempa, *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, 2010. **54**(1): p. 15-44.
3. Glew, M.D., et al., *PG0026 is the C-terminal signal peptidase of a novel secretion system of Porphyromonas gingivalis.* J Biol Chem, 2012. **287**(29): p. 24605-17.
4. Veith, P.D., et al., *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, 2013. **12**(10): p. 4449-61.
5. Page, R.C., et al., *Chronic inflammatory gingival and periodontal disease.* Jama, 1978. **240**(6): p. 545-50.
6. Hajishengallis, G., *Periodontitis: from microbial immune subversion to systemic inflammation.* Nat Rev Immunol, 2015. **15**(1): p. 30-44.
7. Eke, P.I., et al., *Prevalence of periodontitis in adults in the United States: 2009 and 2010.* J Dent Res, 2012. **91**(10): p. 914-20.
8. Eke, P.I., et al., *Update on Prevalence of Periodontitis in Adults in the United States: NHANES 2009 to 2012.* J Periodontol, 2015. **86**(5): p. 611-22.
9. Merchant, A., et al., *Oral hygiene practices and periodontitis in health care professionals.* J Periodontol, 2002. **73**(5): p. 531-5.
10. Batchelor, P., *Is periodontal disease a public health problem?* Br Dent J, 2014. **217**(8): p. 405-9.
11. Grossi, S.G., et al., *Assessment of risk for periodontal disease. II. Risk indicators for alveolar bone loss.* J Periodontol, 1995. **66**(1): p. 23-9.
12. Haffajee, A.D. and S.S. Socransky, *Relationship of cigarette smoking to attachment level profiles.* J Clin Periodontol, 2001. **28**(4): p. 283-95.
13. Duran-Pinedo, A.E. and J. Frias-Lopez, *Beyond microbial community composition: functional activities of the oral microbiome in health and disease.* Microbes Infect, 2015. **17**(7): p. 505-16.
14. *Structure, function and diversity of the healthy human microbiome.* Nature, 2012. **486**(7402): p. 207-14.
15. Paster, B.J., et al., *The breadth of bacterial diversity in the human periodontal pocket and other oral sites.* Periodontol 2000, 2006. **42**: p. 80-7.

16. Perez-Chaparro, P.J., et al., *Newly identified pathogens associated with periodontitis: a systematic review.* J Dent Res, 2014. **93**(9): p. 846-58.
17. Kinane, D.F. and G.J. Marshall, *Periodontal manifestations of systemic disease.* Aust Dent J, 2001. **46**(1): p. 2-12.
18. Hajishengallis, G., *Immunomicrobial pathogenesis of periodontitis: keystones, pathobionts, and host response.* Trends Immunol, 2014. **35**(1): p. 3-11.
19. Genco, R.J. and W.S. Borgnakke, *Risk factors for periodontal disease.* Periodontol 2000, 2013. **62**(1): p. 59-94.
20. Lamont, R.J. and G. Hajishengallis, *Polymicrobial synergy and dysbiosis in inflammatory disease.* Trends Mol Med, 2015. **21**(3): p. 172-83.
21. Bostanci, N. and G.N. Belibasakis, *Porphyromonas gingivalis: an invasive and evasive opportunistic oral pathogen.* FEMS Microbiol Lett, 2012. **333**(1): p. 1-9.
22. Darveau, R.P., G. Hajishengallis, and M.A. Curtis, *Porphyromonas gingivalis as a potential community activist for disease.* J Dent Res, 2012. **91**(9): p. 816-20.
23. Butler, C.A., et al., *The Porphyromonas gingivalis ferric uptake regulator orthologue binds hemin and regulates hemin-responsive biofilm development.* PLoS One, 2014. **9**(11): p. e111168.
24. Aruni, A.W., et al., *The Biofilm Community-Rebels with a Cause.* Curr Oral Health Rep, 2015. **2**(1): p. 48-56.
25. How, K.Y., K.P. Song, and K.G. Chan, *Porphyromonas gingivalis: An Overview of Periodontopathic Pathogen below the Gum Line.* Front Microbiol, 2016. **7**: p. 53.
26. Hajishengallis, G. and R.J. Lamont, *Breaking bad: manipulation of the host response by Porphyromonas gingivalis.* Eur J Immunol, 2014. **44**(2): p. 328-38.
27. Gui, M.J., et al., *Spheres of influence: Porphyromonas gingivalis outer membrane vesicles.* Mol Oral Microbiol, 2015.
28. Deleon-Pennell, K.Y., L.E. de Castro Bras, and M.L. Lindsey, *Circulating Porphyromonas gingivalis lipopolysaccharide resets cardiac homeostasis in mice through a matrix metalloproteinase-9-dependent mechanism.* Physiol Rep, 2013. **1**(5): p. e00079.
29. Nakao, R., et al., *Effect of Porphyromonas gingivalis outer membrane vesicles on gingipain-mediated detachment of cultured oral epithelial cells and immune responses.* Microbes Infect, 2014. **16**(1): p. 6-16.
30. Potempa, J., A. Banbula, and J. Travis, *Role of bacterial proteinases in matrix destruction and modulation of host responses.* Periodontol 2000, 2000. **24**: p. 153-92.
31. Curtis, M.A., et al., *Molecular genetics and nomenclature of proteases of Porphyromonas gingivalis.* J Periodontal Res, 1999. **34**(8): p. 464-72.

