



Terms and Conditions of Use of Digitised Theses from Trinity College Library Dublin

Copyright statement

All material supplied by Trinity College Library is protected by copyright (under the Copyright and Related Rights Act, 2000 as amended) and other relevant Intellectual Property Rights. By accessing and using a Digitised Thesis from Trinity College Library you acknowledge that all Intellectual Property Rights in any Works supplied are the sole and exclusive property of the copyright and/or other IPR holder. Specific copyright holders may not be explicitly identified. Use of materials from other sources within a thesis should not be construed as a claim over them.

A non-exclusive, non-transferable licence is hereby granted to those using or reproducing, in whole or in part, the material for valid purposes, providing the copyright owners are acknowledged using the normal conventions. Where specific permission to use material is required, this is identified and such permission must be sought from the copyright holder or agency cited.

Liability statement

By using a Digitised Thesis, I accept that Trinity College Dublin bears no legal responsibility for the accuracy, legality or comprehensiveness of materials contained within the thesis, and that Trinity College Dublin accepts no liability for indirect, consequential, or incidental, damages or losses arising from use of the thesis for whatever reason. Information located in a thesis may be subject to specific use constraints, details of which may not be explicitly described. It is the responsibility of potential and actual users to be aware of such constraints and to abide by them. By making use of material from a digitised thesis, you accept these copyright and disclaimer provisions. Where it is brought to the attention of Trinity College Library that there may be a breach of copyright or other restraint, it is the policy to withdraw or take down access to a thesis while the issue is being resolved.

Access Agreement

By using a Digitised Thesis from Trinity College Library you are bound by the following Terms & Conditions. Please read them carefully.

I have read and I understand the following statement: All material supplied via a Digitised Thesis from Trinity College Library is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of a thesis is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form providing the copyright owners are acknowledged using the normal conventions. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone. This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.

**The role of *hagB* and *hagC* in the virulence of
Porphyromonas gingivalis and identification of putative
novel virulence factors by Transposon-Seq**

A thesis submitted to the University of Dublin, Trinity College, in fulfilment of the
requirements for the degree Doctor of Philosophy (Ph.D.) by

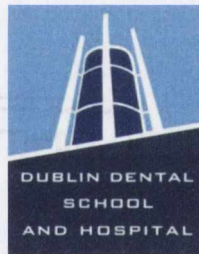
Eimear Connolly

January 2016



**Trinity
College
Dublin**

The University of Dublin

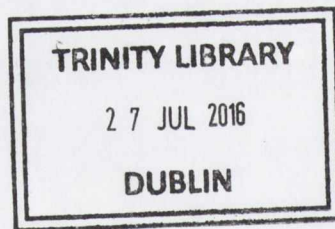


**Microbiology Research Unit,
Division of Oral Biosciences,
Dublin Dental University Hospital,
Trinity College,
Dublin 2.**

Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work.

I agree to deposit this thesis in the University's open access institutional repository or allow the library to do so on my behalf, subject to Irish Copyright Legislation and Trinity College Library conditions of use and acknowledgement.



Thesis 10991

Summary

Porphyromonas gingivalis is a species of bacteria commonly found in the oral cavity. It is associated with severe forms of periodontal disease such as chronic periodontitis which is one of the major causes of tooth loss in adults globally. In order to cause disease the bacteria utilises a number of specialised mechanisms to disrupt the host immune response and facilitate out-growth of dental plaque. One group of factors important for growth and survival in the gingival crevice are a group of cell surface proteins commonly referred to as the haemagglutinins.

HagB and HagC are neglected members of this group of proteins. Their exact role in the disease process has yet to be fully established. They share a high level of homology to one another and form a distinct group separate from the other haemagglutinins. Previous attempts to study their role have yielded successful single mutants of each gene but attempts to create a double $\Delta hagB/\Delta hagC$ mutant have proved unsuccessful.

By first deleting the *hagC* gene and then disrupting the *hagB* gene we were successfully able to create a $\Delta hagB/\Delta hagC$ mutant in *P. gingivalis* strain W83 as well as single mutants of both. Phenotypic analysis was carried out under a range of conditions. One of the strongest phenotypes observed were differences in colonial pigmentation between wild-type and mutant strains in both solid and liquid media. Deletion of one or both of these genes also affected how the bacteria interacted with a number of cell lines and other bacterial species. Changes in adherence and invasion capabilities were found when H357 oral epithelial cells were infected with the mutant strains. The data also showed that there were differences in the immuno-stimulatory properties of the strains when co-cultured with THP-1 cells. When mono and multi-species biofilm formation were examined it was found that the *hagC* mutant and the double mutant had impaired mono-species biofilm formation.

The data also indicated an important role for *hagC* in the formation of multi-species biofilms.

The double $\Delta hagB/\Delta hagC$ mutant exhibited some aberrant phenotypes in adhesion and cytokine stimulation. Quantative real-time polymerase chain reaction (qPCR) was carried out in order to establish if in the absence of *hagB* and *hagC* if compensatory gene expression was activated. A number of candidate genes, such as the gingipains, were identified and screened. It was found that in several cases the mutant strains, particularly the double mutant showed some levels of aberrant expression. The data also indicated that this was not a haem starvation effect, as restoration of HagB to the double mutant genome restored pigmentation but did not alleviate the phenotypic abnormalities. We hypothesise that this result may be due to bi-stable genetic variation present in the *P. gingivalis* population which was selected during the process of mutagenesis. Our data indicate that HagB and HagC are crucial haemagglutinins required for the normal physiology and growth of *P. gingivalis* which play a role in adherence, invasion and biofilm formation.

In conjunction with the work carried out on HagB and HagC we also aided in developing a rapid screening process which could aid in establishing the role of genes of poorly defined or unknown function. This screen would allow for a more targeted approach towards mutagenesis and would substantially speed up the process of establishing the role of genes of previously unknown function.

Abstract

The haemagglutinin genes of *P. gingivalis* are believed to play an important role in the virulence of the organism. A subset of these genes, namely *hagB* and *hagC*, are distinct from the other family members based on nucleotide sequence homology. They encode for proteins whose function in the virulence of *P. gingivalis* is poorly understood. It has been established that they can act as haemagglutinins and adhesins to human coronary arterial endothelial cells (HCAECs). In addition, HagB has been shown to be highly immunogenic with potential as a vaccine candidate. Their role in interactions with oral cells and oral bacteria has yet to be investigated. One reason for the lack of experimental characterisation is that attempts to generate a $\Delta hagB/\Delta hagC$ double mutant have been unsuccessful. In this study, both *hagB* and *hagC* were deleted to create the first $\Delta hagB/\Delta hagC$ mutant as well as single and complemented mutants.

It was found that *hagB* and *hagC* contribute to a number of processes in *P. gingivalis*, including pigmentation on blood agar and agglutination of red blood cells (RBCs). The double $\Delta hagB/\Delta hagC$ mutant exhibited a severe defect in pigmentation and greatly reduced capacity to agglutinate sheep RBCs. When adhesion to and invasion of oral epithelial H357 cells was examined, it was found that both $\Delta hagB$ and $\Delta hagC$ mutants exhibited reduced adhesion relative to wild-type. In addition, deletion of *hagB* in the fimbriated strain ATCC33277 indicated that HagB was a major contributor to adherence in a fimbriated strain of *P. gingivalis*. The role of these genes in strain W83 in single and multispecies biofilm formation was also examined. Unexpectedly, *hagC* mutants were found to exhibit the greatest defects in biofilm formation.

However, paradoxically the $\Delta hagB/\Delta hagC$ double mutant did not exhibit an additive defect in adherence and complementation with *hagB* resulted in a hyper-adherent phenotype. The

$\Delta hagB/\Delta hagC$ double mutant also elicited higher levels of IL-8 and MIP1 β from THP-1 cells, despite removal of these major surface antigens. Examination of gene expression in the $\Delta hagB/\Delta hagC$ mutant using quantitative Real-Time polymerase chain reaction (qPCR) indicated deregulated expression of the gingipains *rgpA* and *rgpB*, which was not complemented by the re-addition of *hagB*. We hypothesise that the $\Delta hagB/\Delta hagC$ mutant may only be viable in subpopulations of the bacterium that express compensatory haemagglutinins and this may partly explain why previous attempts to generate a $\Delta hagB/\Delta hagC$ mutant have not been successful.

In conjunction with the work carried out on the role of *hagB* and *hagC*, we also developed a rapid screening process for adherence and invasion genes using a transposon mutagenesis library and Tn-Seq. By using this technology we developed a screen for genes that might play a role in virulence processes, allowing us to identify where areas of further study should be directed, this will be particularly useful for the study of genes of unknown or poorly defined function.

In summary, this work supports the idea that HagB could be used as a vaccine antigen as antibody responses would block adhesion and biofilm formation by the bacteria. I have also generated a panel of novel candidate genes for future investigation as virulence factors and vaccine candidates.

Table of Contents

Declaration.....	ii
Summary.....	iii
Abstract.....	v
Index of Figures	xiv
Index of Tables	xxiii
Abbreviations.....	xxiv
Acknowledgements.....	xxvii
Chapter 1	1
Introduction.....	1
1.1 Periodontitis	2
1.2 The evolution of the aetiology of periodontitis.....	6
1.3 <i>P. gingivalis</i> ; a keystone pathogen for an ancient disease	11
1.4 <i>P. gingivalis</i> and Dysbiosis	13
1.5 Virulence factors; a nasty side to <i>P. gingivalis</i>	28
1.6 The role of the <i>hag</i> genes in <i>P. gingivalis</i> :	34
1.7 The <i>hagB</i> and <i>hagC</i> genes of <i>P. gingivalis</i>	37
1.8 Defining a role for genes whose function is poorly understood in <i>P. gingivalis</i>	43
1.9 Rationale for this study:	44
Chapter Two	46
Materials and Methods.....	46
2.1 General Microbiology Methods	47

2.1.1 Strains	47
2.1.2 Bacterial culture methods	47
2.1.3 Buffers and solutions	49
2.1.4 Chemicals, enzymes, reagents and antibiotics.....	50
2.1.5 Oligonucleotides	51
2.1.6 Storage of isolates.....	51
2.1.7 Assessment of nucleic acids	54
2.1.8 Agarose gel electrophoresis	54
2.2 DNA techniques:	55
2.2.1 Whole genomic DNA purification.....	55
2.2.2 Isolation of plasmid DNA.....	56
2.2.3 Restriction enzyme digestion of DNA.....	56
2.2.4 Gel purification	57
2.2.5 DNA Ligation	57
2.2.6 DNA sequencing.....	57
2.2.7 Basic Analysis of DNA sequences	58
2.2.8 Preparation of electro-competent cells	58
2.2.9 Bacterial Transformation	59
2.3 Polymerase chain reaction.....	60
2.3.1 Routine polymerase chain reaction (PCR)	60
2.3.2 Quantitative (real time) PCR (qPCR)	61
2.4 Southern Immunoblot Hybridisation.....	63

2.4.1 Amplification and labelling of DNA probe	63
2.4.2 Electrophoresis and Transfer	63
2.4.3 Pre-hybridisation	64
2.4.4 Hybridisation	65
2.4.5 Washing	65
2.4.6 Detection.....	66
2.4.7 Development.....	66
2.5 RNA techniques	67
2.5.1 Freeze fracture method of RNA isolation	67
2.5.2 cDNA synthesis:	68
2.6 Cell culture methods	69
2.6.1 H357 cell culture	69
Chapter 3: Construction and confirmation of a <i>hagB</i> and <i>hagC</i> mutant set in	
<i>Porphyromonas gingivalis</i>	71
3.1 Introduction:	72
3.2 Materials and Methods	75
3.2.1 Generation of <i>hagB</i> mutant strains:	75
3.2.2 Creation of <i>hagC</i> mutant in strain W83:	77
3.2.3 Construction of Δ <i>hagB</i> complemented mutant	80
3.2.4 PCR confirmation of <i>P.gingivalis</i> mutant genotypes:	82
3.2.5 Attempted construction of <i>hagC</i> mutant in strain ATCC33277:.....	84
3.2.6: Characterisation of <i>hagB</i> and <i>hagC</i> expression under haemin rich conditions.....	84

3.3 Results:	86
3.3.1 Disruption of <i>hagB</i> in <i>P. gingivalis</i>	86
3.3.2 Construction of <i>hagC</i> disruption cassette using <i>E.coli</i> restriction endonuclease-mediated cloning:	88
3.3.3 Transformation of <i>hagC</i> disruption cassette into <i>P.gingivalis</i> .	89
3.3.4 Construction of Δ <i>hagB</i> / Δ <i>hagC</i> mutant in <i>P.gingivalis</i>	90
3.2.5 Confirmation of Δ <i>hagB</i> complemented mutant	91
3.2.6 Analysis of <i>hagB</i> and <i>hagC</i> expression in WT and mutant strains	92
3.4 Discussion:	97
Chapter 4: Examining the role of <i>hagB</i> and <i>hagC</i> in pigmentation, growth, haemagglutination and environmental stress sensitivity	102
4.1 Introduction	103
4.2 Materials and Methods	107
4.2.1 Pigmentation studies	107
4.2.2 Growth rate determination	108
4.2.3 Haemagglutination assays	108
4.2.4 Stress sensitivity testing	109
4.3 Results	111
4.3.1 Analysis of the effect of removal of <i>hagB</i> and <i>hagC</i> from the <i>P. gingivalis</i> W83 genome on pigmentation in solid and liquid media	111
4.3.2 Determining the impact removal of <i>hagB</i> and <i>hagC</i> on growth and doubling times in sTSB	115

4.3.3 Examining the role that <i>hagB</i> and <i>hagC</i> in haemagglutination in <i>P. gingivalis</i> W83	117
4.3.4 Exploring the effect mutagenesis of <i>hagB</i> and <i>hagC</i> on the stress response of <i>P.gingivalis</i> W83	117
4.4 Discussion	120
Chapter five: Analysis of the role of <i>hagB</i> and <i>hagC</i> of <i>Porphyromonas gingivalis</i> in adhesion and biofilm formation.	128
5.1 Introduction	129
5.2 Materials and Methods	132
5.2.1 Adherence assay	132
5.2.2 Invasion assay	133
5.2.3 <i>P. gingivalis</i> mono-species biofilm studies	133
5.2.4 Multi-species biofilm studies using a sub-gingival plaque model	134
5.2.5 Analysis of gingipain and <i>hagA</i> expression in mutant strains.....	134
5.2.6 Statistical analysis:	135
5.3 Results	136
5.3.1 Adhesion of WT and mutant strains cultured under haemin rich conditions	136
5.3.4 Examining the role that <i>hagB</i> plays in adherence in strain ATCC33277.....	138
5.3.3 The effect of haemin starvation on adhesion of WT and mutant strains.....	138
5.3.5 <i>P. gingivalis</i> invasion is effected by the removal of <i>hagB</i> and <i>hagC</i>	143
5.3.6 Ability of mutant strains to form mono-species biofilms in comparsion to WT W83.	147

5.3.7 Analysis of the ability of mutant strains to contribute to the formation of multi-species biofilms compared to WT W83.....	147
5.3.8. Analysis of gene expression of mediators in haem acquisition in broth based cultures.....	150
5.3.9. Analysis of gene expression of mediators of haem acquisition in blood agar grown cells.....	157
5.4 Discussion	161
Chapter 6: Examining the role that <i>hagB</i> and <i>hagC</i> play in stimulating the immune responses of THP-1 cells to whole cell <i>Porphyromonas gingivalis</i>	170
6.1 Introduction	171
6.2 Materials and Methods	174
6.2.1 Cell culture methods	174
6.2.2 Multi-analyte enzyme linked immunosorbent assay (ELISA)	174
6.2.3 Macrophage inflammatory protein 1 β ELISA:.....	176
6.2.4 Statistical Analysis.....	176
6.3 Results	177
6.3.1 Multi-analyte assay overall.....	177
6.3.2 Quantitative examination of levels of MIP-1 β produced by THP-1 cells post infection with WT and mutant strains	177
6.4 Discussion	183
Chapter 7: Screening a <i>Porphyromonas gingivalis</i> transposon mutagenesis library for genes involved in adherence, invasion and survival in an epithelial co-culture model	188
7.1 Introduction	189

7.2 Materials and Methods:.....	192
7.2.1 Bacterial culture conditions:.....	192
7.2.2 Cell culture conditions:.....	193
7.2.3 Cell based assays:	193
7.2.4 DNA extraction:	195
7.2.5 Nested semi-random PCR and sequencing.....	196
7.2.6 Data Analysis:	197
7.3 Results:.....	200
7.3.1. Validation and statistical analysis of input and output samples:.....	200
7.3.2 Identifying genes involved in adherence, invasion and survival in an epithelial co-culture model	202
7.4 Discussion:	208
Chapter 8: General Discussion	218
References:.....	231

Index of Figures

Chapter 1

1.1: Diagram showing the differences between a healthy tooth and an diseased tooth, in particular the difference in inflammation and bone level, as well as the presence of dental plaque. Reproduced from (Darveau, 2010).....	3
1.2: Dysbiosis is a microbial shift disease. a) In the healthy mouth a balance is maintained between the oral micro-flora and the host responses. b) the introduction of <i>P. gingivalis</i> into the oral biofilm causes a shift in this balance where the oral micro-flora over grows and protective host responses are inhibited.....	9
1.3: Diagram outlining how <i>P. gingivalis</i> manipulates the host defence system to encourage the dysbiotic state in the oral cavity allowing for the development and progression of periodontitis. After (Darveau et al., 2012).....	15
1.4: Diagram showing how <i>P. gingivalis</i> uses the gingipains to manipulate the complement cascade. RgpA is used to coat itself in C4b shielding itself from immune surveillance mechanisms. The gingipains act in concert with one another to degrade the central components of the complement system preventing opsonisation and the formation of the membrane attack complex.....	18
1.5: a) Using the antagonistic or evasive from of its lipopolysaccharide <i>P. gingivalis</i> binds to TLR4 but prevents the receptor from activating. This inhibits the activation of nitric oxide synthase and the production of free radicals preventing bacterial killing. b) Once internalised <i>P. gingivalis</i> inhibits non-canonical inflammasome activation by binding to the caspase-11 receptor but preventing its oligomerization.....	20
1.6: Figure outlining the mechanism of action used by <i>P. gingivalis</i> to inhibit neutrophil killing. <i>P. gingivalis</i> blocks the TLR2-MyD88 signalling pathway via cross-talk with C5aR. MyD88 undergoes ubiquitination by SMURF-1 and subsequent proteosomal degradation. The cross-talk between the two receptors activates the pro-inflammatory	

TLR2-Mal-PI3K pathway inhibiting phagocytosis (Hajishengallis, 2015).....	222
1.7: <i>P. gingivalis</i> SerB prevents the phosphorylation of p65, a key mediator in the NF-κB pathway. This prevents p65 translocating to the nucleus and activating transcription of <i>IL8</i>	25
5	
1.8: <i>P. gingivalis</i> prevents the transcription of chemokines associated with Th-1 cells, ICAP-10, MIG and ITAC by inhibiting STAT1 and preventing the activation of the transcription factor IRF-1.....	27
1.9: The OMVs produced by <i>P. gingivalis</i> play a role in biofilm formation and bacterial co-aggregation. They can coat the surface of the highly motile <i>T. denticola</i> which the non-motile <i>L. sabburneum</i> uses to adhere to <i>T. denticola</i>	300
1.10: Phylogentic analysis of HagA-HagE in two strains of <i>P. gingivalis</i> (Strain ATCC33277 and W83). Homology and phylogenetic distances were determined using ClustalW.....	35
5	
1.11: The <i>hagB</i> and <i>hagC</i> locus in strain W83 of <i>P. gingivalis</i> showing that the two genes are located chromosomally close together.....	354
1.12: Image showing the amino acid sequence alignment of HagB and HagC to one another. They share 98% identity to one another with differences seen at the N-terminus of the sequence	38
1.13: Image taken from the Artemis comparsion tool (ACT) software package which allows comparison of genomic synteny and homology. Regions in red are highly homologous to one another. Image shows that the <i>hagB</i> and <i>hagC</i> genes are highly conserved genes despite genomic rearrangements which occur around them.....	39

Chapter 3

3.1: a) Diagram showing the positions of the primers used to confirm the junctions of the *ErmAM-ErmF* cassette within *hagB* b) A 0.8% agarose gel of PCR reactions carried out to confirm the junctions of *hagB* transformants, with no template and wild type DNA controls. 1) 1 kb ladder 2) no template control (HagBIF and ErmIR) 3-4) pJW1 transformant 1 (HagBIF and Erm1R) 5-6) Wild type DNA (HagBIF and ErmIR) 7) empty 8) no template control (ErmIF and HagBIR) 9-10) pJW1 transformant 1 (ErmIF and HagBIR) 11-12) Wild type DNA (ErmIF and HagBIR).....76

3.2: Diagram showing the construction of phagCΔ, which was used to delete *hagC* from the *P. gingivalis* W83 genome. The cassette was constructed in pBlueScript; *tetQ* was ligated in first to give pBSTetQ. The region adjacent to the 5' end of *hagC* (RAHC1) was then ligated into pBSTetQ to give pBSTetQHC1. Into this plasmid the region adjacent to the 3' end of the *hagC* gene was then ligated to give plasmid phagCΔ.....78

3.3: a) Diagram showing position of primers used to confirm the integration of *tetQ* within the *hagC* orf. Both primer sets yield an amplicon size of 1200 bp. b) a 0.8% agarose gel showing PCR reactions carried out to confirm the junctions of *hagC* transformants, as well as the presence of *tetQ*. 1) 1Kb ladder 2) blank 3) no template control 4) blank 5) ECM200 DNA screened using HC1_IF and HC1_IR. 6) ECM200 DNA screened using HC2_IF and HC2_IR 7) ECM200 screened for the presence of *tetQ*, using TetQF and TetQR.....79

3.4: Map of the chloramphenicol resistance cassette used to reintroduce the *hagB* gene in strain in ECM233 to generate GMC100. Only relevant restriction sites mentioned in section 3.2.2 are shown.....81

3.5: a) Diagram illustrating the position of the primers used in the confirmatory PCR in wild type W83. b) Diagram illustrating the position of the primers used in the confirmatory PCR in a double knock-out of W83. c) A 0.8% agarose gel confirming differences in size of the *hagB* and *hagC* loci between wild type W83 and ECM233. 1) 1 Kb ladder 2) no template control 3) Wild type W83 screened with DKOBFP and DKOBRP (expected amplicon of 2100 bp) 4) ECM233 screened with DKOBFP and DKOBRP (expected amplicon of 4400bp) 5)1 Kb ladder 6) No template control 7) Wild type W83 screened with DKOCRFP and DKOCRBP (expected amplicon of 3100bp) 8) ECM233 screened with

DKOCCF and DKOCCP (expected product of 4600 bp).....	83
3.6: Southern blot analysis of genomic DNA isolated from Wild type W83, ECM200 and ECM233. Probe designed to share 100% homology to a 275 bp region of both <i>hagB</i> and <i>hagC</i> . Diagram also shows plasmid pJW1 recombination that occurred during the event where the plasmid integrated with the 5' region of the <i>hagB</i> gene and the 3' region of the <i>hagC</i> gene.....	87
3.7: a) A 0.8 % agarose gel confirming the presence of <i>hagB</i> in Δ <i>hagB</i> complemented mutants 1) 1 kb ladder 2) negative control 3) positive control 4-9) screened transformants (expected product size of 2100 bp) b) A 0.8 % agarose gel confirming the absence of the <i>erm</i> cassette in Δ <i>hagB</i> complemented mutants. 1) 1 kb ladder 2) negative control 3) positive control. 4-9) screened transformant.....	94
3.8: a) Diagram showing that at OD ₆₀₀ of 0.7 under haemin rich conditions <i>hagB</i> is expressed. b) There was a statistically significant difference between WT and strain ECM233.....	95
3.9: a) Expression levels of <i>hagC</i> after growth in sTSB to OD ₆₀₀ of 0.7 b) There was a statistically significant difference between strain ECM100 and all other mutant strains.....	96

Chapter 4

4.1: Image showing the pigmentation differences between WT W83 and mutant strains when grown on sTSA anaerobically at 37°C for 4 days.....	113
4.2: Image showing the level of pigmentation in cell pellets from broth cultures of <i>P. gingivalis</i> following haem starvation (top row) and subsequent restoration to haemoglobin or haemin rich broth post starvation for 24 hr or 48 hr. The strain names are shown below.....	114
4.3: Graph showing a comparison of growth rates between WT W83 and mutant strains of <i>P. gingivalis</i> . Doubling times in hr are shown below.....	116

4.4: Haemagglutination assays of *P. gingivalis* with sheep RBCs (2.5% sheep's blood) demonstrating the difference in haemagglutination capabilities between WT W83 and the mutant strains ECM100, ECM200 and ECM233 grown to either O.D₆₀₀ 0.4 or 0.6 in. sTSB.....118

4.5: Results of stress sensitivity testing of WT W83 and mutant strain ECM233 to chloramphenicol (control), 9% vol/vol hydrogen peroxide, crystal violet, diamide and Triton X-100. No statistically significant difference in growth between WT W83 and strain ECM233 was detected (p-value > 0.05). All experiments were carried out in triplicate.....119

Chapter 5

5.1: a) Levels of adherence of *P. gingivalis* WT W83 and mutant derivative strains to H357 cells after 90 min infection following growth in haemin rich media. The level of interaction is expressed as a percentage of the initial inoculum. ANOVA was performed and statistically significant differences were found to be present between the strains, indicated by stars (p-value < 0.0001). b) The results of Dunnett's Multiple Comparison, a post ANOVA test that can be carried out to determine if there are statistically significant differences between the strains compared to the WT strain. In this case there were statistically significant difference between WT and all other strains with the exception of ECM233.....137

5.2: a) Levels of adherence of *P. gingivalis* WT W83, ECM233 and DKO3 to H357 cells after 90 min infection after growth in haemin rich media. The level of interaction is expressed as a percentage of the initial inoculum. b) The results of Dunnett's Multiple Comparison. There were no statistically significant differences found between WT strain and the mutant strains under these conditions.....140

5.3: a) Levels of adherence of the indicated *P. gingivalis* strain ATCC33277 derived strains to H357 cells after 90 min infection after growth in haemin rich media. The level of adherence is expressed as a percentage of the initial inoculum. ANOVA revealed statistically significant differences between the strains, indicated by stars (p-value 0.008). b) The results of Dunnett's Multiple Comparison. There were statistically significant differences found between WT strain and the mutant strains under these conditions (p-value < 0.05).....141

5.4: a) Levels of adherence <i>P. gingivalis</i> WT W83 and mutant derivative strains to H357 cells after 90 min infection following growth in haemin free media. The level of interaction is expressed as a percentage of the initial inoculum. b) The results of Dunnett's Multiple Comparison. There were no statistically significant differences found between WT strain and the mutant strains under these conditions.....	142
5.5: a) Levels of <i>P. gingivalis</i> WT W83 and mutant derivative strains within H357 cells after 90 minutes infection and subsequent kill step. Experiment was carried out after growth of bacteria in haemin rich sTSB media. The level of invasion is expressed as a percentage of the initial inoculum. Statistical significance is indicated by a star. b) The results of Dunnett's Multiple Comparison. In this case there were no statistically significant differences between WT and mutant strains with the exception of GMC100.....	144
5.6: a) Normalisation of levels of invasion of <i>P. gingivalis</i> WT W83 and mutant derivative strains (invasion/adherence) based on 90 min assay results. The levels of invasion is expressed as a percentage of the bacteria that have adhered after 90 min. ANOVA indicated a statistically significant difference between strains, indicated by a star (p-value 0.0122). b) The results of Dunnett's Multiple Comparison. In this case there were no statistically significant differences between WT and mutant strains with the exception of GMC100.....	145
5.7: a) Levels of invasion of <i>P. gingivalis</i> WT W83 and mutant derivative strains within H357 cells after 24 hr infection and subsequent kill step. Experiment carried out after growth in haemin rich media. The level of invasion is expressed as a percentage of the initial inoculum. ANOVA indicated a statistically significant different between the strains indicated with stars (p-value 0.0074). b) The results of Dunnett's Multiple Comparison post ANOVA.....	146
5.8: Biofilm growth of <i>P. gingivalis</i> WT W83 and mutant derivative strains was measured either by crystal violet staining (a) or staining with CFSE and fluorescent intensity measurements over 3 days (b). Significant differences on day 3 were observed when comparing ECM200 and ECM233 biofilm formation to the WT control.....	148
5.9: Mono-species biofilm growth of <i>P. gingivalis</i> WT W83 and mutant derivative strains revealed by staining with CFSE on day 3 of biofilm growth. Differences in fluorescent	

intensity are visible between WT compared to strain ECM200 and ECM233.....149

5.10: Mature biofilm biomass of multi-species biofilms was measured using crystal violet staining. Significant differences were observed between the biomass of biofilms containing *P. gingivalis* WT and those containing strain ECM200.

Data are mean $\pm$ SEM of 3 independent biofilms per group. ***
p<0.0011.....152

5.11: a) The amount of each species within the biofilm was quantified using qPCR. There were no significant differences in the total number of each species present within the biofilms. b) Percentage composition of each biofilm by species. There were no significant differences in the proportions of each species present within the biofilms.....153

5.12: Expression level is based on ΔC_t values as calculated in section 2.3.2 a) Expression levels of *rgpB* after growth in sTSB to an OD₆₀₀ of 0.7 and b) Expression levels of *kgp* after growth in sTSB to an OD₆₀₀ of 0.7. Student t-testing was performed to compare WT to mutant strains individually.....154

5.13: Expression levels of *hagA* in WT W83 and mutant strains grown to an OD₆₀₀ of 0.7 in sTSB. A two-sided student's t-test was carried out to analyse the difference between WT and strain ECM200. There is statistically significant difference between WT and strain ECM20 (p-value 0.0113).....155

5.14: Expression levels of *rgpA* in *P. gingivalis* strains grown to an OD₆₀₀ of 0.7 in sTSB.. A two-sided student's t-test was carried out to analyse the difference between WT and mutant strains. Statistically significant differences were found between WT vs. ECM200 (p-value 0.0174), WT vs. ECM233 (p-value 0.0009), WT vs. GMC100 (p-value 0.0194).....156

5.15: Expression of *rgpA* and *rgpB* in *P. gingivalis* strains were examined after growth on sTSA a) *rgpA* expression levels. There was no statistically significant difference between the strains. b) *rgpB* expression levels. There was no statistically significant difference between the strains.....158

5.16: Expression levels of *kgp* in *P. gingivalis* were assessed after growth on sTSA. On analysis using an unpaired students t-test it was found that there was a statistically significant difference between strain ECM100 and GMC100 (p-value of 0.0422).....159

5.17: Expression levels of *hagA* in *P. gingivalis* were assessed after growth on sTSA. On analysis using an unpaired students t-test it was found that there was a statistically significant difference between strain ECM233 and GMC100 (p-value of 0.0003).....160

Chapter 6

6.1: The results obtained from the multi-analyte ELISA data analysed in two ways. a) Using the corrected absorbance values. b) Using fold differences as a measure of scale.....179

6.2: Mip-1 β expression data from multi-analyte assay a) on corrected raw data values (t-test p-value 0.0021) and b) fold difference (t-test p-value of 0.0036).....180

6.3: a) Levels of Mip-1 β production by THP-1 cells at an M.O.I of 10. b) The results of Dunnett’s multiple comparison. It was found that there were statistically significant differences between WT and strains ECM233.....181

6.4: a) Levels of Mip-1 β production by THP-1 cells at an M.O.I of 50. b) The results of Dunnett’s Multiple Comparison. There were no statistically significant differences found between WT strain and the mutant strains under these conditions.....182

Chapter 7

7.1: *In vitro* model of host cell interaction outlining the methods used in both the adherence and invasion Tn-Seq screening assays.....194

7.2: Diagram outlining the downstream processing of DNA. Samples were sheared and C-tailed before under-going a semi-random PCR process. The samples then underwent Illumina sequencing. The generated sequences then underwent data analysis.....199

7.3: a) Ratio of output/input reads per gene were generated in GeneSpring and graphed showing their distribution. Normalised Ratio = No. Output reads/input reads normalized to median. Genes with normalized ratios 5-fold above or below the median are shaded. b) Numerical data showing the range and distribution of normalised ratios (pre-normalisation).....	201
7.4: Diagram showing the location and abundance of insertions, generated using GenomeView. a) Shows the location and abundance of reads recovered for <i>fimR</i> and <i>fimS</i> in the input and output libraries. b) Shows the location and abundance of reads recovered for <i>serB</i> in the input and output libraries.....	209
7.5: Diagram showing the input and output reads for the <i>fimA</i> gene. It can be seen clearly that the insertions in the output files are predominantly located at the 3'- and 5'- ends of the gene.....	212
7.6: Diagram outlining the potential interactions of PGN_0378, an exopolyphosphatase enzyme. An interaction with Ppk was observed among these interactions.....	214
7.7: Diagram showing the location and abundance of sequence reads in input and output library reads for PGN_1347. It can be seen that there is a substantial reduction in reads in the output samples compared to the input samples.....	215
7.8: Diagram outlining the potential interactions of PGN_1347, a putative TonB dependent exported protein. An interaction with RagA was observed among these interactions.....	216

Chapter 8

8.1: Diagram outlining a two stage model of adherence in <i>P. gingivalis</i> . (a) Stage 1 of the interactions is a long range interaction mediated by the FimA subunit of the major fimbriae interacting with β 1-integrins present on the surface of epithelial cells. (b) Stage 2 occurs after the bacteria has moved closer to the cells and is a short range interaction mediated by HagB and an unknown receptor which acts to stabilise the interaction between the bacterium and the host cell surface.....	228
--	-----

8.2: Diagram showing the indirect effects that mutagenesis may have (a) The development of a compensatory secondary mutation (red) after the primary mutation is introduced (yellow). (b) Mutagenesis can affect the bacterial epi-genome leading to changes in bacterial epigenetics such as alterations to methylation patterns. (c) Mutagenesis may interfere with sRNA molecules and as such RNA regulatory. This will affect key processes where sRNAs play a role such as 1. mRNA processing and 2. sRNA protein binding involved in genomic transcription regulation.....229

Index of Tables

Table 2.1: *P. gingivalis* strains used in this study and their *hagB* and *hagC* genotype.
 Mutant strain names were assigned by the laboratory where the mutants were made.48

Table 2.2: Characteristics of PCR primers used in this study52

Table 2.3: Primers used in qPCR experiments53

Table 4.1: A table listing the reagents used for stress sensitivity testing of *P. gingivalis* W83 and mutant derivative ECM233, showing zones of inhibition for each agent tested.. 110

Table 7.1: Table showing the genes which had a 5 fold or more reduction in the output files compared to the input files.....204

Abbreviations

ACT	Artemis comparsion tool
Akt	Protein kinase B
ANOVA	Analysis of variance
ApoE	Apolipoprotein E
AS	Artificial saliva
ATP	Adenosine triphosphate
BAD	Bcl-2 associated death
BLAST	Basic Local Alignment Search tool
BLASTn	BLAST nucleotide
Bp	Base pair (s)
CaCl ₂	Calcium chloride
CBA	Columbia blood agar
CCL3/MIP-1 α	Chemokine ligand 3/ Macrophage inflammatory protein-1 α
cDNA	Complementary DNA
CFSE	Carboxyfluorescein succinimidyl ester
CPS	Capsule
Ct	Threshold Cycle
CXCL10/ICAP-10	Interferon gamma induced protein
CXCL11/I-TAC	Monokine induced by interferon gamma
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
dTTP	Deoxythymidine triphosphate
e.g.	For example
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
FCS	Foetal calf serum

G	Gravity
GDH	Glasgow Dental Hospital
GECs	Gingival epithelial cells
GM-CSF	Granulocyte macrophage colony stimulating factor
H2O2	Hydrogen peroxide
HbR	Haemoglobin receptor domain protein
HCAE	Human coronary artery endothelial
HCl	Hydrochloric acid
Hr	Hour(s)
HSP60	Heat shock protein 60
HβD3	Human β defensin 3
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
Jak	Janus Kinase
JNK	The c-jun terminal kinase
Kb	Kilobase
kV	Kilovolts
LB	Luria-Bertani
LLRs	Leucine rich repeats
LPS	Lipopolysaccharide
LX	Luria-Bertani X-gal
M	Molar
M.O.I	Multiplicity of infection
MCP-1	Monocyte chemotactic protein 1
MDC	Macrophage derived chemokine
MgCl2	Magnesium chloride
Min	Minute
MIP-1β/CCL4	Macrophage inflammatory protein-1β
mM	Millimolar
mRNA	Messenger ribonucleic acid
MYD-88	Myeloid differentiation response gene 88
NaOH	Sodium Hydroxide
O.D.	Optical density
OMV	Outer membrane vesicle
orf	Open reading frame
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
Pg381	<i>P. gingivalis</i> 381
pH	Decimal logarithm of the reciprocal of the hydrogen ion activity
PI3K	Phosphoinositide 3-kinase
PIPES	Piperazine-N,N'-bis(ethanesulfonic acid); 1,4-piperazinediethanesulfonic acid
PMA	Phorbol 12-Myristate 13-Acetate
poly-P	Inorganic polyphosphate
Ppk	polyphosphate kinase
PPRs	Pathogen recognition receptors

PPX	Exopolyphosphatase
PTFL	Polytetrafluoroethylene
qPCR	Quantative (real-time) polymerase chain reaction
r.p.m	Rotations per minute
RANTES	Regulated on activation, normal T-cell expressed and secreted protein
RBC	Red blood cell
rHagB	Recombinant HagB
RNA	Ribonucleic acid
ROS	Reactive oxygen species
S	Seconds
SerB	Serine phosphatase B
SES	Socio economic status
STAT	Signal transducer and activator of transcription
sRNA	Small ribonucleic acid
sTSB	supplemented tryptic soy broth
TE	Tris EDTA
Th	T-helper
T _{hyb}	Hybridisation temperature
TLR	Toll like receptor
T _m	Melting temperature
TNF- α	Tumour necrosis factor alpha
TRIF	TIR domain containing adaptor inducing interferon β
Tn-Seq	Transposon-Seq
TSA	Tryptic soy agar
UV	Ultra-violet
V	Volt
v/v	Volume per volume
W	Watt
WT	Wild type
w/v	Weight per volume
w/w	Weight per weight
Δ Ct	Delta Ct
$\Delta\Delta$ Ct	Delta Delta Ct
%	Percentage
μ g	Micrograms
μ l	Microlitre(s)
μ M	Micromolar

Acknowledgements

Firstly, I'd like to thank my supervisor Dr. Gary Moran, whose support, feedback, patience, understanding and encouragement were an immense help over the course of this work and without whom I would never have reached this stage. I would also like to thank Dr. Moran for the opportunities to present my work both nationally and internationally. For all of these things I am immensely grateful.

I would also like to extend my thanks to Prof. David Coleman and Dr. Mary O'Donnell for all their help and support throughout my time in the lab, particularly when things became complicated. My particular thanks to Dr. Mary O'Donnell for having an open door policy and for the many random conversations. I could not have asked for a kinder lab manager. I also wish to extend my thanks to Prof. Sullivan for his encouragement and support throughout this project.

A special thanks to the staff in the Microbiology Laboratory at the Dublin Dental University Hospital-I was very lucky to have spent the duration of my study in such a fantastic and supportive environment with such lovely people. Thank you for all the tea, treats and general hilarity.

Particular thanks to Aisling Miller for generally being wonderful about bubbles and life in general. My thanks to her also for "shoo"-ing me back down to my bench when required. Thanks to Dr. John Haran for the bench chats and for taking the time to help when it was needed. My thanks to Aisling for much of the photography featured in this work and to John for sharing his knowledge of qPCR. I am immensely grateful to them both.

Special thanks to Dr. Phillipa Cashin who over the duration of my Ph.D. became an incredible and wonderful friend. I will forever be grateful for the fusion of our senses of

humour while we worked together down the back lab. My thanks to Dr. Peter Hyde for understanding that sometimes words aren't necessary, for making me laugh and for listening to my epic rants about anything and everything. My thanks to Peter Flanagan for his time as a wonderful bench buddy. I will miss our musical renditions. Thanks to Alex, Anna, Brenda, Sarah, Emily, Hannah, Aoife, Peter K, Maria, Hal, Abdrazak and Khalid as well for the chats, help and general banter over the past few years.

I am incredibly lucky in my friends and as such would like to thank Jo, Rebecca, Claire, Amy and Lisa for their ongoing support and love. I would never have been able to do this without you all, may we spend another ten years at least cackling with one each other. Thank you for all the epic adventures and quests throughout the last few years. Special thanks to Claire, Rebecca and Lisa for making sure I got out while I wrote up and to Jo and Amy for taking calls at all hours. I love you all and I think we can now safely say "mischief managed".

To Philip, my partner, thank you for always making me laugh, even if it is mainly at myself. Your support, grace and humour made this more fun than I ever expected it to be. Thank you for the epic adventures and hilarious stories. I'm looking forward to the next adventure with you.

To my mother, thank you for your support, kindness and generosity over the course of my studies, it's greatly appreciated. None of this would have been possible without you.

To Vela, Pippa and Tabby, thank you for generally being adorable.

Chapter 1

Introduction

“For there was never yet a philosopher that could endure toothache patiently”

-William Shakespeare.

1.1 Periodontitis

Toothache, tooth loss and humanity have gone hand in hand together since our earliest times. Teeth are supported by tissue known as the periodontium, which consists of the epithelial, connective and bone tissues that support the tooth (Fig 1.1). The word itself comes from two Greek terms; “peri” meaning around and “odons” meaning tooth (Darveau, 2010). In a healthy oral cavity this tissue exists in a state of balanced harmony with dental plaque which is a poly microbial biofilm made up of a host of microorganisms that build up on the tooth and tooth root (Jenkinson, 2011). Good oral hygiene removes much of this biofilm and prevents the development of dental disease.

The major cause of tooth loss in humans is periodontitis, which is a chronic inflammatory disease of the periodontium in response to the presence of polymicrobial dental plaque that colonises the area between the tooth surface and the marginal gingiva. When left unchecked and untreated it facilitates the destruction of the tooth supporting tissues such as alveolar bone and connective tissue, consequently leading to tooth loss (Fig 1.1) (Darveau, 2010).

The disease can also affect systemic health, as studies suggest that it increases a person's risk of developing atherosclerosis (Saffi et al., 2015) and possibly some autoimmune diseases such as rheumatoid arthritis (Atanasova and Yilmaz, 2014).

There are a number of recognised risk factors for the development of periodontitis. Poor oral hygiene has one of the strongest links with disease development and progression. Levels of periodontitis in those with poor oral hygiene are substantially higher than in those with good oral hygiene practices (Hobdell et al., 2003).

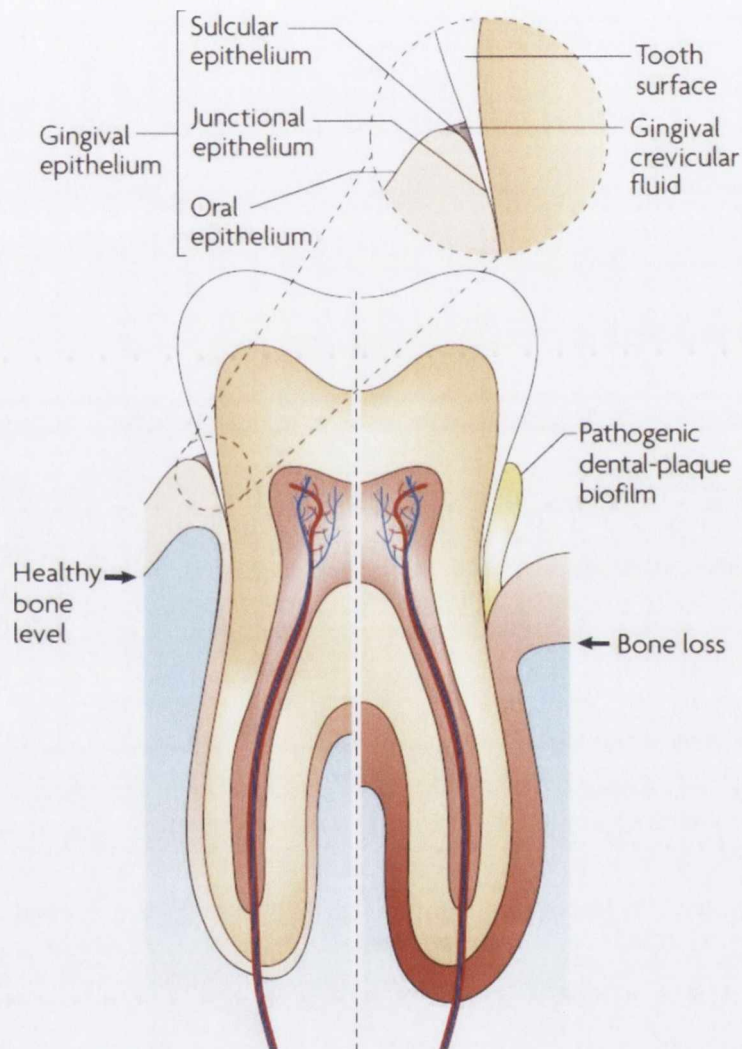


Figure 1.1: Diagram showing the differences between a healthy tooth and a diseased tooth, in particular the difference in inflammation and bone level, as well as the presence of dental plaque. Reproduced from (Darveau, 2010).

The effect of smoking has been extensively examined; there is a positive correlation between prevalence of periodontal disease with increasing severity and the number of packs/years smoked (AlJehani, 2014). Smoking has a number of effects on the oral cavity. It encourages the growth of specific periodontal pathogens and by selecting these organisms through smoking there is an increased risk for the development and progression of periodontitis (Zambon et al., 1996). Studies have shown repeatedly that smoking suppresses the immune system, facilitating this outgrowth. When the cytokine and chemokine profiles of smokers were examined and compared to those of non-smokers it was found that a range of cytokines including interleukin-6, chemokine ligand 3 (CCL3 or MIP-1 α) and interleukin 8 were reduced in smokers compared to the non-smoking controls (Souto et al., 2014, Tymkiw et al., 2011).

Socio economic status (SES) also influences disease development and progression. Those with a higher SES and better access to education have been shown to have a reduced incidence of disease compared to those with a low SES (Hobdell et al., 2003). Hobdell *et al.* (2003) who examined the link between oral health and SES in developed and less developed nations found that the incidence of periodontitis was associated with SES in all regions. This is probably related to access to basic dental care (Hobdell et al., 2003). Additionally, in less developed nations, access to water, toothbrushes and toothpaste may well be linked to SES. It has been shown in Britain that despite universal access to dental care as provided by the National Health Service, oral health inequalities due to SES are still an issue due to the behavioural risk factors associated with it, such as dietary habits, poor oral hygiene, tobacco consumption, alcohol and drug use (Watt and Sheiham, 1999). Periodontitis is also associated with aging, with the risk of developing the disease increasing with age.

A number of factors are likely to contribute to this; the immune system loses potency with aging which facilitates sub-gingival plaque outgrowth if a proper oral hygiene routine isn't maintained, the prevalence of certain diseases with known associations with periodontitis, such as diabetes also increases as the population ages (AlJehani, 2014).

Diabetes (either Type I or Type II) has been shown to be a risk factor for the development of periodontitis. In individuals where diabetes is poorly controlled there is an increased risk of developing periodontitis and the disease may progress more rapidly in those who have either controlled diabetes or who are diabetes free (AlJehani, 2014).

In the U.S.A the World Health Organisation has shown that approximately 47% of adults over 30 years of age have some form of periodontitis. Of that 47%, 8.7% have mild periodontitis, 30% have a moderate form of the disease and 8.7% have a severe form. This is in keeping with global studies which indicate that severe forms of the disease will affect between 5-15% of the adult population globally (WHO, 2003). It is also well established that there is an increase in the prevalence of the disease in the older population. In adults over 65 yrs of age in the U.S.A the percentages are 5.9%, 53% and 11.2% for mild, moderate and severe forms respectively, highlighting that 70.1% of the population which are over 65 are affected by some form of the disease (Frieden, 2013). Given the high percentage of adults affected, particularly those aged over 65, the economic impact of treatment and prevention is significant. In the United States alone periodontal disease is estimated to account for \$4.4 billion in annual expenditure (Brown et al., 2002).

Links between periodontitis and the progression of a number of systemic diseases have also been examined. Evidence that periodontitis drives the development of rheumatoid arthritis has been put forward (Koziel et al., 2014), as well as evidence that periodontitis is a risk factor for cardio-vascular disease (Gu et al., 2011), chronic respiratory infections (Oztekin et al., 2014) and Alzheimer's disease (Abbayya et al., 2015). One hypothesis

suggests that the chronic, low grade, inflammation caused by periodontitis can trigger the development or drive the progression of these diseases under certain circumstances.

1.2 The evolution of the aetiology of periodontitis

Historically periodontitis has been associated with a number of microbiological aetiologies. At the end of the nineteenth century it was widely accepted that dental infections including periodontitis were caused by non-specific out-growth of all bacteria in dental plaque (Black, 1884). This was later called the “Non-specific plaque hypothesis” (Loesche, 1976), it was believed that the levels of plaque directly accounted for the pathogenicity of disease without discriminating between differences in levels of virulent bacteria. This led to mechanical removal of plaque becoming the mainstay in treatment and prevention of dental disease and is still used in treatment today (Rosier et al., 2014).

This theory had largely been abandoned by the mid-twentieth century as techniques for the isolation and identification of bacteria improved. By the 1970s a number of advances had been made in microbiological isolation and identification techniques including the development of the anaerobic hood. This led to the formation of the “specific plaque hypothesis” which held that oral diseases including periodontitis were inflammatory diseases caused by specific periodontopathogens and that treatment with antibiotics would be effective in disease treatment and management (Loesche, 1986a, Loesche, 1986b). Subsequent studies have shown that the introduction of antibiotics into treatment programmes has had only a modest effect on treatment success (Eberhard et al., 2008a, Eberhard et al., 2008b).

In the decade after the “specific plaque hypothesis” a number of potential periodontopathogens were identified, including *Bacteroides* species. The identification of bacteria that could be potentially linked to periodontal disease led to the idea that periodontitis and other oral diseases could be initiated by specific pathogens. This idea was

continuously researched and resulted in the elucidation of Socransky's complexes which include bacterial groups that are linked to periodontitis (Socransky et al., 1998).

By using whole genomic DNA probes and checkerboard DNA-DNA hybridisation Socransky and colleagues were able to characterise the microbial community in periodontal pockets, upon characterisation they placed specific organisms into a colour coded system that reflected cluster analysis, community ordination and associated disease severity (Socransky et al., 1998). One of these complexes is the "red complex". This complex is made up of *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola*. All of these organisms were found to be associated with more severe forms of periodontitis (Hajishengallis and Lamont, 2012).

In the early 1990's the "ecological plaque hypothesis" was proposed which suggested that the development of disease is the result of an imbalance in the total micro-flora due to ecological stress which allows for the enrichment of some oral pathogens (Marsh, 1994). It essentially built on the work of another scientist, Theilades, which held that "increased virulence of plaque is due to plaque ecology that is unfavourable to the host" (Theilade, 1986). Marsh expanded on this idea substantially and linked the changes in microbial composition to alterations in environmental factors such as pH, redox potential and the presence of nutrients. This work is widely respected today and it is still generally accepted that the environment influences dental plaque composition.

More recently, two intrinsically linked theories have been developed to explain the pathogenesis of periodontitis based on recent data from microbiome studies and rodent model experiments. These are the "keystone pathogen theory" and the "polymicrobial synergy and dysbiosis model".

The "keystone pathogen" theory states that some microbial species have a disproportionate effect on their environment relative to its overall abundance in a microbial niche. It

suggests that a low abundance microbial pathogen can trigger inflammatory disease, such as periodontitis, by encouraging outgrowth of the normal micro-flora and by changing its composition. Building on this theory came the “polymicrobial synergy and dysbiosis model”. This is a disease that is caused by a reduction in the level of beneficial symbionts and/or a rise in the number of pathogenic species present. This concept is commonly referred to as dysbiosis (Hajishengallis and Lamont, 2012). Currently the mechanisms that maintain the stability or trigger changes in microbial composition are not fully understood.

Central to the concept of dysbiosis is the role of the keystone pathogen. In the case of periodontitis, one of the most important the keystone pathogens believed to trigger the process is *Porphyromonas gingivalis*. It is believed that it disrupts the normally homeostatic relationship that the microbial community has with the host and alters it to a dysbiotic state (Fig 1.2) (Darveau et al., 2012). As the dysbiotic community expands in size and number there is further disruption of tissue homeostasis and the destruction of periodontal tissue as mediated by pathobionts (Lamont and Hajishengallis, 2015).

Recent evidence suggests that these pathobionts include previously under-appreciated species such as *Filifactor aloci* and species from the genera *Prevotella*, *Megasphaera* and *Desulfobulbus*. Meta-genomic analysis, which focused on using 16s rRNA amplicon sequencing to characterise the subgingival microbiome in healthy and diseased patients, has shown that these species have as strong a correlation with severe forms of disease as the traditional species such as *P. gingivalis* (Dewhirst et al., 2010, Griffen et al., 2012).

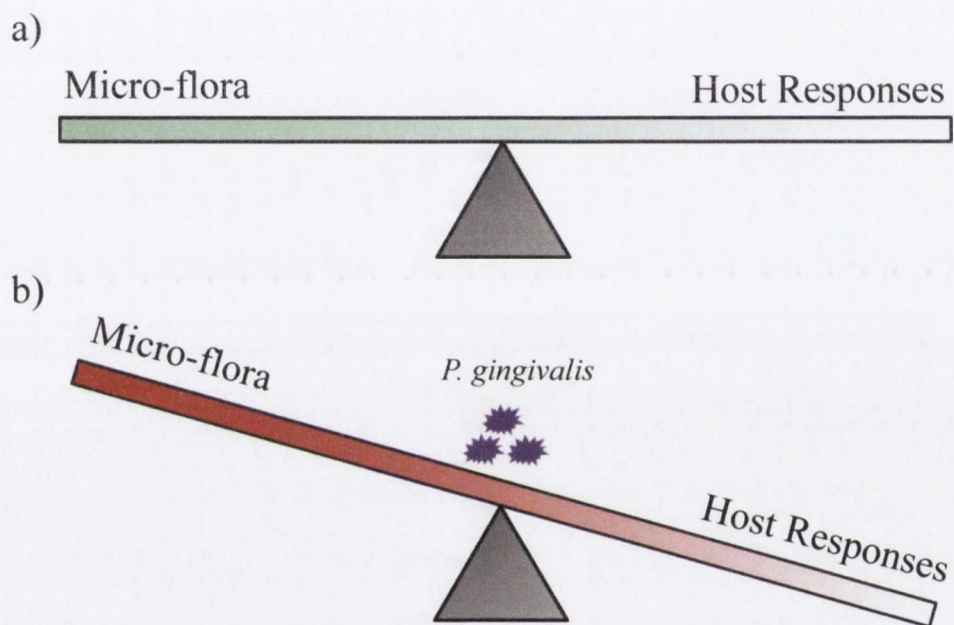


Figure 1.2: Dysbiosis is a microbial shift disease. a) In the healthy mouth a balance is maintained between the oral micro-flora and the host responses. b) The introduction of *P. gingivalis* into the oral biofilm causes a shift in this balance where the oral micro-flora over grows and protective host responses are inhibited.

Another study has highlighted the importance of these species in the virulence of the overall microbial community present within the plaque biofilm. This meta-transcriptomic study, which focused on the gene expression profiles of the human oral microbiome both in health and disease, has shown that a large number of the virulence factors that are upregulated in the microbiome of periodontitis patients come from previously under-appreciated species that were not normally associated with the disease (Duran-Pinedo et al., 2014).

These studies have revolutionised the field in a number of ways; the meta-genomic studies that have been carried out have led to the identification of several previously unknown bacterial species due to the fact that they were unculturable using standard methods. They have also allowed for the detection of species that although culturable may have been overlooked due to low abundance. These studies have facilitated a move away from looking at a few potential causative agents of periodontitis to one where the whole of the microbial community is considered in disease development (Dewhirst et al., 2010). Meta-transcriptomic studies have allowed us to understand how the microbes within the community interact with each other and in turn how the community as a whole interacts with the host, allowing the virulence of the community as a whole to be considered as well as singular species within that community (Duran-Pinedo et al., 2014)

The theory has been put forward that periodontitis requires a keystone pathogen such as *P. gingivalis* for disease initiation but that this pathogen is eventually out-competed in the later stages of the disease by the pathobionts which go on to further exacerbate the condition (Lamont and Hajishengallis, 2015). The behaviour of the periodontal microbiota conforms with the evolutionary biology theory known as the “Black Queen Hypothesis” which suggests that functions that are energetically costly to organisms on an individual level will be retained by a subset of members within the community because they benefit

the community as a whole. Evolution in accordance with this theory produces “beneficiaries” of reduced function and genomic content that are dependent on the small subset of bacteria which retained functionality (Morris et al., 2012). It predicts the emergence of bacteria that could act as keystone organisms such as *P. gingivalis*: those that have kept a function that provides an essential service to other members of the microbiota (Lamont and Hajishengallis, 2015).

1.3 *P. gingivalis*; a keystone pathogen for an ancient disease

P. gingivalis, a gram negative, anaerobic coccobacillus, has been called the vampire of the microbial world (NM et al., 2003). Our journey with it in terms of research has been compared to Dante’s pilgrimage (Yilmaz, 2008). As an organism it is most commonly associated with chronic forms of periodontitis (Lamont and Jenkinson, 1998).

As a pathogen *P. gingivalis* has a substantial clinical impact, a number of studies have highlighted the strong association between the presence of *P. gingivalis* and chronic periodontitis (Colombo et al., 2006, Kato et al., 2014, Liu et al., 2003, Zeng et al., 2012). It has also been associated with the development of aggressive periodontitis in some populations (Sanchez et al., 2015).

P. gingivalis has other disease associations beyond periodontitis. Recent studies have suggested that it may play a role in the development of atherosclerosis and numerous hypotheses have been put forward. *P. gingivalis* DNA has been detected in human atherosclerotic plaque suggesting that it may directly contribute to plaque development (Kozarov et al., 2005). The organism has also been shown to invade human coronary artery endothelial cells (Dorn et al., 1999, Song et al., 2005). Studies carried out in an apolipoprotein E (ApoE) knock-out murine model have shown that peptides from *P. gingivalis* heat shock protein 60 (HSP60) can stimulate T-cell populations that are pro-atherogenic (Jeong et al., 2015). This highlights how *P. gingivalis* can contribute to the

development of systemic diseases through triggering not only local inflammatory responses but systemic responses as well.

Colonisation with *P. gingivalis* is associated with increased gingival pocket depth, receding gums and alveolar bone loss due to resorption (Lamont and Jenkinson, 1998). A landmark study carried out in the 1980's highlighted the importance of *P. gingivalis* in the development of periodontitis. In this study a group of non-human primates were infected with the organism and subsequently monitored. It was found that over the time course of the study the infected animals went on to develop the symptoms of periodontitis (Holt et al., 1988). Studies such as this have suggested that *P. gingivalis* is the most important of the "red complex" organisms suggested by Socransky et al in 1998. However, the pathogenesis of periodontitis is now believed to be substantially more complicated while it is commonly recognised that the three organisms that form the red complex, *P. gingivalis*, *T. denticola* and *T. forsythia* are associated with more severe forms of periodontitis (Zhang et al., 2005a), some studies have found that "red complex" bacteria were below detection levels in diseased sites. This could be because other pathobionts, such as those identified in more recent 16s community profiling studies (Liu et al., 2012), had replaced the red complex organisms and were now driving the disease at these sites (Lamont and Hajishengallis, 2015). Rodent model data (see below) implies that *P. gingivalis* may stimulate outgrowth of other microbes, suggesting that its abundance may decrease as the infection progresses. It is also worth noting that red complex bacteria are frequently detected in low numbers in healthy sites (Bik et al., 2010). Clearly, further studies are required to investigate the dynamic nature of the subgingival microbiome during the development of periodontitis.

More recently *P. gingivalis* has been defined as a keystone pathogen for the development of periodontitis. It is also believed to be central to the development of the dysbiotic state

associated with periodontitis. Numerous studies have been carried out that support both of these theories. In a rodent model of periodontitis it has been shown that the introduction of *P. gingivalis* into plaque at low levels could trigger changes in the composition of the dental plaque and lead to periodontitis (Kesavalu et al., 2007, Love et al., 2000). In germ free mice it has been shown that *P. gingivalis* alone is not sufficient to cause disease and that the presence of other commensal species is required for the initiation of the disease process, highlighting the importance of other species in the development of dysbiosis (Hajishengallis et al., 2011). Other studies carried out in rabbit models (Hasturk et al., 2007) and non human primates have also highlighted the ability of *P. gingivalis* to initiate the development of periodontitis (Page et al., 2007). These studies support the hypothesis that *P. gingivalis* is a key-stone pathogen that requires and effects the growth of other members of the microbial community found in dental plaque during the development of periodontitis (Hajishengallis et al., 2011).

One of the main reasons why *P. gingivalis* is considered central to the “polymicrobial synergy and dysbiosis model” is the fact that it is capable of subverting host immunity. It has three major mechanisms which it uses to do this: (i) *P. gingivalis* is capable of Toll-like receptor (TLR) response manipulation, (ii) it specifically targets key components of the complement response and (iii) subversion of interleukin-8 (IL-8) as well as other chemokines (Rosier et al., 2014).

1.4 *P. gingivalis* and Dysbiosis

P. gingivalis encourages the development of periodontitis by changing the normally symbiotic relationship with the oral micro-flora to a dysbiotic one through immune subversion (Fig 1.3). This facilitates the classic tissue destruction commonly found in periodontitis, where the plaque biofilm has accumulated on the tooth surface leading to the destruction of connective tissue and alveolar bone before finally causing in tooth loss (Fig

1.1). In healthy periodontal tissue the alveolar bone, which supports the tooth root, is covered by the oral epithelium and a specialised epithelium known as the junctional epithelium, which serves to connect it to the tooth surface. The area between the tooth and the epithelium is referred to as the sulcus and is filled with nutrient rich gingival crevicular fluid. In this area a number of select innate immune defence mediators are present such as Toll-like receptors (TLRs), β -defensins, complement and lipopolysaccharide binding protein. These mediators all act in concert with each other and with other signalling and regenerative systems to ensure clearance and killing of overgrowth of bacteria in dental plaque, as well as working in co-operation with one another to ensure tissue homeostasis.

In periodontitis, *P. gingivalis* inhibits the innate host defence system, disrupting tissue homeostasis and facilitating the exposure of host connective tissue to the multi-species biofilm, which leads to further modulation of the host responses. This triggers the classic tissue destruction found in periodontitis leading to the destruction of the connective tissue and alveolar bone, resulting in tooth loss (Darveau, 2010). *P. gingivalis* facilitates the process of changing what would normally be a symbiotic relationship with the oral microflora to a dysbiotic one through immune subversion (Fig 1.3) (Darveau et al., 2012).

Within the oral microbiome there are a variety of interactions that take place. These interactions are facilitated by a number of processes including microbial co-aggregation where the bacteria stick to one another in close communities.

This sticking to each other facilitates the formation of the plaque biofilm which in turn stimulates the host immune response leading to periodontitis and encouraging the development of dysbiosis.

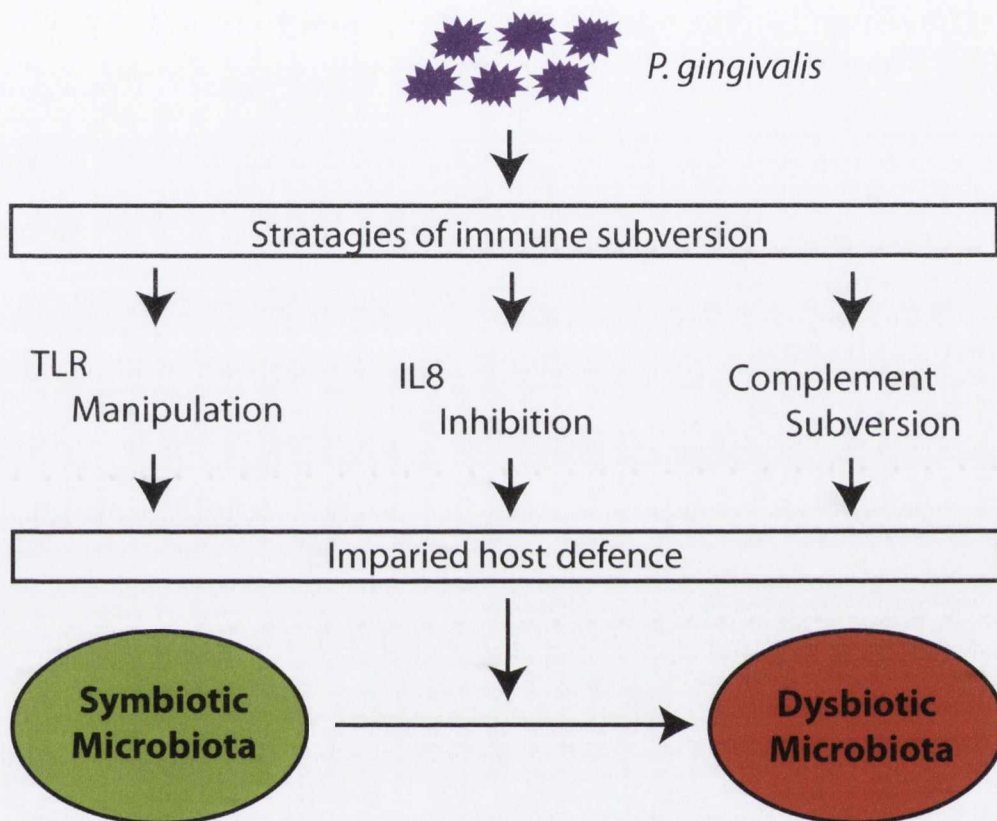


Figure 1.3: Diagram outlining how *P. gingivalis* manipulates the host defence system to encourage the dysbiotic state in the oral cavity allowing for the development and progression of periodontitis. After (Darveau et al., 2012).

P. gingivalis interactions with other species vary across the spectrum of synergy and antagonism. It has been shown that by simply introducing *P. gingivalis* into an oral biofilm the overall virulence of the biofilm increases, even when *P. gingivalis* is introduced in low numbers (Kesavalu et al., 2007, Love et al., 2000). The key to the ability of *P. gingivalis* to subvert host immunity lies in its ability to target key mediators of the host response, as well as how these processes interact with other bacterial species. Proteins of the complement system are present in gingival crevicular fluid at approximately 80% of the levels found in human serum (Hajishengallis, 2010). The system itself is composed of three distinct pathways (classical, alternative or lectin) which converge at the third complement component (C3). The activation of C3 triggers the production and release of effector molecules that have a number of functions (i) they can recruit and activate inflammatory cells (C3a and C5a) (ii) they can mediate microbial opsonisation and phagocytosis (C3b) and (iv) lead to lysis of targeted microbes via the membrane attack complex (Ricklin et al., 2010).

P. gingivalis plays a dual role in terms of complement manipulation, being able to activate and suppress components of the complement system. The main mediators of these processes in *P. gingivalis* are a group of multi-functional proteolytic enzymes referred to as the gingipains. The group consist of three enzymes RgpA, RgpB and Kgp and are encoded for by three separate genes *rgpA*, *rgpB* and *kgp*. All three enzymes cleave the amino acid sequence at amino acids with primary amine groups. RgpA and RgpB are specific for Arg-Xaa bonds, whereas Kgp is specific for Lys-Xaa bonds (NM et al., 2003). All three gingipains target the central component of the complement pathway, C3, successfully. However it has been shown that RgpA and RgpB are more potent than Kgp (Popadiak et al., 2007). By targeting the central component of the complement system *P. gingivalis* prevents deposition of C3b (an opsonin) and the membrane attack complex on the bacterial surface (Fig 1.4) (Lamont and Hajishengallis, 2015).

However as well as having an inhibitory role it has also been shown that the gingipains can activate complement rather than inhibit it. One potential reason for this is that a low level of complement mediated inflammation is beneficial to *P. gingivalis* and the microbial community as a whole. This is because it leads to increased flow of gingival crevicular fluid leading to higher levels of available nutrients (Popadiak et al., 2007). This could be reflective of what happens during early growth within the plaque biofilm, as *P. gingivalis* multiplies the levels of the gingipains could rise sufficiently to switch from stimulators to inhibitors of complement, thus protecting the biofilm as a whole (Lamont and Hajishengallis, 2015). *P. gingivalis* also has a degradation independent mechanism of complement protection. RgpA is capable of capturing C4b-binding protein on its cell surface allowing it to negatively regulate the classical and lectin binding pathways (Fig 1.4) (Potempa et al., 2008). *In vivo* these mechanisms do not act on their own but rather in synergy with other species within the dental biofilm. The gingipains have been shown to act in synergy with karilysin which is produced by *T. forsythia* (Jusko et al., 2012) and interpain A that *Prevotella intermedia* produces (Potempa et al., 2009). *P. intermedia* is also able to activate complement when present in low numbers, aiding *P. gingivalis* in nutrient acquisition (Potempa et al., 2009). These synergistic interactions highlight the importance of the microbial community as a whole in encouraging the development of dysbiosis.

Toll like receptors are a class of pattern recognition molecules that play a major role in innate immunity. They are proteins which span the cellular membrane and have a common structural element in their extracellular region. This element consists of repeats of 24 to 29 amino acids known as a Leucine rich repeat (LRRs). All TLRs contain several LRRs. Each TLR detects a specific repertoire of pathogen molecules that are generally highly conserved (e.g bacterial lipoproteins and peptidoglycans, flagella and double stranded RNA).

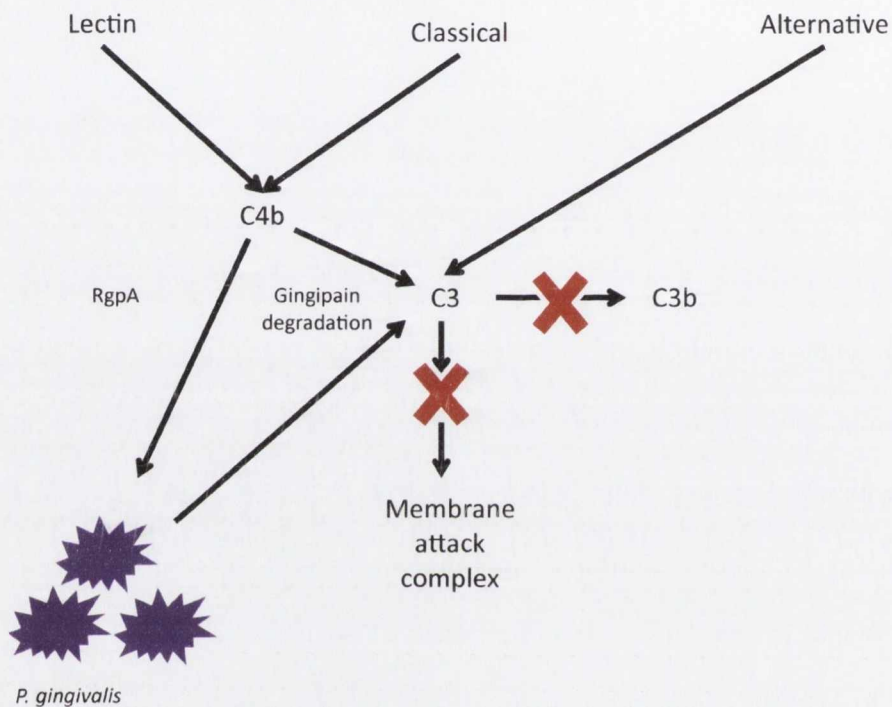


Figure 1.4: Diagram showing how *P. gingivalis* uses the gingipains to manipulate the complement cascade. RgpA is used to coat itself in C4b shielding itself from immune surveillance mechanisms. The gingipains act in concert with one another to degrade the central components of the complement system preventing opsonisation and the formation of the membrane attack complex.

P. gingivalis has developed a number of ways of both evading and manipulating TLR responses in periodontitis. In particular the organisms interactions with TLR4 and TLR2 have been well studied. One of the most unique ways it manipulates the TLR response is through remodelling the structure of its lipopolysaccharide (LPS). The bacterium is capable of altering the lipid A component of its LPS into three distinct forms; a mono-phosphorylated penta-acylated version which acts as a TLR4 agonist, a non-phosphorylated penta-acylated lipid A which is an evasive form and a mono-phosphorylated tetra-acylated lipid A that acts as a TLR4 antagonist. This shift in form is mediated through an enzyme that has lipid A phosphatase activity. By using the TLR4 antagonist or evasive form *P. gingivalis* prevents the activation of nitric oxide synthase thus protecting its self from bacterial killing (Fig 1.5 a) (Darveau, 2010). It has been shown that the switch between forms is regulated *in vitro* by haemin concentration as well as being potentially regulated by temperature (Al-Qutub et al., 2006, Curtis et al., 2011).

Survival within macrophages has been linked to expression of the evasive or antagonistic form of lipid A. A study showed that *P. gingivalis* cells expressing these two forms of lipid A had enhanced survival rates in macrophages compared to those that expressed the agonist lipid A form (Wang et al., 2010). Protection against non-canonical inflammasome activation is also dependent on expression of the antagonistic form of lipid A (Slocum et al., 2014). In order for inflammasome activation to occur via this pathway, caspase-11 must undergo oligomerization. This is a response that can normally be triggered by the LPS of Gram-negative bacteria (Lamont and Hajishengallis, 2015). However in the case of *P. gingivalis* modulation of the structure of its LPS allows it to avoid inflammasome activation, despite its LPS still binding caspase-11. The antagonistic form of lipid A binds to caspase-11 but no oligomerization of the receptor occurs and thus the inflammasome pathway fails to activate despite receptor binding (Fig 1.5 b) (Slocum et al., 2014).

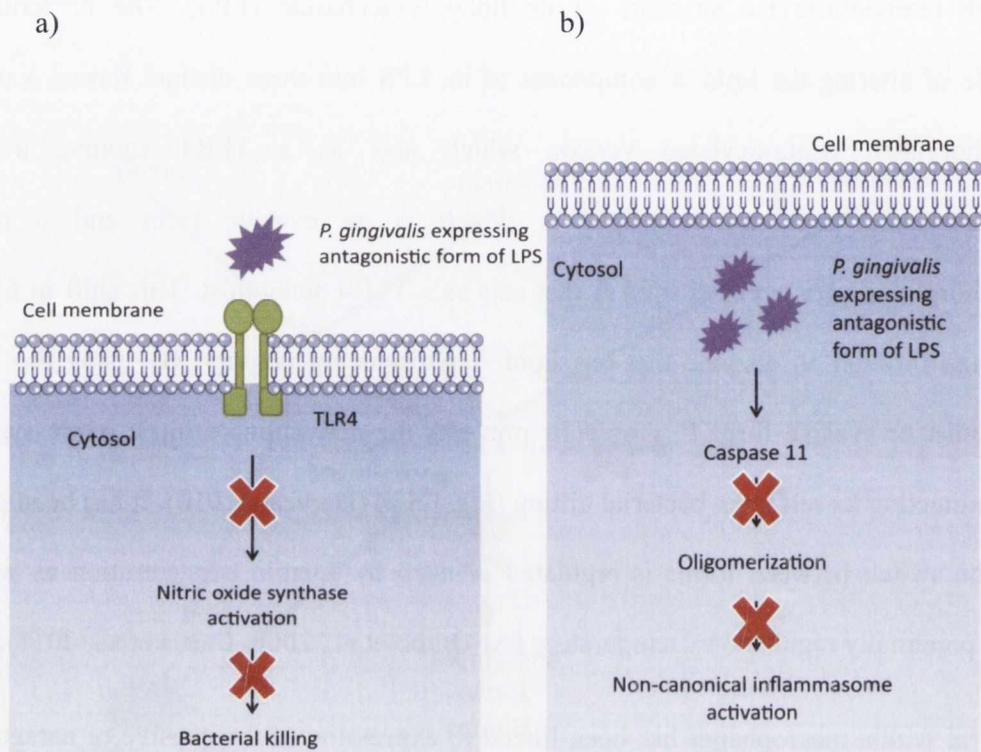


Figure 1.5: a) Using the antagonistic or evasive form of its lipopolysaccharide *P. gingivalis* binds to TLR4 but prevents the receptor from activating. This inhibits the activation of nitric oxide synthase and the production of free radicals preventing bacterial killing. b) Once internalised *P. gingivalis* inhibits non-canonical inflammasome activation by binding to the caspase- 11 receptor but preventing its oligomerization.

P. gingivalis has developed a novel mechanism to avoid neutrophil killing. It has been shown that *P. gingivalis* blocks the TLR2-MyD88 pathway through crosstalk between TLR2 and the receptor for complement fragment C5a (C5aR). This crosstalk triggers ubiquitination and proteosomal degradation of MyD88 thereby blocking the pathways anti-microbial activities. Instead the cross-talk between TLR2 and C5aR activates a pro-inflammatory TLR2-Mal-phosphoinositide 3-kinase pathway which inhibits phagocytosis by interfering with actin polymerisation while also promoting the nutrient rich pro-inflammatory environment preferred by periodontopathogens (Fig 1.6) (Maekawa et al., 2014).

This ability to interfere with TLR signalling is beneficial to the microbial community as a whole. The interference of *P. gingivalis* with TLR2 based neutrophil killing has been shown to protect otherwise at risk species such as *F. nucleatum* (Maekawa et al., 2014). It also protects the biofilm against macrophages by blocking intracellular killing through the production of nitric oxide. Interfering with TLR2 and C5aR helps to inhibit the expression of inducible nitric oxide synthase (iNOS) via a cAMP-dependent mechanism (Wang et al., 2010). This pathway can also be activated through TLR4 which *P. gingivalis* also effectively blocks (Lamont and Hajishengallis, 2015).

Cytokines and chemokines are of vital importance to the immune response. They are cell signalling molecules that act to mediate and regulate immune processes such as the recruitment of immune cells to sites of infection. Cytokines refer to a broad group of proteins, of which chemokines are a subset. Chemokines have a very specific function, to induce chemotaxis, namely the migration of immune cells such as neutrophils to the site of infection. In dysbiosis some key cytokines and chemokines are manipulated by *P. gingivalis* and other species in order to benefit the biofilm as a whole.

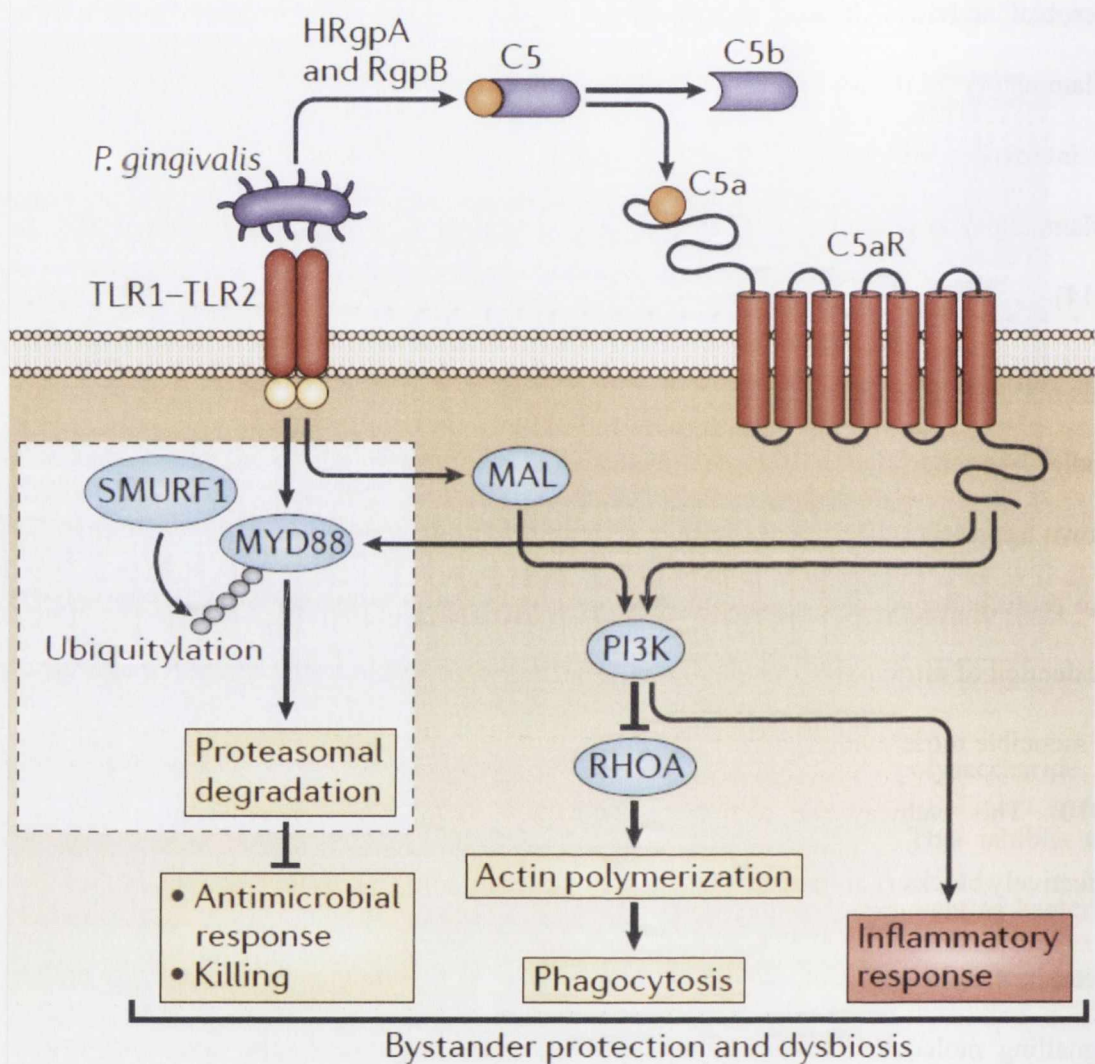


Figure 1.6: Figure outlining the mechanism of action used by *P. gingivalis* to inhibit neutrophil killing. *P. gingivalis* blocks the TLR2-MyD88 signalling pathway via cross-talk with C5aR. MyD88 undergoes ubiquitination by SMURF-1 and subsequent proteosomal degradation. The cross-talk between the two receptors activates the pro-inflammatory TLR2-Mal-PI3K pathway inhibiting phagocytosis (Hajishengallis, 2015).

One of the best studied examples of this is the ability of *P. gingivalis* to down-regulate IL-8 expression and production by gingival epithelial cells (GECs). In a normal healthy epithelium a gradient of IL-8 exists. GECs adjacent to the tooth surface produce IL-8 in response to dental plaque, this IL-8 diffuses into the surrounding area with concentrations found to be highest closest to the tooth surface (Darveau, 2010). The IL-8 acts as a guide for the recruitment of neutrophils to the gingival crevice. The importance of this response is highlighted in those who have genetic defects such as leukocyte adhesion deficiency. Individuals affected by this disorder fail to recruit leukocytes such as neutrophils to the site of infection. A side-effect of this genetic defect is that they suffer from severe and aggressive forms of periodontitis (Hajishengallis and Moutsopoulos, 2014). In periodontitis *P. gingivalis* disrupts this response by preventing the expression of IL-8 by GECs. In a process known as “local chemokine paralysis” it has been shown that GECs exposed to *P. gingivalis* fail to produce IL-8 despite receiving stimulation with bacterial species that are known to be potent inducers of this chemokine (Darveau, 2010, Darveau et al., 1998).

The key mediator of this process in *P. gingivalis* is serine phosphatase B (SerB). SerB is a phosphoserine phosphatase which is essential to both inhibition of IL-8 and internalisation and survival within host cells (Bainbridge et al., 2010, Darveau et al., 1998, Takeuchi et al., 2013). When SerB comes into contact with epithelial cells it is secreted, where it has been shown to affect the cell signal transduction pathways that play a role in the control of the cytoskeleton architecture (Hasegawa et al., 2008). Once *P. gingivalis* is successfully inside the GEC, SerB also suppresses the production of IL-8 by dephosphorylation of RelA and p65 which play major role in the NF- κ B signalling pathway.

By targeting the S536 residue of p65, which is a subunit of NF- κ B, the translocation of this subunit to the nucleus and thus the transcription of the *IL8* gene is prevented (Fig 1.7) (Takeuchi et al., 2013).

This prevents the IL-8 gradient from been established, encourages the development of the dysbiotic state and protects the microbial community as whole.

As with complement based suppression, other species act in synergy with *P. gingivalis* to suppress IL-8 production. *T. denticola* inhibits IL-8 production through its own novel mechanism. *T. denticola* acts very specifically to prevent internalised *F. nucleatum* fusing with lysosomes which may inhibit TLR based responses (Shin et al., 2013). Both *P. gingivalis* and *T. denticola* are acting to reduce the response to pro-inflammatory species that are present in the biofilm such as *F. nucleatum*.

Apart from IL-8 *P. gingivalis* also affects the expression of other chemokines, particularly a number associated with the activation of T-helper 1 (Th-1) cells. It has been shown to inhibit the production of “interferon gamma-induced protein” (ICAP-10/CXCL10), “monokine induced by gamma interferon” (MIG/CXCL9) and “interferon-inducible T-cell alpha chemo-attractant” (I-TAC/CXCL11). These chemokines play an essential role in the recruitment of T-cells to infected sites. It was found that inhibition of these occurred at the genetic level. Inhibition of ICAP-10, MIG and I-TAC was found to be suppressed not by NF- κ B mediated pathways but through the transcription factor IRF-1 which is controlled at a transcriptional level. The transcription factor STAT-1 is a known activator of IRF-1 and was found to be suppressed by *P. gingivalis* (Fig 1.8) (Jauregui et al., 2013) . It is worth nothing that *P. gingivalis* has also been shown to inhibit the expression and production of interleukin-2 (IL-2) which plays an important role in T-cell proliferation (Khalaf and Bengtsson, 2012). .

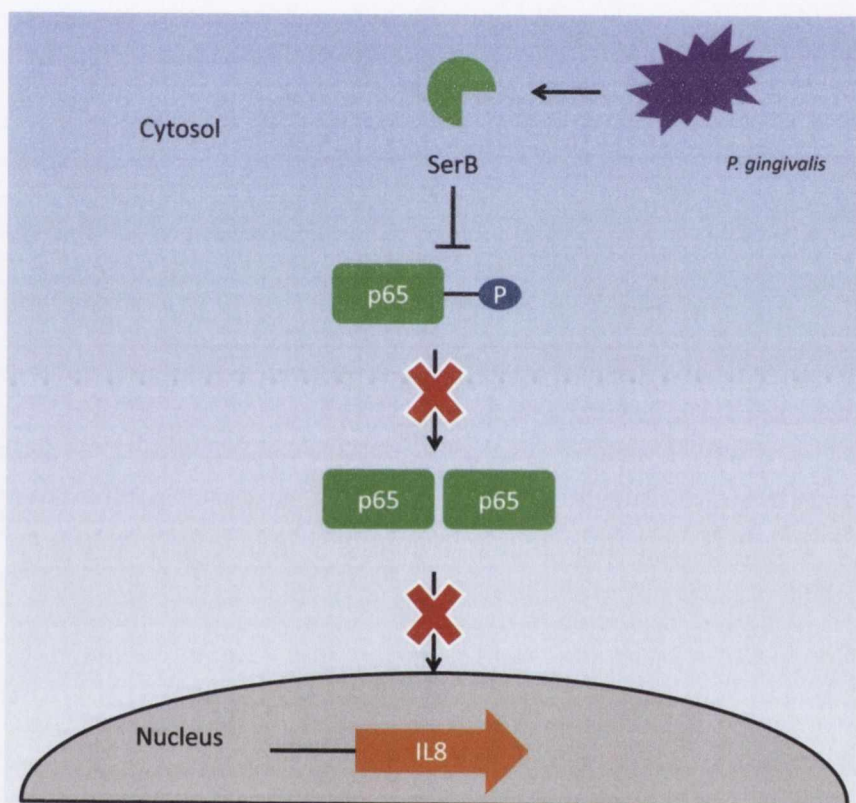


Figure 1.7: *P. gingivalis* SerB prevents the phosphorylation of p65, a key mediator in the NF- κ B pathway. This prevents p65 translocating to the nucleus and activating transcription of *IL8*

As with IL-8 the inhibition of these chemokines is of a high enough level to protect the biofilm as a whole despite the presence of known inducers such as *Fusobacterium nucleatum* (Lamont and Hajishengallis, 2015).

As well as inhibiting the production of chemokines associated with T-cells *P. gingivalis* is also capable of stimulating them. It has the ability to induce interleukin-6 (IL-6) and interleukin-23 (IL-23) both of which have been shown to encourage the development of a Th17 based cell response as opposed to a Th-1 response (Moutsopoulos et al., 2012). By targeting specific cytokines and chemokines *P. gingivalis* may be acting to skew the development of the T-cell response in favour of one that benefits itself and the microbial community as a whole. By reducing the development of Th-1 based immunity and encouraging the development of a Th-17 mediated inflammatory response it is encouraging the development of a specific niche. Th-1 cells have been shown to play an essential role in dictating cell based immunity against periodontal pathogens and by suppressing this response *P. gingivalis* protects itself and the biofilm (Gemmell et al., 2007, Lamont and Hajishengallis, 2015). By encouraging Th-17 responses *P. gingivalis* is creating a niche environment for itself and other member of the biofilm, as studies have shown that Th-17 cells are associated with inflammatory bone destruction commonly seen in periodontal disease (Lamont and Hajishengallis, 2015). This bone destruction opens new environments for *P. gingivalis* and other microbial species to occupy.

Through all of these mechanisms of immune subversion *P. gingivalis* protects not only itself but other microbial species from the immune system, thereby encouraging the development of dysbiosis. Its ability to act in synergy with other microbes to enhance these protective mechanisms highlights its role as a key stone pathogen within the bacterial community as well serving to highlight the importance of the shift from homeostasis to dysbiosis which is of central importance to disease progression and development.

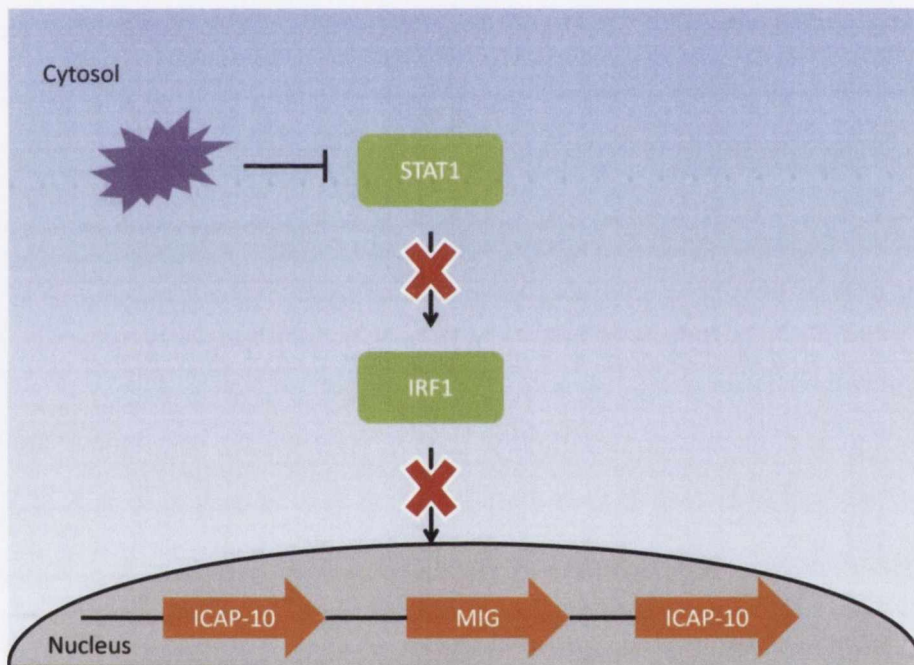


Figure 1.8: *P. gingivalis* prevents the transcription of chemokines associated with Th-1 cells, ICAP-10, MIG and ITAC by inhibiting STAT1 and preventing the activation of the transcription factor IRF-1

1.5 Virulence factors; a nasty side to *P. gingivalis*

Aside from the major mechanisms of immune subversion and evasion as outlined previously, *P. gingivalis* also has a number of other virulence factors which contribute to the development and progression of periodontal disease, as well as dysbiosis. Although these factors may not be major mediators of dysbiosis they do contribute to it and facilitate its establishment in the oral cavity through direct or indirect means.

As outlined previously, *P. gingivalis* uses a modulated form of its LPS to directly contribute to the initiation and development of dysbiosis by interfering with cell signalling pathways. These modifications are carried out by the lipid A phosphatase enzymes. In the absence of lipid A phosphates *P. gingivalis* can't modulate the structure of its LPS. It has been shown that mutants with locked LPS phenotypes due to mutations in lipid A phosphatase genes behave differently compared to the WT parent strain. Work carried out in a rabbit ligature model has shown that the WT strain but not the mutant strains could colonise the oral cavity. The data indicated that lipid A phosphatases are of significant importance in allowing *P. gingivalis* to establish initial colonisation in the oral cavity and once established lipid A phosphatase then goes on to aid the overgrowth of commensal species of bacteria by modulating the form of LPS as required. The same study demonstrated that the mutants with locked profiles and the parent strain all produced disease in the animal model but that the abundance and microbial species found were very different (Zenobia et al., 2014). Murine models of periodontitis have shown that the outgrowth of commensal species is vital to the establishment of disease (Hajishengallis et al., 2011). This serves to highlight the importance of lipid A as a direct mediator and lipid A phosphatases as indirect mediators of dysbiosis by facilitating the establishment of the biofilm early on and then subsequently protecting it from immune responses.

P. gingivalis produces outer membrane vesicles which have been shown contribute to the organism's virulence. It uses them as a means of shipping cargos of virulence factors into the surrounding environment. They are highly virulent, as they contain concentrated volumes of proteins involved in processes such as nutrient acquisition and immune dysregulation. Studies have shown that they were enriched for IhtB and HmuY, both of which play a key role in the acquisition of iron (Veith et al., 2014). They also contain LPS and the gingipains (Nakao et al., 2014). Indeed it has been suggested that OMVs which carry the gingipains may contribute to the overall tissue destruction observed in periodontitis. It has been shown *in vitro* that cellular detachment can be mediated by OMVs but that this detachment is inhibited by treatment of OMVs with anti-gingipain serum prior to cell line exposure (Nakao et al., 2014). OMVs actively contribute to the ecology of sub-gingival plaque; a recent study has shown that the OMVs produced by *P. gingivalis* can mediate the attachment of the non-motile bacterium *Lachnoanaerobaculum sabburreum* to *T. denticola* which is highly motile. By coating *T. denticola* with its OMVs it facilitates the movement of non-motile bacterium into niches of the periodontal pocket that they would otherwise be unable to occupy facilitating development of the biofilm as a whole (Fig 1.9) (Grenier, 2013). All of these functions highlight the importance of OMVs to the overall health of the biofilm and to the development of dysbiosis through indirect means of either shipping key mediators to sites or direct means through tissue destruction and co-aggregation.

The capsule of *P. gingivalis*, also known as CPS or K-antigen, is also recognised as a major virulence factor (Brunner et al., 2010b). There are thought to be at least six different serotypes of *P. gingivalis* (Sims et al., 2001). It should be noted that not all strains of *P. gingivalis* are encapsulated. Its presence is something of a mixed blessing.

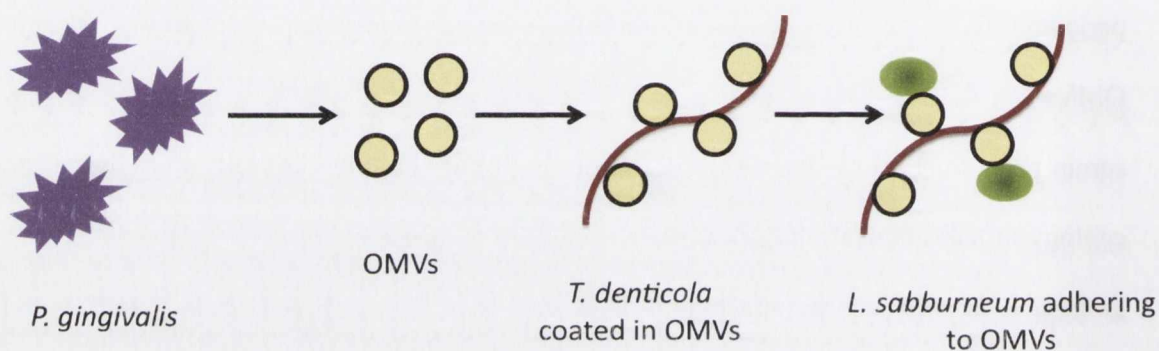


Figure 1.9: The OMVs produced by *P. gingivalis* play a role in biofilm formation and bacterial co-aggregation. They can coat the surface of the highly motile *T. denticola* which the non-motile *L. sabburreum* uses to adhere to *T. denticola*.

Those that are encapsulated have been shown to have increased invasion capabilities compared to non-encapsulated strains. In a murine lesion model it was demonstrated that encapsulated strains could cause systemic infection whereas non-encapsulated strains could only cause localised infection (Laine and van Winkelhoff, 1998). Encapsulated strains have also been found to be more resistant to phagocytosis by polymorphonuclear leukocytes than non-encapsulated strains (Sundqvist et al., 1991). The presence of the CPS may play a role in protecting against the innate immune response. A study has shown that a non-encapsulated mutant strain induced higher levels of cytokine production by human gingival fibroblasts in comparison to the WT parent strain (Brunner et al., 2010a, Brunner et al., 2010b, Laine and van Winkelhoff, 1998). However the presence of the capsule is not always beneficial to the organism. Non-encapsulated strains are substantially better at adherence in comparison to their encapsulated variants, it has been shown that there are differences depending on the serotype of the capsule, with capsule type 4 adhering slightly better than the other variants (Dierickx et al., 2003)

The ability of *P. gingivalis* to adhere to and invade cells is vital to the development of dysbiosis. Without cellular access SerB will not act to down-regulate IL-8 production which is one of the key mechanisms involved in the immune subversion tactics used to establish dysbiosis, as discussed previously. *P. gingivalis* is capable of adhering to and invading a variety of cell types. One of the key mediators of adherence and invasion in *P. gingivalis* is the Type I (major) fimbriae which is encoded for by the *fimA* genes. *P. gingivalis* produces a number of sub-types of fimbriae, with some being more adhesive than others. Strains of *P. gingivalis* can be classified based on the fimbriae encoded for by the *fimA* genes (Gao et al., 2012). It's well established that fimbriae play a role in the ability of *P. gingivalis* to adhere to various oral substrates and molecules, such as proline rich proteins and glyco-proteins, as well as to other microbial species and host cells, all of which are vital in the establishment of the dental biofilm (Amano et al., 2004, Lamont and

Jenkinson, 1998). *P. gingivalis* has been shown to invade gingival epithelial cells by a mechanism that is dependent on the binding of its major fimbriae to β 1-integrin leading to re-arrangements in the actin cyto-skeleton (Yilmaz et al., 2002, Yilmaz et al., 2003). It is also worth noting that the various sub-types of *P. gingivalis* fimbriae are associated with variation in the organism's invasion capability depending on the sub-type the strain possesses (Inaba et al., 2008). Indeed some strains which do not possess fimbriae are considered more virulent than those that do. These strains are still capable of invasion, albeit to a lesser extent (Inaba et al., 2008).