32. Rego, A.T., V. Chandran, and G. Waksman, *Two-step and one-step secretion mechanisms in Gram-negative bacteria: contrasting the type IV secretion system and the chaperone-usher pathway of pilus biogenesis.* Biochem J, 2010. **425**(3): p. 475-88.
33. Costa, T.R., et al., *Secretion systems in Gram-negative bacteria: structural and mechanistic insights.* Nat Rev Microbiol, 2015. **13**(6): p. 343-59.
34. Desvaux, M., et al., *Secretion and subcellular localizations of bacterial proteins: a semantic awareness issue.* Trends Microbiol, 2009. **17**(4): p. 139-45.
35. McBride, M.J. and Y. Zhu, *Gliding motility and Por secretion system genes are widespread among members of the phylum bacteroidetes.* J Bacteriol, 2013. **195**(2): p. 270-8.
36. Nakayama, K., *Porphyromonas gingivalis and related bacteria: from colonial pigmentation to the type IX secretion system and gliding motility.* J Periodontal Res, 2015. **50**(1): p. 1-8.
37. de Diego, I., et al., *The outer-membrane export signal of Porphyromonas gingivalis type IX secretion system (T9SS) is a conserved C-terminal beta-sandwich domain.* Sci Rep, 2016. **6**: p. 23123.
38. Kariu, T., et al., *Inhibition of gingipains and Porphyromonas gingivalis growth and biofilm formation by prenyl flavonoids.* J Periodontal Res, 2016.
39. Shoji, M., et al., *Characterization of hemin-binding protein 35 (HBP35) in Porphyromonas gingivalis: its cellular distribution, thioredoxin activity and role in heme utilization.* BMC Microbiol, 2010. **10**: p. 152.
40. Gorasia, D.G., et al., *Porphyromonas gingivalis Type IX Secretion Substrates Are Cleaved and Modified by a Sortase-Like Mechanism.* PLoS Pathog, 2015. **11**(9): p. e1005152.
41. Shoji, M., et al., *Por secretion system-dependent secretion and glycosylation of Porphyromonas gingivalis hemin-binding protein 35.* PLoS One, 2011. **6**(6): p. e21372.
42. Sato, K., et al., *Identification of a new membrane-associated protein that influences transport/maturation of gingipains and adhesins of Porphyromonas gingivalis.* J Biol Chem, 2005. **280**(10): p. 8668-77.
43. Seers, C.A., et al., *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, 2006. **188**(17): p. 6376-86.
44. Sato, K., et al., *Identification of Porphyromonas gingivalis proteins secreted by the Por secretion system.* FEMS Microbiol Lett, 2013. **338**(1): p. 68-76.
45. Nguyen, K.A., J. Travis, and J. Potempa, *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, 2007. **189**(3): p. 833-43.

46. Taguchi, Y., et al., *Involvement of an Skp-Like Protein, PGN_0300, in the Type IX Secretion System of Porphyromonas gingivalis.* Infect Immun, 2016. **84**(1): p. 230-40.
47. Sato, K., et al., *A protein secretion system linked to bacteroidete gliding motility and pathogenesis.* Proc Natl Acad Sci U S A, 2010. **107**(1): p. 276-81.
48. Chen, Y.Y., et al., *CPG70 is a novel basic metallocarboxypeptidase with C-terminal polycystic kidney disease domains from Porphyromonas gingivalis.* J Biol Chem, 2002. **277**(26): p. 23433-40.
49. McGraw, W.T., et al., *Purification, characterization, and sequence analysis of a potential virulence factor from Porphyromonas gingivalis, peptidylarginine deiminase.* Infect Immun, 1999. **67**(7): p. 3248-56.
50. Petersen, T.N., et al., *SignalP 4.0: discriminating signal peptides from transmembrane regions.* Nat Methods, 2011. **8**(10): p. 785-6.
51. Ross, B.C., et al., *Identification of vaccine candidate antigens from a genomic analysis of Porphyromonas gingivalis.* Vaccine, 2001. **19**(30): p. 4135-42.
52. Veith, P.D., et al., *Porphyromonas gingivalis outer membrane vesicles exclusively contain outer membrane and periplasmic proteins and carry a cargo enriched with virulence factors.* J Proteome Res, 2014. **13**(5): p. 2420-32.
53. Okamoto, K., et al., *Involvement of a lysine-specific cysteine proteinase in hemoglobin adsorption and heme accumulation by Porphyromonas gingivalis.* J Biol Chem, 1998. **273**(33): p. 21225-31.
54. Smalley, J.W., et al., *HmuY haemophore and gingipain proteases constitute a unique syntrophic system of haem acquisition by Porphyromonas gingivalis.* PLoS One, 2011. **6**(2): p. e17182.
55. Lowe, E.C., et al., *A scissor blade-like closing mechanism implicated in transmembrane signaling in a Bacteroides hybrid two-component system.* Proc Natl Acad Sci U S A, 2012. **109**(19): p. 7298-303.
56. Chen, C.K., N.L. Chan, and A.H. Wang, *The many blades of the beta-propeller proteins: conserved but versatile.* Trends Biochem Sci, 2011. **36**(10): p. 553-61.
57. Zhang, Z., Q. Liu, and W.A. Hendrickson, *Crystal structures of apparent saccharide sensors from histidine kinase receptors prevalent in a human gut symbiont.* Febs j, 2014. **281**(18): p. 4263-79.
58. Nelson, K.E., et al., *Complete genome sequence of the oral pathogenic Bacterium porphyromonas gingivalis strain W83.* J Bacteriol, 2003. **185**(18): p. 5591-601.
59. Parker, A.C. and C.J. Smith, *Genetic and biochemical analysis of a novel Ambler class A beta-lactamase responsible for cefoxitin resistance in Bacteroides species.* Antimicrob Agents Chemother, 1993. **37**(5): p. 1028-36.

60. Potempa, J. and K.A. Nguyen, *Purification and characterization of gingipains.* Curr Protoc Protein Sci, 2007. **Chapter 21**: p. Unit 21.20.
61. Chen, Y.Y., et al., *The outer membrane protein LptO is essential for the O-deacylation of LPS and the co-ordinated secretion and attachment of A-LPS and CTD proteins in Porphyromonas gingivalis.* Mol Microbiol, 2011. **79**(5): p. 1380-401.
62. Zhou, X.Y., et al., *Sequence-independent processing site of the C-terminal domain (CTD) influences maturation of the RgpB protease from Porphyromonas gingivalis.* Mol Microbiol, 2013. **89**(5): p. 903-17.
63. Paramonov, N., et al., *Identification of the linkage between A-polysaccharide and the core in the A-lipopolysaccharide of Porphyromonas gingivalis W50.* J Bacteriol, 2015. **197**(10): p. 1735-46.

APPENDIX

A) ABBREVIATIONS

BCA- bicinchoninic acid

bp - base pair

CTD- C-terminal domain

dH_2O - distilled water

DNA - deoxyribonucleic acid

e.g. - exempli gratia (for example)

etc - et cetera (and other things)

g - gram or gravitational force (acceleration)

kb - kilobase pair

M - molar concentration

mA - milliampère

mg – milligram

min - minutes

mL - milliliter

ng - nanogram

PBS- phosphate-buffered saline

Pg- *Porphromonas gingivalis*

TLCK- tosyl-L-lysyl-chloromethane hydrochloride

µg - microgram

µL - microliter

B) Doubling times were calculated from the growth curves for three density points (OD_{600}: 0.5, 0.55 and 0.6) of the logarithmic growth phase.