Once inside the cell *P. gingivalis* can extend the cellular lifespan. It does this in a number of ways: it produces adenosine triphosphate (ATP)-hydrolysing enzymes that inhibits ATP induced apoptosis. The organism produces and secretes an ATP-utilising enzyme, nucleoside diphosphate kinase, which acts in an anti-apoptotic manner to prevent ATP based apoptosis (Yilmaz et al., 2008). The organisms also encourages a pro-survival phenotype for itself by accelerating the cell cycle through the S-phase by modulating pathways that p53 and the cyclins play a key role in (Kuboniwa et al., 2008).

P. gingivalis can also prevent mitochondrial based apoptosis. The organism is capable of preventing the release of cytochrome C, a key mediator in the initiation of mitochondrial apoptosis (Yilmaz et al., 2004). It activates a number of signalling pathways which induce an anti-apoptotic phenotype, such as the "Janus Kinase/ signal transducer and activator of transcription 3" (JAK/STAT3) and "Phosphatidylinositol 3-kinase/Protein kinase B" (PI3K/Akt) pathways. Activation of these pathways in primary gingival epithelial cells has been shown to cause cells to become resistant to cell death from a number of anti-apoptotic molecules including extracellular ATP (Yilmaz et al., 2004). *P. gingivalis* inhibits the function of members of the caspase family which play a role in programmed cell death.

The organism acts on caspase 3 and caspase 9 and it is able to deactivate “bcl-2-associated death” (Bad) signalling, which is pro-apoptotic (Boisvert and Duncan, 2010).

P. gingivalis can also successfully spread from cell to cell through actin cytoskeleton bridges without passing through extracellular spaces. It does this without triggering cell death in GECs and it may act as a means of immune evasion particularly regarding the humoral immune response (Yilmaz et al., 2006).

P. gingivalis has well established proteolytic activity through its gingipains. The maturation of the gingipains is dependent on the POR secretion system which is in turn linked to carbohydrate biosynthesis. This pathway is regulated by several proteins including VimA, VimE and VimF (Vanterpool et al., 2004). VimE is independently expressed of VimA and plays a role in modulating the proteolytic activity of the gingipains in strain W83 (Vanterpool et al., 2004). VimF is essential to gingipain maturation; it is a glycoprotein with galactosyltransferase activity which plays a role in gingipain glycosylation (Muthiah et al., 2013).

The gingipains play an essential role in the virulence of *P. gingivalis*. As outlined previously they degrade members of the complement cascade there by directly contributing to the development of dysbiosis. However they also contribute massively to nutrient acquisition and cellular invasion. Studies have found that RgpA and Kgp gingipains (also termed as HagD & Hag E, section 1.6) have adhesive haemagglutinin encoding domains that play a role in adhesion to a number of cell types including epithelial cells and gingival fibroblasts (Chen and Duncan, 2004) (Andrian et al., 2004). They may also affect vascular permeability at the site of infection. The argine gingipains (RgpA & RgpB) can activate plasma bradykinin and kallikrein or in contrast to this trigger increased levels of thrombin and pro-thrombin. This can lead to increased vascular permeability at the site of infection (Imamura et al., 2001a, Imamura et al., 2001b). Kgp which is a Lys- gingipain has also

been shown to be capable of fibrinogen degradation (Scott et al., 1993). This could encourage increased bleeding at the site of infection and thus an increase in the levels of available nutrients (Bostanci and Belibasakis, 2012).

The role of the gingipains in haem acquisition has been widely studied. They are well documented haemagglutinins and haemolysins that work in conjunction with the Hmu system of haem uptake, acting to facilitate the acquisition of haem from oxygenated haemoglobin through proteolytic mediated mechanisms (Li and Collyer, 2011). The freed haem is then acquired by the dual component Hmu uptake system, which consists of HmuY and its receptor HmuR (Olczak et al., 2008). It is worth noting that liberation of haemoglobin from red blood cells is facilitated by several proteins with hemagglutination activity (HagA- E). The gingipains RgpA and Kgp possess hemagglutination domains which account for a proportion of this activity, with the remaining hemagglutination activity been accounted for by HagA, HagB and HagC, as well as other proteins.

1.6 The role of the *hag* genes in *P. gingivalis*:

P. gingivalis possesses at least eight haemagglutinins, a subset of which are known as the Hag proteins. The Hag proteins are encoded for by five genes, *hagA – hagE*. The *hagD* and *hagE* genes are pseudonyms of *rgpA* (Oralgen, 2009a) and *kgp* (Oralgen, 2009b). The function of the proteins encoded for by both of these genes has been discussed previously (Section 1.5).

Based on amino acid sequence homology, the Hag proteins can be divided into two main sub-families (Fig 1.10). The HagA, HagD and HagE proteins have been shown to have >70% homology each other whereas HagB and HagC have 98.6% homology.

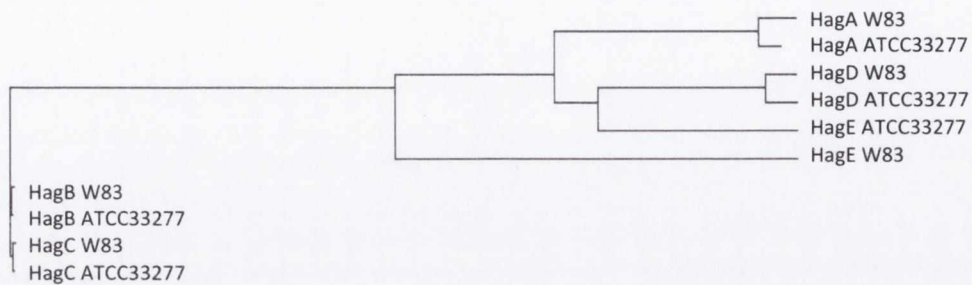


Figure 1.10: Phylogentic analysis of HagA-HagE in two strains of *P. gingivalis* (Strain ATCC33277 and W83). Homology and phylogenetic distances were determined using ClustalW.

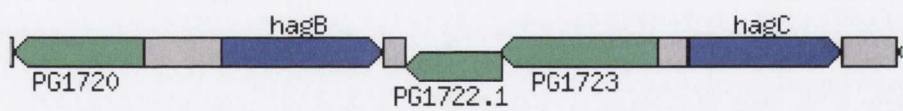


Figure 1.11: The *hagB* and *hagC* locus in strain W83 of *P. gingivalis* showing that the two genes are located chromosomally close together.

The *hagB* and *hagC* genes are located closely together on the chromosome (Fig 1.11) and form a distinctly separate group from other Hag proteins (Fig 1.10) (Lepine et al., 1996)

As a group, the most widely studied members are RgpA and Kgp whose role in virulence and in the development of dysbiosis has been established, as outlined previously. HagA has been shown to play a role in adherence and invasion to a number of cell types including GECs and human coronary artery endothelial cells (HCAEs) which is of note given that *P. gingivalis* has been linked to the development of atherosclerosis (Belanger et al., 2012). HagA potentially plays a role in co-aggregation with *T. denticola*. One study has shown that the interactions of *P. gingivalis* with other bacterial species, such as *T. denticola*, leads to increased adhesive properties through adhesion domain containing proteins including HagA (Meuric et al., 2013).

The expression of *hagA* has been shown to be affected by levels of adrenaline and nor-adrenaline and stimulation with catecholamines leading to higher levels of *hagA* (Graziano et al., 2014). The gene is also up-regulated in response to oxidative stress (Meuric et al., 2008). The HagA protein is highly immunogenic. In a murine model sublingual vaccination with a fusion protein made of *Escherichia coli* maltose binding protein and the functional domain of HagA was shown to be protective against *P. gingivalis* infection in the oral cavity through Th1-type and Th2-type mediated immunity (Yuzawa et al., 2012).

Comparatively little is known about the function of the *hagB* and *hagC* genes. HagB is the more studied of the two and research on the role of *hagC* is sorely lacking. This could be due to the fact that it was assumed that these genes were functionally redundant and potentially essential to the genome.

1.7 The *hagB* and *hagC* genes of *P. gingivalis*

The *hagB* and *hagC* genes form a separate group distinct from the other *hag* genes. They were first identified in 1995 (Progulske-Fox et al., 1995). They encode proteins that are 98.6% identical to one another, with minor difference seen at the N-terminus of the sequence (Figure 1.12). The *hagB* and *hagC* genes are located closely together on the chromosome and are uniformly present amongst strains. This highlights that both genes are highly conserved between strains of *P. gingivalis* (Fig 1.13) and that maintenance of both genes is required, potentially for optimum growth and nutrient acquisition. Both HagB and HagC have haemagglutination activity indicating a role for them in the acquisition of haem, an essential nutrient for *P. gingivalis*.

The genes are under the control of different promoter sequences and are expressed separately to one another. They are differentially regulated by factors including growth cycle phase and environmental iron levels (Lepine and Progulske-Fox, 1996). Expression of *hagB* and *hagC* is directly regulated by HaeR in *P. gingivalis* strain ATCC 33277. This study examined global gene expression under conditions of different haemin concentration. The *hagB* gene was found to be expressed under all conditions whereas *hagC* transcripts were only detected when there was no haemin present in the media (Scott et al., 2013). Another study demonstrated a role for *clpB* in *hagB* expression. Mutant studies showed that in the absence of *clpB*, *hagB* was found to be downregulated. The *clpB* mutant was also found to be less capable of invasion than the WT strain (Yuan et al., 2007), an effect that the down-regulation of *hagB* could contribute to. When expression patterns of genes induced by oxidative stress were examined in *P. gingivalis* using mutant studies, the results showed that *hagB* expression was stable during exposure to air (Meuric et al., 2008).

98.0% identity in 350 residues overlap; Score: 1727.0; Gap frequency: 0.0%

```

HagB,      24 MTVENLRLQRLQNLEHYRFAKNVLTLCRTANIAKLNPKLPELEKAIEMEDLALNPPVANE
HagC,      1 MTAEIFSFSRLQNLEHYRFAKNVLTLCRTANIAKLNPKLPELEKAIEMEDLALNPPVANE
          ** *      *****

HagB,      84 LTPQVIALDEERDRAYQALMSRVRSYAFDEDSQLRNAAARIEDVAARYGNVIRMNYDKET
HagC,      61 LTPQVIALDEERDRAYQALMSRVRSYAFDEDSQLRNAAARIEDVAARYGNVIRMNYDKET
          *****

HagB,     144 AAIENFLTDLKGENIRPLVTKLGVTALVDRLEKNNKAFADFFLRRLSTDQRGKYDVKALR
HagC,     121 AAIENFLTDLKGENIRPLVTKLGVTALVDRLEKNNKAFADFFLRRLSTDQRGKYDVKALR
          *****

HagB,     204 AETDRTLVAVVRMDSIDDMEPSPEIRALIELYNRLVANRRALLARRASYGEAAVEKRRR
HagC,     181 AETDRTLVAVVRMDSIDDMEPSPEIRALIELYNRLVANRRALLARRASYGEAAVEKRRR
          *****

HagB,     264 EIAEMLRPLLARIVEEKKTAVFAGRTLGTGKNRHYLITFVAENGDEEDRWYRINGEQLVY
HagC,     241 EIAEMLRPLLARIVEEKKTAVFAGRTLGTGKNRHYLITFVAENGDEEDRWYRIDGEQLVY
          *****

HagB,     324 VPEDELPKPKKKKKPASSTDTPSEPPVLPDPSQGGSSSGGGEQGSTGGGL
HagC,     301 VPEDELPKPKKKKKPASSTDTPSEPPVLPDPSQGGSSSGGGEQGSTGGGL
          *****

```

Figure 1.12: Image showing the amino acid sequence alignment of HagB and HagC to one another. They share 98% identity to one another with differences seen at the N'-terminus of the sequence.

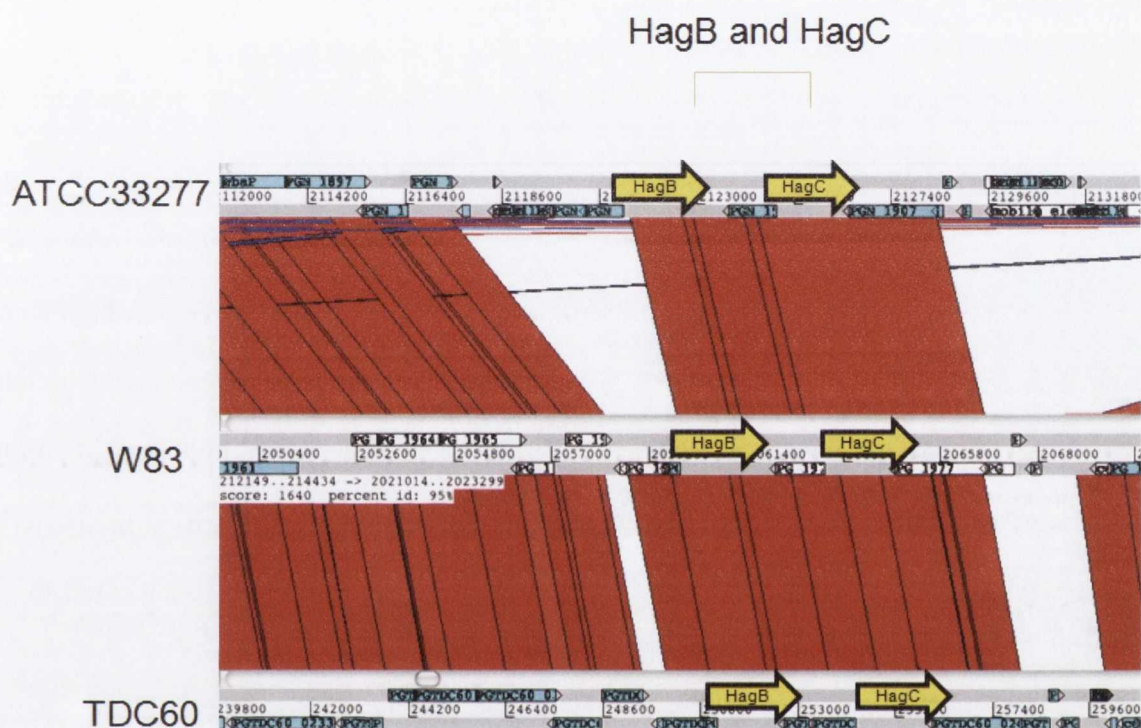


Figure 1.13: Image taken from the Artemis comparison tool (ACT) software package which allows comparison of genomic synteny and homology. Three strains of *P. gingivalis* were compared, strain ATCC33277, W83 and TDC60. Regions in red are highly homologous to one another. Image shows that the *hagB* and *hagC* genes are highly conserved genes despite genomic rearrangements which occur around them.

It is believed that *hagB* and *hagC* are both expressed at various stages during the infectious processes as demonstrated using an *in vivo* expression technology vector termed pGIVET in strain Pg381 (Lee et al., 1996). The fact that these genes are expressed during the infectious process highlights their role as potential virulence factors and that they may potentially play a role in the pathogenesis of periodontitis.

The *hagB* and *hagC* genes encode for 39-kDA polypeptides that share ~ 98% identity to one another, HagB has a pI of 8.42. Transmission electron microscopy has confirmed its presence on the surface of *P. gingivalis* and HagB can be expressed on the surface of a number of bacterial species including *E. coli* and *Salmonella* as well. Given the high levels of homology between *hagB* and *hagC* it is likely that they are both located on the cellular surface of *P. gingivalis* (Borgwardt et al., 2014, Isoda et al., 2007, Lepine et al., 1996, Song et al., 2005). Aside from knowing that these proteins are expressed on the bacterial surface other important aspects such as their structure and how they are anchored to the cell wall remain to be elaborated on.

Recent studies have shown that binding of antimicrobial peptides to HagB may be important in the attenuation of the pro-inflammatory immune response. Recombinant HagB can bind to human-beta-defensin 3 (H β D3) which is an anti-microbial peptide found in the oral cavity. It is present in saliva and gingival crevicular fluid at a concentration of 6.2 μ g/ml and it is recognised as playing a role in inflammation through the stimulation of cytokine production (Harvey et al., 2013).

In human myeloid dendritic cell cultures, binding of recombinant HagB (rHagB) by H β D3 attenuates the production of interleukin -6 (IL-6), interleukin-10 (IL-10) and granulocyte macrophage colony stimulating factor (GM-CSF). This binding was also found to attenuate the ERK1/2 response, which is a cell-signalling pathway closely associated with inflammation (Pingel et al., 2008). The attenuation of this response is because the binding

of H β D3 to rHagB alters but does not inhibit the binding of *P. gingivalis* HagB to the surface of human dendritic cells. This alteration in binding prevents rHagB from penetrating the dendritic cells. The receptor rHagB uses for this binding has yet to be established (Van Hemert et al., 2012).

In studies carried out to establish how H β D3 regulates the chemokine and cytokine response to pro-inflammatory response rHagB was found to be as immuno-reactive as *E. coli* 055:B5 endotoxin. It elicited a pro-inflammatory response from myeloid dendritic cells, with a strong response in 21 chemokines and cytokines (Harvey et al., 2013).

It has been shown that rHagB is a TLR4 agonist for human dendritic cells (Gaddis et al., 2009). Immunization with rHagB leads to the triggering of a rHagB-specific CD4⁺ T cell response. This response can in turn be shaped by TLR4 signalling through components of the TLR4 signalling cascade. The myeloid differentiation response gene 88 (MYD88) was found to play a role in shaping the T-cell response to rHagB, while TIR domain containing adaptor inducing interferon β (TRIF) actively participates in the co-ordination of memory inflammatory cytokine production after HagB ligates TLR4. The study also highlighted the ability of rHagB to act as a TLR4 agonist and as an immunogen as opposed to a super antigen (Gaddis et al., 2011). This is an important consideration given rHagB status as a potential vaccine candidate against *P. gingivalis* infection.

In a murine model it has been demonstrated that rHagB has vaccine potential. Mice immunized with rHagB showed increased levels of Hag serum immunoglobulin (Ig) G and salivary IgA (Dusek et al., 1994). This effect was enhanced when mono-phosphoryl lipid A was used as an adjuvant to potentiate a specific antibody response to rHagB. Post intranasal vaccination in mice it was found that in the presence of the adjuvant better immunity was induced than to rHagB alone, with increased levels of IgG being produced (Yang et al., 2002). Another study carried out in a rat model showed that immunization

with rHagB significantly reduced bone loss (Katz et al., 1999). Given that these genes are highly homologous to one another it is likely that HagC has similar potential, though as of yet very little work has been carried out on it in this area.

HagB has also been shown to mediate the attachment of *P. gingivalis* to HCAEs. Expression studies carried out in *E. coli* showed that the surface expression of HagB allowed the bacterium to attach to the cells. The attachment of *P. gingivalis* strain 381 or the *E. coli* strain expressing rHagB to HCAEs was inhibited by antibodies to HagB or rHagB itself in a dose dependent manner when antibodies to HagB or rHagB were used (Song et al., 2005).

The exact role of *hagB* or *hagC* in the process of dysbiosis has yet to be fully established. Previous attempts to generate a double *hagB/hagC* mutant were unsuccessful suggesting that these genes may be essential to the growth of the bacterium (Lepine et al., 1996). Another possible reason for this may be the high homology between the two genes, which could mean that it is very difficult to create specific, sequential knock out mutants. Due to the high homology between *hagB* and *hagC*, it is likely that these genes are functionally redundant, indicating that a double mutant is required in order to fully appreciate the phenotypic effects of a Hag mutant. It is hoped that the work carried out here will elaborate further on the individual function of each gene. Previous studies of the single *hagB* or *hagC* mutants have shown a defect in haemagglutination but analysis of host cell interactions or biofilm formation have not been investigated. A double *hagB/hagC* mutant would allow us to fully investigate the role of these proteins in these processes and determine if a vaccine induced antibody response to these antigens could effectively inhibit colonisation by *P. gingivalis*.

1.8 Defining a role for genes whose function is poorly understood in *P. gingivalis*

While many important genes that play a role in the infectious process of *P. gingivalis* have been identified in recent years, there are still many genes within the *P. gingivalis* genome where function is poorly understood. It is essential that we elaborate on the role of these genes so that their role in the infectious process can be more clearly defined.

A common technique used to establish gene function is insertional mutagenesis, where the function of the gene is interrupted and it ceases to code for a viable protein product. The effect that this mutation has on phenotype is then studied e.g. how the mutant interacts with cell lines in comparison to the WT parent strain. It is a technique that has been commonly used to establish the role of genes in the virulence of *P. gingivalis* (Simpson et al., 2000, Vanterpool et al., 2004). However this technique is not without limitations: (i) the function of only a small number of genes can be studied at any given time, and (ii) it is a lengthy process with cassette creation and mutagenesis taking time which is exacerbated by the slow growth rate of *P. gingivalis*

Transposon mutagenesis offers an alternative approach to traditional methods outlined above and is an important tool for creating inactivation mutants. The value of transposon mutagenesis is based on the fact that the transposable element inserts randomly into sites throughout the host's genome, while giving only one insertion per mutant strain (Hayes, 2003).

Early attempts to use this technique in *P. gingivalis* had limited success. They were hindered by both using transposons based on Tn4435 and Tn4400 which preferentially inserted into certain regions of the chromosome ("hot-spots") and the limited range of *P. gingivalis* strains that were available for mutagenesis (Chen et al., 2000, Takada and Hirasawa, 1998). However huge advances have been made in the field using a Mariner transposon in *P. gingivalis* (Klein et al., 2012). This system has been shown to be highly

effective in a diverse range of species (Lampe et al., 1999, Lampe et al., 1996). A recent major advance in the analysis of bacterial transposon libraries, including Mariner based systems, has been the application of massively parallel sequencing technology to quantitatively identify the location of the transposons in a library pool by sequencing the transposon-genome junction. This strategy is commonly known as “Tn-Seq”. Using this method the lag between gene discovery and gene function can be addressed as it allows for putative gene function to be established, as well as facilitating higher order genome organisation (van Opijnen and Camilli, 2013). It has already been used to identify the essential genes of *P. gingivalis* (Klein et al., 2012) and we believe it has the potential to establish the role of genes of unknown function through an adaptive screening method that can implicate processes that the genes are involved in, thus elaborating on their function.

1.9 Rationale for this study:

As outlined earlier the exact role of *hagB* and *hagC* in the virulence of *P. gingivalis* has yet to be established. Although single mutants have been made previously there has been no successful attempts to generate a double mutant of these genes (Lepine et al., 1996), which is vital to enhancing our knowledge of the role that they play in the development of periodontitis and how vaccine induced blocking antibody responses could protect from *P. gingivalis* colonisation.

A further section of the study focuses on developing a novel screening method that would allow for rapid analysis of genes involved in virulence processes linked to cellular invasion. By screening a transposon mutagenesis library through our established adherence and invasion assay protocols and downstream sequencing technology we were able to identify candidate genes which may play a substantial role in both of these virulence processes.

Specific aims of the project are:

- Develop a mutagenesis procedure to generate a *hagB/hagC* double mutant
- Characterise the role of *hagB* and *hagC* in haem acquisition and host cell interactions
- Develop a Tn-Seq screen to identify *P. gingivalis* genes required for interaction with host cells.

Chapter Two

Materials and Methods

2.1 General Microbiology Methods

2.1.1 Strains

The *P. gingivalis* strains used in this study are outlined in Table 2.1. The majority of strains were acquired from the Dublin Dental University Hospital microbiological strain collection at Dublin Dental University Hospital, University of Dublin, Trinity College, Lincoln Place, Dublin 2.

Plasmid pJW1 was obtained from Prof. A. Progulske-Fox, University of Florida. The ATCC 33277 *fimA* mutant was obtained from Hua Xie, School of Dentistry Meharry Medical College, Nashville. The ATCC 33277 transposon mutagenesis library was obtained from Brian Klein Department of Molecular Biology and Microbiology, Tufts University Sackler School of Biomedical Sciences, Boston.

2.1.2 Bacterial culture methods

2.1.2.1 *P. gingivalis*:

P. gingivalis strains (Table 2.1) were grown and maintained routinely on Tryptic Soy Agar (TSA) supplemented with 5 g/l yeast extract (Sigma-Aldrich, Co. Wicklow, Ireland), 0.50 g/l L-cysteine hydrochloride (Sigma-Aldrich), 5.0 µg/ml hemin (Sigma-Aldrich) and 1.0 µg/ml menadione (Sigma-Aldrich) and 5% horse blood (Oxoid Limited, Wade Road, Basingstoke, Hampshire, England). Bacteria were incubated at 37°C in BBL gas jars under anaerobic conditions generated by AnaeroGen gas packs (Oxoid), when required media was supplemented with 5.0 µg/ml erythromycin, 0.25-2.0 µg/ml tetracycline (Sigma-Aldrich) or 10 µg/ml chloramphenicol (Sigma-Aldrich).

Table 2.1: *P. gingivalis* strains used in this study and their *hagB* and *hagC* genotype.

Mutant strain names were assigned by the laboratory where the mutants were made.

Strain	Genotype	Parent strain
<i>P. gingivalis</i> W83	<i>hagB/hagC</i>	n/a
ECM100	$\Delta hagB::ErmF$	W83
ECM200	$\Delta hagC::TetQ$	W83
ECM233	$\Delta hagB::ErmF, \Delta hagC::TetQ$	ECM200
DKO3	$\Delta hagB::ErmF, \Delta hagC::TetQ$	ECM200
GMC100	<i>hagB::cat</i>	ECM233
<i>P. gingivalis</i> ATCC33277	<i>hagB/hagC</i>	n/a
($\Delta hagB$) ATCC33277	$\Delta hagB::ErmF$	ATCC33277
($\Delta fimA$) ATCC33277	<i>hagB/hagC</i>	ATCC33277

For broth cultures supplemented Tryptic Soy Broth (sTSB) containing 5 g/l yeast extract (Sigma-Aldrich), 0.50 g/l L-cysteine hydrochloride (Sigma-Aldrich), 5.0 µg/ml hemin (Sigma-Aldrich) and 1.0 µg/ml menadione (Sigma-Aldrich) was used.

2.1.2.2 *E.coli*:

Escherichia coli strain XL10-gold (endA1 glnV44 recA1 thi-1 gyrA96 relA1 lac Hte Δ (mcrA) 183 Δ (mcrCB-hsdSMR-mrr) 173 tet^R F'[proAB lacI^qZΔM15 Tn10 (Tet^R Amy Cm^R)] was used as the host strain for all plasmids. All strains were maintained on Luria-Bertani (LB) agar and were incubated aerobically at 37°C. For liquid cultures, strains were incubated aerobically at 37°C with shaking at 200 r.p.m in LB broth. When required media was supplemented with 100 µg/ml ampicillin (Sigma-Aldrich), 300 µg/ml erythromycin (Sigma-Aldrich), 5 µg/ml tetracycline (Sigma-Aldrich) or 10 µg/ml chloramphenicol (Sigma-Aldrich).

Identification of recombinant plasmids in *E.coli* XL-10-gold was facilitated by blue/white screening (indicating insertional disruption of the *lacZ* gene). Transformed cells were selected onto Luria Bertani X-gal (LX) plates.

LX plates were made from 500 ml LB agar containing 200 µl of X-Gal (Sigma-Aldrich) from a 100 mg/ml stock, 500 µl ampicillin (Sigma-Aldrich) from a 100 mg/ml stock and 100 µl IPTG (Sigma-Aldrich) from a 200 mg/ml stock.

2.1.3 Buffers and solutions

Formulations for specific buffers and solutions used are described in the relevant experiments during which they were used. When required solutions were sterilised by filtration or autoclaving as described below.

All solutions were made up to a final volume with sterile, ultra-pure (Milli-Q) water processed using a MilliQ Biocel system (Merck-Millipore, MA, USA), unless otherwise stated.

When a solution was required to be a specific pH, pH was measured using a Thermo Scientific Orion 3-Star bench-top pH meter (Thermo-Fischer Scientific, Ballycoolin, Dublin 15, Ireland) and adjusted to higher pH using 5 M Sodium Hydroxide (NaOH) (Sigma-Aldrich) or lower pH using 37% (v/v) Hydrochloric acid (HCl) (Sigma-Aldrich).

Sterilisation by filtration based methods involved passing a solution through a Steritop Vacuum-driven filtration system with a 0.22 μ M membrane (Merck-Millipore) assisted by a vacuum pump, into previously sterilised glassware. Sterilisation of solutions by autoclaving was carried out in a Tomy SX-700 portable autoclave (Digital Biology, Tokyo, Japan), which was set to 121°C for 15 min. The success of autoclaving was monitored with defend autoclave tape (MyDent, International, NY USA).

Tris-EDTA (TE) Buffer was routinely used in experimentation at a 1 X concentration. 1 X TE buffer was made of 10 mM Trizma base (Sigma-Aldrich) brought to pH 8.0 with HCl as well as 1 mM Ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich).

2.1.4 Chemicals, enzymes, reagents and antibiotics

Suppliers for specific chemicals, enzymes, reagents and antibiotics are given in the relevant sections of this study. The main suppliers are detailed below.

The chemicals, reagents, enzymes and antibiotics used were all of molecular biology grade and sourced from the following companies: Promega (Promega Corporation, Madison, Wisconsin USA), Qiagen (GmbH, D-40724 Hilden), Sigma-Aldrich (Sigma-Aldrich Inc, 3050 Spruce St, St Louis, MO 63103 USA 314-7771-5765).

2.1.5 Oligonucleotides

All oligonucleotides were designed as part of this study unless otherwise stated (Tables 2.2 and 2.3). Prior to purchase, specificity of oligonucleotide sequences were assessed using BLAST nucleotide (BLASTn). The oligonucleotide sequence was uploaded to the server and tested against the relevant genome in order to ensure specificity for the intended target.

Custom synthesized oligonucleotides (primers) were produced by Sigma-Aldrich for this study through their online ordering system. Oligonucleotides were received in a lyophilised form before being re-suspended in the appropriate volume of sterile molecular grade water (Sigma-Aldrich) to make stock concentrations of 100 μ M.

2.1.6 Storage of isolates

All strains used in this study were stored for future laboratory use. Strains were stored at -80°C and were processed the same way regardless of species. Single colonies from pure cultures on fresh agar plates were used to inoculate 1.5 ml vials containing cryogenic beads and cryopreservatives (Microbank, Pro-Lab Diagnostics, Cheshire, UK). The vial was gently shaken to ensure homogenisation. After homogenisation, excess cryopreservative was removed from the vial. Vials were labelled and stored at -80°C in an ultra-low temperature freezer.

In order to reactivate cultures, vials were removed from storage and a single “cryobead” was removed from the vial and placed onto suitable fresh agar for incubation as outlined in section 2.1.2

Table 2.2: Characteristics of PCR primers used in this study

Primer	Sequence (cut sites underlined)	Amplicon length (bp)
HagBIF	ATATTGGAGGTATCCGTACCT	922
ErmIR	GCTATCTTTGCTTTATTGTC	922
ErmIF	TTTCATCCTTCGTAGTTCAAAG	2500
HagBIR	GCATTTTCTTACTTGCGTC	2500
HC1_IF	CAATAGTATTGTTGTGCCAG	1200
HC1_IR	CTATATCCATAGAGTCCGTTATG	1200
HC2_IF	CAGCTGACCCCTTATGTCTTCA	1200
HC2_IR	ATTATAGCGGAGGGAAATTGCT	1200
TetQF	GGGGGATCCGGGGGGAAATACTAAATGC (underlined: <i>Bam</i> HI)	2700
TetQR	GGG <u>ACTAGT</u> AGCTCGTCTATTTTTTTTATT (underlined: <i>Spe</i> I)	2700
HagCF1	GGGCTCGAGACCTGCAGAGTATGTA (underlined: <i>Xho</i> I)	313
HagCR1	GGGAAGCTTTTTGAATTAAGGGGTTAAGTC (underlined: <i>Hind</i> III).	313
HC2_F	GGGTCTAGAAAGGAGGCAGCAGTAGCGGCG (underlined: <i>Xba</i> I)	411
HC2_R	GGGGCGGCCGCTTCACGGATTATCCCTTTTA (underlined: <i>Not</i> I)	411
DKOBFP	CAGGAAGCCGTTGTCACG	WT: 2100 ECM233: 4400
DKOBRP	ATCTCTGGCAACAACATTGGGATAG	WT: 2100 ECM233: 4400
DKOCFP	GCCGATCTTCGAAATTCCAAG	WT: 3100 ECM233: 4600
DKOCRP	AGAGCGTTCACCGATTAT	WT: 3100 ECM233: 4600
HagsF1	GCTTTCGACGAGGACAGCCAG	275
HagsR1	GCCTTCACGTCATATTTGCCTCG	275
ATCCHagC2F	GGGCGGCCGCTACACACCTGTTTCATGACCGTACGCTA (underlined: <i>Not</i> I)	300
ATCCHagC2R	GGGTCTAGACACAGGCAACCGAGTATAAAAAGAC (underlined: <i>Xba</i> I)	300

Table 2.3: Primers used in qPCR experiments

Primer	Sequence (5'-3')	Amplimer Lenght (bp)	R ²	Reference
16S rRNA F	AGGCAGCTTGCCATACTGCG	404	0.9967	(Dou et al., 2010)
16S rRNA R	ACTGTTAGCAACTACCGATGT	404	0.9967	(Dou et al., 2010)
HagA F	GTGGTAGAGGCCGGTAAGACTTATCA	312	0.9995	(Dou et al., 2010)
HagA R	CGTCACCTGTATGAGTAGCAGTG	312	0.9995	(Dou et al., 2010)
Kgp F	AATACGCCGGTTCGTATGCT	226	0.98	(Hiratsuka et al., 2008)
Kgp R	TGTCACCAACCAAAGCCAAG	226	0.98	(Hiratsuka et al., 2008)
RgpA F	TCAAGAACAATCTCGGCCTGACCG	275	0.9962	(Hiratsuka et al., 2008)
RgpA R	AGCACGCTTACCCTTACAGTAGTT	275	0.9962	(Hiratsuka et al., 2008)
RgpB F	TCGCTGATGAAACGAAGTTGACG	230	0.9985	(Hiratsuka et al., 2008)
RgpB R	TTCGAATACCATGCGGTTCTTAGC	230	0.9985	(Hiratsuka et al., 2008)
HagB F	GACTGTAGAAAATTTGCGTCTGCAGC	205	0.9976	n/a
HagC F	GAAATTTTCTCGTTTTCCC	198	0.9973	n/a
HagS R	GTCGAGGGCTATGACCTGAG	HagB: 205 HagC: 198	0.9976 0.9973	n/a

2.1.7 Assessment of nucleic acids

Prior to downstream applications, it was necessary to assess nucleic acids in solution in terms of both quality and quantity. This was carried out for both dsDNA and RNA using a Nanodrop spectrophotometer (Thermo-Fischer Scientific). The solution in which the nucleic acids were suspended was used to blank the Nanodrop spectrophotometer to a baseline absorbance. A 1 µl volume of DNA or RNA was subsequently loaded onto the Nanodrop spectrophotometer stage and the concentration of nucleic acids was quantified in ng/µl by Nanodrop 2000c software (Thermo-Fischer Scientific) on a PC running Windows XP (Microsoft, CA, USA). The 260 nm / 280 nm and 260 nm/ 230 nm absorbance ratios were used to assess purity of DNA/RNA.

2.1.8 Agarose gel electrophoresis

Agarose (Sigma-Aldrich) was prepared in 0.5 X TBE (5.4 g/l Trizma Base (Sigma-Aldrich), 2.75 g/l Boric Acid (Sigma-Aldrich), 1 mM EDTA (Sigma-Aldrich) pH 8.0) and melted in a microwave (Bosch, Stuttgart, Germany) to molten agarose. Gel Red (Biotium, CA, USA) was added to a final concentration of 100 µl/l in order to fluorescently stain the DNA for visualisation. After the addition of gel red molten agarose was cast in gel tanks (Thermo-Fischer Scientific). Well spacing combs (Thermo-Fischer Scientific) with a range of spacing from 5 to 25 were placed in the casts and the gels were allowed to set. Once solid, the tanks were filled with enough 0.5 X TBE to submerge the gel and the comb was removed.

A 2 µl volume of Green/Blue loading dye (Promega) and 5 µl of sterile water (Sigma-Aldrich) were added to 5 µl of PCR product and loaded into the gel. For size estimations either a 100 bp or 1 kb DNA ladder (Promega) was run in a single lane of the gel. The gel was run at 100 V, 20 W on a high-power electrophoresis power supply unit (Mason Technologies, Dublin, Ireland) until the loading dye fronts had reached the end of the gel.

All gels were imaged on an Alpha Imager Mini (Alpha-Innotech c/o Protein Simple c/o Medical Supply Company, Mulhaddart, Dublin, Ireland) using ultra-violet light at wavelength 320 nm to allow visualisation of the fluorescently stained DNA.

2.2 DNA techniques:

2.2.1 Whole genomic DNA purification

P. gingivalis cultures were grown overnight in selective broth in sTSB. Cells were harvested from 1.5 ml culture in a sterile 1.5 ml micro-centrifuge tube. The media was removed and pellet was re-suspended in 200 µl of breaking buffer (2 % Triton X-100, 1% Sodium dodecyl sulphate, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (Sigma-Aldrich), and 100 mM NaCl (Sigma-Aldrich)). The cell suspension was then added to a 2 ml screw capped tube (Starstedt type) containing 0.3 g glass beads (0.1 mm diameter) and 200 µl of phenol-chloroform-isoamyl alcohol (24:24:1). Cells were disrupted by agitation for 1 min in a bead beater (Bio 101, Vista, CA).

The resulting lysate was centrifuged at 10,000 x g speed for 10 min. The upper aqueous phase was removed and extracted with an equal volume of chloroform-isoamyl alcohol (24:1). The extraction was then centrifuged for 2 min at 10,000 x g; the aqueous phase was removed to a fresh 1.5 ml micro-centrifuge tube. Nucleic acids were then precipitated using an ethanol precipitation method by the addition of one tenth volume of sodium acetate buffer (Sigma-Aldrich) (pH 5.2) and 3 volumes of cold 100 % ethanol (Sigma-Aldrich). The sample was allowed to stand at -20°C for at least one hour and then centrifuged for 15 min at the 10,000 x g in a 4°C micro-centrifuge (Eppendorf). The supernatant was removed and the resulting pellet air dried and re-suspended in 20-50 µl of sterile water.

Genomic DNA was also isolated using GenElute Bacterial Genomic DNA kit (Sigma-Aldrich) as per the manufacturer's instructions. DNA concentration was established using a Nanodrop 2000 (Thermo-Fischer Scientific).

2.2.2 Isolation of plasmid DNA

Plasmids used in this study were maintained in *E. coli* strain XL10-gold (endA1 glnV44 recA1 thi-1 gyrA96 relA1 lac Hte Δ (mcrA) 183 Δ (mcrCB-hsdSMR-mrr) 173 tet^R F'[proAB lacI^qZ Δ M15 Tn10 (Tet^R Amy Cm^R)] as outlined in section 2.1.2.2.

Strains were grown on L-agar at 37°C overnight. The following day 3 ml cultures of *E. coli* were inoculated using single colonies from these plates and incubated overnight at 37°C with agitation at 200 r.p.m. A 1 ml volume of this culture was transferred to a micro-centrifuge tube (Eppendorf) and centrifuged at 13,000 x *g* for 2 min in a bench top centrifuge (Eppendorf). This step was repeated so that the final volume spun down was 2 ml. The supernatant was discarded and the plasmid was purified using The Pureyield Plasmid Miniprep System (Promega) according to the manufacturer's instructions. DNA concentration was established using a Nanodrop 2000 (Thermo-Fischer Scientific).

2.2.3 Restriction enzyme digestion of DNA

DNA was prepared and cleaned prior to digestion as outlined in sections 2.2.1 and 2.2.2 depending on the DNA required.

DNA fragments that contained the appropriate cut sites were digested with appropriate restriction endonucleases (Promega) either for 3 hr at 37°C or overnight at 4°C. Digestion reactions were carried out in 20 μ l volumes containing 1 X of the appropriate buffer, 2 μ l of each of appropriate enzymes (5-10 U/reaction), 2 μ l BSA and DNA template for digestion. The final volume was made up with sterile water where required. The success of

DNA digestion was determined by agarose gel electrophoresis as outlined in section 2.1.8, when required fragments underwent gel purification.

2.2.4 Gel purification

Restriction endonuclease generated DNA fragments and occasionally PCR products were purified from 0.8 % agarose gels in TBE. Fragments were electrophoresed in agarose gels and viewed on a UV transilluminator as outlined in section 2.2.8. The fragment of interest was extracted from the gel with a sterile scalpel and purification was carried out using the Wizard SV Gel and PCR Clean-Up System (Promega) according to the manufacturer's instructions.

2.2.5 DNA Ligation

DNA ligations were carried out in micro-centrifuge tubes (Eppendorf). DNA fragments were ligated into pBlueScript II KS (-) phagemid or pUC19 phagemid digested with the appropriate restriction endonucleases. Ligation reactions were carried out in a 20 µl volume with a 2:1 ratio of insert to vector DNA in 1 X ligase buffer (300mM Tris-HCL [pH 7.8 at 25°C], 100 mM DTT and 10 mM ATP) with 1 U of T4 DNA ligase (Promega). Reactions were either carried out at 37°C for 3 hr or overnight at 4°C. In order to sequence some products plasmid pGEM-T Easy (Promega) was occasionally used. DNA was ligated directly into plasmid pGEM-T Easy as per the manufacturer's instructions.

2.2.6 DNA sequencing

All sequencing required for this study was carried out commercially in St. James Hospital, Dublin on behalf of the Oral Biosciences Division, Dublin Dental University Hospital, by Source Bioscience (Nottingham UK). The method used was a single-read Sanger sequencing on an ABI 3730x1 DNA analyser (Applied Biosystems, Life Technologies). Sequence reads were given via email in the form of .seq and .ABI files.

2.2.7 Basic Analysis of DNA sequences

Raw sequence files obtained from sequencing in the form of .ABI files were imported as chromatograms and analysed using ApE Plasmid Editor (Jorgensen Laboratory, Department of Biology, University of Utah, Salt Lake City, UT, USA). The validated sequences were then exported as .txt files and imported into genetic analysis software of choice.

In most cases in this study the software used was ApE Plasmid Editor (Jorgensen Laboratory) or SerialCloner 2.1 Freeware (Frank Perez, Curie Institute, Paris, France). Previously published sequences were obtained from Oralgen (Los Alamos National Laboratory) or Human Oral Microbiome Database (The Forsyth Institute, Cambridge, UK) in FASTA formatting and uploaded to ApE Plasmid Editor (Jorgenson Lab).

2.2.8 Preparation of electro-competent cells

2.2.8.1 *E. coli*

A 2 ml volume of LB broth was inoculated with *E. coli* and grown overnight at 37°C with agitation at 200 r.p.m. This culture was used to inoculate 400 ml of fresh culture the following day and the resulting culture was grown until mid-log phase (OD₅₅₀ nm 0.4-0.5). The cells were then chilled on ice for 1 hr. Cells were then decanted into two sterile 250 ml sorvall bottles and spun at 3,000 x g for 10 min at 4°C.

The media was removed and the cells were gently re-suspended in 30 ml of ice cold 100 mM MgCl₂ and centrifuged to form a pellet again. The supernatant was removed and the pellet was re-suspended in 100 ml of ice-cold 100 mM CaCl₂ buffer (60 mM CaCl₂, 10 mM PIPES, 15% glycerol). This cell suspension was again pelleted by centrifugation and re-suspended in 20 ml ice-cold CaCl₂ buffer. Ice cold 80 % glycerol was gradually added to a final concentration of 10%.

The suspension was then aliquoted into 1 ml amounts and placed on ice. Aliquots were then snap frozen in liquid nitrogen and immediately placed at -80°C in a pre-chilled container.

2.2.8.2 *P. gingivalis*:

A 10 ml volume of supplemented TSB was inoculated with *P.gingivalis* from a 3 to 4 day blood agar plate. Broth was then incubated at 37°C anaerobically overnight. A 100 ml volume of supplemented TSB was inoculated with sufficient overnight culture to obtain an initial OD₆₀₀ of 0.1. Broths were incubated at 37°C anaerobically until culture reached a final absorbance of 0.55-0.65 at OD₆₀₀. Cells were centrifuged for 10 min at 3,000 x g at 4°C, in sterile centrifugation tubes.

The supernatant was removed and cells were gently re-suspended in 100 ml cold electroporation buffer and centrifuged again. Cells were re-suspended in 50 ml cold electroporation buffer, centrifuged as described above. The final pellet was gently re-suspended in 1 ml cold electroporation buffer. Electrocompetent cells were divided into 100 µl aliquots and stored at -80°C.

2.2.9 Bacterial Transformation

2.2.9.1 Transformation of *E.coli*

For each transformation a 200 µl aliquot of competent cells was thawed on ice. Transforming DNA was then added to each tube and mixed by gentle swirling. Tubes were stored on ice for 30 min. The following controls were included; competent bacteria that received no DNA, competent bacteria that received a known amount of super-coiled plasmid DNA. The tubes were transferred in a rack to a circulating water bath that had been pre-heated to 42°C.

They were left in the water bath for exactly 90 sec. The tubes were rapidly transferred to an ice bath and allowed to chill for 1-2 min.

A 3 ml volume of LB broth was added to each tube. Cultures were incubated overnight at 37°C aerobically with shaking. The appropriate volume of culture was transferred onto LX plates or LB agar plates containing the appropriate antibiotics. Plates were then incubated aerobically overnight at 37°C

2.2.9.2 Electroporation of *P. gingivalis*

For each transformation, a vial containing 100 µl of electrocompetent *P. gingivalis* was thawed on ice. Transforming DNA was pipetted into a sterile electroporation cuvette (0.2 cm gap). Cells were then pipetted into the same cuvette and mixed by gently tapping the cuvette. The cuvette was placed into the electroporation chamber of the Bio-Rad (Bio-Rad Laboratories Ltd. Bio-Rad house, Maxted Road, Hemel Hempstead, Hertfordshire HP2 7DX) minipulser and electroporated using the following settings: 2.5 kV, 400 Ω and 25 µF for 5.0 msec. immediately after electroporation 1 ml of pre-reduced, pre-warmed sTSB was added and the cells were transferred to a sterile micro-centrifuge tube. The cell suspension was incubated anaerobically at 37°C overnight.

The following day the entire 1 ml from the transformation was plated onto ten blood agar plates (100 µl per plate) containing the appropriate antibiotics. Plates were incubated anaerobically until *P. gingivalis* antibiotic resistant colonies were visible on the plate (up to 10 days).

2.3 Polymerase chain reaction

2.3.1 Routine polymerase chain reaction (PCR)

Template DNA was diluted to a concentration of between 50-100 ng/µl using sterile MilliQ water (Merck-Millipore). A master mix was prepared from the required reagents

and added so that the final contents of each tube were as follows: 1 X Taq reaction buffer, 2.5-3.5 mM Magnesium chloride (MgCl_2), 2.5 U Taq DNA Polymerase (Promega), 2.5 μl (10 pmol) of both forward and reverse primer (Sigma-Aldrich) and 0.2 Mm dATP, dTTP, dCTP and dGTP deoxynucleotide triphosphates (Promega) (DNTPs). Sterile nuclease free water (Sigma-Aldrich) was used to bring the final volume up to 50 μl . All reactions were set up in 0.2 ml polystyrene tubes (Molecular BioProducts).

Reactions were carried out using a thermal cycler (Kyratech, Queensland, Australia). An initial denaturation step was carried out at 95°C for 2 min before 30 cycles of the following: denaturation at 95°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 1 min/kilobase of amplicon length. A final extension step was then carried for 5 min at 72°C before the cycle went into an indefinite holding stage at 4°C. Both the annealing temperatures and the extension times were adjusted in accordance to what required for each experiment, the former being dependent on the melting temperature of the primers being used.

Amplification products were separated by electrophoresis on 0.8 % (w/v) agarose gels as outlined in section 2.1.8

2.3.2 Quantitative (real time) PCR (qPCR)

For quantitative PCR (qPCR) cDNA was prepared as outlined in section 2.7.2. Primer pairs specific for the desired target were designed as per section 2.1.5 or taken from the literature as outlined in Table 2.3.

A comprehensive BLAST search was carried out to ensure primer complementarity was specific for the gene of interest. Most primer sets have been previously validated in the literature as indicated in Table 2.3. Primers were designed to meet stringent criteria, which included a melting temperature of 59°C – 60°C with a short amplicon length.

Reactions were carried out on MicroAmp Fast Optical 96-well reaction plates (Applied Biosystems) in 15 µl reactions using 1 X Fast SYBR Green PCR Master Mix (Applied Biosystems), 150 nM of each oligonucleotide primer, 1 µl of diluted template and sterile water. The cycling conditions used were 95°C for 20 sec, followed by 40 cycles of 95°C for 3 sec and 60°C for 30 sec.

Before carrying out qPCR experiments the amplification efficiency of each primer set was assessed and compared to the internal control primers for the 16s gene. The performance of each primer set was analysed using standard curve based methods. Ten fold serial dilution of WT W83 genomic DNA were used as template in a series of reactions for each primer pair and a standard curve generated by plotting the Ct values versus template concentration. Using a proprietary algorithm (Applied Biosystems), the Threshold Cycle (Ct) value is computed for each reaction. The Ct value is the point of intersection between the threshold line and the amplification curve, with the average being calculated over technical replicates. The reliability of a run was determined by analysing the R² value and standard deviation between replicates. The R² value for each primer set is shown in Table 2.3. R² values > 0.98 were considered to show good efficiency in amplification.

Gene expression was calculated using a relative quantification method, which involves the detection of a control target gene in tandem with the experimental target detection. The “endogenous control” target is a gene that has a relatively constant level of expression and allows experimental samples to be compared to it. In the case of *P. gingivalis*, it has been shown that the 16s rRNA gene is a suitable endogenous control (Dou et al., 2010).

On completion of the PCR, target gene expression was compared to endogenous control to generate a ΔCt value as follows:

$$\Delta Ct = Ct (\text{Experimental Target}) - Ct (\text{Endogenous control})$$

The results were presented by transforming the data logarithmically to the 2^{-ΔCt}, which makes an increase in value equal to an increase in expression by inverting the data.

2.4 Southern Immunoblot Hybridisation

All chemicals used in this protocol were purchased from Sigma-Aldrich unless otherwise stated. All buffers were sterilised before use as described in section 2.1.3

2.4.1 Amplification and labelling of DNA probe

The probe was made using standard PCR methods as outlined in section 2.3.1 and checked on a 0.8 % agarose gel. It was stored at - 20°C until use. The HagsF and HagsR primers were used to amplify the probe; these primers gave a product of 275 bp, and were designed against the central region of both *hagB* and *hagC*. The probe was denatured before use by incubating it at 95°C for 5 min in a PCR machine. It was immediately placed on ice for 5 min before being added to pre-warmed hybridisation buffer.

2.4.2 Electrophoresis and Transfer

Genomic DNA was isolated as described in 2.2.1 and digested with *Bgl*II (Promega) overnight as per 2.2.3. Digests were run on a 0.8 % agarose gel alongside 5 µl of 'DIG-labelled molecular weight marker III' (Roche-Diagnostics) at 20 mV for 20 hr. The gel was visualised on the trans-illuminator to ensure good separation.

The gel was depurinated by incubation in 250 mM Hydrochloric acid (HCl) in a clean glass dish at room temperature with gentle shaking for 10 min. It was then rinsed in water and incubated in denaturing solution (500 mM NaOH, 1.5 M NaCl) for 15 min with mild agitation. This step was repeated using fresh denaturing solution. The gel was rinsed briefly in water before being placed in neutralisation solution (500 mM Tris, 1.5 M NaCl, pH 7.5) for 15 min with gentle shaking. This step was repeated. It was then equilibrated in 20 X SSC (3 M NaCl, 300 mM Sodium Citrate, pH 7.0) for 30 min before setting up the capillary sandwich.

The capillary sandwich was set up as follows; a glass dish, which served as a reservoir, was filled with 20 X SSC buffer. On a suspended glass plate, a filter paper bridge was built using Whatmann 3 mm blotting paper (Whatmann, GE Healthcare Life Sciences, Wien, Austria) pre-soaked in 20 X SCC. The gel was placed face-down on the bridge, followed by a piece of positively charged Nylon membrane (Roche Diagnostics) which had been cut to fit the size of the gel. Being careful not to capture air bubble, 3 pre-soaked blotting paper layers were placed on the bridge, followed by two paper towel stacks (>10 cm high). The towels were placed evenly across the top to absorb and draw the liquid from the reservoir. The sandwich was weighted using books and the entire of the capillary sandwich was wrapped in cling film to prevent evaporation. The apparatus was then left to transfer overnight.

The next day the transfer sandwich was dismantled. The membrane was placed on Whatmann 3 mm paper (GE Healthcare Life Sciences) that had been soaked in 2 x SSC (0.3 mM NaCl, 30 mM Sodium Citrate, pH 7.0). It was then exposed to 0.120 Joules of UV light for 1-3 min to crosslink the DNA to the membrane. This was carried out for both sides of the membrane; it was then rinsed with MilliQ ultrapure water (Millipore-Merck) and allowed to air dry.

The orientation of the membrane was noted and the membrane was stored dry at 4°C until proceeding to hybridisation. Transfer efficiency was assessed by visualising the remaining DNA on the agarose gel.

2.4.3 Pre-hybridisation

The membrane was placed in a hybridisation roller bottle (Thermo-Fischer Scientific). A 10 ml volume of reconstituted DIG Easy-Hyb (Roche Diagnostics) was added. The bottle was sealed and incubated with slow rotation in a hybridisation oven (Thermo-Fischer

Scientific). It was equilibrated for 30 min – 3 hr at the appropriate hybridisation temperature.

Hybridisation temperature (T_{hyb}) was calculated based on the melting point of the probe-target hybrid (T_{m}) using the formulae below.

$$T_{\text{m}} = 49.82 + (0.41 \times \% \text{ GC of probe}) - 600/\text{lenght of homology in bp}$$

$$T_{\text{hyb}} = T_{\text{m}} - (22.5^{\circ}\text{C})$$

2.4.4 Hybridisation

Immediately before hybridisation the DIG-labelled DNA probe was denatured at 95°C for 5 min in a thermal cycler (Kyratech). After denaturation it was placed immediately on ice for 5 min. The roller bottle was gently decanted and 3.5 ml of DIG Easy Hyb (Roche Diagnostics) was added per 100 cm² of blot. A 2 µl volume of denatured DNA probe was added per ml of DIG Easy Hyb solution. The bottle was resealed and incubated for 16 hr at the previously calculated T_{hyb} in a hybridisation oven (Thermo-Fischer Scientific) with gentle rotation.

2.4.5 Washing

All buffers were prepared fresh. The membrane was removed from the hybridisation solution and washed with 200 ml of Low Stringency Buffer (2 X SCC, 0.1% w/v SDS) for 10 min with vigorous shaking on a Unimax 2010 mixing tray (Heidolph, Germany). This was repeated with fresh buffer for another 10 min. The membrane was then transferred to a fresh roller bottle (Thermo-Fischer Scientific). A 200 ml volume High Stringency Buffer (0.5 X SCC, 0.1 % w/v SDS) which had been pre-warmed to 65°C was added and incubated at 65°C for 15 min with rotation. This step was repeated with fresh buffer.

2.4.6 Detection

The membrane was incubated in 100 ml of Wash Buffer (1% w/v Maleic Acid Buffer (100 mM Maleic Acid, 150 mM NaCl, pH 7.5), 0.3% w/v Tween-20) in a clean glass dish with gentle shaking. The Wash Buffer was decanted and the membrane was incubated with 100 ml of 2% w/v Blocking Reagent (Roche Diagnostics) suspended in Maleic Acid Buffer in a roller bottle (Thermo-Fischer Scientific) in a hybridisation oven (Thermo-Fischer Scientific) with slow rotation for 2 hr at room temperature.

This solution was discarded and replaced with 2% w/v Blocking Reagent (Roche Diagnostics) re-suspended in Maleic Acid Buffer with the addition of 2 µl of Anti-Digoxigenin-AP, fragment-antigen-binding (Fab) fragments (Roche Diagnostics), henceforth referred to as the antibody. The membrane was incubated with this solution in the hybridisation oven (Thermo-Fischer Scientific) for 30 min at room temperature, it was transferred to a clean glass dish containing 100 ml Wash Buffer and washed vigorously. This step was repeated twice more.

The membrane was placed between two unsealed transparent polystyrene sheets. A 1 ml volume of CDP-Star (Amersham, GE Healthcare Life Sciences) was added, drop-wise to the surface of the blot for every 100 cm² of membrane. The two sheets were carefully closed over and allowed to incubate in the dark for 5 min. Excess liquid was removed and the sheets were sealed using a vacuum sealer.

2.4.7 Development

The sealed membrane was exposed to BioMax Light film (Kodak, c/o Sigma Aldrich) for varying lengths of time (2 min – 25 min) in a light cassette (Kodak). Image development was carried out in the dark as follows: 30 sec in developer solution (Kodak), 30 sec in distilled water, 30 sec in fixing solution (Kodak) and 1 min in cool distilled water. The X-ray film was then air dried and viewed.

2.5 RNA techniques

2.5.1 Freeze fracture method of RNA isolation

Relevant strains were grown anaerobically in sTSB to an OD₆₀₀ of 0.7 or lawned onto sTSA plates and cultured anaerobically for four days at 37°C. After incubation, plate or broth grown bacteria were transferred to polystyrene Falcon tubes (Greiner Bio-one) and centrifuged using a swing-bucket bench-top centrifuge (Eppendorf) for 10 min at 2,500 x g. The supernatant was decanted, leaving a 1 ml volume in the tube which was used to re-suspend the pellet. The resulting “slurry” of culture was added drop-wise to 20 ml of liquid nitrogen (Calor Gas, Dublin, Ireland) in 50 ml polystyrene Falcon tubes, with gentle mixing to prevent aggregation of droplets. Immediately after vaporisation of the liquid nitrogen, the frozen droplets were stored at -80°C.

Prior to RNA extraction, the apparatus required for mechanical disruption of cells were soaked in RNase Away (Molecular BioProducts) for 15 min at 21°C. The apparatus was subsequently rinsed with sterile ultrapure water treated with 0.1 % (v/v) diethylpyrocarbonate (DEPC) (Sigma-Aldrich). Apparatus were left to air dry and were then pre-cooled in liquid nitrogen. To extract the RNA the frozen pellets of culture were transferred to polytetrafluorethylene (PTFL) vessels (Sartorius Mechatronics, Goettingham, Germany) containing a tungsten-carbide grinding ball (Sartorius Mechatronics). The PTFL vessels were sealed and processed on a Mikro-Dismembrator S series (Sartorius Mechatronics) for 2 min at 1,500 r.p.m. The PTFL vessels were then opened and the grinding ball removed for cleaning.

A 600 µl volume of RLT Buffer (Qiagen, West Sussex, UK) containing 1% (v/v) of β-mercaptoethanol (Sigma-Aldrich) was then added to the PTFL vessel containing finely ground bacterial culture. The mixture was allowed to thaw for 2 min before being transferred to a 2 ml microcentrifuge tube (Eppendorf).

It was equilibrated with an equal volume of 70% (v/v) ice-cold ethanol. The equilibrated sample was transferred to an RNeasy spin column with collection tube (Qiagen) and centrifuged for 30 sec at 10,000 x g. The liquid flow-through was discarded. This step was repeated until all the sample had passed through the column. A 700 µl volume of Buffer RW1 (Qiagen) was added to the spin column and centrifuged at 10,000 x g for 30 sec. The liquid flow-through was discarded and 500 µl of Buffer RLT (Qiagen) was added to the spin column and centrifuged at 10,000 x g for 2 min. The flow-through was discarded. The RNeasy spin column was transferred to a new collection tube (Qiagen) and centrifuged at 16,000 x g for 1 min to ensure removal of excess ethanol.

The spin column was transferred to a new 1.5 ml microcentrifuge tube (Eppendorf) and 50 µl of sterile Milli-Q ultrapure water (Merck-Millipore) was added directly to the column membrane. The column was incubated for 5 min at room temperature before centrifugation at 16,000 x g for 1 min to elute the RNA.

To remove genomic DNA, a 5 µl volume of TURBO DNase Buffer (Ambion, Life technologies, USA) and 1 µl of TURBO DNase (Ambion) was added to the sample and gently mixed. The sample was incubated at 37°C for 30 min in a water bath, a 5 µl volume of DNase inactivation reagent (Ambion) was added to the sample followed by thorough mixing by pipetting. This mixture was incubated at 21°C for 5 min with mixing every 90 sec. The samples were centrifuged at 10,000 x g for 1.5 min and the supernatant was transferred to a new micro-centrifuge tube (Eppendorf). This sample consisted of DNA-free bacterial total RNA, quality was assessed using agarose gel electrophoresis as described in section 2.1.8. The sample was subsequently used for cDNA synthesis.

2.5.2 cDNA synthesis:

First strand synthesis of cDNA was performed using the Superscript III First strand synthesis system for real time PCR (Life Technologies). A 100 ng quantity of RNA was

used as the template and was added to a 0.2 ml thin-walled tube (Molecular BioProducts) with 10 mM of each dATP, dTTP, dCTP, and dGTP as well as 50 μ M Random hexamers (Promega) and sterile water up to 10 μ l. The reaction was incubated in a thermal cycler (G-Storm) at 65°C for 1 hr and then 72°C for 15 min followed by immediate incubation on ice. The resulting cDNA was stored at -20°C.

2.6 Cell culture methods

2.6.1 H357 cell culture

H357 cells were grown in 5% CO₂ at 37°C (Sanyo CO₂ Incubator) in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) supplemented with 1/3 Hams-F12 (Sigma-Aldrich), 10 ng/ml Epidermal Growth Factor (Sigma-Aldrich), 0.5 μ g/ml Hydrocortisone (Sigma-Aldrich), 10 ng/ml Cholera toxin (Sigma-Aldrich), 5 μ g/ml Insulin (Sigma-Aldrich), 200 IU/ml penicillin (Sigma-Aldrich), 200 μ g/ml streptomycin (Sigma-Aldrich) and 5 μ g/ml fungizone (Sigma-Aldrich) in T-75 flasks (Corning). To this, a final volume of 10% foetal calf serum was added. Media was replaced every 2 to 3 days depending on cell density. Cells were split when they reached 70 - 80% confluency.

Confluent cultures were trypsinized in 3 ml trypsin for up to 10 min. Cells were checked microscopically to ensure full trypsinization before the addition of 7 ml media. The detached cells were then spun at 3,000 x g for 3 min. The supernatant was removed and the pellet re-suspended in a 1 ml volume of media.

When cell counting was required it was performed using the Trypan Blue (Sigma-Aldrich) exclusion method and a Neubauer Haemocytometer (Marienfeld Glassware, Germany). A 10 μ l volume of Trypan Blue was added to a sterile micro-centrifuge tube (Eppendorf); to this a 10 μ l volume of cell suspension was added. A 10 μ l volume of this solution was added directly to the central chamber of a clean Haemocytometer (Marienfeld Glassware)

and covered with a 0.13 – 0.17 mm cover-slip (Thermo-Fischer Scientific). The cells were counted using a light microscope (Nikon instruments, c/o/ Aquilant Scientific Dublin) at 400 x magnification. In total 5 separate and non-adjacent 0.20 mm x 0.20 mm squares of the central region of the haemocytometer were counted. An estimation of the total number of cells per ml was calculated by the used of the following formula:

$$\text{Cells/ml} = \text{Total cells counted} \times \frac{\text{dilution factor}}{0.2} \times 10,000 \text{ cells/ml}$$

**Chapter 3: Construction and confirmation of a
hagB and *hagC* mutant set in *Porphyromonas*
gingivalis.**

3.1 Introduction:

P. gingivalis produces an array of virulence factors such as lipopolysaccharide and the gingipains, many of which play a role in the development and progression of periodontal disease (Darveau, 2010, Lamont and Jenkinson, 1998, Yoshimura et al., 2009). They have a unique ability to promote a dysbiotic micro-flora in the oral cavity due to their ability to subvert the host immune response and interfere with the inflammatory process (Darveau et al., 2012).

One large class of virulence factors that *P. gingivalis* produces are the haemagglutinins. This group of proteins play an essential role in a number of aspects of the infectious process, in particular with regards to nutrient acquisition. *P. gingivalis* is a haem auxotroph, meaning that it does not possess the enzymes required for haem biosynthesis. In addition the lack of a siderophore scavenging system means that it must rely on alternative mechanisms of haem uptake (Gao et al., 2010, Smalley et al., 2011). The haemagglutinins play a key role in facilitating the acquisition of haem through erythrocyte binding. These proteins facilitate the absorption of haem by *P. gingivalis* through the haemagglutination and lysis of erythrocytes. The proteolytic activity of the gingipains releases the haem from the haemoglobin molecule, allowing for its uptake by HmuY which then binds to HmuR (its receptor) to facilitate internalisation. They form a dual component uptake system, which is encoded for by the *hmu* operon and regulated by environmental levels of iron (Olczak et al., 2008).

P. gingivalis possess at least eight haemagglutinins, a subset of these eight are encoded for by a group of five genes known commonly as the *hag* genes, *hagA* - *hagE*. They have varying levels of homology to one another, with *hagA* and *hagD* having 73.8% homology to one another and *hagA* and *hagE* having 93% homology.

The genes *hagB* and *hagC* form a distinct sub-group from the other hemagglutinins. They are located closely together on the chromosome and share ~98% amino acid sequence identity. Although *hagB* and *hagC* were first identified in 1995 (Progulske-Fox et al., 1995), not much is known about the exact role of these genes. Single mutant studies carried out in *P. gingivalis* have shown that both *hagB* and *hagC* possess haemagglutination activity but they are not the sole mediators of this process (Lepine et al., 1996). Expression studies carried out in *Escherichia coli* and a *Salmonella* vaccine vector have shown that HagB at least is trafficked to the bacterial cell surface (Isoda et al., 2007, Lepine et al., 1996) and HagB has been shown to be highly immunogenic (Dusek et al., 1994, Katz et al., 1999). Given the high levels of homology between the two genes and high amino acid sequence identity, it is likely that HagC shares similar immunogenic and virulence factor properties.

Although the genes are 98.57% homologous they appear to be expressed differentially to each other. Reverse transcription PCR has shown that the levels of *hagB* and *hagC* mRNA varied according to both growth phase and haemin concentration. It was also found that HagB mRNAs were detected in the early logarithmic growth phase whereas HagC transcripts were detected in the mid-exponential phase (Lepine and Progulske-Fox, 1996). It has been shown that they are both expressed *in vivo* in a mouse abscess model of infection, particularly early in the infectious process (Lee et al., 1996).

Single mutants of *hagB* and *hagC* have been made previously in strain *P. gingivalis* 381 (Pg381) (Lepine et al., 1996). However attempts to create a double mutant of both of these genes in this strain failed leading to the belief that these genes were essential to the survival of *P. gingivalis*, and while one gene could be removed safely it was impossible to remove both. This also fuelled the belief that these genes may be redundant in function, something that the high levels of homology on a genetic level also concurred with.

In order to fully understand the exact role of these genes in the virulence of *P. gingivalis* a double mutant of both genes is required.

Since the role of *hagB* and *hagC* both *in vitro* and *in vivo* has yet to be clearly elucidated on we undertook to inactivate the *hagB* and *hagC* loci in *P. gingivalis* strain W83, which is a robust, non-fimbriated virulent strain of *P. gingivalis*. Deletions were constructed using insertional mutagenesis based methods that had been previously used to create single mutants of both *hagB* and *hagC*. By using and adapting these techniques we were able to create a mutant strain set, allowing us to study the phenotypic effect that the removal of these genes had on the organism.

An attempt was also made to create mutants in the ATCC33277 strain, a fimbriated strain which is classified as a less virulent strain of *P. gingivalis* (Chen et al., 2004). This was to allow for comparison of the effects that removal of these genes would have between the two strains. While it was possible for a *hagB* mutant to be produced in this strain, we were unable to obtain a *hagC* mutant and as such a double mutant of these genes in this background. There are a number of potential reasons for this, which are outlined in detail in the discussion of this chapter.

3.2 Materials and Methods

3.2.1 Generation of *hagB* mutant strains:

Plasmid pJW1 was used to disrupt the *hagB* gene of *P. gingivalis*. Plasmid pJW1 contains the *hagB* gene interrupted by the *ermAM-ermF* genes, which encode for erythromycin resistance in *E. coli* and *P. gingivalis*, respectively. This disruption cassette has been used previously to interrupt *hagB* in Pg381. Plasmid DNA was introduced into *P. gingivalis* strain W83 by electroporation and transformants were selected for by plating cells onto agar containing 50 µg/ml gentamicin and 5 µg/ml erythromycin.

Integration of plasmid pJW1 into the chromosomal *hagB* locus was confirmed by PCR using GoTaq (Promega, Promega Corporation, Madison, Wisconsin, USA) and junctional primers. HagBIF is located in the region before the *ermAM:ermF* cassette insertion. It was paired with ErmIR located within the *ermAM:ermF* cassette (Fig 3.1 a). Expected amplicon size was 922 bp. ErmIF is located within the *ermAM:ermF* cassette, it was paired with HagBIR (Fig 3.1 a) with an expected amplicon size of 2500 bp. Transformants were subsequently analysed by DNA sequencing which confirmed the Δ *hagB* genotype in the derivative of strain of W83 which was subsequently termed strain ECM100

The same method and cassette that were used to make strain ECM100 were also used to create a *hagB* mutant in strain ATCC 33277. This mutant was confirmed using the same PCR based methods and termed strain Δ *hagB* ATCC33277.

(a)

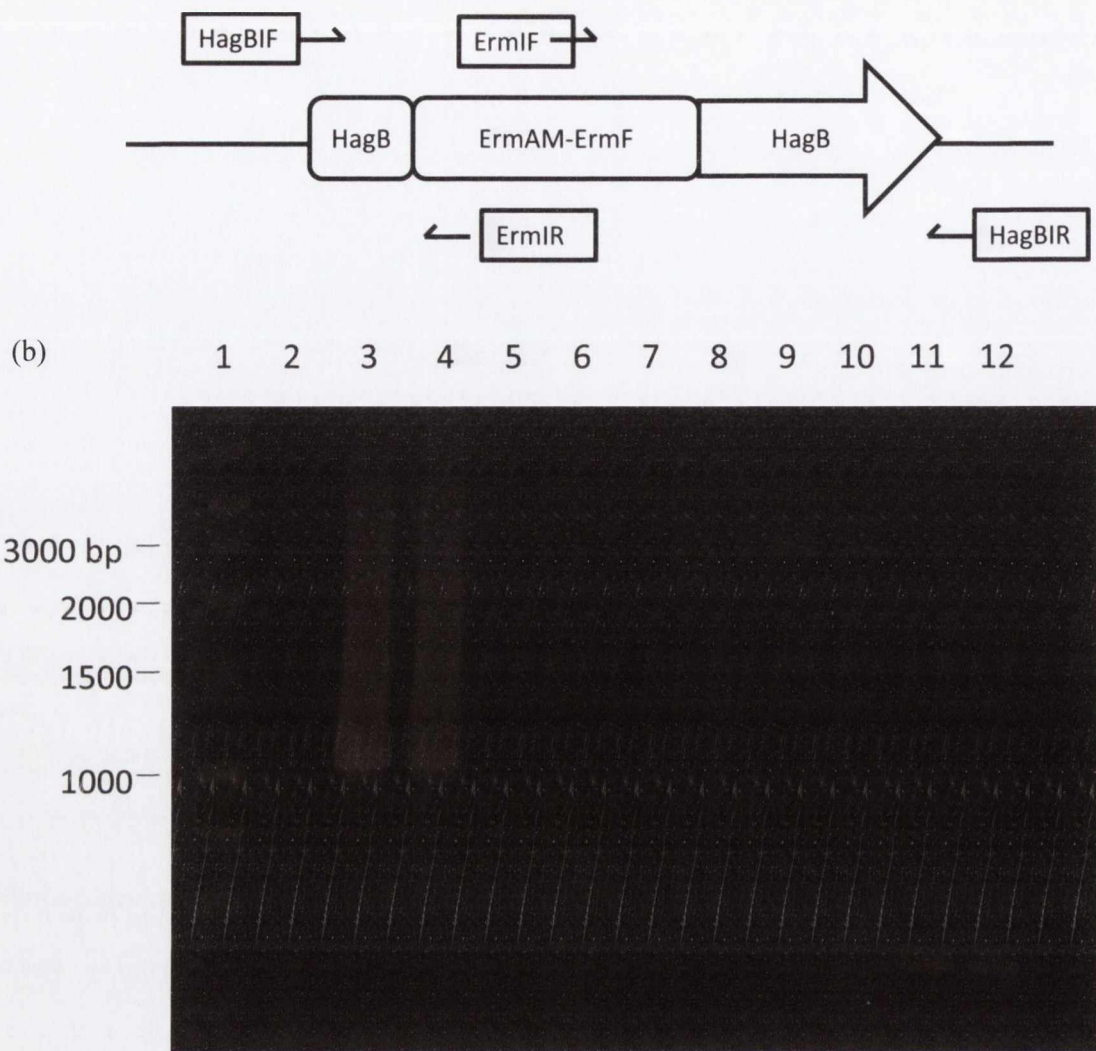


Figure 3.1: a) Diagram showing the positions of the primers used to confirm the junctions of the *ErmAM-ErmF* cassette within *hagB* b) A 0.8% agarose gel of PCR reactions carried out to confirm the junctions of *hagB* transformants, with no template and wild type DNA controls. 1) 1 kb ladder 2) no template control (HagBIF and ErmIR) 3-4) pJW1 transformant 1 (HagBIF and ErmIR) 5-6) Wild type DNA (HagBIF and ErmIR) 7) empty 8) no template control (ErmIF and HagBIR) 9-10) pJW1 transformant 1 (ErmIF and HagBIR) 11-12) Wild type DNA (ErmIF and HagBIR)

3.2.2 Creation of *hagC* mutant in strain W83:

The *tetQ* gene was amplified using Expand High Fidelity DNA polymerase (Roche Life Sciences) from plasmid pT-COW. The PCR amplicon was digested with *Bam*HI and *Spe*I enzymes and ligated to *Bam*HI/*Spe*I digested pBlueScript, to generate pBSTetQ.

A 313 bp region adjacent to the 5' end of *hagC* (RAHC1) was amplified with HagCF1 and HagCR1. The PCR amplicon was digested with enzymes *Xho*I and *Hind*III and ligated to *Xho*I/*Hind*III digested pBSTetQ, to generate pBSTetQHC1. A 411 bp region adjacent to the 3' end of *hagC* (RAHC2) was amplified with HC2_F and HC2_R. The PCR amplicon was digested with enzymes *Xba*I and *Not*I and ligated into *Xba*I/*Not*I digested pBSTetQHC1 to generate phagCΔ. The resultant phagCΔ plasmid contained the *tetQ* gene flanked on either side by regions directly upstream and downstream of the *hagC* gene (Fig 3.2). Plasmid phagCΔ was used to create *P. gingivalis* mutant ECM200 by electroporation. Successful transformants were selected for by plating onto agar containing a 5 µg/ml tetracycline. To confirm integration of plasmid phagCΔ and removal of *hagC* from its chromosomal locus confirmatory PCR was carried out using junctional primers. Primer HC1_IF is located upstream of *hagC* and the *tetQ* insertion site and in conjunction with HC1_IR which is located within the *tetQ* gene (Fig 3.3 a) would be expected to generate an amplicon of 1200 bp following correct insertion of the cassette. Similarly, primer HC2_IF which is located within *tetQ*, when used in conjunction with primer HC2_IR, which lies in a region downstream from *hagC* (Fig 3.3 a), would be expected to generate an amplicon 1200 bp following correct insertion of the cassette. As further confirmation transformants were screened for the presence of *tetQ* by PCR using TetQ_F and TetQ_R, with an expected amplicon size of 2700 bp. These PCR amplicons were then ligated to plasmid pGem-T-easy and were then sent for DNA sequencing using plasmid encoded M13 F and M13 R primer binding sites.

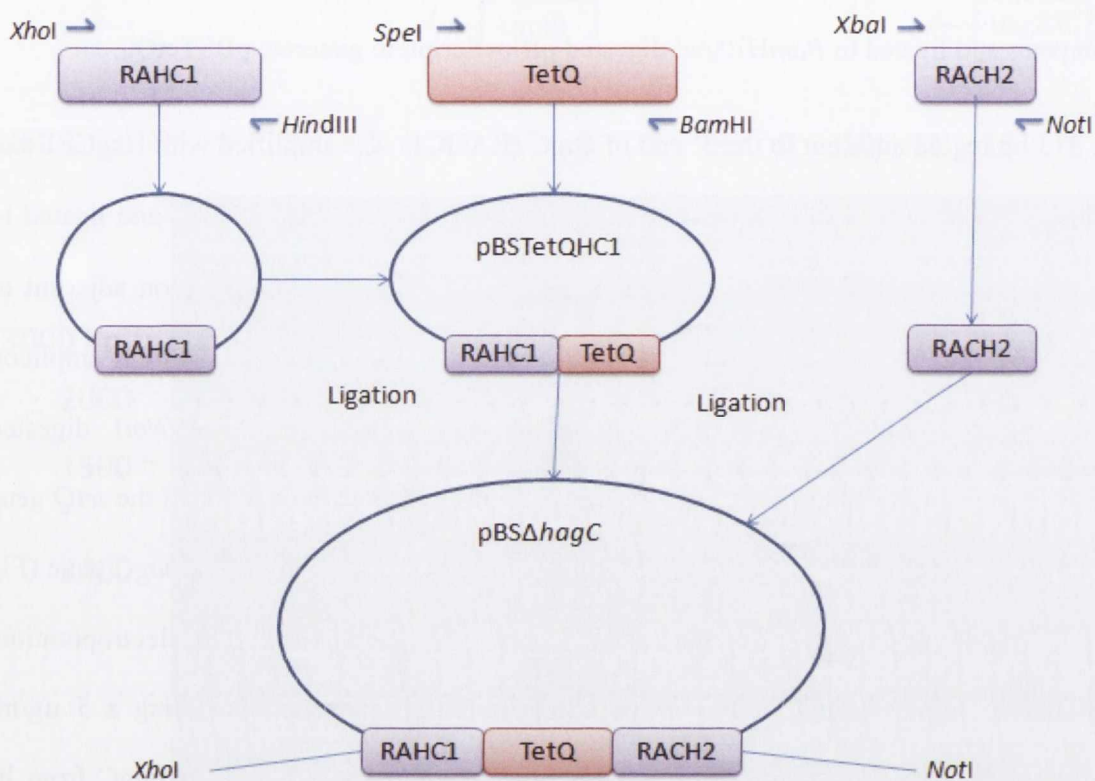
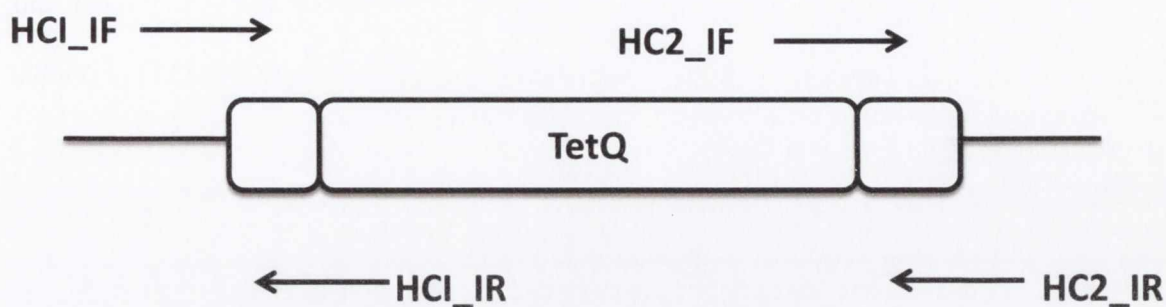


Figure 3.2: Diagram showing the construction of phagCΔ, which was used to delete *hagC* from the *P. gingivalis* W83 genome. The cassette was constructed in pBlueScript; *tetQ* was ligated in first to give pBSTetQ. The region adjacent to the 5' end of *hagC* (RAHC1) was then ligated into pBSTetQ to give pBSTetQHC1. Into this plasmid the region adjacent to the 3' end of the *hagC* gene was then ligated to give plasmid phagCΔ

(a)



(b)

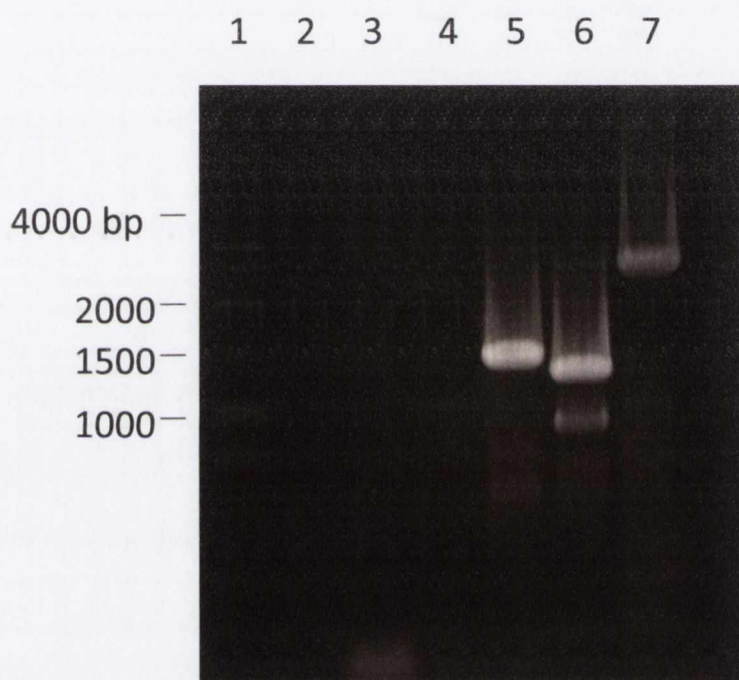


Figure 3.3: a) Diagram showing position of primers used to confirm the integration of *tetQ* within the *hagC* orf. Both primer sets yield an amplicon size of 1200 bp. b) a 0.8% agarose gel showing PCR reactions carried out to confirm the junctions of *hagC* transformants, as well as the presence of *tetQ*. 1) 1Kb ladder 2) blank 3) no template control 4) blank 5) ECM200 DNA screened using HCl_IF and HCl_IR. 6) ECM200 DNA screened using HC2_IF and HC2_IR 7) ECM200 screened for the presence of *tetQ*, using TetQF and TetQR.

After initial sequence reads were obtained, primer walking was carried out to confirm the sequence of the region in full. This sequence analysis confirmed the deletion of the *hagC* gene from its locus in strain ECM200. Post construction of ECM100 and ECM200 plasmid pJW1 was transformed into ECM200 electrocompetent cells to create ECM233, a double mutant of both the *hagB* and *hagC* genes.

3.2.3 Construction of Δ *hagB* complemented mutant

In order to reintroduce *hagB* to strain ECM233, a strategy involving construction of a chloramphenicol resistance marker was employed. This cassette was constructed by Dr Gary Moran. Briefly, a fusion between the promoter of the *P. gingivalis tnaA* gene and the gene encoding chloramphenicol acetyl transferase (*catI*) was generated using fusion PCR. This fusion product was digested with *EcoRI* and *HindIII* and ligated to pBlueScript II which already contained the downstream sequences of the *tnaA* gene on a *HindIII/XhoI* fragment. A *SacII/NotI* fragment from phagB containing the entire *hagB* gene plus upstream sequences was ligated upstream of this *tnaA-catI* fusion. A *HindIII/XhoI* fragment containing sequences downstream of *hagB* were ligated downstream of the *tnaA-catI* to generate phagB-CAT (Fig 3.4). The entire construct was sequenced in order to ensure the correct orientation of the fragments and to confirm that no mutations had been introduced in the *catI* sequences during PCR amplification. The entire construct was released from the plasmid backbone by digestion with *SacII* and *Apal* and used to transform ECM233. Transformants were selected on sTSB plates containing 10 µg/ml chloramphenicol.

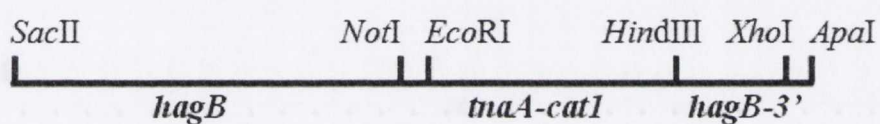


Figure 3.4: Map of the chloramphenicol resistance cassette used to reintroduce the *hagB* gene in strain in ECM233 to generate GMC100. Only relevant restriction sites mentioned in section 3.2.3 are shown.

3.2.4 PCR confirmation of *P.gingivalis* mutant genotypes:

PCR confirmation of *P. gingivalis* mutants was carried out using Expand High Fidelity PCR reagents (Roche Applied Science, Roche Diagnostics Ltd, Charles Avenue, Burgess Hill, West Sussex, RH159RY, U.K.). Two pairs of primers that flanked the *hagB* (DKOBFP/DKOBPR) and *hagC* (DKOCFP/DKOCRP) genes respectively were designed. Primers were designed to allow discrimination between wild-type genes and genes containing insertions (Fig 3.5 a & b). The insertion of pJW1 in *hagB* resulted in an increase in amplicon size from 2,100 bp to approximately 4,400 bp and the insertion of *tetQ* in *hagC* resulted in an increase in amplicon size from 3,100 bp to 4,600 bp.

In order to confirm the genotype of strain ECM233, genomic DNA was obtained using GenElute Bacterial Genomic DNA kit (Sigma-Aldrich) as per the manufacturer's instructions and PCR was carried out to amplify the *hag* gene loci using primers DKOBFP and DKOCRP. This fragment was then cloned into plasmid pGEM T-easy and sequenced using the dye terminator method (Source BioScience, DNA Sequencing, Dublin, Ireland)

Southern blot analysis was also carried out on mutant strains using a probe that was specific for both the *hagB* and *hagC* genes. Due to the results from this blot, all strain were confirmed using PCR and sequencing methods to be plasmid pJW1 integrated correctly into *hagB*.

Confirmation of the complementation of *hagB* back into the *hagB* locus was performed in a number of ways. A number of single colony clones were assessed to confirm that restoration of *hagB* was succesful. The resistance profile of each colony was assessed by growth on agar containing 10 µg/ml chloramphenicol, 5 µg/ml erythromycin or 2 µg/ml tetracycline.

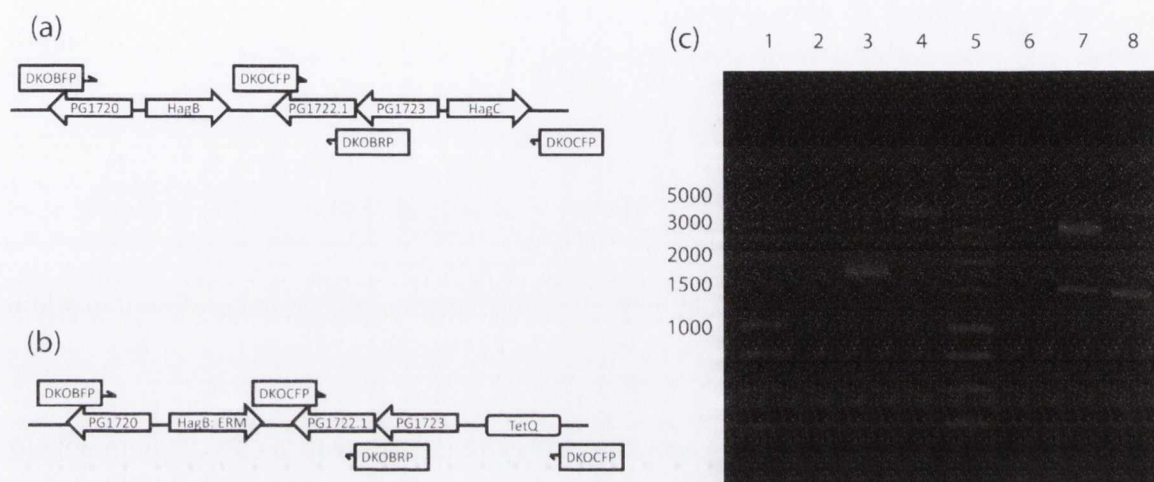


Figure 3.5: a) Diagram illustrating the position of the primers used in the confirmatory PCR in wild type W83. b) Diagram illustrating the position of the primers used in the confirmatory PCR in a double knock-out of W83. c) A 0.8% agarose gel confirming differences in size of the *hagB* and *hagC* loci between wild type W83 and ECM233. 1) 1 Kb ladder 2) no template control 3) Wild type W83 screened with DKOBFP and DKOBRP (expected amplicon of 2100 bp) 4) ECM233 screened with DKOBFP and DKOBRP (expected amplicon of 4400bp) 5) 1 Kb ladder 6) No template control 7) Wild type W83 screened with DKOCRFP and DKOCRFP (expected amplicon of 3100bp) 8) ECM233 screened with DKOCFP and DKOCRFP (expected product of 4600 bp).

Successful transformants were expected to be resistant to tetracycline and chloramphenicol while been sensitive to erythromycin due to the removal of the *ermAM:ermF* cassette.

PCR profiling was also carried out. Each clone was assessed by PCR for the presence of *hagB* and *ermF*. In complemented mutants it was expected that the clones would be negative for the presence of the *ermF* gene and positive for the presence of *hagB*. Appropriate positive and negative controls were included for all PCR experiments.

3.2.5 Attempted construction of *hagC* mutant in strain ATCC33277:

An attempt was made to apply the same method of mutant production as was used in strain W83. Due to the homology between the strain only one of the regions adjacent to the 3' end needed to be amplified and ligated into pBSTetQHC1 to create a cassette homologous to the *hagC* gene in strain ATCC33277.

A 300 bp region adjacent to the 3' end of *hagC* was amplified with primers ATCC HagC2F and ATCC HagC2R. The PCR amplicon was digested with enzymes *Xba*I and *Not*I and ligated into *Xba*I/*Not*I digested pBSTetQHC1 to generate pBSΔ*hagCATCC*. The resultant plasmid phagCΔATCC contained the *tetQ* gene flanked on either side by regions directly upstream and downstream of the *hagC* gene homologous to strain ATCC33277.

Several attempts were made to transform both digested and undigested forms of the plasmid into strain ATCC33277, however all failed to yield successful transformants.

3.2.6: Characterisation of *hagB* and *hagC* expression under haemin rich conditions

Expression levels of *hagB* and *hagC* during exponential growth in sTSB was assessed by qPCR as described in Chapter 2 Section 2.3.2. The primers used were designed to specifically target the only region of low homology between *hagB* and *hagC*. The *16srRNA* gene acted as the control gene for this experiment.

All statistical analysis was carried out using Graphpad Prism 5 software (Graphpad Software Inc., 7825 Fay Avenue, Suite 230, La Jolla, CA 92037, USA). Differences between WT and mutant strains were determined by analysis of variance (ANOVA) or by t-test based methods. When ANOVA was carried out Dunnett's Multiple Comparison was also performed. For all data p -values of < 0.05 were considered statistically significant.

3.3 Results:

3.3.1 Disruption of *hagB* in *P. gingivalis*

In order to disrupt the *hagB* orf plasmid pJW1 was obtained from the laboratory of Ann Progulske-Fox (University of Florida). This plasmid contains *hagB* interrupted by the *ermAM* and *ermF* genes, encoding for erythromycin resistance in *E. coli* and *P. gingivalis*, respectively. Plasmid pJW1 has been used previously to interrupt *hagB* in Pg381. Transformants were selected for by plating onto agar containing 50 µg/ml gentamicin and 5 µg/ml erythromycin.

Unexpectedly, transformation of the parent strain with pJW1 resulted in a Southern blot banding pattern indicating a disruption of *hagB* but also deletion of *hagC*. This pattern suggested that pJW1 recombined with the 5' end of *hagB* and the 3' end of *hagC* to delete the entire *hag* locus (Fig 3.6). As such further colonies obtained from the transformation were screened by both PCR and DNA sequencing, as outlined below to obtain strain ECM100.

Integration of plasmid pJW1 into the chromosomal *hagB* locus was confirmed by PCR amplification of the chromosome-cassette junctions, where one primer was placed within the selected resistance gene and another within the bacterial genome adjacent to the insertion (Fig 3.1 a). Expected PCR amplicons of 922 bp and 2500 bp were generated from strain ECM100 (Δ *hagB*) and observed on 0.8% agarose gels. Amplimers of the expected size were detected in 4 of the 8 transformants analysed (Fig 3.1 b).

Further confirmation was carried out by DNA sequencing. The region containing the *hagB-ermAM-ermF* cassette integration was amplified using the primers DKOBFP and DKOBRP and cloned into plasmid pGEM T-Easy.

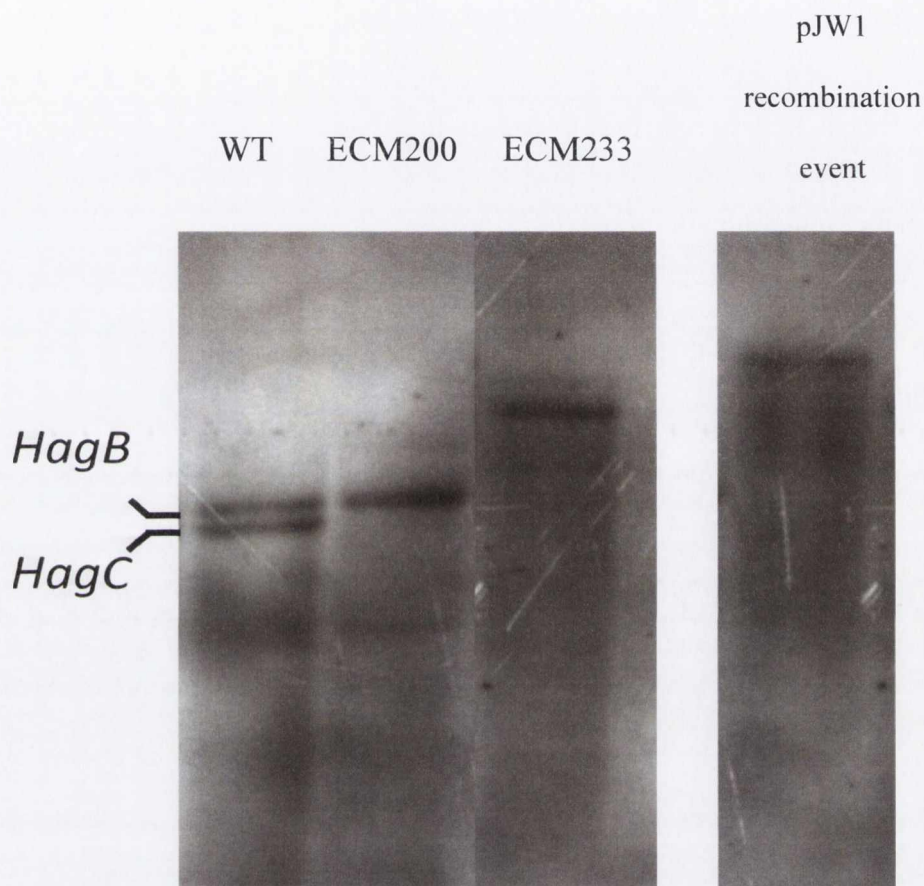


Figure 3.6: Southern blot analysis of genomic DNA isolated from Wild type W83, ECM200 and ECM233. Probe designed to share 100% homology to a 275 bp region of both *hagB* and *hagC*. Diagram also shows plasmid pJW1 recombination that occurred during the event where the plasmid integrated with the 5' region of the *hagB* gene and the 3' region of the *hagC* gene. (ECM200 = Δ *hagC*, ECM233 = Δ *hagB/hagC*).

Primers M13 F and M13 R were used to carry out initial sequencing followed by primer walking based on the reads obtained from M13 F and M13 R. The overall sequencing results verified that *ermAM-ermF* had successfully disrupted *hagB* to yield *P. gingivalis* strain ECM100.

Plasmid pJW1 was also used to interrupt *hagB* in strain ATCC33277. The same confirmation process was used to confirm the integration of the *ermAM:ermF* cassette into the chromosomal *hagB* locus in ATCC33277 as was carried out for strain W83. This strain was labelled strain Δ *hagB* ATCC33277.

3.3.2 Construction of *hagC* disruption cassette using *E.coli* restriction endonuclease-mediated cloning:

To disrupt the *hagC* gene, a cassette with homology to the *hagC* flanking regions from the *P. gingivalis* genome was designed. The *hagC* gene was predicted to be replaced with *tetQ*, which encodes for tetracycline resistance. *TetQ* was amplified from plasmid pT-COW using the TetQ_F and TetQ_R primers, which contained the restriction enzyme sites for *Bam*HI and *Spe*I. This facilitated ligation to *Bam*HI and *Spe*I digested plasmid pBlueScript, giving plasmid pBSTetQ. pBSTetQ was then sent for sequencing to confirm the sequence of the *tetQ* gene. Two fragments from the *P. gingivalis* genome were also amplified by PCR. The 5'-region adjacent to *hagC* was amplified using primers HagCF1 and HagCR1 to give a product of 313 bp. These primers contained the restriction endonuclease recognition sequences for *Xho*I and *Hind*III. This allowed for ligation into plasmid pBSTetQ giving pBSTetQHC1.

The 3' adjacent region of *hagC* was amplified using the primers HC2_F and HC2_R to give a product of 411 bp. *Xba*I and *Not*I recognition sites were designed in the primers to facilitate ligation to *Xba*I and *Not*I digested plasmid pTetQC1 to yield pBS Δ *hagC* (Fig 3.2). The structure of the completed plasmid was confirmed by DNA sequencing.

The *tetQ* gene encodes for tetracycline resistance in both *E. coli* and *P. gingivalis*. Culture of *E. coli* transformants of pBSΔ*hagC* in LB-broth supplemented with 5μg/ml tetracycline confirmed the functionality of the *tetQ* gene, as growth only occurred in broths that were inoculated with bacteria that contained the plasmid phagCΔ.

Using the same methodology a cassette was constructed to delete *hagC* from the ATCC33277 genome. The degree of DNA sequence homology between strains W83 and ATCC33277 meant that only the 3' region of the cassette used in strain W83 needed to be replaced in order for the cassette to function in strain ATCC33277. Primers ATCC HagC2F and ATCC HagC2R were used to amplify a 300 bp region which was then ligated to pBSTetQHC1 to give phagCΔATCC. This cassette was then used in transformation to delete *hagC* from the genome of ATCC33277.