	Strain	**OD_{600} - 0.6 reached at time [h]**	**Doubling duration [min]**	**SD [min]**
CONTROL	W83	6.8	**232**	±21
	Δ1604*	12.0	**260**	±25
PG1604 mutants at N-terminus of CTD	DM007 F677ins	14.1	**274**	±21
	AT001 Q678ins	9.4	**322**	±29
	AG001 S683sub	9.4	**232**	±21
	AL004 A686sub	8.8	**218**	±17
	AL005 L689sub*	8.1	**218**	±17
	AL006 D690ins	8.8	**210**	±17
PG1604 mutants at C-terminus of CTD	AL002 I770ins	8.9	**213**	±21
	DM006 I770sub*	13.4	**305**	±29
	DM008 R776ins	11.0	**207**	±21

*** mutants nagative in gingipain activity**

CURRICULUM VITA

Apurva Tadimari Prabhakar

787 Eastern Parkway, Apt 4
Louisville, Kentucky 40217
502.202.0981

tadimariapurva@gmail.com
a0tadi01@louisville.edu

Summary

I am a foreign trained dentist from India, currently enrolled at the University of Louisville as a Master's in oral biology candidate. I aim to pursue a career as a clinician-researcher. I have research interests in host immunity, pathogenesis and auto-immunity. I am starting my Ph.D. in oral health research at the Virginia Commonwealth University School of Dentistry, Fall 2016.

EDUCATION

1. **UNIVERSITY OF LOUISVILLE DENTAL SCHOOL** Louisville, Kentucky
   08/2014-2016
   Master's In Oral Biology **GPA- 3.742**

2. **M.S. RAMAIAH DENTAL COLLEGE** Bangalore, India
   08/2007 - 09/2012
   Bachelor of Dental Surgery

ECE **GPA- 3.94/4.00**
01/08/2013

3. **Continued dental education program**

ESTHETIC AND RESTORATIVE ADVANCEMENTS by Bangalore, India 04/2013
Dr. Terry Douglas, University of Texas Health Center

EXTERNSHIP:

4. **St.Luke's Dental Hospital, General Practice Residency** Bethlehem PA, U.S.A 06/2013 – 10/2013

25 hours/week of observership, shadowing Attendings and Residents, attending lectures. I was exposed to advanced restorative, prosthodontics, periodontal, surgical and implant cases

EXAMINATIONS:

NBDE Part 1 **Status- PASS**

Taken on 08/14/2013

TOEFL iBT **Score- 104**
Taken on 03/11/2016

READING	26
LISTENING	30
SPEAKING	24
WRITING	24

WORK EXPERIENCE

1. **UNIVERSITY OF LOUISVILLE SCHOOL OF DENTISTRY** Louisville, Kentucky 2015-2016

STUDENT RESEARCHER, Dr.Potempa Lab, Department of Oral Immunology and Infectious Diseases

Lab Techniques Proficient in

i) Gel Electrophoresis
ii) Ion Affinity Chromatography Ni Sepharose High Performance
iii) Protein Purification
iv) BCA Analysis
v) Gingipain Enzymatic Activity Assay
vi) Acetone Precipitation
vii) Western-blot
viii) Clone Manager 9 Program

viii) Basic Molecular Biology Techniques, a few to mention:

- Master plasmid construction
- Primer design
- Restriction Enzyme Digestion, Ligation
- Plasmid Purification
- Gel Purification
- Transformation
- Mutagenesis

Lab Safety Training Completed Louisville, Kentucky 09/2015

1. Blood Borne Pathogen Training
2. Laser Safety Training
3. Refresher Training for Radiation Users
4. Biosafety Training (BSL1/BSL2)
5. NIH Guidelines
6. Select Agents
7. Formaldehyde Training
8. Lab Safety Training (09/11/2014)
9. Animal Training Level I and II

2. UNIVERSITY OF LOUISVILLE SCHOOL OF DENTISTR Louisville, Kentucky 01/2015 – 4/2015

STUDENT ASSISTANT, SIMULATION LAB

3. SMILE AVENUE Bangalore,India 12/2011 – 06/2014

DENTIST

- Anterior and posterior teeth Root Canal treatment.
- Class I, Class II, Class V cavity preparations Composite restorations, GIC and silver amalgam restorations.
- Direct and indirect pulp-capping.
- Fabrication of complete dentures and RPDs, Tooth preparation for FPDs.
- Oral prophylaxis.
- Coronoplasty.
- Pit and fissure sealant placements.
- Other preventive procedures.
- Extraction of anterior and posterior primary and permanent teeth.
- Fabrication of retainers.
- Taking intra-oral periapical radiographs, ortho-pantamographs and bitewing radiographs.

4. MACULA HEALTHCARE PVT. LTD Bangalore, India
02/2014 –08/2014

Subject Expert- Prosthodontics, Entrance books, comprehensive self-study portal

- Contributor for the development of the Dental Entrance Examination Self-study portal, Entrance books.