3.3.3 Transformation of *hagC* disruption cassette into *P.gingivalis*.

Disruption of *hagC* in *P. gingivalis* was carried out with digested and undigested phagCΔ DNA. The plasmid DNA was digested with *Xho*I and *Not*I to release the *hagC* cassette, allowing *tetQ* to integrate into the *P. gingivalis* genome.

As with strain ECM100, PCR of the predicted cassette-chromosomal junctions was used to confirm integration of phagCΔ into the chromosomal *hagC* locus. Primers were designed so that one primer in each pair sat adjacent to the *hagC* orf, while the second primer sat within *tetQ* (Fig 3.3 a).

Following correct integration, amplification with HC1_IF and HC1_IR was predicted to yield a 1200 bp product. The predicted product size of amplimers obtained with primers HC2_IF and HC2_IR was also 1200 bp. PCR amplimers matching the predicted sizes were detected in successfully transformed colonies (Fig 3.3 b). Further confirmation was also carried out by DNA sequencing. The sequencing results confirmed the deletion of *hagC*

and the presence of *tetQ*, yielding strain ECM200. The same methods were used to transform phagCΔATCC into strain ATCC33277. After multiple attempts the experiment failed to yield any transformants.

3.3.4 Construction of Δ*hagB*/Δ*hagC* mutant in *P.gingivalis*

Due to the high levels of homology between *hagB* and *hagC*, creation of a Δ*hagB*/Δ*hagC* double mutant first required that the entire *hagC* orf be deleted with phagCΔ, followed by targeted disruption of *hagB* with pJW1.

Electrocompetent cells of ECM200 were prepared and transformed with plasmid pJW1. Transformants were selected for on agar containing 50 μg/ml gentamicin and 5 μg/ml erythromycin for 10 – 14 days. Eight colonies were observed on the plates all of which were stored for further investigation.

In order to confirm that the plasmids phagCΔ and pJW1 had incorporated correctly into *P. gingivalis*, PCR and Southern blot analysis were carried out.

A number of PCR reaction were carried out in order to confirm the genotype of Δ*hagB*/Δ*hagC* using primers that were previously used to characterise strains ECM100 and ECM200. A second confirmatory PCR experiment was also designed to identify the difference in size in the *hagB* and *hagC* encoding regions of the chromosome following correct integration of the cassettes (Fig 3.5 a & b). It was predicted that these regions would be smaller in size in wild type W83 compared to the Δ*hagB*/Δ*hagC* mutant. The predicted amplicons were observed in both strains (Fig 3.5 c).

The PCR products from Δ*hagB*/Δ*hagC* were subsequently gel purified and ligated into plasmid pGEM T-Easy in accordance with the manufacturer's instructions. The plasmids were then digested using *NotI*, which has two cut sites in pGEM T-Easy, to allow for the

presence of the correct inserts to be confirmed. Expected band sizes of 4.4 kb for the disrupted *hagB* region and 3.1 kb for the deleted *hagC* region were observed.

Plasmid DNA was sent for sequencing using M13 F and M13 R primers, subsequent primer walking was then carried out to obtain the full sequence of the region. Sequencing confirmed that both genes had been successfully mutated to create $\Delta hagB/\Delta hagC$, which was designated as strain ECM233. The original PCR primers had been designed to facilitate the creation of a full sequence map of entire chromosomal region between orfs pgin_c_17_261 and pgin_c_1_7249 which correspond to *hagB* and *hagC* loci in ECM233. Southern blot analysis was performed using a probe designed against a central region with 100% homology to both the *hagB* and *hagC* genes in *P. gingivalis* strain W83 (Fig 3.6). Genomic DNA from wild type W83, ECM100, ECM200 and ECM233 was isolated and digested with *Bgl*III. The digested fragments were then transferred to nylon membranes after electrophoresis. In the parent strain W83, two bands were expected, a 4 kb band representing *hagC* and a 5.3 kb band representing *hagB*. For strain EMC200 the 4 kb was expected to be deleted entirely but the *hagB* band to be identical to wild-type. In $\Delta hagB/\Delta hagC$ the *hagB* band was expected to shift upwards, with no band present for *hagC* (Fig 3.6).

3.2.5 Confirmation of $\Delta hagB$ complemented mutant

The reinsertion of *hagB* and the removal of *ermAM:ermF* cassette was confirmed through antibiotic resistance profiling and PCR. Transformants were plated onto agar containing 10 µg/ml chloramphenicol, 5 µg/ml erythromycin or 2 µg/ml tetracycline. All transformants grew on the media contain 2 µg/ml tetracycline as expected.

It was anticipated that successful transformants would fail to grow on the media containing erythromycin and would successfully grow in the presence of chloramphenicol.

This was the case for transformant number 2 but unequivocal resistance profiles were found for the other transformants so it was decided to confirm the complementation further through PCR based methods.

A number of transformants were screened for the presence of both *hagB* and the *erm* genes. The expected *hagB* product of ~2100 bp was seen in all transformants with the exception of transformant 5 (Fig 3.7 a). The colonies were then screened for the presence of the *erm* cassette which would be predicted to be absent from transformants with the correct insertion. (Fig 3.7 b). Transformant 2 was both positive for the presence of *hagB* and negative for the presence of the *erm* genes (Fig 3.7 a and b) indicating the correct genotype and was designated GMC100

3.2.6 Analysis of *hagB* and *hagC* expression in WT and mutant strains

Expression studies of the *hagB* and *hagC* gene were carried out to profile expression during exponential phase as well as to ensure that expression levels in the mutants were as expected.

The expression of *hagB* was found to be high during this stage in the growth cycle. As the *hagB* cassette is a disruption cassette as opposed to a deletion cassette we expected to observe some expression for all strains. The highest levels were observed for WT and strain GMC100 both of which have a fully functional *hagB* gene. Interestingly strain ECM200 which also has a *hagB* was expressed at a lower level compared to both of these strains with its levels been equivalent to strain ECM100 and ECM233, which is reflected in the statistical analysis of the data. In the case of this data set, an unpaired student t-test was carried out to assess the difference between the WT and each mutant strain individually. There were statistically significant differences between WT and strain ECM100 (p-value 0.0366), WT and strain ECM200 (p-value 0.0413) and WT and strain ECM233 (p-value 0.0195) (Fig 3.8).

Expression levels of *hagC* were assessed. These were found to be substantially lower than that of *hagB*. There was a statistically significant difference between strain ECM100 and the other mutant strains, with strain ECM100 showing a significantly higher level of expression. Minimal levels of expression were detected in all strains where *hagC* had been deleted (Fig 3.9). This was as expected given the absence of the gene.

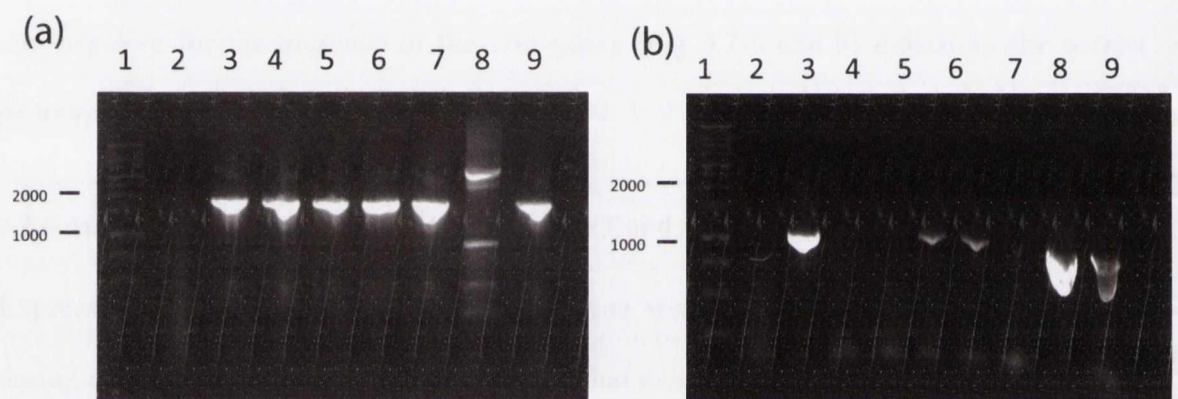


Figure 3.7: a) A 0.8 % agarose gel confirming the presence of *hagB* in Δ *hagB* complemented mutants 1) 1 kb ladder 2) negative control 3) positive control 4-9) screened transformants (expected product size of 2100 bp) b) A 0.8 % agarose gel confirming the absence of the *erm* cassette in Δ *hagB* complemented mutants. 1) 1 kb ladder 2) negative control 3) positive control 4-9) screened transformant

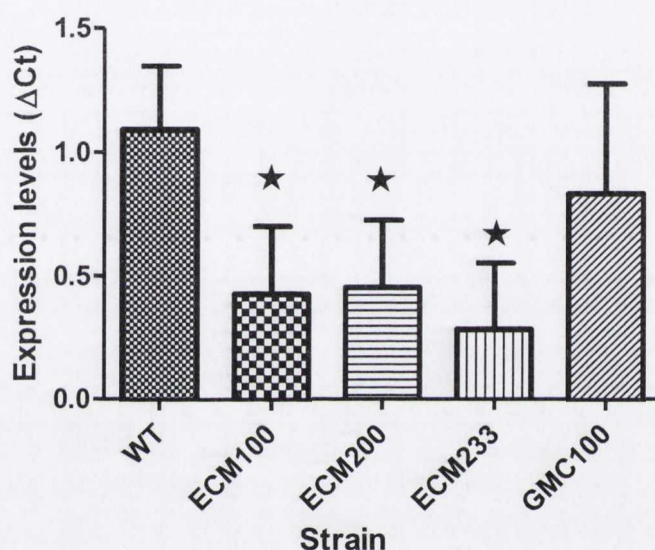
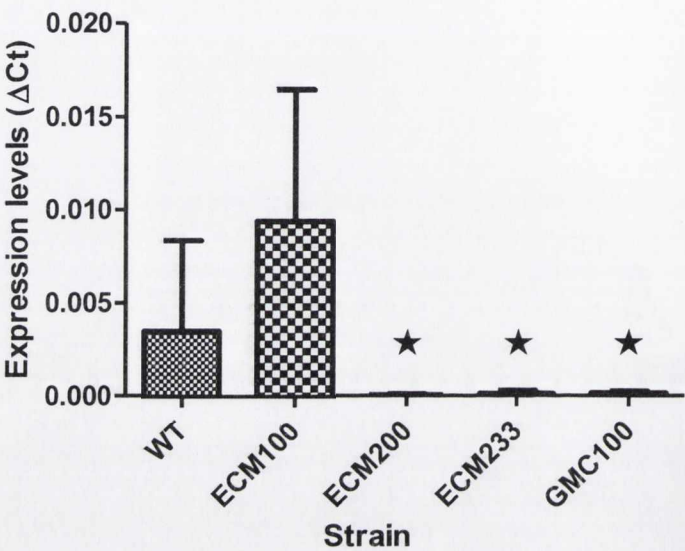


Figure 3.8: Diagram showing that at OD₆₀₀ of 0.7 under haemin rich conditions *hagB* is expressed. Expression level is based on ΔC_t values as calculated in section 2.3.2. Student t-tests were used to compare WT to each mutant strain individually it was found that there were statistically significant differences between WT and strain ECM100 (p-value 0.0366), WT and strain ECM200 (p-value 0.0413) and WT and strain ECM233 (p-value 0.0195). * indicates a significant value between 0.01 and 0.05. (ECM100 = $\Delta hagB$, ECM200 = $\Delta hagC$, ECM233 = $\Delta hagB/hagC$, GMC100 = $\Delta hagC$, *hagB::cat*)

(a)



(b)

Dunnett's Multiple Comparison Test	q	Significant? P < 0.05?
ECM100 vs. WT	1.886	No
ECM100 vs. ECM200	2.997	Yes
ECM100 vs. ECM233	2.974	Yes
ECM100 vs. GMC100	2.963	Yes

Figure 3.9: a) Expression levels of *hagC* after growth in sTSB to OD₆₀₀ of 0.7. Expression level is based on ΔCt values as calculated in section 2.3.2 b) Statistical analysis was carried out using ANOVA with Dunnet’s Multiple Comparson carried out post ANOVA. There was a statistically significant difference between strain ECM100 and all other mutant strains. * indicates a significant value between 0.01 and 0.05. (ECM100 = Δ*hagB*, ECM200 = Δ*hagC*, ECM233 = Δ*hagB/hagC*, GMC100 = Δ*hagC*, *hagB::cat*)

3.4 Discussion:

P. gingivalis is considered to play a central role in the development of periodontitis despite its presence in low abundance. It is currently considered to be a key-stone pathogen for the development of the disease whereby through immune subversion mechanisms it helps to facilitate and maintain the development of a dysbiotic microbiota (Darveau et al., 2012). It is well known that the organism possess a number of virulence factors that actively contribute to the development of chronic periodontitis (Bainbridge et al., 2010, Darveau et al., 1998). A pathogenic role for haemagglutinins HagB and HagC has been postulated but remains to be fully established. Previous studies have shown that both genes are expressed during the infectious process in strain Pg381 (Lee et al., 1996). However whether expression varies between strains is unknown. There are extensive genomic rearrangements between strains of *P. gingivalis* which may influence expression (Naito et al., 2008). However it is important to note that the HagB and HagC loci appear to be highly conserved between strains, whether or not genetic rearrangements upstream or downstream of these loci could lead to variations in expression has yet to be established.

In order for our understanding of the virulence of *P. gingivalis* to develop further it is essential that the role of HagB and HagC in pathogenicity is fully elaborated on. As of yet the role of these two proteins in the development of periodontitis has yet to be established. However, HagB has been shown to play a role in adhesion to human coronary artery endothelial cells, giving it a potential role in the events that trigger the development of atherosclerotic plaque (Song et al., 2005). It has also been identified as been highly immunogenic molecule, as such this means that it posses vaccine potential, which has been shown in a variety of animal studies that have been carried out (Dusek et al., 1994, Katz et al., 1999). Given the high homology between the two proteins on a genetic level it is likely that HagC would have similar properties.

The aim of this section of the study was to create the first $\Delta hagB/\Delta hagC$ mutant in *P. gingivalis* so that the role of these genes in the virulence of *P. gingivalis* could be established more clearly.

Upon transformation of plasmid pJW1 into strain W83 it was found that a number of outcomes could be obtained. The plasmid could, as expected target the *hagB* gene, or as was seen in the Southern blot pJW1 could recombine with the 5' end of *hagB* and the 3' end of *hagC* to delete the entire Hag locus. This is interesting to note as it highlights that these genes are not essential to this strain of *P. gingivalis*.

It is also in contrast to previous deletion studies carried out in strain Pg381 with plasmid pJW1 which did not result in this type of double disruption event, suggesting that *hagB* and *hagC* are essential in strain Pg381, but not in strain W83 which was used in the present study.

It meant that as we were using plasmid pJW1 to create strain ECM233 careful consideration of the *hagB* and *hagC* genes was required and approaches tailored to overcome the high sequence homology as well as plasmid pJW1 insertion capabilities. For any attempt to be successful it was vital that one of the genes was fully removed from the *P. gingivalis* genome prior to any attempts to disrupt the remaining gene. For this reason, we created phagC Δ , a deletion cassette designed to remove *hagC* completely from the genome and replace it with *tetQ*, thus ensuring that the second plasmid used, plasmid pJW1, would selectively disrupt the *hagB* gene only.

A number of methods were used to confirm strain ECM233 including Southern blot analysis, a number of PCRs and DNA sequencing. This was carried out in order to ensure that both plasmids had inserted as expected. The expected results were seen for all confirmation studies carried out.

In order to complete the strain set a complemented *hagB* mutant was created using a re-insertational cassette which also contained the chloramphenicol resistance gene. The expected resistance phenotype for this mutant upon successful insertion of the cassette was chloramphenicol resistant and erythromycin sensitive, as the erythromycin resistance genes would be completely removed when *hagB* was restored. This was the case for some of the transformants but not all, some still showed the presence of erythromycin resistance.

In order to confirm the mutant more firmly it was decided to use PCR based methods to check for the presence of *hagB* and the absence of the *erm* cassette (Fig 3.7). As was expected most transformants were PCR positive for the presence of *hagB*, with the exception of transformant 5, instead of the expected band of ~2100 bp a band that was substantially larger (~4000 bp) was observed. The transformants were also screened for the presence of the *erm* genes. Of the five mutants screened, only two were fully PCR negative for the presence of *erm*. Transformant number two matched the expected antibiotic resistance and PCR profiles; as such it was labelled strain GMC100 and used in all further experiments. There are a number of reasons that some of the transformants may have been screen positive for the presence of the *erm* genes and why transformant number 5 gave a substantially larger band when screened for *hagB*. The cassette used to create strain GMC100 is capable of inserting into the *P. gingivalis* genome in a number of ways, which would have allowed for the results seen.

An attempt was also made to create a *hagB* and *hagC* mutant strain set in the ATCC33277 background, as this would have allowed for comparison between a non-fimbriated virulent (W83) and a fimbriated strain that is considered less virulent (ATCC33277). While we were able to use plasmid pJW1 to create a *hagB* knock-out we were unable to remove *hagC* from the genome.

This could be because the *hagC* gene is essential to strain ATCC33277 and thus removing it from the genome was fatal for the bacterium. Also strain ATCC33277 is not as robust a strain as strain W83, which in my experience recovers more rapidly post transformation. This lack of robustness could have contributed to its inability to survive the transformation as removing *hagC* would have weakened the strain in comparison to wild type.

It is interesting to note that in previous studies a double *hagB/hagC* mutant could not be created in strain Pg381 either, although single mutants were viable. This suggests that these genes are essential to strain Pg381. From ATCC33277 our data would suggest that *hagC* is essential for function and that removal of the gene is lethal to the bacterium, *hagB* appears to play a less important role. Both of these strains are fimbriated but strain Pg381 is classed as a virulent strain of *P. gingivalis*, whereas ATCC33277 is not, at least in the abscess model of infection. It is worth noting that both of these strains are fimbriated and perhaps the reason that a double mutant cannot be created is that HagB and HagC potentially play a role that is linked to the production or transport of fimbriae. One way to assess this further would be to take another non-fimbriated strain of *P. gingivalis* and see if the *hagB* and *hagC* can be safely removed from the genome without the mutation proving fatal.

The expression of *hagB* and *hagC* during the exponential phase of bacterial growth has been examined previously (Lepine and Progulske-Fox, 1996). This study found that *hagB* was expressed during the early exponential phase while *hagC* was detected later in the growth cycle. Our study reflects these findings with high levels of *hagB* transcripts been detected and low levels of *hagC* transcripts. It is worth noting that expression of *hagC* was higher in WT and ECM100 compared to all other strains. This is the expected result given the deletion of the *hagC* gene and also serves to highlight the sensitivity of the primers used in the study.

This is the first study where a $\Delta hagB/\Delta hagC$ mutant has successfully been created in *P. gingivalis*. One of the reasons that our attempt was successful could be our choice of strain. In this study we used strain W83 which is a robust strain of *P. gingivalis* that grows faster than other strains such as ATCC33277 and Pg381. Its more efficient growth rate may make its post-transformation recovery easier than what it would be for other strains, an idea that is supported by the inability to successfully remove hagC from strain ATCC33277. Here we show that neither *hagB* nor *hagC* are essential for growth and survival in strain W83, evidence which is further corroborated by the ability of plasmid pJW1 to recombine with both genes and remove the entire locus from the *P. gingivalis* genome.

Chapter 4: Examining the role of *hagB* and *hagC*

in pigmentation, growth, haemagglutination and environmental stress sensitivity.

4.1 Introduction

Porphyromonas gingivalis propagates in a very specific niche in the oral cavity, the gingival crevice, where its nutritional requirements are met and there is reduced oxygen tension (Lamont and Jenkinson, 1998). Haem, a proto-porphyrin, is an essential requirement for the growth of *P. gingivalis*. It is a component of haemoglobin found in red blood cells (Lamont and Jenkinson, 2000). *P. gingivalis* is missing most of the genes required for *de novo* porphyrin biosynthesis, and as such it must acquire this essential molecule from the environment (Olczak et al., 2005). In addition, the bacterium does not produce siderophores meaning that it cannot acquire iron via this scavenging mechanism and instead must rely on its haem uptake systems for iron accumulation.

The molecules required for haem uptake include a number of specific outer membrane receptors, lipoproteins and haemagglutinins in addition to a group of proteases required for haemoglobin breakdown, known as the gingipains. This latter group is composed of three proteolytic enzymes RgpA, RgpB and Kgp which are encoded for by three separate genes *rgpA*, *rgpB* and *kgp*, respectively. These enzymes are arginine and lysine specific extracellular proteinases that act in conjunction with the Hmu haem uptake system. RgpA and RgpB are specific for Arg-Xaa bonds, whereas Kgp is specific for Lys-Xaa bonds (Smalley et al., 2011). The gingipains facilitate proteolytic mediated haem acquisition from oxygenated haemoglobin. The freed haem is then acquired using a dual component uptake system consisting of HmuY and HmuR. The haem molecule is bound to HmuY, which then binds to HmuR (its outer-membrane receptor) to facilitate internalisation of the acquired haem molecule. Both are encoded for by the *hmu* operon, which is in turn regulated by environmental levels of iron (Olczak et al., 2008).

A second haem acquisition system has also been identified in *P. gingivalis*, termed the HusA system. The system has yet to be fully defined but the HusA protein was found to

have a higher binding affinity for haem than HmuY. HusA is expressed and released from the bacteria's cellular surface and has been shown to be essential for *P. gingivalis* growth under haem limited conditions (Gao et al., 2010).

The acquisition of haem from the surrounding environment gives *P. gingivalis* its classic black pigmentation when grown on blood agar. *P. gingivalis* stores haem on its surface in the form of μ -oxo bishaem (Lamont and Jenkinson, 1998), the μ -oxo bishaem molecule is composed of two proto-porphyrin IX rings bridged by an oxygen atom and is commonly referred to as dimeric haem. It is generated by *P. gingivalis* through the reaction of haemoglobin-derived iron (II) or iron (III) protoporphyrin IX and oxygen from both oxy- and deoxy-haemoglobin and subsequently deposited on the bacterial cell surface (Smalley et al., 2002). The presence of μ -oxo-bishaem accounts for both the initial greenish colour of *P. gingivalis* as seen on blood agar in the early stages of growth, as well as the black pigmentation seen later, which is due to accumulation of μ -oxo bishaem over time on the bacterial surface (Smalley et al., 2004).

The generation of μ -oxo bishaem serves as an important virulence factor for *P. gingivalis*. It allows for free oxygen to be absorbed and in doing so it prevents free monomeric haem groups from taking part in the production of reactive oxygen species (ROS). The coating also has inherent catalase activity meaning that it has protective capabilities against oxidative attack by immune cells releasing hydrogen peroxide (H_2O_2) (Smalley et al., 2004).

Haemagglutinins are important virulence factors for *P. gingivalis* as they facilitate the acquisition of haem from red blood cells and feed into the haem acquisition systems outlined previously. *P. gingivalis* possesses at least eight haemagglutinins; a subset of which is known as the Hag proteins. The haemagglutinins have varying levels of homology to one another with HagA and HagD sharing 73.8% amino acid sequence identity and

HagA and HagE sharing 93% identity. The highest levels of homology can be seen between HagB and HagC proteins, which are 98.6% identical. The genes encoding *hagB* and *hagC* are 98.57% identical at the nucleotide sequence level and the genes which encode for them are located closely together on the chromosome (Lepine and Progulske-Fox, 1996). There is no significant homology between the HagA, HagD and HagE proteins and HagB and HagC.

The *P. gingivalis* stress response has been well examined. The effect of oxygen on the growth and physiology of the organism has been studied previously, where it was found that the organism is oxygen tolerant and the level of tolerance increases in the presence of increasing haemin concentrations in the media (Diaz and Rogers, 2004). The effect of haemin on the growth of *P. gingivalis* has been clearly studied, it is well established that *P. gingivalis* grows poorly in the absence of haemin (Lamont and Jenkinson, 2000). The impact that temperature has on the phenotype of the organism has also been investigated. It was found that *P. gingivalis* grew well at all temperatures ranging from 37°C to 41°C, however optimal growth was seen between 37°C and 39°C. As temperature increased a reduction in gingipain activity was seen, with an 82% reduction in arginine specific gingipain function and a 72% reduction in lysine gingipain function been seen at 41°C (Percival et al., 1999).

Environmental stresses such as these are important to examine in both WT and mutant strains given that the oral cavity is in a continuous state of flux, with environmental changes taking place constantly. It is essential to understand bacterial responses to such stresses in order to elaborate further on their survival mechanisms.

Single *hagB* and *hagC* mutants have been described previously which have indicated a role for these genes in haemagglutination in Pg381 (Lepine et al., 1996).

Here, we sought to build on this work by creating a unique double mutant of *hagB* and *hagC* in *P. gingivalis* W83. Creation of this mutant allows us to study the role of these genes in the virulence of *P. gingivalis* in more detail, granting us the opportunity to define their role more clearly. It was predicted that this double *hagB/hagC* mutant would have a weaker haemagglutination pattern than either of the single mutants. We also sought to demonstrate the effect that removing *hagB* and/or *hagC* from the *P. gingivalis* genome has on pigmentation, growth and the bacterial stress response, all of which have been previously unstudied.

4.2 Materials and Methods

4.2.1 Pigmentation studies

4.2.1.1 Examination of pigmentation differences on solid media

Overnight broth cultures of *P. gingivalis* W83 and mutant derivatives were grown in haemin free sTSB anaerobically at 37°C. A 100 µl aliquot of each culture was spread onto one half of a sTSA agar plate and the plates were incubated anaerobically at 37°C. Plates were monitored for growth every day with any differences in pigmentation and colony size recorded.

4.2.1.2 Examination of pigmentation difference in liquid media

Cultures of *P. gingivalis* W83 and its mutant derivatives were cultured in 7 ml haemin free sTSB and allowed to grow overnight at 37°C anaerobically. These cultures were used to sub-culture subsequent haemin free sTSB broths and incubated anaerobically at 37°C overnight. This process was repeated for five days to ensure that the *P. gingivalis* strains had used up all of its stored haem reserves. A 1 ml volume of culture was taken and spun at 10,000 x g for 1 min in a 1.5 ml sterile centrifuge tube (Eppendorf). The subsequent pellets of bacteria were examined and photographed for pigmentation differences. This was carried out for each strain.

After five days the broth cultures were subcultured into either sTSB which contains 5 µg/ml (76.9 nM) of haemin or into sTSB containing 10 µM human haemoglobin (Sigma-Aldrich). The broth cultures were then incubated anaerobically at 37°C overnight before being examined for differences in pigmentation as outlined previously. A 1 ml volume of each culture was subcultured into fresh media daily and development of pigmentation was examined every 24 hr.

4.2.2 Growth rate determination

P. gingivalis W83 and the mutant strains were cultured in 20 ml haemin free sTSB for 48 hr. From these starter cultures, three separate cultures were set-up for each strain at the following optical densities (OD); OD₆₀₀ of 0.2, OD₆₀₀ of 0.4 and OD₆₀₀ of 0.6. The optical density of each broth was measured hourly and noted. A final 24 hr measurement was taken from the OD₆₀₀ 0.6 cultures. The data generated from each of the cultures was amalgamated to generate a continuous growth curve for each strain over a 24 hr period. The doubling times for each strain were calculated using Graphpad Prism 5 software (Graphpad Software Inc., 7825 Fay Avenue, Suite 230, La Jolla, CA 92037, USA). This experiment was carried out in triplicate.

4.2.3 Haemagglutination assays

A 2 ml volume of defibrinated sheep's blood was pipetted into a sterile 50 ml falcon tube and spun at 1,500 x g for 10 min. The supernatant was then removed and the red blood cell (RBC) pellet was re-suspended in 6 ml sterile 1 X PBS (Sigma- Aldrich) and centrifuged at 1,200 x g for 5 min. This wash was repeated with 6 ml of PBS. The supernatant was aspirated from the resulting pellet and the size of the pellet was measured using a sterile 10 ml pipette. A sufficient volume of PBS was then added to the pellet to yield a 10% vol/vol suspension of RBCs. In the haemagglutination assay, a final working solution of 2.5% vol/vol RBCs was used. This was made by making a 1:4 dilution on the 10% vol/vol solution in PBS. Cultures of WT and mutant strains were grown in haemin rich sTSB to the desired OD₆₀₀ measured using a spectrophotometer. Sterile haemin rich sTSB was used to blank the spectrophotometer. Once the desired OD₆₀₀ was reached a 1 ml volume of culture was added to a sterile micro-centrifuge tube and pelleted at 10,000 x g for 1 min. The media was then removed and the cell pellet was re-suspended in 1 ml of 1 X PBS.

The pellet was washed twice in 1 ml PBS by centrifugation at 10,000 x g for 1 min. The cells were re-suspended in PBS to an OD₆₀₀ of 0.7 which has a known bacterial count of ~ 5 x 10⁸ cells/ml. Two-fold serial dilutions of the bacterial suspension were prepared in PBS. A 100 µl aliquot of each bacterial dilution was then mixed with 100 µl of 2.5% sheep's blood in a column of 8 wells in 96-well microtitre plate with round bottom wells. Triplicate columns were inoculated for each bacterial strain. The plate was then incubated at room temperature for 18 hr before reading. Assays were read visually, with positive and negative reactions determined by eye.

4.2.4 Stress sensitivity testing

The susceptibility of *P. gingivalis* W83 and mutant derivatives to the stress inducing chemicals listed in Table 4.1 was determined. For these experiments, WT W83 and the *hagB/hagC* mutant ECM233 were grown in haemin free sTSB overnight. Blank antibiotic disks (Oxoid) were soaked in the chemical being tested for 2 min to ensure that the disk was saturated. A 1 ml volume of bacterial culture of WT W83 or ECM233 was spread onto a sTSA plate. The disk soaked with the agent being tested was placed in the centre of each of the inoculated plates (Table 4.1). Solutions of 10 X PBS and 1 M NaCl were used to test halotolerance. Hydrogen peroxide and diamide were used to generate oxidative stress. Crystal violet is a known antimicrobial believed to interfere with the bacterial cell wall. Ferrozine is an iron chelator and was used to assess susceptibility to iron limitation. Both ionic (10 % N- laurolysarcosine) and non-ionic (Triton X-100) detergents were used to assess membrane stability. Plates were incubated anaerobically for seven days at 37°C and the zone of clearance around each disk was then measured. As a positive control for inhibition, disks soaked in 10 µg/ml of chloramphenicol were used. For a negative control 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was used.

Table 4.1: A table listing the reagents used for stress sensitivity testing of *P. gingivalis* W83 and mutant derivative ECM233, showing zones of inhibition for each agent tested.

Agent tested	Sensitivity (measured by zone of clearance in mm)	
	WT W83	ECM233
Control: Chloramphenicol (10µg/ml)	34.33 (± 2.081)	35.66 (±1.527)
HEPES solution	0	0
10% N-laurolysarcosine	0	0
10 X Phosphate buffer saline	0	0
1M NaCl	0	0
Ferrozine	0	0
9% vol/vol Hydrogen peroxide	11.66 (±0.577)	12.33 (±0.577)
Crystal violet	37 (±2.645)	35 (±2.00)
Diamide	28.3 (±2.886)	28 (±1.732)
Triton X-100	26 (±1.00)	27.6 (±0.577)

4.3 Results

4.3.1 Analysis of the effect of removal of *hagB* and *hagC* from the *P. gingivalis* W83 genome on pigmentation in solid and liquid media

To determine the role *hagB* and *hagC* in the pigmentation of *P. gingivalis* the effect of mutation of *hagB* and *hagC* was assessed. This analysis was carried out in comparison to the WT strain using both plate and broth based methods.

After four days growth on sTSA plates there were observable differences in pigmentation (Fig 4.1). By this time, WT W83 exhibited the classic black pigmentation, whereas ECM100 and ECM200 were a pale brown/green colour in comparison. ECM233, in which both genes have been disrupted, was white/cream in colour. Colonies of ECM233 were also smaller compared to WT W83 following 4 days incubation, indicating a slower growth rate (Fig 4.1). Restoration of *hagB* to strain ECM233 (strain GMC100) resulted in partial restoration of WT pigmentation to a similar level observed in ECM200. After further incubation for another seven days it was found that all mutant strains had darkened in their pigmentation, with ECM100, ECM200 and GMC100 exhibiting darker pigmentation compared to ECM233. However all mutant strains remained a dark brown/green colour and colonies never exhibited the classic black pigmentation seen in WT W83.

Following analysis of pigmentation using plate based methods it was decided to establish if the effect seen on agar plates also occurred in liquid media (Fig 4.2). Broths of each strain were set up and incubated anaerobically in haemin free TSB and after 48 hr it was found that all pigmentation had been removed from the bacterial pellet. The bacteria were then sub-cultured and haemin starved for a further 3 days to ensure depletion of all iron reserves. Pigmentation was checked daily and all pellets remained creamy white in colour. Bacteria were still viable throughout the sub-culturing process.

The bacteria were then restored to either sTSB containing 5 µg/ml (76.9 nM) haemin or sTSB containing 10 µM haemoglobin. This haemin concentration has been shown to support the growth of *P. gingivalis* (Belanger et al., 2007) and is widely used (Gao et al., 2012, Wright et al., 2014, Yuan et al., 2007). Unexpectedly, restoration of the strains to haemin containing media did not restore pigmentation. The bacterial pellets remained creamy white in colour for the duration of the experiment.

However, restoration of the bacteria to haemoglobin did restore pigmentation to the cell pellet. After 24 hr, WT W83 and GMC100 exhibited the greatest amount of pigmentation with cell pellets that were a deep brown colour, while strains ECM100, ECM200 and ECM233 were a light shade of brown (Fig 4.2). After 48 hr all strains had darkened further with strain ECM100, ECM200 and ECM233 showing regions of deep brown pigmentation (Fig 4.2).

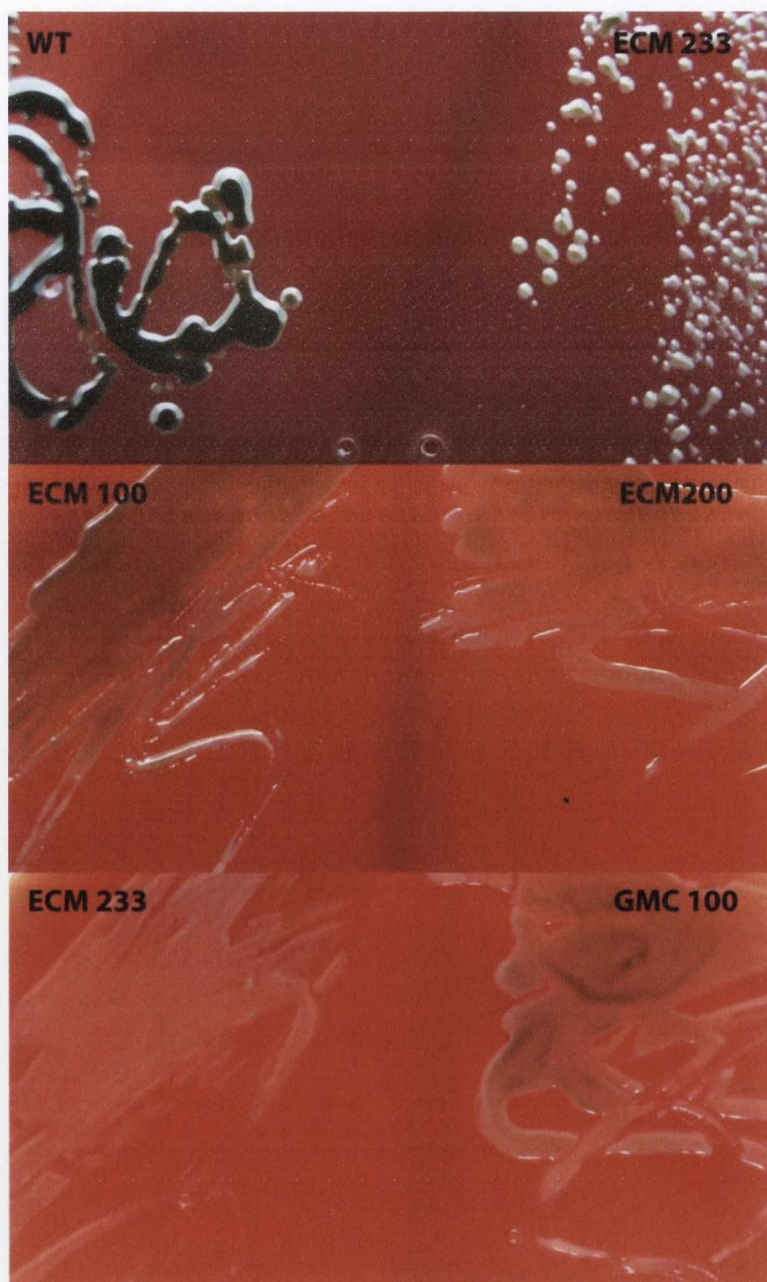


Figure 4.1: Image showing the pigmentation differences between WT W83 and mutant strains when grown on sTSA anaerobically at 37°C for 4 days. (ECM100 = $\Delta hagB$, ECM200 = $\Delta hagC$, ECM233 = $\Delta hagB/hagC$, GMC100 = $\Delta hagC$, $hagB::cat$)

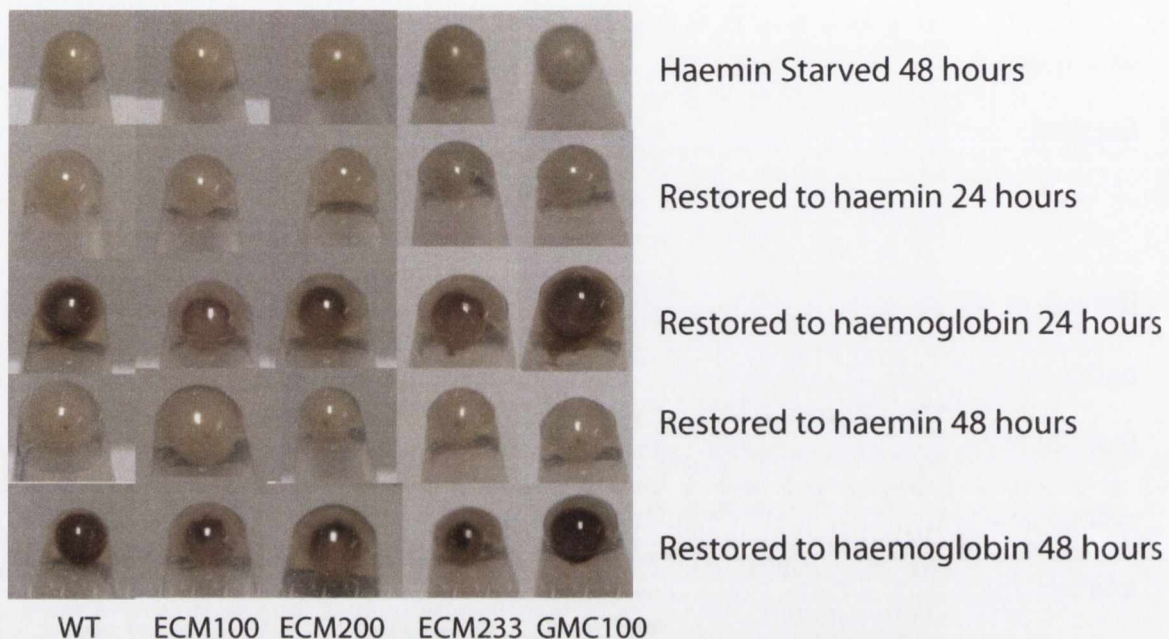
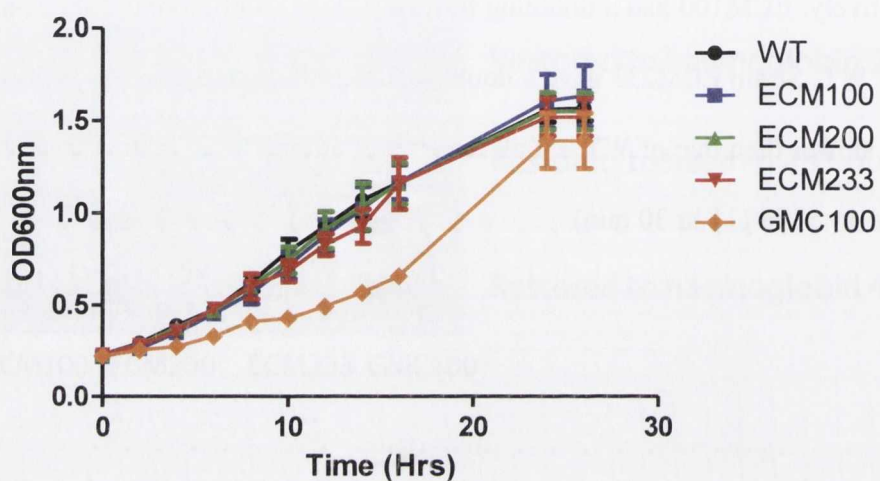


Figure 4.2: Image showing the level of pigmentation in cell pellets from broth cultures of *P. gingivalis* following haem starvation (top row) and subsequent restoration to haemoglobin or haemin rich broth post starvation for 24 hr or 48 hr. The strain names are shown below, (ECM100 = $\Delta hagB$, ECM200 = $\Delta hagC$, ECM233 = $\Delta hagB/hagC$, GMC100 = $\Delta hagC$, *hagB::cat*).

4.3.2 Determining the impact removal of *hagB* and *hagC* on growth and doubling times in sTSB.

Growth curves were generated in sTSB liquid cultures following haemin starvation to see if the removal of *hagB* and *hagC* affected the ability of *P. gingivalis* to grow in broth culture (Fig 4.3). A number of differences were observed between the strains. WT W83 and ECM200 had similar doubling times of 7.3 hr (7 hr 18 min) and 7.2 hr (7 hr 12 min), respectively. ECM100 had a doubling time of 6.89 hr (6 hr 53 min) which was faster than that of WT. Strain ECM233 gave a doubling time of 8.73 hr (8 hr 44 min) which is almost 1.5 hr slower than that of WT. Of all the strains, strain GMC100 had the longest doubling time of 11.53 hr (11 hr 30 min).



	WT	ECM100	ECM200	ECM233	GMC100
Doubling Time	7.302	6.892	7.293	8.732	11.53

Figure 4.3: Graph showing a comparison of growth rates between WT W83 and mutant strains of *P. gingivalis*. Doubling times in hr are shown below. (ECM100 = $\Delta hagB$, ECM200 = $\Delta hagC$, ECM233 = $\Delta hagB/hagC$, GMC100 = $\Delta hagC, hagB::cat$)

4.3.3 Examining the role that *hagB* and *hagC* in haemagglutination in *P. gingivalis* W83

Haemagglutination assays were carried out to determine if there were differences in the ability of WT W83 to haemagglutinate red blood cells compared to mutant strains (Fig 4.4). Clear differences in haemagglutination were observed between WT W83 and ECM233 cultures grown to OD₆₀₀ 0.4 or 0.6. It was found that WT had a titre of 16 while ECM233 had a titre of 2, indicating a strong defect in haemagglutination. At OD₆₀₀ 0.6 WT W83 had the same score as strain ECM200 (16), however at the lower optical density of 0.4, the score for ECM200 dropped from 16 to 8. Strains ECM100 and ECM233 consistently gave titres 1 to 2 dilutions lower than WT W83.

4.3.4 Exploring the effect mutagenesis of *hagB* and *hagC* on the stress response of *P.gingivalis* W83

The ability of the strain ECM233 to cope with exposure to environmental stress was compared to WT W83 (Fig 4.5). All strains were grown in broth culture overnight before being lawned on sTSA plates with disks of the agent being tested as described in section 4.2.4. After several days of growth, differences in the zones of clearances were measure in mm (Table 4.1). Each test was carried out in triplicate. There were a number of reagents to which WT W83 and mutant strains of *P. gingivalis* exhibited intrinsic resistance to, including 1M NaCl, Ferrozine and N-laurolysarcosine. Where sensitivity was detected, there was found to be no statistically significant difference between WT W83 and strain ECM233 (p-value > 0.05).

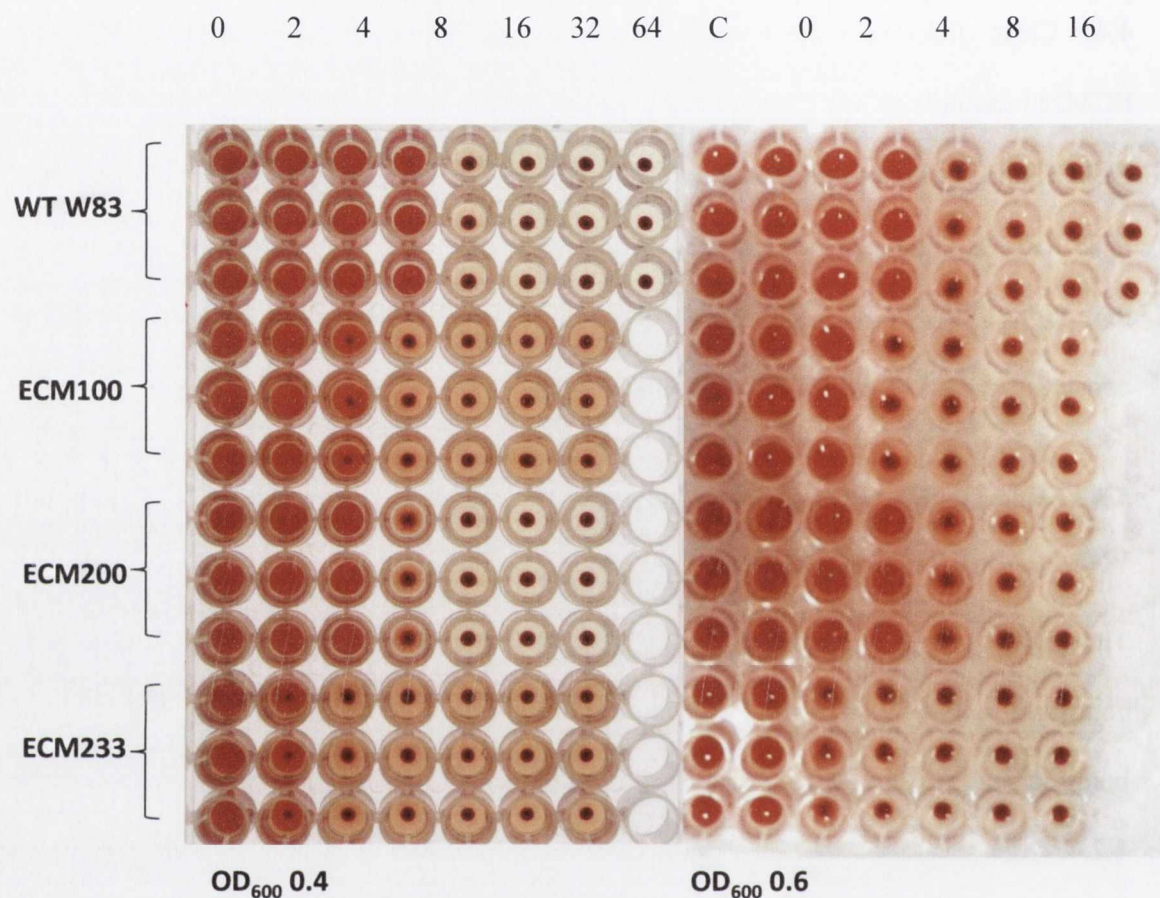


Figure 4.4: Haemagglutination assays of *P. gingivalis* with sheep RBCs (2.5% sheep's blood) demonstrating the difference in haemagglutination capabilities between WT W83 and the mutant strains ECM100, ECM200 and ECM233 grown to either O.D₆₀₀ 0.4 or 0.6 in. sTSB. (ECM100 = $\Delta hagB$, ECM200 = $\Delta hagC$, ECM233 = $\Delta hagB/hagC$).

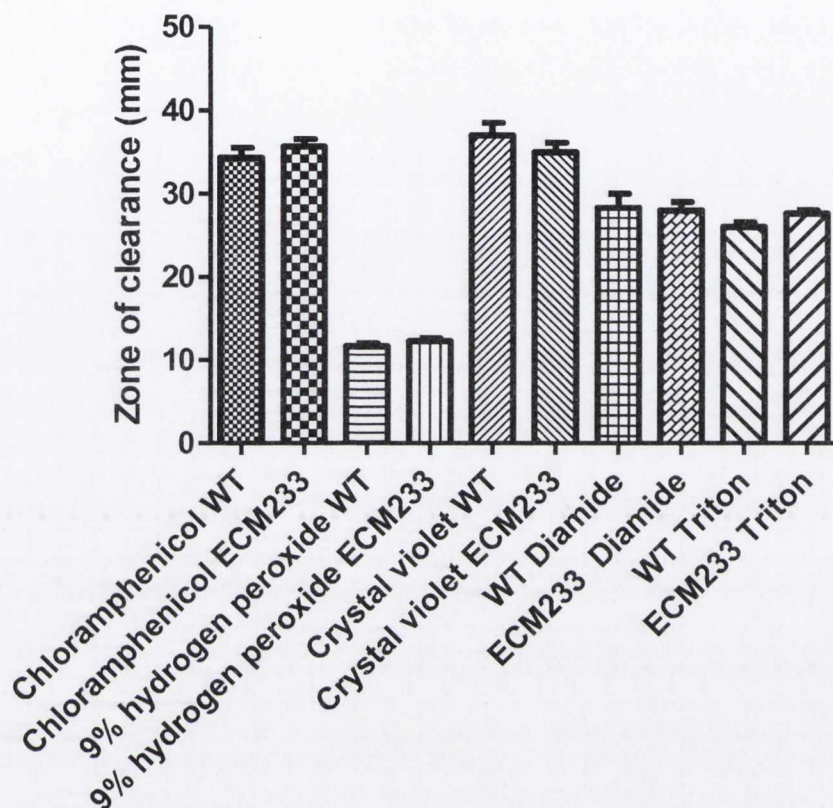


Figure 4.5: Results of stress sensitivity testing of WT W83 and mutant strain ECM233 ($\Delta hagB/C$) to chloramphenicol (control), 9% vol/vol hydrogen peroxide, crystal violet, diamide and Triton X-100. No statistically significant difference in growth between WT W83 and strain ECM233 was detected (p -value > 0.05). All experiments were carried out in triplicate.

4.4 Discussion

Understanding the role *hagB* and *hagC* play in processes that are vital for the survival of *P. gingivalis* is an important contribution to our understanding of the bacteria as a whole. Pigmentation, haemagglutination, and the ability to survive in the presence of environmental stressors are all essential characteristics that allow *P. gingivalis* to thrive during infection. The role of the *hagB* and *hagC* genes and their encoded proteins in some of these processes has been partially characterised. However, in contrast the roles of the other hemagglutinins *hagA*, *hagD* and *hagE* in these processes have been extensively characterised. Previously, the ability of HagA and HagB to act as haemagglutinins has been shown in single mutant studies (Lepine et al., 1996). However no study carried out to date has examined the impact that removal of both *hagB* and *hagC* would have on bacterial survival mechanisms. Here we sought to examine the effect that the removal of these genes would have on some of the basic processes that *P. gingivalis* requires for survival.

It has been shown previously that *hagB* and *hagC* have haemagglutination activity but are not the sole mediators of the process in *P. gingivalis* (Lepine et al., 1996). We show here that mutants of one or both of the *hagB* and *hagC* genes have a reduced capacity to capture red blood cells. The double mutant strain ECM233 has the weakest haemagglutination pattern of the strains tested and strains ECM100 and ECM200 were shown to be defective compared to WT. It is worth noting that the double *hagB/hagC* mutant described here has a similar pattern of haemagglutination to the *rgpA/kgp* mutant described by Shi *et al.* (1999). This suggests that *hagB* and *hagC* are as essential to this process as *kgp* and *rgpA*. In both our study and the work of Shi *et al.* it can be seen that individual mutants of *hagB*, *hagC*, *rgpA*, *kgpB* or *hagA* results in a 2-fold reduction in haemagglutination titre relative to their respective wild-type parental isolates.

A double *rgpA/kgp* mutant exhibited an 8-fold reduction in titre relative to wild-type whereas the double *hagB/hagC* mutant described here exhibits a 4-fold reduction in titre. These data confirm that *hagB* and *hagC* play a major role in haemagglutination, at least as important as the gingipains *rgpA*, *kgp* and *hagA*. It also refutes the claim that *rgpA*, *kgp* and *hagA* are the sole mediators of haemagglutination in *P. gingivalis* (Shi et al., 1999), as our study shows that *hagB* and *hagC* make significant contributions to this process.

Given that the mutants are defective in their ability to capture red blood cells and as such access the protoporphyrin ring, it is perhaps not surprising that they are defective in pigment production. The classic black pigmentation displayed by *P. gingivalis* when grown on blood agar plates has a number of functions. It represents a means of nutrient storage for the bacteria as well as a means of protection against free radicals released by white blood cells such as neutrophils. In this study, analysis of pigment accumulation was determined following haemin starvation to remove residual pigmentation. After four days of growth on sTSA some very strong pigmentation differences were observed between the strains (Fig 4.1). WT W83 reached its normal levels of pigmentation with colonies appearing black and glossy on plates, in comparison the single mutants were a pale, murky brown/green colour. The double mutant, strain ECM233 was a pale creamy colour and formed colonies that were significantly smaller than WT W83. Reintroduction of *hagB* to ECM233 partially restored pigmentation to a level similar to that observed in the single mutants confirming the role of *hagB* in this phenotype. After a further five days incubation all strains darkened in pigmentation, however the mutant strains never developed the classic black pigmentation associated with *P. gingivalis* instead remaining a dark brown/green shade.

Similar results have been observed in studies of gingipain mutants. Shi *et al.* (1999) found that a *kgp* mutant had reduced pigmentation capabilities, similar to those observed in our

single mutant strains during early growth. Interestingly an *rgpA/rgpB* double mutant did not exhibit reduced pigmentation. However when all three gingipains were deleted a severe pigmentation defect developed (Shi *et al.*, 1999), akin to what was observed in the current study during the early stages of growth of strain ECM233. While our mutant strains darken over time (~ 7 days) the various gingipains mutants had failed to do so. It is however possible that the plates were not incubated for long enough to allow for pigmentation to develop. This suggests that *hagB* and *hagC* play a similar role in the development of pigmentation as the gingipains and that they are equally as important to the process.

While pigmentation is defective in our mutant strains, all of the mutants darken following extensive incubation (~7 days). This suggests that in the absence of *hagB* and/or *hagC* the ability of the organisms to acquire haem is reduced but not completely lost. The likely reason for this could be the reduced ability of mutant strain to capture red blood cells via haemagglutination. This would slow the rate at which pigmentation develops substantially as rates of haem uptake would be directly affected. The fact that the colonies are green/brown in colour suggests that μ -oxo bishaem is still been formed by *P. gingivalis* (Smalley *et al.*, 2004). However, it could be also be true that removal of *hagB* or *hagC* affects the ability of the organism to store μ -oxo bishaem on its surface, preventing the same level of storage that is seen in WT that gives the classic black pigmentation. We know from our qPCR studies that the mutant strains are expressing the key gingipains proteins involved in this process, indeed in some cases they appear to be up-regulating them (Chapter 5)

Broth based pigmentation studies revealed similar results (Fig 4.2). Restoration to media containing haemin did not restore pigmentation, while restoration to media containing 10 μ M haemoglobin did. The lack of pigmentation in haemin broth is most likely due to the fact that the levels of haemin used in the media (76.9 nM) are too low to allow for the

development of pigmentation (John Smalley, Personal Communication). However, higher levels of haemin are toxic to the bacteria, making it difficult to analyse pigment restoration in broth cultures containing haemin as the source of porphyrin (John Smalley, Personal Communication).

The concentration of haemoglobin used in these experiments was 10 μ M, which is a substantially lower level of haem than is present in human biological systems, given that the final concentration of haemoglobin in human blood is 2 to 3 mM. The concentration of haemoglobin we used is $\sim$ 100 times lower than that of human blood. It is worth noting that even with this low level of haemoglobin, pigmentation in the haemoglobin broth cultures still occurred. Restoration to haemoglobin resulted in the formation of a brown pigmentation (Fig 4.2) as opposed to the black or green/black pigmentation observed when the strains are grown on sTSA post starvation. This suggests a different manner of defect where μ -oxo bis-haem is not been formed and that the pigmentation observed is likely the result of storage of the brown-pigmented molecule Fe (III) proto-porphyrin IX species (Smalley *et al.*, 2004). However it is also possible that the colonies were not incubated for long enough in the haemoglobin rich media to allow for full pigmentation to develop as these cultures were only incubated for 48 hr. One way to fully establish the exact nature of the pigment present would be through ultra-violet visible spectroscopy based methods used by Smalley *et al.* (2004). These methods would allow for the exact biochemical nature of the iron based pigments present on the surface of each mutant to be established.

It is also worth noting that restoration of *hagB* in strain GMC100 generated a strain that developed pigmentation in haemoglobin sTSB just as quickly as WT W83, which is unexpected as GMC100 is genotypically similar to the pigment defective strain EMC200 (Δ *hagC*). It has been postulated that the removal of *hagB* and *hagC* from the genome could trigger the activation of compensatory mechanisms. This is illustrated further in Chapter 5

where the qPCR data suggests that strain ECM233 has higher levels of gingipain expression in comparison to the WT strain (Chapter 5). *RgpA* in particular exhibits higher levels of expression in ECM233 and has been shown previously to contribute to the expression of haemoglobin receptor domain protein (HbR) which has the ability to bind haemoglobin (Shi et al., 1999). In strain GMC100, where *hagB* has been restored to strain ECM233, it is possible that there is an additive effect of *hagB* restoration and over-expression of gingipains which allows for faster development of pigmentation.

Growth curve analysis was carried out on all of the strains in sTSB after haemin starvation for 48 hr. A number of differences were found between the strains. WT and ECM200 had similar doubling times while strain ECM100 grew faster than WT. This could be because removal of this gene which is highly expressed in sTSB allows the bacterium to redirect the energy it would spend producing and exporting HagB into other proteins essential for growth, allowing the bacteria to grow more quickly. As the bacterium does not need to agglutinate and lyse red blood cells in sTSB, there appears to be no negative consequences associated with deletion of *hagB* in liquid media. It is also interesting to note that in a study carried out on gingipain mutants, an *rgpA/rgpB* mutant and an *rgpA/rgpB/kgp* triple mutant also grew faster than the WT parent strain, which in this case was strain ATCC33277 (Shi et al., 1999).

Strains ECM233 and GMC100 grew slower in comparison to the other strains. In the case of ECM233 the doubling time was almost 1.5 hr longer than that of WT W83. This suggests that removal of both *hagB* and *hagC* does affect the ability of *P. gingivalis* to grow in sTSB. However, the effect on growth may be indirect. In the absence of *hagB* and *hagC*, strain ECM233 is expressing compensatory mechanisms which require large amounts of energy to produce, including the gingipains. As noted above, Shi *et al* (1999) have shown that expression of the gingipains has a considerable effect on growth rate. The

energy that is being fuelled into the production of compensatory proteins may slow the growth of the organism down, leading to the increased doubling time seen in the strain. Unexpectedly strain GMC100, the complemented mutant, has a significantly extended doubling time which was ~ 4 hr longer than that of WT W83. It was assumed that re-addition of *hagB* into the *P. gingivalis* genome would restore doubling times to a level similar to the single *hag* mutants. As strain ECM233 is already funnelling energy into other compensatory mechanisms to facilitate bacterial survival, it could be that re-addition of *hagB* back into the genomes adds to the burden of protein production. This means that the bacterium is now spending energy not only producing *hagB* but potentially other compensatory proteins as well, thus slowing the doubling time of the strain down significantly. All of these data suggest that production of the proteins required for haem acquisition by *P. gingivalis* is an energetically expensive process, highlighting its importance for bacterial growth.

Environmental stress tests were carried out on the WT W83 and strain ECM233 using a number of known stressors. *P. gingivalis* was found to be halophilic as both 1M NaCl, 10 X PBS had no effect on growth (Sigma-Aldrich). This suggests that the bacteria are capable of surviving in the presence of high salt concentrations. This has been demonstrated previously in the case of iron salts. In this study, carried out in broth culture *P. gingivalis* was found to grow well in the presence of high levels of metal sulphides (Milner et al., 1996). Ferrozine (Sigma-Aldrich) also known as 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-*p,p'*-disulfonic acid monosodium salt hydrate was also tested. This is an iron chelating agent which binds Fe^{2+} . *P. gingivalis* did not exhibit any susceptibility to this agent which was not unexpected as *P. gingivalis* acquires its iron from haem sources. Both strains tested were also found to be resistant to 10% N-laurolysarcosine which is another sodium salt compound as well as an ionic detergent which would be capable of interfering with protein structures on the bacterial cell surface.

It has been shown previously that *P. gingivalis* is able to adapt to the presence of oxidative stress. Studies have shown a role for PG2212 zinc finger protein in the management of oxidative stress. It is believed to be a transcriptional regulator that plays an important role in oxidative stress resistance in strain W83 (Dou et al., 2014). Using plate based methods we wanted to establish if removal of *hagB*, *hagC* or both interfered with the oxidative stress response in *P. gingivalis*, particularly in light of the pigmentation and haemagglutination assay results. Both strains were equally susceptible to 9% vol/vol hydrogen peroxide. Similarly both strains had similar susceptibilities to the ROS generating chemical diamide. This reagent has been used in previous *P. gingivalis* studies to examine survival under stress conditions (Yanamandra et al., 2012).

Crystal violet (gentian violet) is routinely used in microbiology as a component of the Gram staining process. It is a dye that is composed of a mixture of violet rosanillinis with antibacterial, antifungal and antihelminthic properties (Docampo and Moreno, 1990). The dye dissociates in physiological conditions and retains a positive charge. This charge is attracted to the negative cell surface components such as LPS and peptidoglycan where in close proximity the dye forms a covalent bond with the bacterial cell surface. It has weaker anti-microbial activity against Gram negative organisms possibly due to the fact that it has to penetrate through a lipid barrier in order to access the microbial cell wall in Gram negative organisms (Maley and Arbiser, 2013). We found no difference in sensitivity to this agent in either strain, suggesting that there is no major difference in cell wall structure between the two strains. This was also suggested by the similar susceptibility patterns of both strains to Triton X-100.

Here we clearly show that *hagB* and *hagC* play a role in the development of pigmentation in strain *P. gingivalis* W83, with a reduced rate of pigmentation observed in mutant strains in both plate and broth based experiments. In agreement with previous work carried out the

role of *hagB* and *hagC* as haemagglutinins in this strain was highlighted, with strain ECM233 having the poorest ability to bind red blood cells. This finding indicates that HagB and HagC are co-expressed under the conditions examined and play an additive role in the process of haem acquisition. This finding indicates that the gene duplication event that resulted in the creation of the *hagB/hagC* locus conferred *P. gingivalis* with enhanced capacity to acquire haem and is likely to have been an important evolutionary development, paralleling the expansion of the gingipain family.

Chapter five: Analysis of the role of *hagB* and *hagC* of *Porphyromonas gingivalis* in adhesion and biofilm formation.

5.1 Introduction

The ability of some pathogenic bacteria to adhere to and invade host cells is vital for their survival. These processes allow bacteria to acquire nutrients, to shield themselves from immune surveillance systems, expand their own life-span and facilitate replication.

P. gingivalis is capable of adherence to and invasion of a variety of cell types including gingival epithelial cells (GECs) (Lamont et al., 1995). One of the major mediators of the adherence process is the fimbriae, of which there are a number of sub-types. Variance of fimbrial sub-types between strains has been shown to effect the ability of the bacteria to adhere to and invade host cells and some fimbrial sub-types promote invasion more than others (Enersen et al., 2013). Studies have shown that strains which possess type II fimbriae are most commonly isolated from severe forms of disease and that type II fimbriae are associated with a more invasive phenotype (Gao et al., 2012). In GECs it has been demonstrated that binding of the major fimbriae to the $\beta 1$ integrin receptor activates cell signalling pathways which lead to remodelling of the actin cytoskeleton, allowing the bacteria to enter into the cell (Yilmaz et al., 2002, Yilmaz et al., 2003).

Studies have shown that *P. gingivalis* is commonly associated with gingival crevicular epithelial cells in patients with chronic periodontitis. In one study it comprised 42% of the bacteria detected either attached to or inside cells recovered from patients with chronic periodontitis (Colombo et al., 2006). This is interesting to note, as the most common form of treatment is root scaling, which removes the bacterial biofilm from around the tooth surface. However if bacteria are present inside host cells, this treatment may be inefficient at eliminating infection by certain invasive bacteria and may lead to a reoccurrence of infection.

Once inside the cell *P. gingivalis* can replicate and it is capable of extending the host cellular life-span by secreting an ATP-hydrolysing enzyme, preventing ATP-induced

apoptosis (Yilmaz, 2008). This mechanism allows it to maximise its own survival (Gouin et al., 2005) (Yilmaz et al., 2006). Another bacterial protein of vital importance to cellular invasion is SerB which plays a role in cytoskeleton remodelling of GECs to facilitate the invasion process (Tribble et al., 2006).

It is worth noting that the ability to adhere to and invade cells varies depending on the strain of *P. gingivalis*. In the current study we examine the role of *hagB* and *hagC* in strain W83. Strain W83 is classed as a virulent strain of *P. gingivalis* but its ability to adhere to and invade cells is relatively poor compared to other strains. The main reason for this is that strain W83 lacks fully functional fimbriae, which are a major adhesin for this bacterium (Nishikawa and Duncan, 2010). However, due to this phenotype, W83 is a useful background to study non-fimbrial adhesins such as HagB and HagC in host cell interactions.

The role that HagB and HagC play in adherence and invasion of oral cell lines has yet to be fully established. Studies have shown that heterologously expressed HagB plays a role in adherence and invasion of human coronary artery endothelial cells, highlighting the fact that it has adhesive properties (Song et al., 2005). HagB and HagC are known haemagglutinins meaning that they can adhere to red blood cells, it has been previously shown that many haemagglutinins can act as adhesins for mucosal surfaces in many bacterial species including *Bordetella pertussis* (Villarino Romero et al., 2014). In the current study we analyse adherence to H357 cells, a human oral squamous cell carcinoma derived from the tongue of a 74 yr old male patient. These cells have been used in previous studies to examine the ability of *P. gingivalis* to adhere to and invade host cells (Suwannakul et al., 2010).

The ability of *P. gingivalis* to co-aggregate with a number of other bacterial species contributes to the formation of the dental biofilm commonly referred to as plaque. Plaque

consists of a number of bacterial species which bind to each other in succession. The ability of *P. gingivalis* to bind with a number of bacterial species, including *Fusobacterium nucleatum*, *S. oralis* and *S. gordonii*, enables the organism to associate with plaque biofilms (Kuboniwa and Lamont, 2010). Within the oral biofilm itself there are a number of different species, many of which act in synergy with one another, while others will have an antagonistic relationship (Jenkinson, 2011). It has been found that the addition of *P. gingivalis* to the subgingival biofilm increases the virulence of dental plaque. The inoculation of *P. gingivalis* into the oral cavities of rats was shown to induce increased levels of alveolar bone resorption; an effect was not seen in uninfected rodents (Kesavalu et al., 2007).

In the current study we sought to establish the role that *hagB* and *hagC* play in adhesion and invasion of the oral squamous H357 cell line. This was carried out because although HagB has been shown to bind to HCAE cells, the exact role of HagB or HagC in interacting with oral cells has not been established. We also examined the role that *hagB* plays in strain ATCC33277, which has markedly better adherence and invasion capabilities compared to strain W83 due to the presence of fully formed and functional fimbriae (Tribble et al., 2013).

The role of *hagB* and *hagC* in co-aggregation and the effect that removal of these genes had on the formation of mono and multi-species biofilms was also investigated. Due to the results that were obtained from our phenotypic and cell based assays it was also decided to use qPCR to determine if in the absence of *hagB* and *hagC* compensatory mechanisms were activated to replace the function of these genes.

5.2 Materials and Methods

5.2.1 Adherence assay

A 24 well plate (Cellstar, Greiner Bio-one GmbH, Bad Haller Str. 32, 4550 Kremsmünster, Austria) was seeded with 5×10^3 H357 cells/well and incubated for 48 hr in 5% CO₂ at 37°C by which time they had reached 1×10^5 cells per well (~80% confluency). The media used was antibiotic free Dulbecco's MEM supplemented with 1/3 Hams-F12 (Sigma-Aldrich), 10 ng/ml Epidermal Growth Factor (Sigma-Aldrich), 0.5 µg/ml Hydrocortisone, 10 ng/ml Cholera toxin (Sigma-Aldrich), 5 µg/ml Insulin (Sigma-Aldrich) and 10 % foetal calf serum.

Bacteria were grown in sTSB for 24 hr under anaerobic conditions. The optical density (OD) of the broths was adjusted to OD₆₀₀ of 0.7. In order to generate a suspension of 1×10^7 bacteria the cultures were diluted using a 1:50 dilution.

Approximately 1×10^7 bacteria were added to each well of a 24 well plate (Cellstar, Greiner Bio-one) giving a multiplicity of infection (M.O.I) of 100. Each bacterial strain was evaluated in triplicate. The plate was spun at 800 x g (Sorvall Legend-T centrifuge) for 5 min. The infected cells were incubated for 90 min in 5% CO₂ at 37°C as per the methods of Song *et al.* (2005). The cells were washed with sterile PBS three times before being lysed in 1 ml sterile water for 20 min. The lysates, which consisted of cells and adherent bacteria were serially diluted tenfold and plated onto Columbia blood agar (CBA) (Cruinn Diagnostics Ltd., 5b/6b Hume Centre, Park West Industrial estate, Nangor Road, Dublin 12, Ireland). Plates were incubated anaerobically at 37°C for seven days before counting. The percentage of adherence was calculated based on the percentage of bacteria recovered post-H357 cell lysis compared to the original inoculum.

For strain W83 and its derivatives, this assay was carried out with bacteria grown in sTSB and bacteria grown in haemin free sTSB.

5.2.2 Invasion assay

Cells and bacteria were seeded and grown in an identical fashion to that described for the adherence assay protocol. To each well of a 24 well plate (Cellstar, Greiner Bio-one) approximately 1×10^7 bacteria were added, giving an M.O.I of 100. This was carried out in triplicate for each strain. The plate was spun at $800 \times g$ (Sorvall Legend-T centrifuge) for 5 min. Infected cells were incubated for either 90 min as per Suwannakul *et al.* (2010) or 24 hr in 5% CO₂ at 37°C. Cells were then washed with sterile PBS three times before being incubated in cell media containing 200 µg/ml metronidazole to kill any extracellular bacteria. Epithelial cells were lysed in sterile water for 20 min. The lysates which consisted of cells and bacteria which had successfully invaded the H357 cells were serially diluted ten-fold and plated onto CBA (Cruinn Diagnostics Ltd.). Plates were incubated anaerobically at 37°C for seven days before counting. The level of invasion was calculated as a percentage based on the number of bacteria recovered post-H357 cell lysis compared to the original inoculum.

5.2.3 *P. gingivalis* mono-species biofilm studies

Biofilm formation was assessed by Ms. Emma Millhouse at Glasgow Dental Hospital (GDH). *P. gingivalis* W83 and its derivatives were grown on plates under anaerobic conditions and cultured in Schaedler's anaerobic broth for 48 hr before washing and standardizing to 1×10^8 bacteria/ml. Bacteria were diluted to 1×10^7 bacteria/ml in artificial saliva (AS) consisting of porcine stomach mucins (0.25% w/v), sodium chloride (0.35% w/v), potassium chloride (0.02% w/v), calcium chloride dehydrate (0.02% w/v), yeast extract (0.2% w/v), lab lemco powder (0.1% w/v) and protease peptone (0.5% w/v) in sterile H₂O. Urea was diluted in phosphate buffered saline (40% w/v) and added to the AS to a final concentration of 0.05% (v/v).

A 1 ml volume of standardized bacterial culture was added to the well of a 24 well plate (Corning, NY, USA) to measure biofilm formation over three days (Sherry et al., 2013). At each time point biofilm growth was measured either by crystal violet staining or staining with carboxyfluorescein succinimidyl ester (CFSE) and fluorescent intensity measured.

5.2.4 Multi-species biofilm studies using a sub-gingival plaque model

Multi-species biofilms consisted of *S. mitis* strain ATCC12261, *F. nucleatum* strain ATCC10953, *A. actinomycetemcomitans* strain ATCC43718 and *P. gingivalis* (both WT W83 and *hag* mutants). All bacterial species were standardised to 1×10^7 c.f.u/ml in AS. Biofilms were prepared in 24 well plates (Corning) containing customised Themonox™ coverslips (13 mm, Fischer Scientific). A standardised solution of each bacterial species in 500 µl of AS was prepared and added to the biofilm over time. *Streptococcus mitis* was added first and grown for 24 hr. The old media was removed and a standardised solution of *Fusobacterium nucleatum* was added. The plate was then incubated anaerobically for 24 hr. The supernatant was removed and standardised solutions of *A. actinomycetemcomitans* and a *P. gingivalis* strain were added. The plates were then incubated anaerobically, at 37°C for a further four days.

Mature biofilm biomass was measured using crystal violet. Composition of the biofilms was assessed using real time PCR as has been described previously (Millhouse et al., 2014, Sherry et al., 2013).

5.2.5 Analysis of gingipain and *hagA* expression in mutant strains

RNA was isolated and processed as outlined in section 2.5.1. This RNA was converted to cDNA as per section 2.5.2. Expression of the gingipains was assessed using qPCR based methods as outlined in section 2.3.2. The primers used were 16S rRNA F and R, HagA F and R, Kgp F and R, RgpA F and R and RgpB F and R, details of which can be found in

table 2.3. The 16S rRNA gene acted as the housekeeping gene and control for the experiments, all of which were carried out in triplicate.

5.2.6 Statistical analysis:

Differences between WT and mutant strains were determined by analysis of variance (ANOVA) or by t-test based methods. When ANOVA was carried out Dunnett's Multiple Comparison was also performed. For all data p-values of < 0.05 were considered statistically significant.

When t-test methods were carried out an unpaired, two-tailed t-test with a 95% confidence interval was used. This method was used to examine statistical differences between two specific strains where the data indicated that a statistically significant difference was present. For all data p-values of < 0.05 were considered statistically significant.

All statistical analysis was carried out using Graphpad Prism 5 software (Graphpad Software Inc., 7825 Fay Avenue, Suite 230, La Jolla, CA 92037, USA).

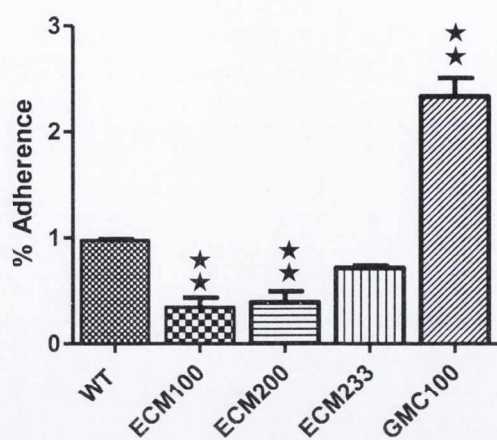
5.3 Results

5.3.1 Adhesion of WT and mutant strains cultured under haemin rich conditions

On average, approximately 1% the initial inoculum of WT W83 bacteria exhibited stable adherence to the H357 epithelial cells following 90 min incubation (Fig 5.1). Other studies of *P. gingivalis* strain W83 have indicated similar adherence rates. For example, Watanabe *et al.* (1992) showed that adherence of W83 to human gingival fibroblasts was ~ 1.4%, which is in keeping with our study (Watanabe *et al.*, 1992). It was found that mutation of either *hagB* or *hagC* affected the ability of *P. gingivalis* W83 to adhere to H357 cells (Fig 5.1). Strains ECM100 and ECM200, which are single mutants of *hagB* and *hagC* showed a statistically significant reduced capacity to adhere compared to the WT strain, exhibiting levels of adherence of approximately 0.35% and 0.4% of the initial inoculum, respectively.

Paradoxically strain ECM233, with a mutation in both *hagB* and *hagC*, exhibited levels of adherence that were greater than either of the single *hagB* or *hagC* mutants and were not statistically significantly different to WT (Fig 5.1). The average level of adherence of ECM233 was found to be 0.71%. Interestingly, when *hagB* was restored to strain ECM233 to generate strain GMC100, this resulted in a hyper adherent phenotype when compared to the WT strain. The average level of adherence in GMC100 was 2.3% compared to 1% in wild-type cells (Fig 5.1). ANOVA showed that there were statistically significant differences between the groups (p-value < 0.0001). When Dunnett's multiple comparison was carried out using WT as a control it highlighted that there were statistically significant differences between WT versus ECM100, ECM200 and GMC100 (p-value < 0.05).

(a)



(b)

Dunnett's Multiple Comparison Test	q	Significant? P < 0.05?
WT vs. ECM100	4.430	Yes
WT vs. ECM200	4.102	Yes
WT vs. ECM233	1.828	No
WT vs. GMC100	9.540	Yes

Figure 5.1: a) Levels of adherence of *P. gingivalis* WT W83 and mutant derivative strains to H357 cells after 90 min infection following growth in haemin rich media. The level of interaction is expressed as a percentage of the initial inoculum. ANOVA was performed and statistically significant differences were found to be present between the strains, indicated by stars (p-value < 0.0001). b) The results of Dunnett's Multiple Comparison, a post ANOVA test which determines statistically significant differences between the strains compared to the WT strain. In this case there were statistically significant difference between WT and all other strains with the exception of ECM233. ** indicates p-values between (0.001 – 0.01) (ECM100 = $\Delta hagB$, ECM200 = $\Delta hagC$, ECM233 = $\Delta hagB/hagC$, GMC100 = $\Delta hagC$, $hagB::cat$)

Due to the unexpected results from the adherence assays involving strain ECM233, it was decided to evaluate adhesion in a second independent *hagB/hagC* double mutant (DKO3). Additional rounds of assays were carried out comparing strain ECM233 to a second mutant clone (DKO3) and WT W83. It was found that strain DKO3 exhibited a similar phenotype to ECM233 and exhibited adherence levels that were not statistically different from wild-type. WT levels of adherence were approximately 1.1%, strain ECM233 was approximately 0.83% and DKO3 was 0.80% (Fig 5.2).

5.3.4 Examining the role that *hagB* plays in adherence in strain ATCC33277

In order to determine if HagB played any role in the adherence of fimbriated strains of *P. gingivalis*, we generated a *hagB* mutant in strain ATCC33277 and compared its relative contribution to adherence in comparison to *fimA*. It was found that mutation of *hagB* significantly affected the ability of ATCC33277 cells to adhere to H357 cells. This mutation resulted in an approximately 10-fold reduction in adherence of ATCC33277 from ~ 10% of the inoculum to approximately ~ 1% in the *hagB* mutant. A *fimA* mutant of ATCC33277 was shown to exhibit a severe defect in its ability to adhere to H357 cells reducing adherence to ~ 0.15% of the inoculum (Fig 5.3).

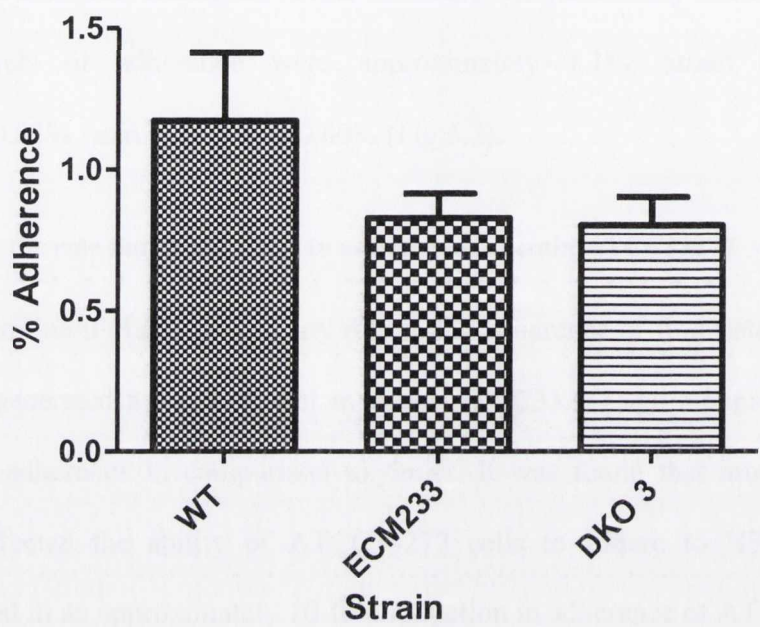
Analysis of variance showed highly statistically significant differences between the strains (p-value 0.0008). Dunnet's multiple comparison highlighted the differences between the WT and mutant strains further by comparing both to WT as a control (p-value < 0.05).

5.3.3 The effect of haemin starvation on adhesion of WT and mutant strains

It is well established that *P. gingivalis* regulates the expression of a number of key adhesion molecules in response to haemin levels including fimbrial components and haemagglutinins (Lamont and Jenkinson, 1998, Lamont and Jenkinson, 2000, Xie et al., 1997).

Here we sought to study the effect that haemin starvation would have on our mutant strains. After growth in haemin starved conditions it was found that the ability of all strains to adhere to H357 cells was substantially reduced. There were no statistically significant differences between the any of the strains (Fig 5.4).

(a)

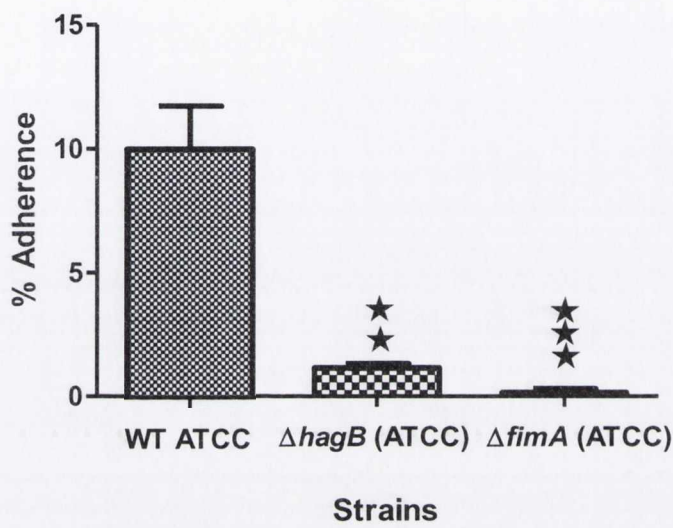


(b)

Dunnett's Multiple Comparison Test	q	Significant? P < 0.05?
WT vs. ECM233	1.542	No
WT vs. DKO 3	1.662	No

Figure 5.2: a) Levels of adherence of *P. gingivalis* WT W83, ECM233 and DKO3 to H357 cells after 90 min infection after growth in haemin rich media. The level of interaction is expressed as a percentage of the initial inoculum. b) The results of Dunnett's Multiple Comparison. There were no statistically significant differences found between WT strain and the mutant strains under these conditions. (ECM233 = Δ *hagB/C*, DKO3 = Δ *hagB/C*).

(a)

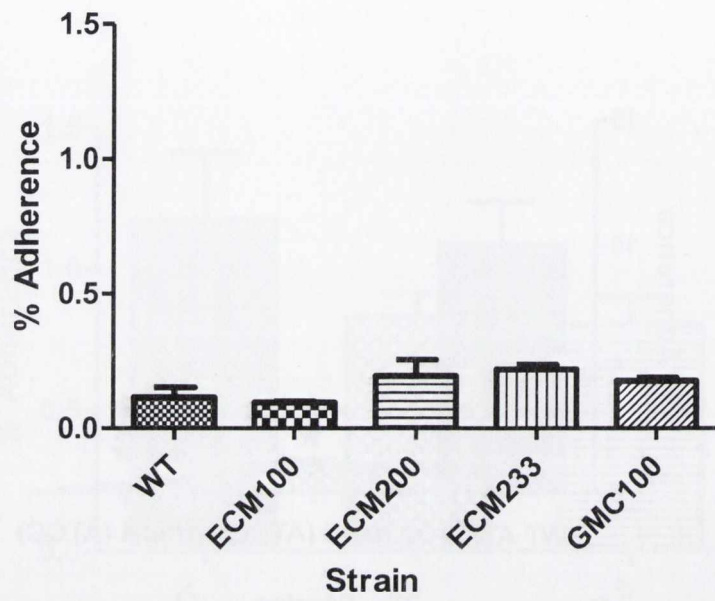


(b)

Dunnett's Multiple Comparison Test	q	Significant? P < 0.05?
WT ATCC vs. ΔhagB (ATCC)	6.198	Yes
WT ATCC vs. ΔFimA (ATCC)	6.894	Yes

Figure 5.3: a) Levels of adherence of the indicated *P. gingivalis* strain ATCC33277 derived strains to H357 cells after 90 min infection after growth in haemin rich media. The level of adherence is expressed as a percentage of the initial inoculum. ANOVA revealed statistically significant differences between the strains, indicated by stars (p-value 0.008). b) The results of Dunnett’s Multiple Comparison. There were statistically significant differences found between WT strain and the mutant strains under these conditions (p-value < 0.05).

(a)



(b)

Dunnett's Multiple Comparison Test	q	Significant? P < 0.05?
WT vs. ECM100	0.3940	No
WT vs. ECM200	1.741	No
WT vs. ECM233	2.278	No
WT vs. GMC100	1.361	No

Figure 5.4: a) Levels of adherence *P. gingivalis* WT W83 and mutant derivative strains to H357 cells after 90 min infection following growth in haemin free media. The level of interaction is expressed as a percentage of the initial inoculum. b) The results of Dunnett’s Multiple Comparison. There were no statistically significant differences found between WT strain and the mutant strains under these conditions. (ECM100 = $\Delta hagB$, ECM200 = $\Delta hagC$, ECM233 = $\Delta hagB/hagC$, GMC100 = $\Delta hagC$, $hagB::cat$).

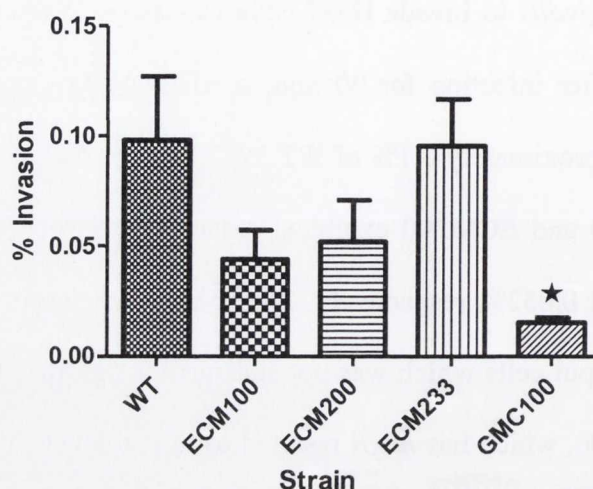
5.3.5 *P. gingivalis* invasion is effected by the removal of *hagB* and *hagC*

The ability of *P. gingivalis* to invade H357 cells was assessed after a 90 min and 24 hr incubation period. After infection for 90 min, a relatively low number of bacteria had invaded the cells. Approximately 0.1% of WT W83 bacteria had successfully invaded the cells. Strain ECM100 and ECM200 exhibited reduced levels of invasion, with invasion levels of 0.045% and 0.052% respectively. Strain ECM223 levels of invasion were, on average 0.095% of input cells which was not statistically significantly different from WT levels. Strain GMC100, which has *hagB* restored to it, had levels of invasion of 0.0155% which was substantially lower than that of WT and strain ECM200 (Fig 5.5). This finding was unexpected, given that the strain was found to be hyper-adherent.

The data was then normalised against the 90 min adherence assay to determine if the altered invasion levels were a direct consequence of altered adhesion. It was found that on normalisation GMC100 was still hypo-invasive compared to other strains (Fig 5.6). ANOVA was carried out and there was found to be a statistically significant difference between strain GMC100 and all other strains (p-value 0.0122). Further analysis by Dunnet's multiple comparison established that there was a statistically significant difference between WT and strain GMC100 (p-value < 0.05).

Following 24 hr incubation, substantially more bacteria had successfully invaded the H357 cells, with greater than ten-fold higher levels of invasion seen when compared to the 90 min assay. A similar pattern of phenotypes to that seen in the 90 min assay was observed following 24 hr incubation. Strains ECM100, ECM200 and GMC100 showed reduced levels of invasion, with average levels of 0.8%, 0.93% and 0.35% respectively, when compared to WT, whose level of invasion was 1.45% of inoculated bacteria (Fig 5.7).

(a)

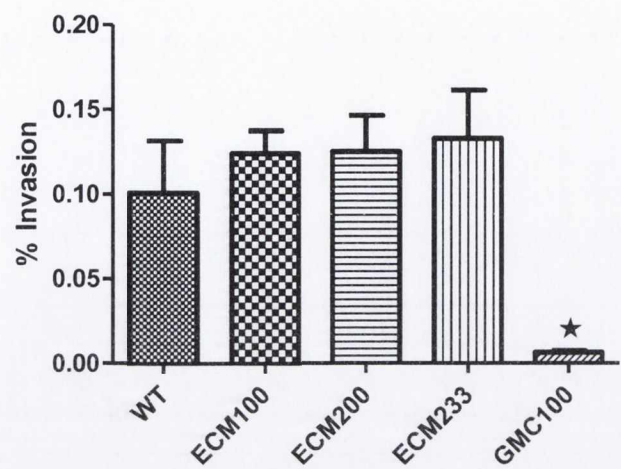


(b)

Dunnett's Multiple Comparison Test	q	Significant? P < 0.05?
WT vs. ECM100	2.004	No
WT vs. ECM200	1.708	No
WT vs. ECM233	0.09899	No
WT vs. GMC100	3.075	Yes

Figure 5.5: a) Levels of *P. gingivalis* WT W83 and mutant derivative strains within H357 cells after 90 minutes infection and subsequent kill step. Experiment was carried out after growth of bacteria in haemin rich sTSB media. The level of invasion is expressed as a percentage of the initial inoculum. Statistical significance is indicated by a star. b) The results of Dunnett's Multiple Comparison. In this case there were no statistically significant differences between WT and mutant strains with the exception of GMC100. (ECM100 = $\Delta hagB$, ECM200 = $\Delta hagC$, ECM233 = $\Delta hagB/hagC$, GMC100 = $\Delta hagC$, $hagB::cat$).

(a)

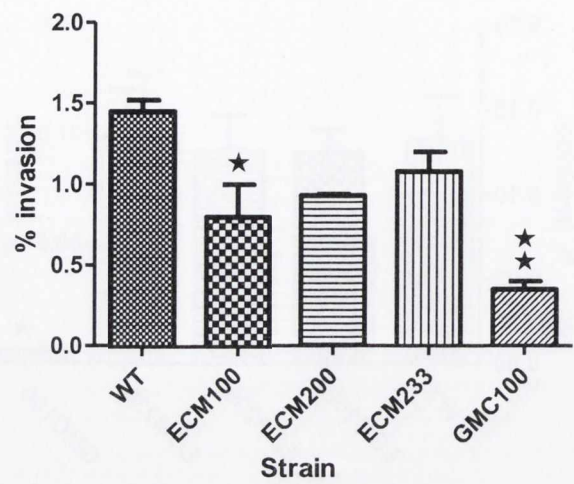


(b)

Dunnett's Multiple Comparison Test	q	Significant? P < 0.05?
WT vs. ECM100	0.7522	No
WT vs. ECM200	0.7854	No
WT vs. ECM233	1.029	No
WT vs. GMC100	3.063	Yes

Figure 5.6: a) Normalisation of levels of invasion of *P. gingivalis* WT W83 and mutant derivative strains (invasion/adherence) based on 90 min assay results. The levels of invasion is expressed as a percentage of the bacteria that have adhered after 90 min. ANOVA indicated a statistically significant difference between strains, indicated by a star (p-value 0.0122). b) The results of Dunnett’s Multiple Comparison. In this case there were no statistically significant differences between WT and mutant strains with the exception of GMC100. (ECM100 = $\Delta hagB$, ECM200 = $\Delta hagC$, ECM233 = $\Delta hagB/hagC$, GMC100 = $\Delta hagC$, $hagB::cat$)

(a)



(b)

Dunnett's Multiple Comparison Test	q	Significant? P < 0.05?
WT vs. ECM100	4.131	Yes
WT vs. ECM200	3.305	No
WT vs. ECM233	2.351	No
WT vs. GMC100	6.991	Yes

Figure 5.7: a) Levels of invasion of *P. gingivalis* WT W83 and mutant derivative strains within H357 cells after 24 hr infection and subsequent kill step. Experiment carried out after growth in haemin rich media. The level of invasion is expressed as a percentage of the initial inoculum. ANOVA indicated a statistically significant difference between the strains indicated with stars (p-value 0.0074). b) The results of Dunnett's Multiple Comparison post ANOVA. In this case there were statistically significant differences between WT, strain ECM100 and WT and GMC100. (ECM100 = $\Delta hagB$, ECM200 = $\Delta hagC$, ECM233 = $\Delta hagB/hagC$, GMC100 = $\Delta hagC, hagB::cat$)

The results of ANOVA testing showed that there were statistically significant differences between the strains (p-value 0.0074). When Dunnet's Multiple comparison was carried out which compares the mutant strains to a specific control column, in this case the WT strain, it was found that the differences between WT and ECM100 and WT and GMC100 were statistically significant. Strain ECM233, whose level of invasion was 1% was similar to that of WT (Fig 5.7).

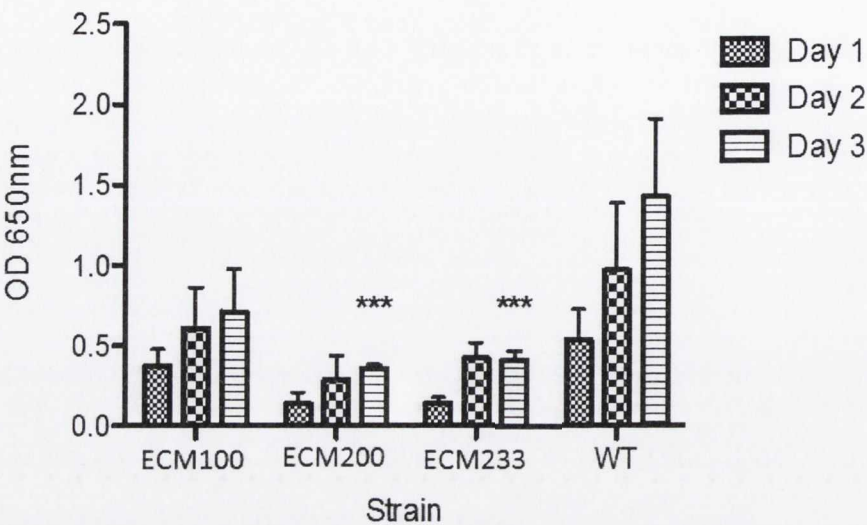
5.3.6 Ability of mutant strains to form mono-species biofilms in comparison to WT W83.

The ability of *P. gingivalis* W83 and mutant derivatives to form a single species biofilm on plastic surfaces in AS was analysed over the course of three days using both crystal violet staining and CFSE staining (Fig 5.8). It was found that on day 3 that there were significant differences between the strains analysed. When crystal violet was used to measure biomass it was found that strains ECM200 and ECM233 formed significantly lower levels of biofilm compared to WT W83 (p-values < 0.001). Using CFSE fluorescence to measure biomass it was also found that there was a statistically significant reduction in biofilm formation in ECM200 (p-value < 0.05) and ECM233 (p-value < 0.01) compared to WT W83 (Fig 5.8). All data are the mean $\pm$ SEM of six independent biofilms per group. Fluorescent microscopic analysis of CFSE stained biofilms confirmed the reduced levels of biofilm in the mutant strains relative to WT W83 (Fig 5.9)

5.3.7 Analysis of the ability of mutant strains to contribute to the formation of multi-species biofilms compared to WT W83

Multi species biofilms were grown following the protocol developed at GDH containing *S. mitis*, *F. nucleatum*, *A. actinomycetemcomitans* and *P. gingivalis* (both WT and *hag* mutants). Mature biofilm biomass was measured using crystal violet.

(a)



(b)

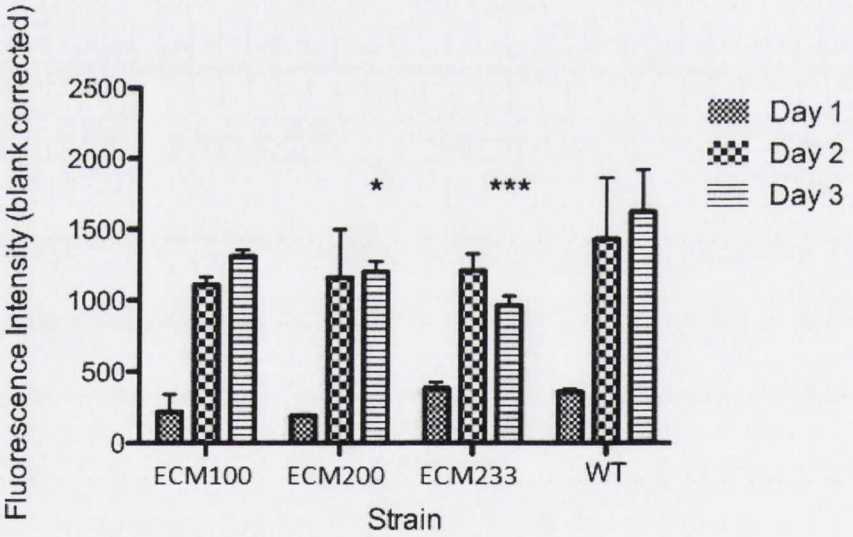


Figure 5.8: Biofilm growth of *P. gingivalis* WT W83 and mutant derivative strains was measured either by crystal violet staining (a) or staining with CFSE and fluorescent intensity measurements over 3 days (b). Significant differences on day 3 were observed when comparing ECM200 and ECM233 biofilm formation to the WT control. (ECM100 = $\Delta hagB$, ECM200 = $\Delta hagC$, ECM233 = $\Delta hagB/hagC$)

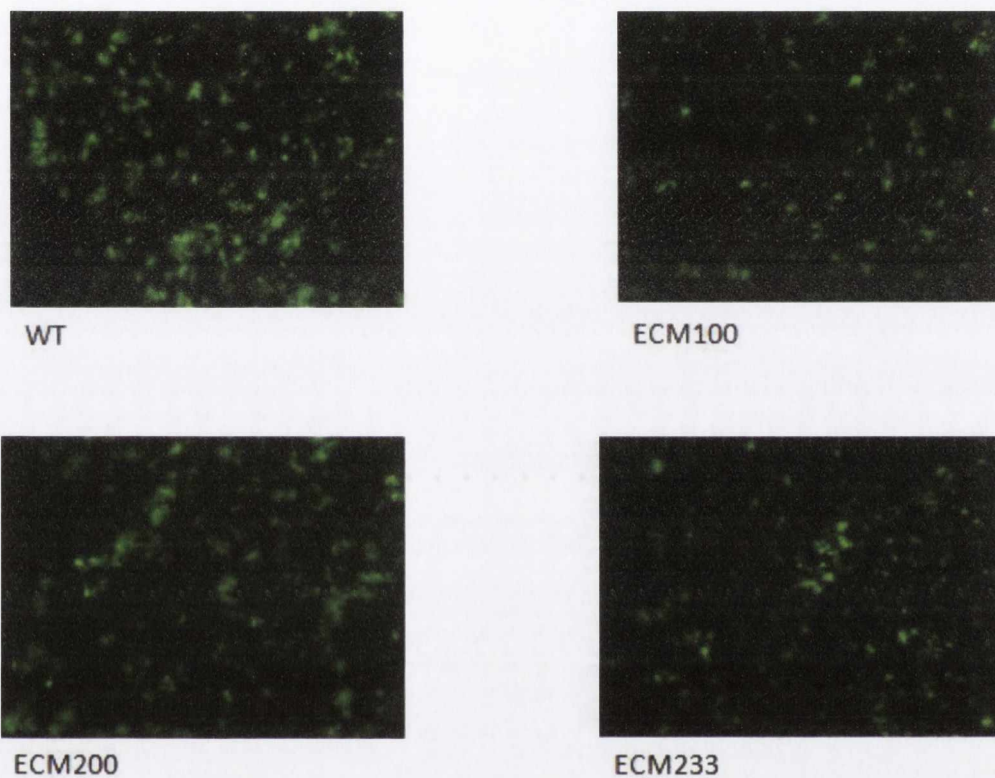


Figure 5.9: Mono-species biofilm growth of *P. gingivalis* WT W83 and mutant derivative strains revealed by staining with CFSE on day 3 of biofilm growth. Differences in fluorescent intensity are visible between WT compared to strain ECM200 and ECM233. (ECM100 = $\Delta hagB$, ECM200 = $\Delta hagC$, ECM233 = $\Delta hagB/hagC$)

Significant differences were observed between the biomass of biofilms containing *P. gingivalis* WT and those containing *P. gingivalis* *hagC* mutant ($p < 0.001$; Fig 5.10). Data are mean $\pm$ SEM of 3 independent biofilms per group.