5. M.S. RAMAIAH DENTAL COLLEGE Bangalore, India
08/2011 – 09/2012

Department Rotating Internship in following departments

- Oral Medicine & Radiology
- Public Health Dentistry
- Conservative Dentistry & Endodontics
- Periodontics
- Oral & Maxillofacial Surgery
- Prosthodontics
- Pedodontics
- Orthodontics
- Oral Pathology

6. KAIWARA RURAL DENTAL HEALTH CENTRE Kolar District, India
03/2012 – 05/2012
Residential Posting

Dental care to people in the inaccessible areas.
I have also been part of screening and treatment camps.

7. M.S. RAMAIAH MEMORIAL MEDICAL HOSPITAL Bangalore, India
02/2012 – 03/2012
Internship - Accident and Emergency Department

8. M.S. RAMAIAH MEMORIAL MEDICAL HOSPITAL
Basic Life Support (BLS) Training Bangalore, India
11/2011 -12/2011

9. COMMUNITY OUTREACH PROGRAM Kolar District, India
01/2012 – 02/2012
01/2010 – 02/2010

Facilitator
Conducted treatment screening and treatment camps
and oral survey

10. MOCK FIRE DRILL RALLY BY GOVERNMENT OF KARNATAKA 11/2011
Triage Supervisor

POSITIONS HELD:

- **Co-Chair** of Health Science Cluster at Graduate Student Council at University of Louisville ,2015-2016

- **Vice-President** for Student Research Group at University of Louisville School of Dentistry, 2015-2016

- **Student Body General Secretary**, M.S.Ramaiah Dental College, 2010 – 2011

- **Science Club Secretary**, M.S.Ramaiah Dental College, 2009 – 2010

PAPER AND POSTER PRESENTATION

- Poster Presentation on the topic "Cargo Proteins of Type 9 Secretion System in Porphyromonas gingivalis" at the Graduate Student Council Research Symposium, University of Louisville, April 2016.
- A. Tadimari Prabhakar ; A. Goel; B. Potempa; D. Mizgalska; K.Nguyen; J. Potempa; A. Lasica, *J Dent Res* Vol 95 Spec Iss A: 2410758, 2016. Poster Presentation at the International Association of Dental Research Conference- Poster Presentation to be held at Los Angeles, March 2016.
- Research Louisville 2015 on the topic "Cargo Proteins Of Type 9 Secretion System " Apurva Tadimari Prabhakar, Jan Potempa ,Anna Lasica ,Barbara Potempa, Oral Immunology And Infectious Diseases
- **Best paper presentation Award** in the National B.D.S seminar in Oral Medicine and Radiology, held in Chennai, India, on the topic "Diagnostic tests for cancer detection."

CONFERENCES ATTENDED

GRADUATE STUDENT COUNCIL RESEARCH SYMPOSIUM, U of L
LOUISVILLE, KY 04/2016

IADR/AADR 45th ANNNUAL MEETING
LOS ANGELES, CA 03/2016

UPENN- PENN PERIODONTAL CONFERENCE
PHILADELPHIA, PA 06/2015

IDA COLGATE FUTURE DENTAL PROFESSIONAL PROGRAM
BANGALORE, INDIA 05/2012

IDA COLGATE FUTURE DENTAL PROFESSIONAL PROGRAM
BANGALORE, INDIA 05/2011

NATIONAL B.D.S SEMINAR IN ORAL MEDICINE AND RADIOLOGY
CHENNAI, INDIA 12/2010

ACADEMIC AWARDS AND NOMINATIONS

- **Summer Research Scholarship**, from University of Louisville School of Dentistry, 2016 and 2015

- School of Interdisciplinary and Graduate Studies- **Oral Biology program tuition scholarship award**, 2016 and 2015

- **Graduate Student Council travel award**, University of Louisville, 2016

- **Graduate Merit Scholarship**, University of Louisville 2016

- **Best Outgoing Student 2007- 2012 award** from M.S. Ramaiah Dental College.

- Public health dentistry, General Surgery and Oral Pathology and Microbiology **Subject Topper Awards**.

- Awarded **Certificates of Merit** for being the topper in all the 4 years of Bachelor of Dental Surgery, 2007- 2012, M.S.Ramaiah Dental College.

- **IDA Scholarship for the year 2009-2010** for being the topper of the class.

- Nominated for the Pierre Fauchard Academy UG Student Certificate of Merit Award 2012, from M.S. Ramaiah Dental College.

- Nominated for the Indian Association of Public Health Dentistry (IAPHD)'s subject topper award for the year 2010 from the Department of Public Health Dentistry, M.S. Ramaiah Dental College.