The abundance of each species within the biofilm was also examined by qPCR (Fig 5.11). It was found that there were no significant differences in the total number of each species present. This was also found to be the case when percentage composition of each biofilm was calculated.

5.3.8. Analysis of gene expression of mediators in haem acquisition in broth based cultures

In order to determine whether deletion of *hagB* or *hagC* had any effect on gene expression, we analysed the expression of a number of genes (*hagA*, *rgpA*, *rgpB* and *kgp*) involved in haem uptake and adherence following growth in sTSB to OD₆₀₀ 0.7. Under these conditions it was found that expression of these genes was considerably lower compared to the expression levels detected following growth on blood agar plates (5.3.9). It was found that there was no statistically significant difference between WT W83 and the mutant strains when expression of *rgpB* was examined (Fig 5.12). Student t-tests were performed to compare WT to mutant strains individually, statistically significant differences between WT W83 and strain ECM200 (p-value 0.0022), WT vs. ECM233 (p-value 0.0185) and Wt vs. GMC100 (p-value 0.031). These strains all showed a statistically significant increase in expression of *kgp* compared to WT. Expression levels of *hagA* were found to be higher on average in strains ECM100 and ECM200 compared to WT W83. ECM200 had a substantial increase in *hagA* expression compared to WT and a student t-test could confirm the statistical significance of this difference (p-value of 0.0113) (Fig 5.13).

The expression of *rgpA* was also examined (Fig 5.14). It was found that there was a statistically significant increase in expression in ECM200, ECM233 and GMC100 in comparison to WT. An unpaired, two-sided student's t-test was used to assess these differences. WT vs. ECM200 had a p-value of 0.0174, WT vs. ECM233 had a p-value of (0.009) and WT vs. GMC100 had a p-value of (0.0194).

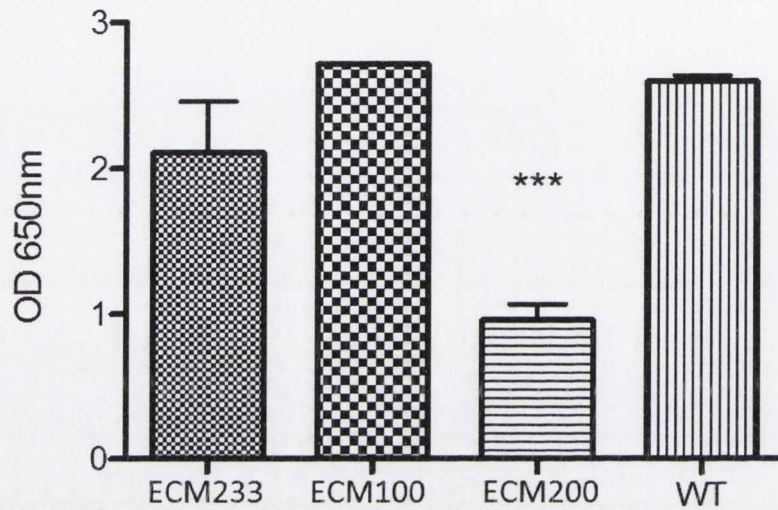
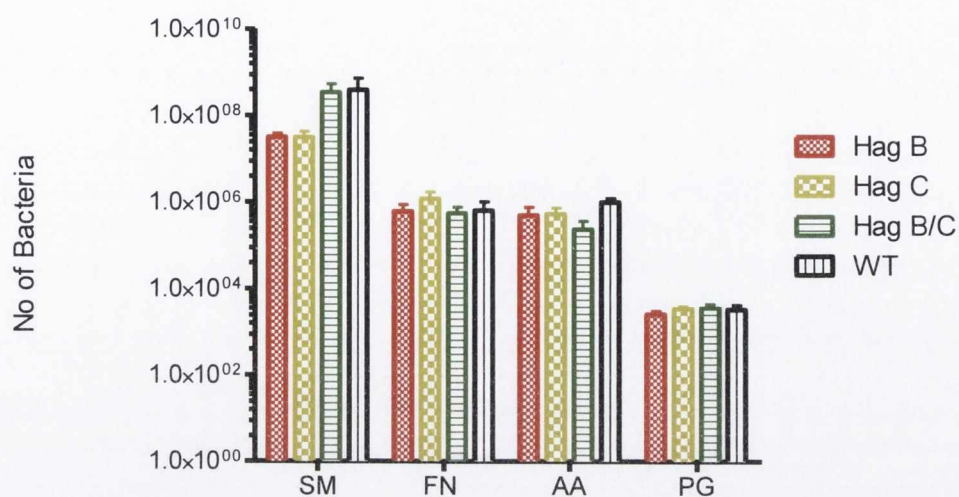


Figure 5.10: Mature biofilm biomass of multi-species biofilms was measured using crystal violet staining. Significant differences were observed between the biomass of biofilms containing *P. gingivalis* WT and those containing strain ECM200. Data are mean $\pm$ SEM of 3 independent biofilms per group. *** $p < 0.001$, (ECM100 = $\Delta hagB$, ECM200 = $\Delta hagC$, ECM233 = $\Delta hagB/hagC$).

(a)



(b)

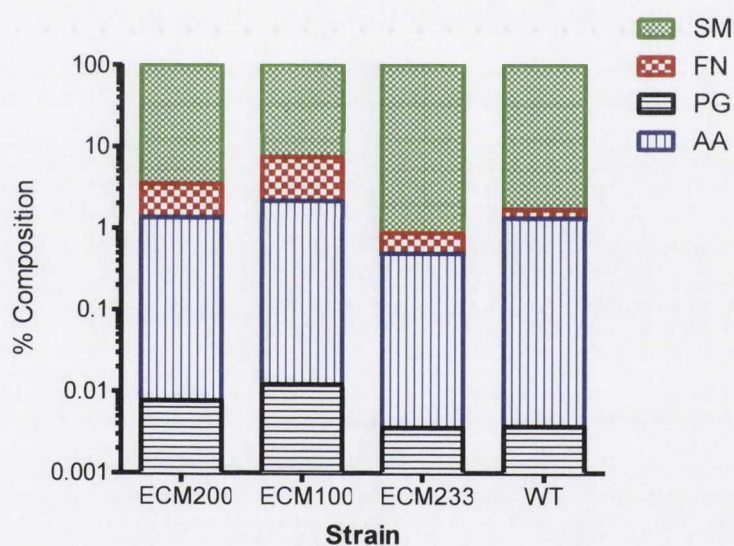
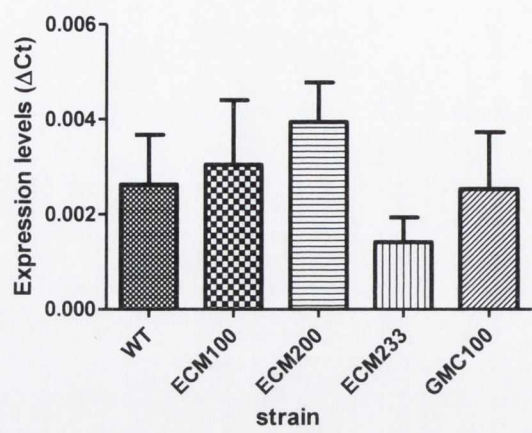


Figure 5.11: a) The amount of each species within the biofilm was quantified using qPCR. There were no significant differences in the total number of each species present within the biofilms. b) Percentage composition of each biofilm by species. There were no significant differences in the proportions of each species present within the biofilms. (ECM100 = $\Delta hagB$, ECM200 = $\Delta hagC$, ECM233 = $\Delta hagB/hagC$).

(a)



(b)

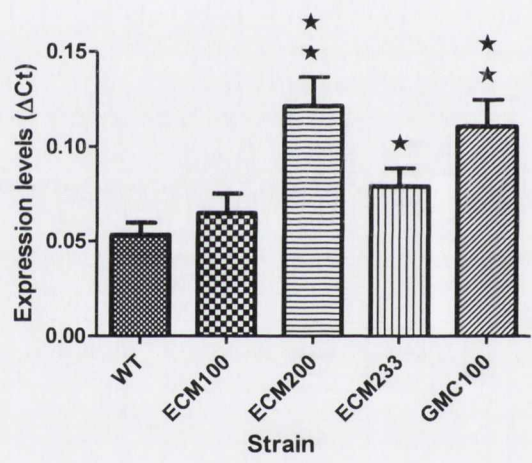


Figure 5.12: Expression level is based on ΔCt values as calculated in section 2.3.2 a) Expression levels of *rgpB* after growth in sTSB to an OD_{600} of 0.7 and b) Expression levels of *kgp* after growth in sTSB to an OD_{600} of 0.7. Student t-testing was performed to compare WT to mutant strains individually. Statistically significant differences were found found between WT vs. ECM200 (p-value 0.0022), WT vs. ECM233 (p-value 0.0185) and WT vs. GMC100 (p-value 0.031). * indicates a p-value between 0.01 and 0.05, ** indicates a value between 0.001 and 0.01. (ECM100 = $\Delta hagB$, ECM200 = $\Delta hagC$, ECM233 = $\Delta hagB/hagC$, GMC100 = $\Delta hagC$, *hagB::cat*)

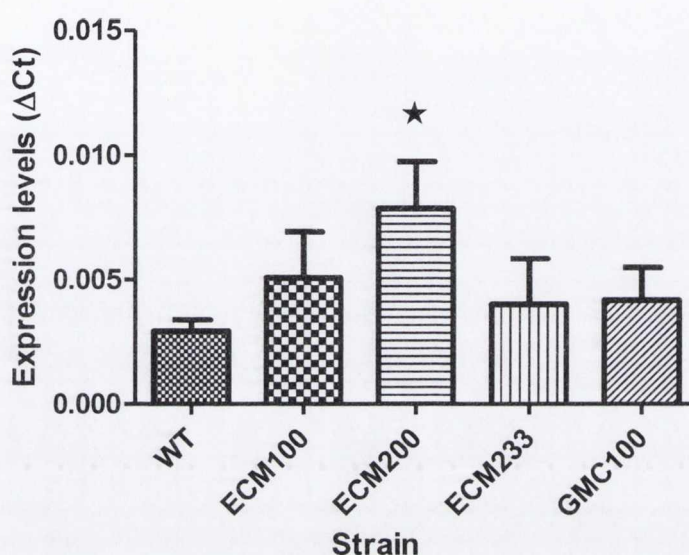


Figure 5.13: Expression levels of *hagA* in WT W83 and mutant strains grown to an OD₆₀₀ of 0.7 in sTSB. Expression level is based on ΔCt values as calculated in section 2.3.2. A two-sided student's t-test was carried out to analyse the difference between WT and strain ECM200. There is statistically significant difference between WT and strain ECM200 (p-value 0.0113). * indicates a p-value between 0.01 and 0.05. (ECM100 = Δ*hagB*, ECM200 = Δ*hagC*, ECM233 = Δ*hagB/hagC*, GMC100 = Δ*hagC*, *hagB::cat*).

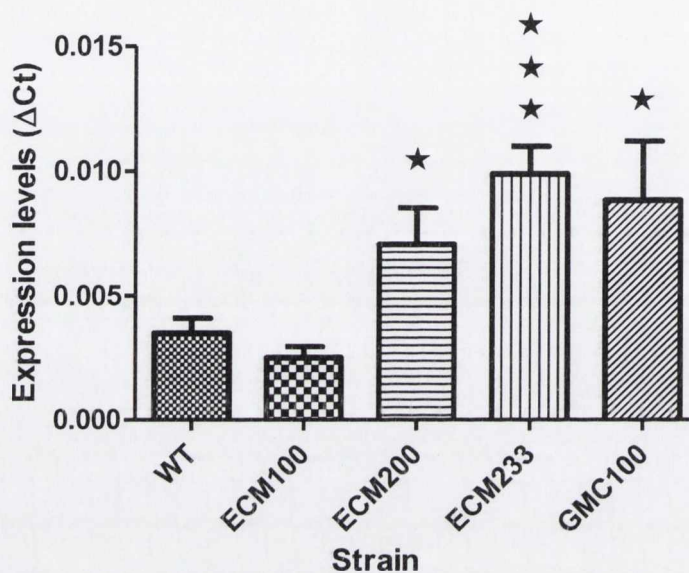


Figure 5.14: Expression levels of *rgpA* in *P. gingivalis* strains grown to an OD₆₀₀ of 0.7 in sTSB. Expression level is based on Δ Ct values as calculated in section 2.3.2. A two-sided student's t-test was carried out to analyse the difference between WT and mutant strains. Statistically significant differences were found between WT vs. ECM200 (p-value 0.0174), WT vs. ECM233 (p-value 0.0009), WT vs. GMC100 (p-value 0.0194) * indicates a p-value between 0.01 and 0.05, *** indicates a value between 0.0001 and 0.001. (ECM100 = Δ *hagB*, ECM200 = Δ *hagC*, ECM233 = Δ *hagB/hagC*, GMC100 = Δ *hagC*, *hagB::cat*).

5.3.9. Analysis of gene expression of mediators of haem acquisition in blood agar grown cells

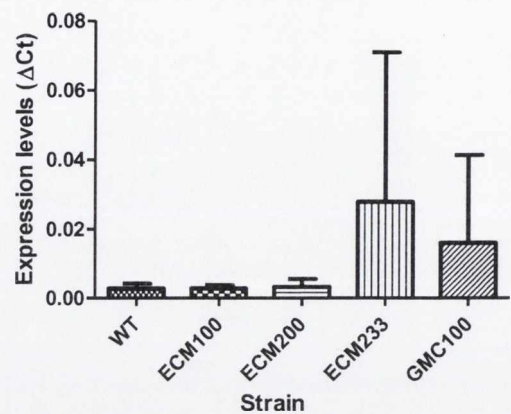
RNA was extracted from bacterial lawns that had been grown on sTSA blood agar plates and converted to cDNA. Genetic expression was then investigated by qPCR. In general, expression levels of the selected genes were much higher relative to sTSB grown cells.

In the case of *rgpA* and *rgpB* it was found that the double mutant ECM233 and the complemented strain exhibited on average higher levels of gene expression compared to WT W83 (Fig 5.15). However, due to the level of variance in the data this increase was not statistically significant. Interestingly, there was far less variance in the gene expression data generated from WT W83 and the two single mutants ECM100 and ECM200 (Fig 5.15).

When *kgp* expression levels were assessed, it was found that WT W83, ECM100 and ECM200 exhibited similar levels of expression. ECM200 exhibited higher levels of expression but these were not found to be statistically significant. However, GMC100 exhibited greatly reduced expression relative to WT W83 (p-value 0.0422) (Fig 5.16).

Expression of *hagA* was reduced in the mutant derivatives relative to WT W83 and this was most significant in GMC100 when compared to its parental strain ECM233 (p-value 0.0003) (Fig 5.17).

(a)



(b)

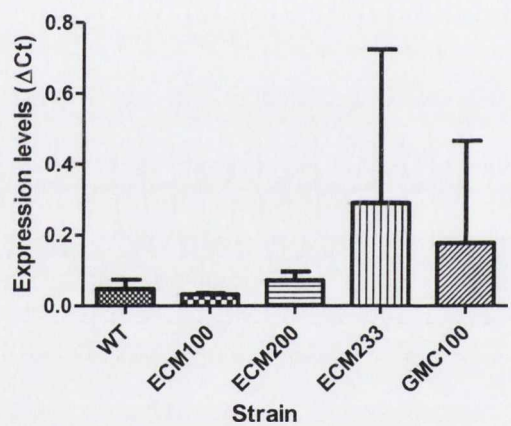


Figure 5.15: Expression of *rgpA* and *rgpB* in *P. gingivalis* strains were examined after growth on sTSA. Expression level is based on ΔCt values as calculated in section 2.3.2 a) *rgpA* expression levels. There was no statistically significant difference between the strains. b) *rgpB* expression levels. There was no statistically significant difference between the strains. (ECM100 = $\Delta hagB$, ECM200 = $\Delta hagC$, ECM233 = $\Delta hagB/hagC$, GMC100 = $\Delta hagC$, *hagB::cat*).

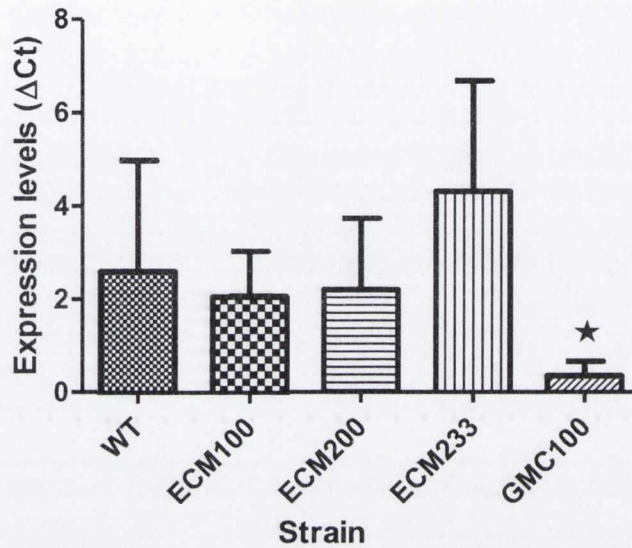


Figure 5.16: Expression levels of *kgp* in *P. gingivalis* were assessed after growth on sTSA. Expression level is based on ΔCt values as calculated in section 2.3.2. On analysis using an unpaired students t-test it was found that there was a statistically significant difference between strain ECM100 and GMC100 (p-value of 0.0422). * indicates a significant p-value between 0.01 and 0.05. (ECM100 = $\Delta hagB$, ECM200 = $\Delta hagC$, ECM233 = $\Delta hagB/hagC$, GMC100 = $\Delta hagC$, *hagB::cat*).

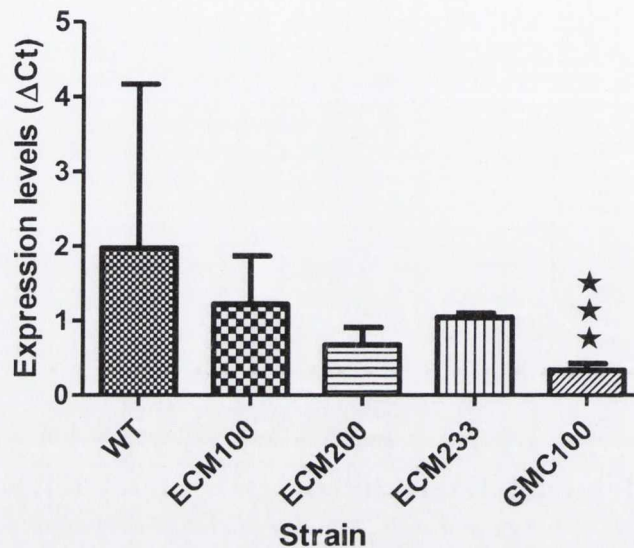


Figure 5.17: Expression levels of *hagA* in *P. gingivalis* were assessed after growth on sTSA. Expression level is based on ΔCt values as calculated in section 2.3.2. On analysis using an unpaired students t-test it was found that there was a statistically significant difference between strain ECM233 and GMC100 (p-value of 0.0003). *** indicates a value between 0.0001 and 0.001 (ECM100 = $\Delta hagB$, ECM200 = $\Delta hagC$, ECM233 = $\Delta hagB/hagC$, GMC100 = $\Delta hagC$, *hagB::cat*).

5.4 Discussion

The ability of *P. gingivalis* to adhere to and invade host cells is vital for the virulence of this pathogen. Adherence and invasion allows *P. gingivalis* to alter host responses from within cells, as well as shielding itself from host immune responses, all of which contributes to the development of dysbiosis within the microbial community (Hajishengallis and Lamont, 2012, Yilmaz, 2008).

Prior to the current study, how HagB and HagC contribute to adherence and invasion of oral cell lines, such as the H357 cells used in this study, was unexamined. Song et al. (2005) have shown that HagB does possess adhesive properties, and that heterologously expressed HagB plays an important role in adherence to HCAE.

One of the aims of this branch of study was to establish through use of our mutant strains, whether *hagB* and *hagC* play a role in adherence or invasion of an oral cell line and how the absence of one or both of these genes would affect the processes involved. The effect of growing the strains in haemin free media prior to cellular infection was also examined in strain W83.

The methods used in this study are growth-dependent based methods which give a strong indication of trends present in adherence and invasion. Alternative growth-independent methods could be used to carry out a more detailed analysis of these trends. These methods include combining the use of fluorescent dyes with flow cytometry to analysis the dynamics of the adherence and invasions processes in more detail.

In *P. gingivalis* flow cytometric methods have been used to elaborate on the differences in cell binding between strains. It has been shown that strain W50 binds to cells as either discrete cells or very small aggregates, whereas strain ATCC33277 bound only as large aggregates to cells (Pathirana et al., 2007).

Similar methods were also used to elaborate on the mechanisms of intercellular spreading used by *P. gingivalis* in gingival epithelial cells (Yilmaz et al., 2006).

Our overall findings highlight that *hagB* and *hagC* do potentially play a role in adherence to H357 cells. It also highlights that these genes and others involved in adherence are regulated by the presence of haemin and require haemin for expression, as highlighted by the results of the haemin starved adherence assay. In the absence of haemin it was found that overall adherence levels in all strains dropped substantially (Fig 5.4). This is not altogether surprising as it has been shown that a substantial number of *P. gingivalis* genes including those involved in adherence and invasion are differentially regulated in response to haemin levels (Dashper et al., 2009), micro-array studies have also demonstrated this (Kiyama-Kishikawa et al., 2005).

Strain W83 is a non-fimbriated strain and as such its ability to adhere to cells is reliant on non-fimbrial adhesins such as the gingipains, which have been shown to be downregulated in the absence of haemin (Kesavalu et al., 2003).

Under normal haemin conditions removal of either *hagB* or *hagC* affected the ability of *P. gingivalis* to adhere to cells. Statistically significant differences were found between WT, strain ECM100 and ECM200 after a 90 min adherence assay was carried out (Fig 5.1). These differences were also apparent for both the 90 min and 24 hr invasion assay, though they were not statistically significant in all cases (Fig 5.5 & 5.7). These results highlight the importance of these proteins in adherence.

The effect that mutation of *hagB* had on the ability of strain ATCC33277 to adhere to cells was also examined. The *hagB* mutant strain was compared to a *fimA* mutant as well as to the WT parental strain (Fig 5.3). FimA acts as a major adhesion for *P. gingivalis* and is a known adhesin for strain ATCC33277. Previous studies have shown that deletion of *fimA* from this strain greatly affected the ability of this bacterium to adhere to an epithelial cell

line (Njoroge et al., 1997). In the current study, removal of *hagB* from the ATCC33277 genome was found to have a substantial affect on the ability of the organism to adhere to H357 cells. Rates of adherence showed a 10-fold decrease in the absence of *hagB*, highlighting that *hagB* plays an important role in this process in this strain, a fact that was previously unknown. Our results for the *fimA* mutant confirm previous data that *fimA* encodes a major adhesin in this strain, with adherence the rate dropping to ~0.16% of the inoculum compared to 10% in the WT strain. These data suggest that adhesion to the H357 cell line is a multi-factorial process involving fimbrial and non-fimbrial adhesins. Although the data indicate that FimA plays an essential role in mediating adherence, it also indicates that HagB is required to stabilise this interaction. Adherence may be a two stage process where fimbriae are required for initial long-range interactions and non-fimbrial adhesins such as HagB mediate stable, short-range interactions.

Strain ECM233, a derivative of strain W83 that has both *hagB* and *hagC* mutated in its genome, produced some unexpected results in both the adherence and invasion assays. It was predicted that due to the loss of both genes that an additive defect in adhesion would be observed in this strain. Instead it was found that in all assays, the level of adherence and invasion in strain ECM233 was comparable to that of the WT parent strain (Fig 5.1, 5.5 & 5.7). In order to exclude the possibility that a random mutation had occurred during the generation of strain ECM233 which conferred additional adhesive capabilities, we characterised a second independent *hagB/hagC* mutant and observed an identical adhesive phenotype. The results of the adherence and invasion assays for the *hagB* complemented strain GMC100 were also unexpected. This strain exhibited a hyper adherent phenotype but was compromised in terms of its invasion capabilities. GMC100 is almost twice as adherent as WT W83 (Fig 5.1), it was postulated that restoration of *hagB* to the genome of ECM233 would generate a strain with similar adherence characteristics of strain ECM200 (Δ *hagC*). This was due to the fact the restoration of *hagB* to strain ECM233 restored

pigmentation to similar levels as those observed in strain ECM200 and it was expected that this gene would complement other phenotypes in a similar fashion. Instead it was found that strain GMC100 was hyper-adherent and hypo-invasive compared to all other strains (Fig 5.5, 5.6 & 5.7).

As noted earlier, previous attempts to generate a double *hagB/hagC* mutant were unsuccessful, suggesting that this combination of mutations is very stressful for the bacterium. The severity of the pigmentation defect on blood agar supports this hypothesis. Therefore, in order for this mutant to remain viable, up-regulation of additional factors may be required to compensate for the loss of *hagB* and *hagC*. The qPCR data support this hypothesis and this up-regulation may be result of a haem starvation response. If this is the case, then restoration of *hagB* to the mutant should restore haem uptake and alleviate this stress response. However, the data indicate that even when *hagB* is restored to the genome of strain ECM233 in GMC100, that the putative compensatory mechanisms activated this strain have remained active, as adhesion does not return to the levels observed in the single mutants. Restoration of *hagB* appears to have an additive effect on adhesion where the combination of *hagB* and other up-regulated genes make the strain hyper adherent. This finding suggests that the compensatory up-regulation of adhesion and haem acquisition factors in strain ECM233 may be the result of a stable, heritable, epigenetic modification rather than a transient response to haem starvation, which would be reversed by complementation with *hagB*. This suggests that the double *hagB/hagC* deletion was constructed in a background where these factors may have already been upregulated. Indeed, such a background may be required for this transformation to yield viable results as previous attempts to generate a *hagB/hagC* mutant in strain Pg381 have been unsuccessful. The fact that our attempts to generate a *hagB/hagC* double mutant in strain W83 were very successful, yielding multiple, independent transformants similar to ECM233, also suggests that a subpopulation of cells in the W83 population may exhibit

this hyper-adherent phenotype and remain viable following deletion of *hagB* and *hagC*. Support for this hypothesis comes from the work of Suwannakul et al., (2010), who have shown that *P. gingivalis* populations are heterogenous and consist of subpopulations of cells that differ in the expression of genes including *rgpB*, *hmuR* and *sodB*. These genes were identified among a set of 19 genes that were differentially expressed in high- and low-invasive sub-populations of *P. gingivalis* strain W50 (Suwannakul et al., 2010). In the current study, it is likely that the double *hagB/hagC* mutation was generated in cells belonging to an adherent sub-population of W83, similar to that described by Suwannakul et al., (2010), which would have been selected during the construction of strain ECM233. Although this phenomenon has been characterised in strain W50 and NCTC11834, it is not known whether all strains exhibit this phenomenon or if all strains exhibit variability in the same subset of genes. The absence of this phenomenon in some strains of *P. gingivalis* may explain why attempts to generate *hagB/hagC* mutant were not successful in strains Pg381 (Lepine et al., 1996) and ATCC33277 (this study).

We hypothesised that due to the selection of these hyper-adherent variants during the construction of strain ECM233, that the gingipains and *hagA* were likely to be expressed at higher levels to compensate for the loss of *hagB* and *hagC*. RNA was extracted from broth grown cells and blood agar grown cells, and expression of the selected genes was found to be higher in the haemoglobin rich environment of the blood agar. Under these conditions (Fig 5.15 & 5.16), which are those under which the mutants were selected, strain ECM233 exhibited increased levels of *rgpA*, *rgpB* and *kgp*, supporting our hypothesis of compensatory gene expression. What is also noticeable from these data is that the level of expression of *rgpA* and *rgpB*, which is extremely reproducible in WT W83 and the single *hag* mutants, exhibits greater levels of variation in ECM233 and GMC100, suggesting that within these strains backgrounds there may exist different sub-populations of cells that differ in expression of these genes.

This increase in expression of the gingipains could account for some of the phenotypes seen in strains ECM233 and GMC100. A role for the gingipains in adherence has been demonstrated (Chen and Duncan, 2004). It has been postulated that these proteins act in concert with fimbriae to increase overall adherence by cleaving extracellular matrix proteins from the cell surface to reveal fimbrial binding sites (Chen et al., 2001a, Kontani et al., 1997). Increased expression of *rgpA* combined with insertion of *hagB* back into the genome could lead to an additive affect that could account for the rapid development of pigmentation in haemoglobin based media as discussed previously. This additive affect could also account for the increased adherence levels seen in strain GMC100 compared to WT. Although GMC100 was hyper-adherent, we did not observe any increase in cellular invasion. However, up-regulation of adherence factors does not necessarily guarantee improved invasion capabilities, particularly as strain W83 is non-fimbrial and has reduced invasion capabilities relative to other strains. Another factor that may account for the reduced invasion capabilities of GMC100 is that in comparison to ECM233, it exhibits greatly reduced expression levels of *kgp* and *hagA* (Fig 5.16 & 5.17). This suggests that the higher levels of *kgp* and *hagA* expression that are being selected and maintained by culture on blood agar in strain ECM233 are perhaps being lost in strain GMC100. This may be due to the fact that complementation with *hagB* has restored the hemagglutination functions that were being compensated for by increased levels of *kgp* and *hagA*. If this is the case, then by a process of stochastic drift of bistable populations, strain GMC100 may be losing certain gene expression patterns inherited from ECM233. Stochastic drift in this case is referring to what can also be called genetic drift. It refers to a change in the frequency of a genotype occurring within the bacterial population due to random sampling of the bacterial population as a whole. This process can lead to selection with one genotype becoming more prevalent over the others.

It is also important to note that when the adherence assay was carried out under haemin free conditions, GMC100's ability to adhere reduced significantly and was not statistically significantly different from the other strains, as was ECM233. This suggests that any genes that are up-regulated in these strains are reliant on the presence of haemin for their expression. In the absence of haemin the bacteria are unable to activate these remunerative abilities.

Alternatively the adherence and invasion phenotypes observed in this study could be due to indirect effects of mutagenesis. In bacterial species the effect of deleterious mutations, such as those introduced to *hagB* and *hagC* can be reduced by secondary site mutations also called compensatory mutations in other genes (Schulz zur Wiesch et al., 2010). It has been suggested that bacterial cells when stressed may enter into a hyper-mutable state where the DNA damage caused by mutagenesis can lead to error-prone repair, leading to secondary mutations arising at another site. Mutations that benefit the bacteria by enabling it to survive are kept, while neutral ones are lost due to bacterial cell death (Hall, 1988, Hall, 1990). This could account for some of the phenotypes observed in our mutant strains, as if beneficial secondary mutations arose during the mutagenesis it is possible they were selected out for, as compensated mutants can be enriched for through population bottlenecks such as *in vitro* serial passaging (Schulz zur Wiesch et al., 2010).

Alterations to the bacterial epigenome may also contribute. The epigenome consists of modifications to DNA nucleotides with small molecules such as methylation. These modifications can change protein-DNA binding and as such can alter the biochemical landscape of the DNA, directly impacting phenotype (Sanchez-Romero et al., 2015). The effects of altering DNA methylation patterns in bacteria have been shown to alter bacterial pathogenesis, as well as virulence. Strains of *Salmonella enterica* where the *dam* gene had been mutated were found to a massively attenuated response compared to WT strains in a

murine model due to altered methylation patterns (Heithoff et al., 1999). In terms of oral pathogens, changes to methylation patterns have been seen to have a direct impact on the virulence of both *S. mutans* (Banas et al., 2011) and *A. actinomycetemcomitans* (Marinus and Casadesus, 2009). It is possible that mutation of the *hagB* and/ or *hagC* genes affects the bacterial genome on an epigenomics level, this could be investigated further using methylome analysis techniques such as single molecule real-time sequencing to assess for differences between WT and mutant strains.

The mutagenesis process may have also interfered with regulatory RNAs such as small non-coding RNAs (sRNAs). These molecules play an important role in bacterial function by altering the physiological response of the bacteria in response to stress. They act by modulating both protein synthesis (mRNA transcription etc) and the activity of specific proteins by binding to them (Ghaz-Jahanian et al., 2013). It is possible that by altering *P. gingivalis* genome and as such the bacterial proteome we have interfered with this nuanced regulatory network which could account for some of the phenotypes seen here.

Our data also supports a role for the *hag* genes in biofilm formation. When mono-species biofilm formation was examined it was found that there was a statistically significant difference in the ability of strains ECM200 and ECM233 to form a mono-species biofilm compared to the WT parent strain. It is worth noting that when CFSE staining was carried out strain ECM233 ($\Delta hagB/\Delta hagC$) was the most statistically significantly different in terms of biofilm formation when compared to WT. In addition strain ECM200 ($\Delta hagC$) was also found to be statistically significantly different. ECM233 lacks the *hagB* and *hagC* genes and its poor ability to form a mono-species biofilm highlights that these genes play an important role in mono-species biofilm formation. The fact that strain ECM200 also shows a statistically significant reduction highlights that *hagC* may play a highly important role in the process.

Another interesting result that highlights the importance of *hagC* in co-aggregation is the multi-species biofilm formation results. While there was no difference in the overall composition of each of the biofilms (Fig 5.11) when the biomass of each of the mature multi-species biofilms was assessed it was found that multi-species biofilms incorporating strain ECM200 (Δ *hagC*) exhibited a statistically significant reduction in biomass compared to the other biofilms (Fig 5.10). The fact that there is a decrease in the overall biomass without species composition being affected suggests that *hagC* may have additional roles in biofilm formation such as the development of the exopolysaccharide matrix or quorum sensing. Further studies such as transcript profiling would be necessary to determine if *hagC* is required for stimulating expression of genes required for these processes.

Here we clearly show the importance of *hagB* and *hagC* in adherence to H357 cells. In the absence of one of the genes adherence rates are directly affected. We have also demonstrated a role for *hagC* in both mono- and multi-species biofilm formation, highlighting the importance of this gene as a virulence factor in its own right separately from *hagB*. This data indicates that our knowledge of *hagC* and not just *hagB* needs to be expanded upon as it appears that they may play separate roles in key virulence process such as biofilm formation. We also show that removal of these genes from the genome of *P. gingivalis* W83 is only possible in variants that express increased levels of *hagA* and the gingipains, a fact which could account for some of the aberrant phenotypes we see in our mutant strains and the fact that previous attempts to generate a double mutant have been unsuccessful. The full extent of this effect could be studied in more detail using RNAseq based methods as it is likely that more genes are selected by the absence of *hagB* and *hagC*. Overall we have highlighted the importance of these genes in virulence processes such as adhesion and biofilm formation.

Chapter 6: Examining the role that *hagB* and *hagC* play in stimulating the immune responses of THP-1 cells to whole cell *Porphyromonas gingivalis*.

6.1 Introduction

It has been shown that *P. gingivalis* inhibits the innate host defence system during periodontitis; disrupting tissue homeostasis and facilitating further exposure of the host connective tissue to the microbiological agents present in plaque. This leads to the classic tissue destruction commonly associated with chronic periodontitis, where plaque has accumulated on the tooth surface, leading to the eradication of the supporting connective tissue and alveolar bone, resulting in tooth loss (Darveau, 2010).

Through its strategies of immune subversion *P. gingivalis* facilitates the conversion of a symbiotic relationship with the oral micro-flora to a dysbiotic one, despite the fact that it's present in low numbers within dental plaque and is not a dominant species within the microbial biofilm (Darveau et al., 2012). *P. gingivalis* actively encourages dysregulation of the immune response, leading to a non-responsive, non-productive form of inflammation. It does this through the production of various virulence factors that interact with the host.

One important feature of the gingival epithelium is its ability to produce interleukin-8 (IL-8) which is pro-inflammatory and acts as a chemokine for neutrophils recruited to the site of infection, by producing this chemokine the gingival epithelium sets up a gradient for the recruitment of neutrophils to the gingival crevice. *P. gingivalis* interferes with this process through the production of serine phosphatase B (SerB), which inhibits the production of IL-8, which in turn delays the recruitment of neutrophils to the infected site and allows for easier colonisation of the periodontium tissues (Hasegawa et al., 2008, Takeuchi et al., 2013).

The gingipains of *P. gingivalis* have complement C5 convertase like activity, which leads to high levels of C5a locally, which in turn activates the C5a receptor on leukocytes. C5aR signalling plays a role in crosstalk with toll like receptor 2 (TLR2), which is activated by *P. gingivalis* surface ligands as well. This crosstalk leads to enhanced inflammation at the

site of infection but impaired killing activity by leukocytes, aiding in the establishment of a non-functional and non-responsive form of inflammation.

P. gingivalis LPS is believed to be a key virulence factor for the initiation and progression of periodontitis, triggering some of the original inflammation and subsequent tissue damage. It's a major antigenic protein that is known to stimulate immune responses. In order to avoid this stimulation, *P. gingivalis* has adapted its LPS through lipid A remodelling. This remodelling allows *P. gingivalis* to alter the structure of its lipid A from a Toll-like receptor 4 (TLR4) antagonist at temperatures of 37°C, to a TLR4 agonist at higher temperatures. It does this through the loss of monophosphorylated and nonphosphorylated tetra-acylated lipid A in favour of monophosphorylated, penta-acylated lipid A as the temperature rises (Curtis et al., 2011).

Limited investigation of the potential of HagB as an immune regulator has been carried out. Studies have shown that recombinant HagB (rHagB) binds to human β defensin three (HBD3), which is an antimicrobial peptide and attenuates the induction of Interleukin-6 (IL-6), Interleukin-10 (IL-10), and granulocyte macrophage colony stimulating factor (GM-CSF) (Pingel et al., 2008). It has also been shown that rHagB activates mouse peritoneal macrophages and stimulates the production of both pro and anti-inflammatory cytokines through activation of extracellular signal-regulated kinases (ERK), the c-jun terminal kinases (JNK) and p38 family of kinases (p38). Inhibition of ERK or p38 was found to have distinct effects on the rHagB induced cytokine production in these cells (Zhang et al., 2005a).

For human myeloid dendritic cells it has been shown that rHagB is a TLR4 agonist (Gaddis et al., 2009). HBD3 has been shown to alter but not inhibit the binding of HagB to the surface of dendritic cells (Van Hemert et al., 2012). Immunisation with rHagB in a murine model has been shown to lead to the induction of a rHagB-specific CD4⁺ T cell

response which can then be shaped by toll like receptor 4 (TLR4) signalling. The same study highlighted that MyD88 was the major adaptor molecule that shapes the T cell response to rHagB, Toll/IL-1R domain-containing adapter inducing IFN- β (TRIF) was also found to participate in the regulation of inflammatory memory cytokine production following ligation of TLR4 by rHagB (Gaddis et al., 2011).

Macrophage inflammatory protein – 1 β (MIP-1 β), also known as CCL4, is a small 8-10 kDA protein produced and secreted by a number of cell types including macrophages, T-cells, B-cells, dendritic cells, neutrophils and mast cells. Production can be stimulated by pro-inflammatory agents, such as LPS, as well as by pro-inflammatory cytokines such as IL-1 and TNF- α and that anti-inflammatory cytokines such as IL-10 down-regulate its production. It is initially synthesized as a 92 amino acid pre-cursor before peptidases cleave the hydrophobic signal peptides to produce the mature protein (Maurer and von Stebut, 2004).

MIP-1 β is commonly produced by activated macrophages in order to recruit further pro-inflammatory cells to the site of inflammation. The protein mediates its effects by binding cell surface chemokine receptors. This binding can then trigger a wide range of responses depending on cell type such as chemotaxis, degranulation, phagocytosis and mediator synthesis. Binding of the protein to its receptor acts on a number of cell-signalling pathways including the JAK/STAT pathway (Maurer and von Stebut, 2004).

In vitro studies have shown that MIP-1 β is produced in response to *P. gingivalis* HagB protein. Binding of histatin 5 to immobilised rHagB was shown to alter the HagB induced chemokine responses. One of the chemokines affected was MIP-1 β , which showed a 12.5% decrease in production when rHagB was bound by histatin 5 (Borgwardt et al., 2014).

THP-1 cells are derived from the peripheral blood of a one year old child with acute monocytic leukaemia. It is a monocyte cell line that can be differentiated into macrophage like cells using Phorbol 12-Myristate 13-Acetate (PMA). THP-1 cells have Fc and C3b receptors but lack surface and cytoplasmic immunoglobulins. They have previously been used to study monocyte and macrophage responses to *P. gingivalis* (Diya et al., 2008, Foey and Crean, 2013, Yee et al., 2012).

Here, we sought to demonstrate the effect that removing *hagB* and *hagC* from the *P. gingivalis* genome has on the cytokine and chemokine profile of THP-1 cells by using an *in vitro* mono-species infection model.

6.2 Materials and Methods

6.2.1 Cell culture methods

Monocytic THP-1 cells were grown in 5% CO₂ at 37°C (Sanyo CO₂ Incubator) in RPMI containing 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, 1.5 g/L bicarbonate (Gibco), to this a final volume of 10% foetal calf serum (Sigma) and 0.05 mM 2-mercaptoethanol was added. When it was required media was supplemented with 2 µl of Phorbol 12 – Myristate 13 – acetate (PMA) (Sigma). The cells were cultured as a single cell-suspension and maintained at a cell concentration between 5 x 10⁴ and 8 x 10⁵ cells/ml. The media was replaced every 2 to 3 days depending on cell density. The cells were split 1:4 once a week. Cell viability was assessed using Trypan blue (Sigma) exclusion as outlined in Chapter 2.

6.2.2 Multi-analyte enzyme linked immunosorbent assay (ELISA)

The THP-1 cells were plated at 1 x 10⁶ cells/well of a 6 well plate in 3 ml of media containing 1 ng/L PMA. On exposure to the PMA the cells differentiated into adherent

macrophage like cells. Prior to the assay been carried out cells were washed with PMA free media and incubated for a further 24 hr.

Cultures of *P. gingivalis* W83 and ECM233 were grown up anaerobically overnight at 37°C in hemin rich sTSB. Using these overnight cultures, 8 ml cultures were inoculated to an OD₆₀₀ of 0.45 and re-incubated for 2 hr until they reached an OD₆₀₀ of 0.6 ($\sim 2 \times 10^9$ bacteria).

The cultures were spun down at 3,200 $\times$ g (Centrifuge 5810 R, eppendorf) for 10 min in sterile falcon tubes. The broth was removed from the cultures using a sterile pipette and the bacterial pellets were re-suspended in 8 ml of 1 X Dulbecco's phosphate buffered saline (PBS) (Sigma). The optical density of each bacterial suspension was re-measured to ensure that it was OD₆₀₀ of 0.6. The bacteria were diluted 1:100 in antibiotic free THP-1 media.

To each well of a 6 well plate 1 ml of bacterial culture was added to give a multiplicity of infection (m.o.i) of 20. The plate was then incubated for 20 hr at 37°C in 5 % CO₂. The media was removed from each well after 20 hr and spun at 3,000 $\times$ g to pellet the bacteria. The supernatant was aspirated off and stored at - 20°C until use.

The Human TLR- induced Cytokines II: Microbial-induced (QIAGEN) kit was used to examine the effect that the absence of HagB and HagC has on THP-1 cells cytokine production. The cytokines and chemokines represented by this array are Tumour necrosis factor-alpha (TNF α), Interleukin-1B (IL-1B), Interleukin-6 (IL-6), interleukin-12 (IL-12), Interleukin-8 (IL-8), monocyte chemotactic protein-1 (MCP-1), regulated on activation, normal T-cell expressed and secreted protein (RANTES), macrophage inflammatory protein-1-alpha (MIP-1 α), macrophage inflammatory protein-1-beta (MIP-1 β), macrophage derived chemokine (MDC) and Eotaxin. The experiment was carried out as per the manufacturer's instructions. This experiment yielded semi-quantative data that allowed for the selection of cytokines for further study.

6.2.3 Macrophage inflammatory protein 1 β ELISA:

Cells and bacterial cultures were prepared as described previously for multi-analyte ELISA. Based on the results from the Multi-analyte ELISA, the chemokine Macrophage inflammatory protein-1 β (MIP-1 β /CCL4) was selected for further analysis. The experiment was carried out on WT, ECM233, ECM100 and ECM200. The kit used was the CCL4 / MIP-1 β kit (R & D systems). The experiment was carried out on cellular supernatants as per the manufacturer's instructions.

It yielded quantitative data that allowed for differences in MIP-1 β production to be assessed in response to wild type W83 and its mutant derivatives. Supernatants from cells that were not exposed to *P. gingivalis* acted as a control. This assay was carried out at M.O.I's of 10 and 50.

6.2.4 Statistical Analysis

For the multi-analyte assay data sets were assessed using an unpaired student's t-test.

For the MIP-1 β ELISA differences between WT and mutant strains were determined by analysis of variance (ANOVA). When ANOVA was carried out Dunnett's Multiple Comparison was also performed. For all data *P*-values of < 0.05 were considered statistically significant. All statistical analysis was carried out using Graphpad Prism 5 software (Graphpad Software Inc., 7825 Fay Avenue, Suite 230, La Jolla, CA 92037, USA).

6.3 Results

6.3.1 Multi-analyte assay overall

This assay was a semi-quantitative assay carried out on *P. gingivalis* WT W83 and ECM233 ($\Delta hagB/\Delta hagC$) to assay changes in expression of multiple protein targets induced by TLR receptor activation. In this case the cytokines and chemokines represented by this array are TNF α , IL-1 β , IL-6, IL-12, IL-8, MCP-1, RANTES, MIP-1 α , MIP-1 β , MDC and Eotaxin.

Of the twelve cytokines and chemokines assessed it was found that there were statistically significant changes between the levels of IL-8 and MIP-1 β . These differences were visible when both the corrected absorbance values and the fold differences were examined (Fig 6.1). Detailed statistical analysis revealed that the most statistically relevant differences were found when MIP-1 β was examined. It was found that THP-1 cells that had been exposed to WT as opposed to ECM233 had lower levels of absorbance for MIP-1 β (p-value 0.0021) (Fig 6.2a). These differences were also demonstrated when fold difference was examined (p-value of 0.0036) (Fig 6.2b). This suggests a lower level of production of MIP-1 β by the THP-1 cells that have been exposed to WT compared to those that have been exposed to ECM233.

In order to verify if this was the case it was decided to carry out a fully quantitative ELISA for MIP-1 β alone, using all mutant strains constructed at the time.

6.3.2 Quantitative examination of levels of MIP-1 β produced by THP-1 cells post infection with WT and mutant strains

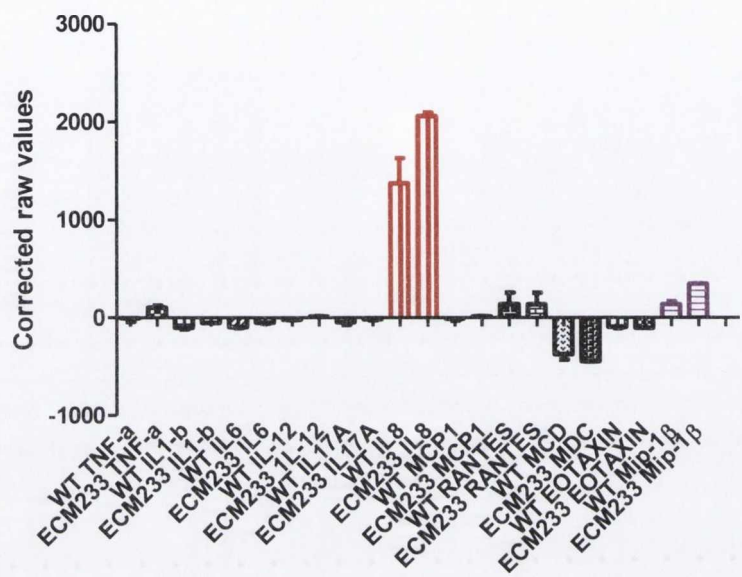
This assay was fully quantitative for the levels of MIP-1 β produced by THP-1 cells on exposure to the different mutant strains. This is due to the fact that as part of the protocol a

standard curve is carried out which on completion allows for the absorbance values to be converted to a pg/ml value. The experiment was carried out at M.O.I of 10 and 50.

It was found that at an M.O.I of 10 there were statistically significant differences between WT and ECM233 (Fig 6.3).

There were no statistically significant differences between WT, ECM100 or ECM200. At M.O.I 50 it was found that while there were differences between WT, ECM100, ECM200 and ECM233, they were not statistically significant (Fig 6.4).

(a)



(b)

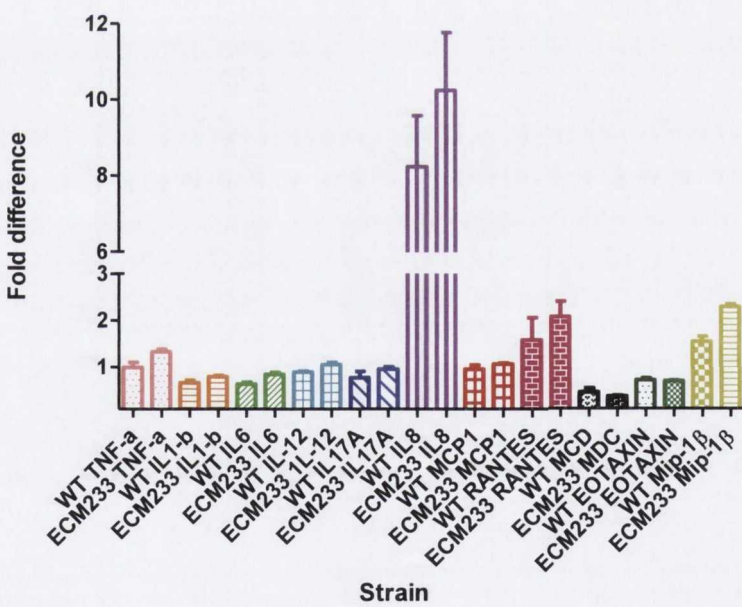
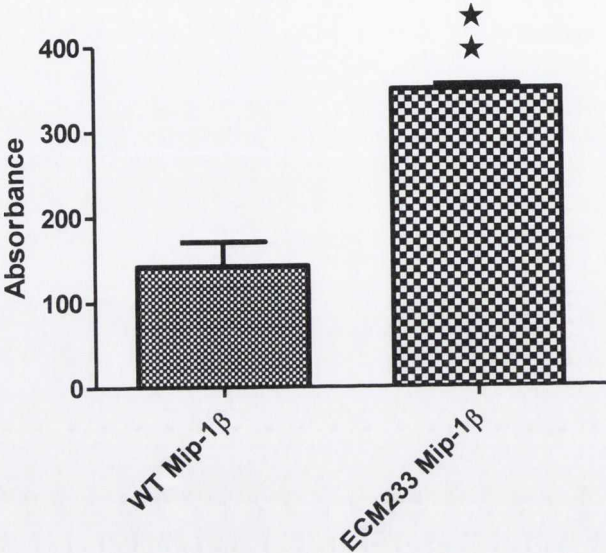


Figure 6.1: The results obtained from the multi-analyte ELISA data analysed in two ways.
a) Using the corrected absorbance values. b) Using fold differences as a measure of scale.

(a)



(b)

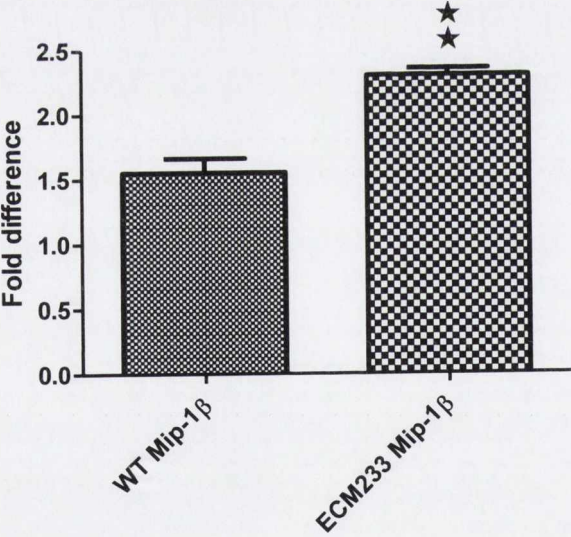
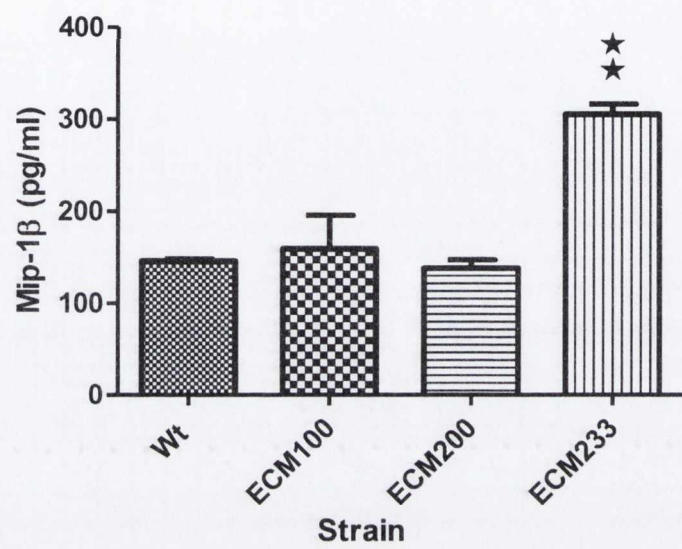


Figure 6.2: Mip-1 β expression data from multi-analyte assay a) on corrected raw data values (t-test p-value 0.0021) and b) fold difference (t-test p-value of 0.0036). (ECM233 = Δ *hagB/hagC*).

(a)

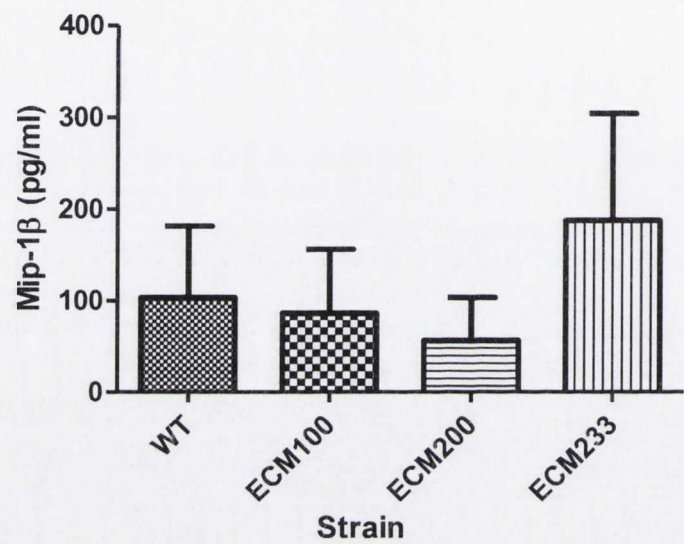


(b)

Dunnett's Multiple Comparison Test	q	Significant? P < 0.05?
Wt vs. ECM100	0.4690	No
Wt vs. ECM200	0.2766	No
Wt vs. ECM233	5.737	Yes

Figure 6.3: a) Levels of Mip-1β production by THP-1 cells at an M.O.I of 10. b) The results of Dunnett’s multiple comparison. It was found that there were statistically significant differences between WT and strains ECM233. (ECM100 = $\Delta hagB$, ECM200 = $\Delta hagC$, ECM233 = $\Delta hagB/hagC$).

(a)



(b)

Dunnett's Multiple Comparison Test	q	Significant? P < 0.05?
WT vs. ECM100	0.1688	No
WT vs. ECM200	0.4676	No
WT vs. ECM233	0.7532	No

Figure 6.4: a) Levels of Mip-1β production by THP-1 cells at an M.O.I of 50. b) The results of Dunnett’s Multiple Comparison. There were no statistically significant differences found between WT strain and the mutant strains under these conditions.

6.4 Discussion

The fact that HagB is immunogenic has already been well documented in a number of studies, including rodent models of infection (Gaddis et al., 2011, Zhang et al., 2005a). However the vast majority of these studies have been carried out using purified rHagB as opposed to whole cell bacteria, thus the effect that the removal of the HagB gene on the immunogenicity of *P. gingivalis* as a whole has yet to be fully established

Given the high levels of homology between HagB and HagC, it is likely that HagC has similar immunogenic properties as that of HagB. To date though, no studies have been carried out assessing the ability of HagC to stimulate the immune response, for this reason it is important to look at a HagC mutant. Due to the previously mentioned homology between the two genes, and the potential for redundancy of function between the two examining the effects of removal of both *hagB* and *hagC* from the *P. gingivalis* genome is essential to elaborate on the immunogenicity of the bacteria as a whole.

One of the primary ways immunity is activated in response to bacteria is through recognition of pathogen-associated molecular patterns (PAMPs) by pathogen recognition receptors (PRRs) which are found on a number of cells involved in innate immunity (Takeda and Akira, 2005). One group of PRRs which have been well studied are the toll like receptors (TLRs) found on cells such as macrophages, monocytes and dendritic cells, all of which act as sentinel cells. Different TLRs recognise different PAMPs, for example TLR2 recognises bacterial peptidoglycan and TLR4 recognises bacterial LPS (Hans and Hans, 2011). TLRs are known to play a dual role in terms of oral health. It has been shown that they are important for maintaining oral homeostasis however they also play a role in the development of periodontitis (Hans and Hans, 2011).

In the healthy state TLRs are continuously exposed to the bacteria present in plaque and are thus in a steady state of low activation (Kusumoto et al., 2004). Under this low level of activation they trigger the release of antimicrobials such as β -defensin into the oral cavity, as well as the recruitment of phagocytic cells. The antimicrobials and white cells act in tandem with one another to prevent bacterial out-growth of plaque, as well as keeping levels of invasion to a minimum (Hans and Hans, 2011).

Gingival epithelial cells serve as a barrier layer, preventing bacteria entering the tissues underneath. If this layer is breached TLRs that were dormant become triggered which facilitates the development of an exaggerated immune response that ultimately leads to tissue destruction and tooth loss. When the response is no longer kept in check by anti-inflammatory signals a non-responsive, non-productive inflammation is established, the levels of plaque increase, the periodontal pockets deepen, connective tissue is destroyed, bone resorption occurs and ultimately the teeth in the affected area will be lost.

TLRs are important for both oral health and in disease progression. In order to understand how TLRs respond in terms of cytokine production to the absence of HagB and HagC from the surface of *P. gingivalis*, it was decided to study cytokine production in a cell line which produces a wide variety of TLRs, namely THP-1 cells.

The multi-analyte assay used here to investigate Human TLR-induced cytokines is a qualitative and semi-quantitative assay. The reason the assay is only semi quantitative is because the absorbance values for each protein assessed are not converted to a concentration of the protein, as no standard curve is carried out to allow for that. As such assessment of the data is based on the corrected absorbance values only, these values can be compared between samples to determine whether there are changes in protein levels in terms of their absorbance values only.

The semi-quantitative multi-analyte assay was performed on WT and strain ECM233, this was due to the fact that ECM233 has both genes missing, allowing for the best comparison. The results showed that there were statistically significant differences in absorbance values between WT and ECM233 when IL-8 and MIP-1 β were examined (Fig 6.1). Of the chemokines flagged by the multi-analyte assay, MIP-1 β was considered to be the most interesting. This is due to the fact that *P. gingivalis*'s interactions with IL-8 have already been well characterised (Champaiboon et al., 2014, Hasegawa et al., 2008, Liu et al., 2014, Takeuchi et al., 2013).

A statistically significant difference was found between WT and strain ECM233 when absorbance and fold difference for MIP-1 β were examined. When the corrected raw data values were studied, it was found that WT had an absorbance level of 200, while the level for ECM233 was 400. An unpaired Student's t-test was performed, which gave a significant p-value of 0.0021 (Fig 6.2a). The fold differences of MIP-1 β were also studied to identify differences between the two strains. Using an unpaired Student's t-test it was found that there was a statistically significant difference between the two strains, with a p-value of 0.0036 (Fig 6.2b).

The detection of these differences suggested that it would be worthwhile to examine the levels of MIP-1 β produced in response to all four mutant strains constructed in this study. A single analyte assay was carried out on WT, ECM100, ECM200 and ECM233. A standard curve was also generated meaning that the assay was fully quantitative for MIP-1 β production, thus allowing differences in concentration of the analyte to be examined. The experiment was carried out at two different multiplicity of infections, an M.O.I of 10 and an M.O.I of 50.

At an M.O.I of 10 it was found that there were no statistically significant differences between WT, ECM100 and ECM200. There was a statistically significant difference in

WT and strain ECM233 (Fig 6.3). The results indicate that more MIP-1 β is produced in response to ECM233 compared to WT, suggesting that in the absence of *hagB* and *hagC* the strain becomes more immunogenic. One possible reason for this could be that in the absence of *hagB* and *hagC*, other cell surface proteins are up-regulated which are highly immunogenic. This theory was supported by our qPCR data as it was found that ECM233 produced high levels of RgpA compared to the other strains when grown under similar conditions (Chapter 5). Exposing the THP-1 cells to ECM233, which is potentially upregulating RgpA could account for the differences in immunogenicity seen, as RgpA is an immunogenic protein. These genes could be upregulated to functionally compensate for HagB and HagC. This would also account for the increased levels of MIP-1 β seen here.

The immunogenicity of HagB has previously been studied, most recently in studies involving the binding of histatin 5, which has anti-microbial activity. It has been shown that the binding of histatin 5 to immobilised rHagB leads to a 12.5% reduction in MIP-1 β produced by dendritic cells (Borgwardt et al., 2014). This study suggests that HagB may bind host proteins to mask its immunogenicity and possibly the immunogenicity of the whole cell surface, a trait which is commonly seen in a variety of bacteria (Potempa et al., 2008). In the absence of HagB and HagC, this could lead to decreased binding of host proteins and increased exposure of bacterial cell surface moieties. The mutant strains examined here generally exhibit reduced pigmentation following growth in haem rich medium, suggesting that the surface haem layer is reduced, which may also lead to increased exposure of bacterial cell surface moieties resulting in increased levels of IL-8 and MIP-1 seen here as there is increased recognition of PAMPs by PRRs.

When the experiment was carried out at an M.O.I of 50, differences were detected but they were not significant (Fig 6.4). One reason for this is that this is a high multiplicity of infection to use against THP-1 cells; each cell is effectively swarmed with 50 bacteria.

This level of exposure to bacteria can trigger apoptotic pathways leading to cell death. It is probable that the assay was carried out on a partially apoptotic population. This was found to be the case when the cells were examined microscopically, with evidence of cell death found in the notable rounding of the cells and the presence of cell debris. It has been previously shown that at an M.O.I of 50 *P. gingivalis* strain W83 does have cyto-toxic effects on THP-1 cells (Yee et al., 2012).

This study shows that the absence of both of these genes from the genome effect the bacterium's immunogenicity, creating a strain that is more immuno-stimulatory than WT. This could be because of up-regulation of other proteins such as RgpA to compensate for HagB and HagC on the bacterial cell surface or because the proteins themselves possess either proteolytic activity or bind host proteins to mask the bacterial cell surface, which in their absence leads to increased levels of cytokines such as MIP-1 β and IL-8 in the double mutant.

Chapter 7:

**Screening a *Porphyromonas gingivalis* transposon
mutagenesis library for genes involved in
adherence, invasion and survival in an epithelial
co-culture model**

7.1 Introduction

Many important genes which play a role in the pathogenesis of *P. gingivalis* infection have been identified in recent years. However there are still a significant number of genes within the *P. gingivalis* genome where function is either poorly understood or completely unknown. In order to fully understand this organism it is vital that we elaborate on the role that these genes and the proteins they encode for play in the infectious process so our understanding of their function can develop and become more clearly defined.

One of the most commonly used techniques to establish gene function is insertional mutagenesis, where the gene of interest is either interrupted or removed from the genome and replaced with an antibiotic resistance gene. By interrupting or deleting the DNA of the gene of interest it ceases to code for a viable protein product. The phenotypic effect of the mutation is then studied e.g. does the mutant differ in growth rate compared to the wild-type parent strain.

The technique has been previously used to establish the role of a number of genes and their subsequent proteins in the virulence of *P. gingivalis*, including the gingipains, *hmuR* and the *vimE* gene (Shi et al., 1999, Simpson et al., 2000, Vanterpool et al., 2004). The technique does have limitations as the function of only a small number of genes can be studied at any given time and the mutagenesis process is lengthy, something that is exacerbated by the slow growth rate of *P. gingivalis* itself.

Transposon mutagenesis is a technique which has been used to identify genes involved in virulence and other bacterial processes. It offers an alternative, high-throughput approach to the traditional methods and is an important tool for creating inactivation mutants. Transposon mutagenesis makes use of transposable elements which are discrete segments of DNA capable of relocating between genomic sites. They are ubiquitous in nature and are found in Archaea, Eubacteria and Eukarya, including humans. When used for

mutagenesis studies the most preferable transposable elements are those that insert randomly or near randomly (Hayes, 2003). The value of using transposons as genetic tools is essentially based on this factor, the transposable elements can insert randomly into sites throughout the host's genome, while giving only one insertion per mutant strain (Klein et al., 2012). Bacterial transposable elements range from simple insertion sequence elements made of a gene for transposition bound by inverted repeat sequences, to complex conjugative transposons which exhibit hybrid properties of transposons, bacteriophages and plasmids (Hayes, 2003).

Two previous attempts have been made to use transposon systems in *P. gingivalis*, with limited success. The systems were based on Tn4435 and Tn4400 which preferentially inserted into certain regions of the chromosome ("hot-spots") essentially limiting the distribution of interrupted genes. The attempts were also limited by the range of *P. gingivalis* strains that were available for mutagenesis (Chen et al., 2000, Hayes, 2003, Takada and Hirasawa, 1998). However huge advances have been made in the field using a Mariner transposon in *P. gingivalis* (Klein et al., 2012). The Mariner transposon was first identified and described in *Drosophila melanogaster* and it has been shown to be highly effective for *in vitro* bacterial mutagenesis in a diverse range of species (Lampe et al., 1999, Lampe et al., 1996). It has been shown that Mariner transposons insert preferentially into "TA" nucleotide sites which are abundant throughout genomes. In the case of *P. gingivalis* ATCC33277 genome, the strain used for this part of the study, it has been shown that there are only four NCBI annotated genes that lack a TA site. They are less than 40 amino acids long and encode for hypothetical proteins (Klein et al., 2012).

Recently major advances in the analysis of bacterial transposon libraries, including Mariner based systems have been made; researchers are now pairing transposon mutagenesis with massively parallel sequencing technology to quantitatively identify the

location of the transposons in a library pool by sequencing the transposon-genome junction. This strategy is commonly known as “Tn-Seq” and it has the potential to accelerate considerably the lag between gene discovery and gene function, as it allows for putative gene function to be established, as well as facilitating higher order genome organisation (van Opijnen and Camilli, 2013). Complex Mariner transposon libraries either teamed with Tn-Seq technology or other methods have already been used to identify the essential genes of *P. gingivalis* (Klein et al., 2012) as well as the essential genome of several other bacterial species including *Campylobacter jejuni*, *Streptococcus pneumonia* and *Haemophilus influenza* (Gawronski et al., 2009, Molzen et al., 2011, Stahl and Stintzi, 2011) . It is our belief that it has the potential to establish the role of genes of unknown function through an adaptive screening method that can implicate processes that the genes are involved in, thus elaborating on their function.

7.2 Materials and Methods:

7.2.1 Bacterial culture conditions:

A transposon mutagenesis library of *P. gingivalis* strain ATCC33277 was kindly donated by Brian A. Klein of Tufts University, Boston. The bacterial library was snap frozen in liquid nitrogen and shipped on dry ice. Upon arrival, the samples were gently thawed and a 200 μ l volume of library was plated onto fresh sTSA supplemented with 50 μ g/ml gentamicin and 10 μ g/ml haemin. Gentamicin was added to prevent contamination. Plates were incubated at 37°C until a lawn of growth was observed. In order to generate library stocks for future use, a 5 ml volume of 20% (v/v) glycerol in TSB was added to each plate. The lawn of growth was then removed with a cell scraper and batches of bacteria from different plates were pooled, aliquoted in 500 μ l volumes and stored at -80°C.

To activate the library for use in assays, frozen aliquots of bacteria (2 x 500 μ l) were removed from the -80°C freezer and thawed at room temperature. A 100 μ l volume was diluted 10-fold to 10^{-4} in sTSB and 100 μ l aliquots were spread on sTSA to assess recovery by plate count. The remainder of the stock (900 μ l) was plated without dilution on sTSA plates containing twice the normal haemin concentration to aid recovery.

Plates were incubated for 10 days and the titre of the library was assessed from the serial dilutions and this was used to estimate the number of mutant clones recovered from the aliquots. Growth was removed from the remaining plates by scraping to yield at least 10^7 individual mutant clones in each assay. The library contains 3.5×10^4 individual insertions (B. Klein, personal communication), meaning that each pool contained at $\sim 3 \times 10^2$ individual copies of each mutant strain. Growth from the plates was suspended in sTSB and pooled for use in the assay.

7.2.2 Cell culture conditions:

H357 cells were maintained as outlined in section 2.6.1. All experiments were carried out in T-75 flasks (Corning).

7.2.3 Cell based assays:

The bacterial suspensions described in section 7.2.1 were added to a sterile 50 ml tube (Greiner Bio-One) and spun at $3,200 \times g$ for 10 min. The supernatant was removed and the pellet was re-suspended in sterile 1 X PBS. The solution was then spun at $3,200 \times g$ for 10 min and the supernatant removed. This wash step was carried out three times in total. After the final wash the bacterial cells were re-suspended in PBS to an OD_{600} of 5. The bacteria were then diluted 1/10 (to give 2.5×10^9 /ml) and then 1/2 (to give 1.25×10^9 /ml). A 2 ml volume of the dilute bacterial solution was set aside to facilitate counting and further down-stream analysis. A 1 ml volume was then added to each T-75 flask, giving an M.O.I of ~ 100 . Bacterial samples were set aside at various stages of each assay to allow for DNA extraction (Fig 7.1).

7.2.3.1 Adherence assays:

Infected cells were incubated at 37°C for 90 min. The media containing non-adherent bacteria was removed and stored for DNA extraction. The epithelial cells were gently washed 3 x times in sterile PBS to remove non-bound bacteria. After washing the cells were incubated at 37°C for 20 min in 10 ml sterile molecular grade H_2O for 20 min in order to lyse the H357 cells. The lysate was then spun down at $3,200 \times g$, the supernatant was removed and the remaining pellet was re-suspended in a 1.1 ml volume of sterile PBS and moved to a sterile micro-centrifuge tube (Eppendorf). A 100 μl volume was taken and serial diluted ten-fold to allow for bacterial plate counting.

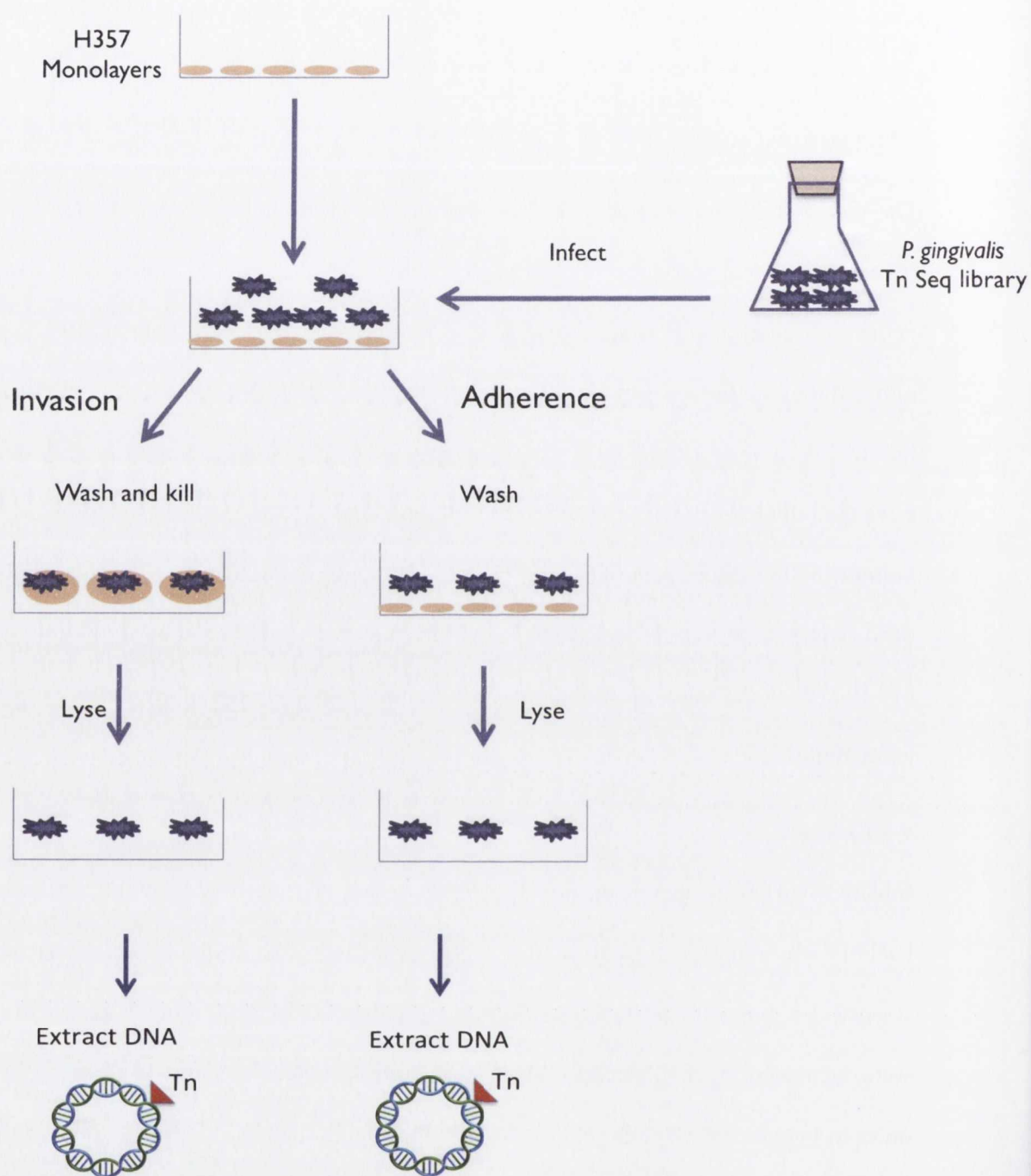


Figure 7.1: *In vitro* model of host cell interaction outlining the methods used in both the adherence and invasion Tn-Seq screening assays.

The remaining 1 ml volume was centrifuged at 10,000 x *g* for 2 min, the supernatant removed and the pellet stored at -20°C for DNA extraction (Fig 7.1).

Samples for DNA extraction were spun down at 3,200 x *g* for 10 min. The remaining supernatant was removed and the pellet was re-suspended in 1 ml sterile PBS and placed in a sterile micro-centrifuge tube (Eppendorf). The sample was then spun at 10,000 x *g* for 2 min, the supernatant removed and the pellet stored at -20°C for DNA extraction. This bacterial DNA is referred to hereon as the adherence output sample.

7.2.3.2 Invasion assays:

Infected cells were incubated at 37°C for 3 hr. The infected cell media containing non-invasive bacteria was removed and stored. Fresh media containing 30 µg/ml of metronidazole was added in order to kill all non-internalised bacteria. Cells were incubated in this media for 1 hr. The cells were then washed 3 x times in sterile PBS. After washing the cells were lysed in 10 ml of sterile molecular grade H₂O at 37°C for 20 min. The lysate was removed from each flask, spun at 3,200 x *g* and the supernatant removed and the pellet re-suspended in a 1.1ml volume of sterile PBS. A 100 µl volume of this suspension was taken and used for counting as outlined in the adherence assay protocol. The remaining 1 ml volume was transferred to a sterile micro-centrifuge tube (Eppendorf), spun at 10,000 x *g*, the supernatant removed and the remaining pellet stored at -20°C for DNA extraction and down-stream analysis (Fig 7.1). This bacterial DNA is referred to hereon as the invasion output sample.

7.2.4 DNA extraction:

Genomic DNA was also isolated using GenElute Bacterial Genomic DNA kit (Sigma-Aldrich) as per the manufacturer's instructions. The quality and the quantity of the DNA was then assessed by nano-drop as per section 2.1.7 before been snap frozen in liquid nitrogen and sent to Tufts University Boston for down-stream analysis.

7.2.5 Nested semi-random PCR and sequencing

All PCR and sequencing reactions were carried out by Brian A. Klein in Tufts University Boston. Genomic DNA was first eluted in 100 μ l elution buffer (Qiagen) and then sheared with a Branson 450 sonifier for 2 min (10 sec on 5 sec off duty cycle, 100 % intensity) using a high intensity cup horn that was cooled by a circulating water-bath (4°C). C-tails were then added to 1 μ g of sheared DNA. The 20 μ l reaction contained 0.5 μ l TdT enzyme (Promega), 4 μ l 5 X TdT reaction buffer (Promega), 475 μ M dCTP and 25 μ M dideoxy CTP. The dideoxy CTP acts to limit the length of the poly-C tails by functioning as a chain terminator. Samples were incubated at 37°C for 1 hr before a heat inactivation step was carried out at 75°C for 20 min, dideoxy CTP and other small molecules were removed by filtration using a Performa gel filtration cartridge (Edge Biosystems). Transposon containing fragments were then amplified in a 50 μ l PCR reaction which contained the following; 5 μ l C-tailed template DNA, 600 nM C-tail specific primer, 600 nM transposon specific primer, 400 μ M dNTPs, 5 μ l 10 X buffer and 1 μ l Easy-A DNA polymerase mix (Agilent). The reaction had an incubation at 95°C for 120 sec followed by 24 cycles that were carried out using 30 sec denaturation steps at 95°C, 30 sec annealing steps at 60°C and 120 sec extension steps at 72°C and a final extension of 120 sec at 72°C. A second PCR was performed to amplify the specific transposon-genomic DNA junctions and add the additional sequences needed for Illumina sequencing and indexing. A 50 μ l reaction was carried out which contained 1 μ l of template from the PCR #1, 600 nM transposon end specific primer, 600 nM indexing primer, 400 μ M dNTPs, 5 μ l 10 X buffer and 1 μ l easy-A DNA polymerase mix (Agilent). The reactions were carried out as follows; sandwiched by an initial incubation at 95°C for 120 sec and a final extension at 72°C, 12 cycles were performed using 30 sec denaturation steps at 95°C, 30 sec annealing steps at 60°C, and 120 sec extension steps at 72°C. Samples were then run for 51 cycles in a single end sequencing reaction on a single lane of an Illumina Genome Analyzer II (Tufts University)

using the custom sequencing primer pSAM3 and the standard Illumina index sequencing primer. The sequences for the primers used here, as well as a more detailed account of the methods can be found in Klein *et al.*, (2012) (Fig 7.2).

7.2.6 Data Analysis:

All read mapping and primary data analysis was carried out on the Galaxy server (Tufts University) in combination with Microsoft Office software. In brief, sequencing files are imported in FASTQ format. Using the adapter clipping script the C-tails are removed from these sequences. The sequences are then filtered based on quality with low quality sequencing reads being removed. The clipped and filtered sequences are aligned to their reference genome in this case *P. gingivalis* strain ATCC33277; accession number AP009380.1, using “Bowtie” with its default setting. The Bowtie output was used to create a custom file termed “hopcount”, which tabulated the transposon insertion sites and the number of times an insertion site was sequenced. From this data, a Microsoft Excel spreadsheet file was generated which indicated the position in the genome, the locus (assuming the insertion occurs within a gene) that an insertion maps to, the strand of DNA associated with the insertion, as well as the frequency of reads for each insertion site. Using this excel output it was possible to compare the abundance of specific insertion mutants in the assay input (initial inoculation) and output (washes and lysates) samples. The presence or absence of an insertion mutant in the input or output samples was estimated based on the number of times an insertion site was sequenced. By generating a ratio of output reads/input reads it was possible to determine which insertion mutants exhibited reduced abundance in the output samples (adherence and invasion samples) relative to the input library. For statistical analysis, these data were imported into GeneSpring GX 13.0 (Agilent Technologies, Santa Clara, CA, U.S.A.).

Ratio values were normalised to the median of the ratios and a subjected to a Mann-Whitney test using the “Storey with Bootstrapping” post-hoc test for multiple testing correction. Insertion mutants with a Q value of < 0.05 that exhibited a 5-fold change in abundance were selected for further analysis. In order to visualise the data, a BED file was generated which contained insertion site and frequency data and can be visualised using the GenomeView software package. The *P. gingivalis* ATCC33277 reference genome for GenomeView visualisation were downloaded from NCBI GenBank in FASTA format. The BED files were then used as input for GenomeView. Further bioinformatic analysis was carried out using the UniProt database (EMBL-EBI), the SIB Swiss Institute of Bioinformatics and the Protein Information Resource (PIR).

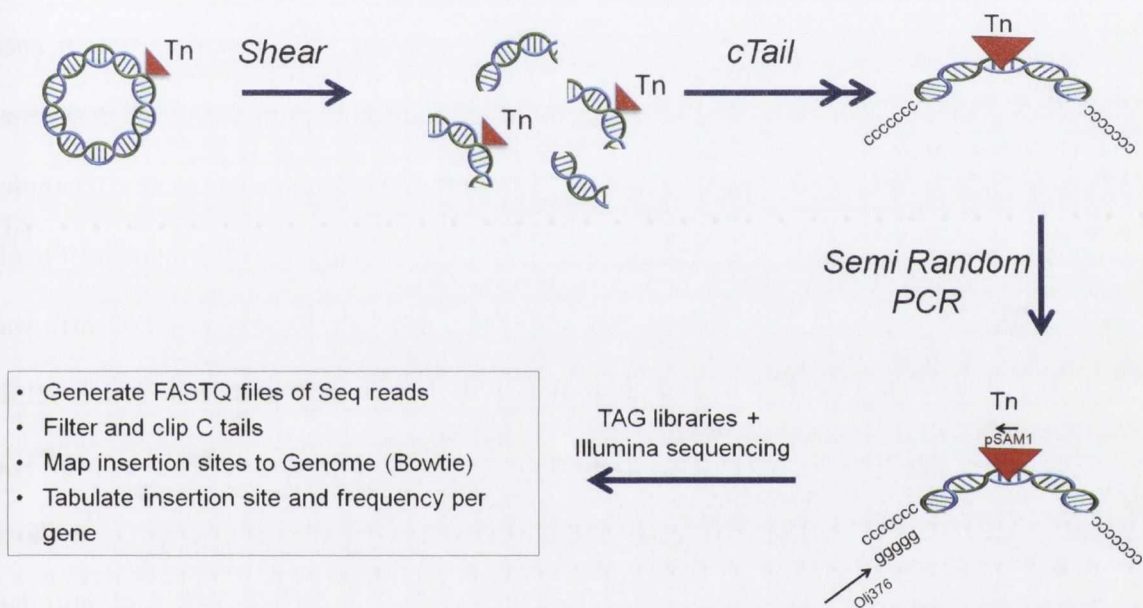


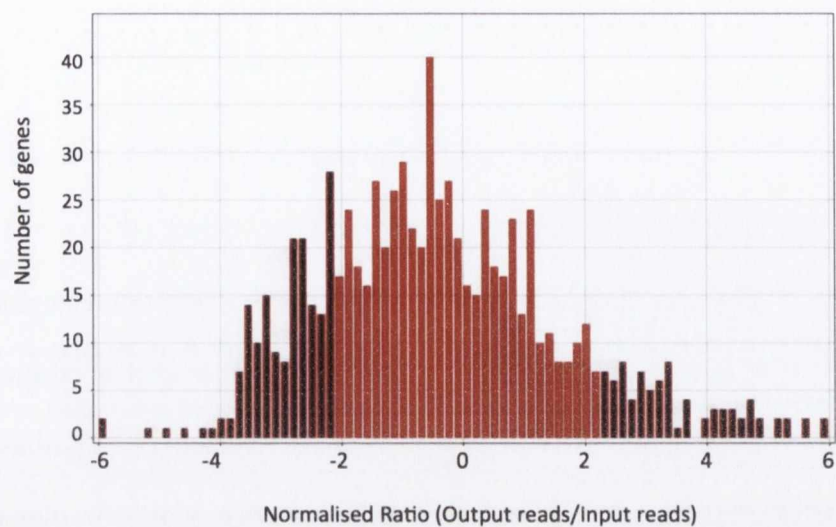
Figure 7.2: Diagram outlining the downstream processing of DNA. Samples were sheared and C-tailed before under-going a semi-random PCR process. The samples then underwent Illumina sequencing. The generated sequences then underwent data analysis.

7.3 Results:

7.3.1. Validation and statistical analysis of input and output samples:

Following Illumina sequencing and data processing, it was found that insertion reads (mutations) were identified in 1192 *P. gingivalis* ATCC33277 genes. This represents 73% of the non-essential genome identified by Klein *et al.* (2012). The numerical data on insertion frequency contained in the “hopcount” file was exported to GeneSpring GX13.0. This file indicated the position in the genome of the insertions, the locus that an insertions map to, strand orientation and the frequency of reads for each insertion site in each experimental condition. Initial examination of the data in GeneSpring identified 390 genes that had <200 sequence reads in both control (input) and experimental (output) samples and these were “flagged absent” and excluded from further analysis as the number of reads was deemed too low for significance analysis. The data for the remaining 802 orfs was included for further analysis. In GeneSpring the ratio of reads per gene in the output libraries (i.e. DNA recovered post-screen) versus the input libraries (inoculum control) was calculated. Data from the adherence and invasion assays was combined as a “total interaction” assay in order to increase the statistical power of the analysis. The ratio data was normalised to the median of the ratios and the normalised ratio values were used to graphically illustrate the distribution of the data. Normalised ratio value corresponds to the number of output reads/input reads normalised to the median ratio for an individual gene (Fig 7.3). In order to identify statistically significant changes in abundance between input and output samples, a two tailed Mann-Whitney test was carried out with an additional post hoc test ($Q < 0.05$) to confirm the differences detected by the Mann-Whitney test. Genes that passed this test with a 5-fold change in abundance were selected for further analysis. A total of 128 genes had a significant (5-fold) decrease in abundance following *in vitro* passage.

(a)



(b)

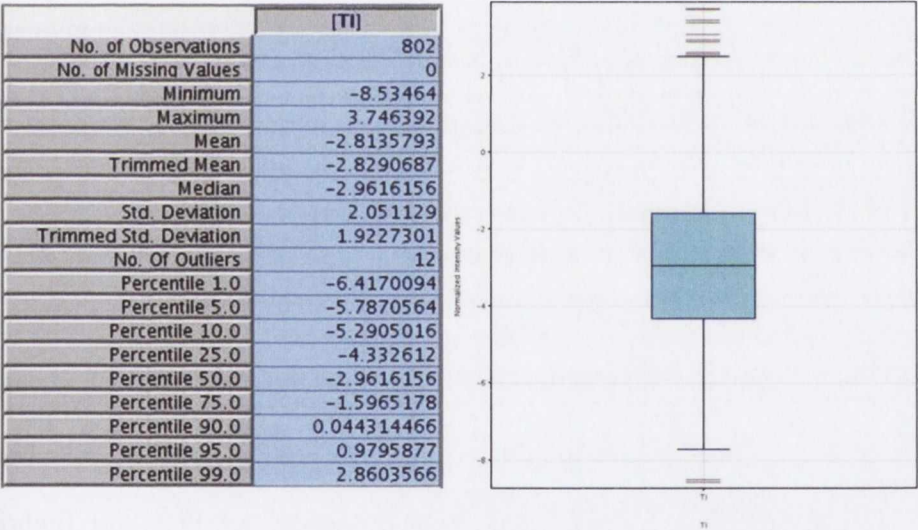


Figure 7.3: a) Ratio of output/input reads per gene were generated in GeneSpring and graphed showing their distribution. Normalised Ratio = No. Output reads/input reads normalized to median. Genes with normalized ratios 5-fold above or below the median are shaded. b) Numerical data showing the range and distribution of normalised ratios (pre-normalisation).

Of the genes assessed 27 genes were found to have a significant increase in abundance (Fig 7.3 b). For the purpose of this study we focused on those that were found to have a significant reduction.

7.3.2 Identifying genes involved in adherence, invasion and survival in an epithelial co-culture model

In order to establish proof of principle for this screen it was decided to examine shifts in abundance in genes that have a known function in adherence and invasion including haemagglutinins (*hagA*, *hagB*, *hagC*) fimbrial subunits including *fimA*, the *fimR/fimS* encoded response regulator genes and the epithelial modifying enzyme *serB*. Our hypothesis was that these genes should exhibit low output/input ratios due to their roles in adherence and or invasion or epithelial cells.

The *fimR/fimS* (PGN_0903/PGN_0904) operon encodes a two component signal transduction system which plays a central role in the regulation of *fimA* expression (Hayashi et al., 2000). The *fimS* gene encodes for a histidine kinase that in conjunction with *fimR*, encoding the cognate response regulator, controls the production of major fimbriae. Studies have shown that FimR controls the expression of several genes clustered around the *fimA* locus. FimR binds to the first gene in this cluster which encodes a key regulatory protein for *fimA* expression (Nishikawa et al., 2004). FimR has also been shown to influence the expression of *mfal* gene, which encodes for the short fimbriae of *P. gingivalis*, by binding directly to the *mfal* promoter region (Wu et al., 2007). Substantial reductions in output reads were seen in *fimR* and *fimS* insertion mutants and *fimR* was shown to have a normalised ration of -2.8. The *fimS* gene (PGN_0904) while not identified in our group of significantly affected genes was shown to exhibit a ~ 50% decrease in abundance in output libraries.

We examined the ratios of the other members of the *fim* gene family which encode the structural subunits of the major fimbriae. We found that *fimC* and *fimE* had normalised ratios of -2.48 and -2.4 showing a significant reduction in abundance in output populations compared to the input samples. *FimA*, which encodes the major adhesive subunit of the fimbriae showed a decrease in abundance of ~ 50 % relative to the input samples.

The gene *serB* encodes for a well described invasin in *P. gingivalis* (Bainbridge et al., 2010, Enersen et al., 2013, Hasegawa et al., 2008). The normalised ratio for *serB* was found to be -3.76 indicating that insertion mutants were significantly less abundant in the output library. We also examined the data recovered for the *hagB* and *hagC* genes. Unfortunately due to the low level of reads (<50) in the input samples data from these genes was flagged absent and was excluded from subsequent analysis.

A total of 128 genes (Table 7.1) showed a 5-fold or greater reduction in abundance following *in vitro* passage. Of these 128 genes, many were found to encode for proteins with hypothetical functions based on homology or proteins with completely unknown function. Of these genes a number encoded putative TonB-dependent outer membrane receptors and a TonB dependent receptor exported protein. Also of potential interest is PGN_1611 (normalised ratio -3.4) which encodes for a protein with homology to internalin.

Table 7.1: Table showing the genes which had a 5 fold or more reduction in the output files compared to the input files.

Name	Normalised Ratio	Description	End	Start	Strand
PGN_0379	-6.43464	hypothetical protein	414665	413733	+
PGN_0313	-6.381278	peptide chain release factor 3	341703	340123	-
PGN_2026	-5.607772	abortive infection protein	2269615	2268314	-
PGN_1712	-5.022053	endonuclease III	1911989	1911315	+
PGN_1347	-4.6955647	TonB-dependent receptor exported protein	1504520	1502016	+
PGN_1247	-4.5232877	hypothetical protein	1397213	1397064	+
PGN_t0026	-4.3139132	tRNA	1142708	1142632	+
PGN_0942	-4.2203945	hypothetical protein	1048531	1047725	+
PGN_0228	-4.1147064	coenzyme F390 synthetase	248828	247494	+
PGN_1185	-4.0775665	hypothetical protein	1316775	1316212	-
PGN_1306	-4.0683125	hypothetical protein	1449152	1448901	-
PGN_1953	-4.0593337	TonB-dependent outer membrane receptor	2176587	2174086	+
PGN_1425	-3.9407166	partial Type II restriction enzyme	1588271	1587756	+
PGN_0858	-3.916292	ABC transporter ATP-binding protein	944314	943616	+
PGN_1690	-3.902369	exported fucosidase	1889381	1887561	+
PGN_0938	-3.887125	8-amino-7-oxononanoate synthase	1045118	1043970	+
PGN_0193	-3.8637103	polysaccharide deacetylase	206364	205717	+
PGN_0159	-3.852226	thiamin-phosphate pyrophosphorylase	174263	172320	-
PGN_0533	-3.8486947	quinolinate synthetase	583429	582503	-
PGN_1738	-3.835968	long-chain-fatty-acid-CoA ligase	1953537	1951864	+
<i>serB</i>	-3.7677516	phosphoserine phosphatase	752459	751218	-
PGN_1992	-3.7609147	helicase	2233058	2230773	-
PGN_0714	-3.7487954	pyrazinamidase/nicotinamidase	776920	776408	+
PGN_0194	-3.748396	hypothetical protein	207076	206390	+
PGN_0139	-3.687785	rRNA large subunit methyltransferase	144061	143588	+
PGN_0882	-3.666457	hypothetical protein	976837	975971	-
PGN_1976	-3.6547836	hypothetical protein	2214194	2211615	+
PGN_0226	-3.647629	hypothetical protein	246413	245244	+
PGN_0811	-3.6311616	hypothetical protein	892635	890017	-
PGN_0011	-3.6275414	hypothetical protein	14986	14216	+
PGN_0949	-3.626822	ABC transporter ATP-binding protein	1059334	1057580	+
PGN_1231	-3.604422	hypothetical protein	1376336	1376094	-
Name	Normalised Ratio	Description	End	Start	Strand

PGN_0230	-3.5679807	serine acetyltransferase	250162	249728	+
PGN_0922	-3.5392446	hypothetical protein	1027813	1026608	+
PGN_0271	-3.521741	endopeptidase PepO	293103	291034	-
PGN_1361	-3.5199527	hypothetical protein	1515843	1515640	+
<i>dps</i>	-3.507471	DNA-binding protein from starved cells Dps	2282128	2281649	+
PGN_0691	-3.4994244	hypothetical protein	751206	750904	-
PGN_0940	-3.4942874	partial transposase in ISPg2	1045856	1045629	+
PGN_0378	-3.4646515	exopolyphosphatase	413634	412726	+
PGN_1611	-3.3983745	hypothetical protein	1802886	1801429	+
PGN_1617	-3.38938	metallophosphoesterase	1809699	1808230	+
PGN_0905	-3.3872117	dihydroorotate dehydrogenase	1007921	1007223	-
PGN_0973	-3.3838176	hypothetical protein	1085742	1083418	-
PGN_0009	-3.380213	glycosyl hydrolase family 3	13130	10119	+
PGN_1928	-3.3131846	hypothetical protein	2152509	2151763	-
PGN_1520	-3.29218	hypothetical protein	1703017	1700387	-
PGN_1524	-3.280363	hypothetical protein	1708322	1705857	+
PGN_0888	-3.2640647	hypothetical protein	983234	983139	+
PGN_0784	-3.252403	hypothetical protein	857338	856085	+
PGN_0150	-3.209242	ATP-dependent RNA helicase	164488	163205	-
PGN_0283	-3.2035283	exonuclease	304260	303478	+
PGN_1671	-3.196377	chromate transport protein	1867179	1866586	-
PGN_0946	-3.1910542	hypothetical protein	1055405	1053000	+
PGN_1112	-3.1808576	PhoH-like protein	1238342	1237008	+
PGN_1317	-3.1558484	hypothetical protein	1466599	1465109	-
PGN_1379	-3.1538986	hypothetical protein	1534478	1534341	+
PGN_0688	-3.148541	hypothetical protein	749461	748877	+
PGN_1697	-3.142384	hypothetical protein	1897149	1896118	-
PGN_1362	-3.107001	exported transglycosylase protein	1517539	1515974	+
PGN_0929	-3.103872	hypothetical protein	1034606	1032705	+
PGN_1581	-3.100394	DNA mismatch repair protein MutS	1774245	1771723	-
PGN_0099	-3.090054	peptidase	106588	105575	+
PGN_1691	-3.0676464	cysteine desulfurase	1890648	1889518	+
PGN_1920	-3.0442676	hypothetical protein	2143727	2141322	-
PGN_0862	-3.027629	Type III restriction enzyme, res subunit	949814	947073	-
PGN_1086	-3.027194	hypothetical protein	1210057	1209872	+
Name	Normalised Ratio	Description	End	Start	Strand
PGN_0144	-3.025141	hypothetical protein	149463	148204	-
PGN_1115	-3.018089	hemagglutinin	1240605	1239496	+

PGN_0448	-3.0097603	ABC transporter ATP-binding protein	490628	489969	-
PGN_1313	-3.0017795	hypothetical protein	1458199	1456472	+
<i>fimR</i>	-2.9793014	LuxR family transcriptional regulator	1005132	1004449	-
PGN_1294	-2.9605135	hypothetical protein	1438501	1438283	-
PGN_1964	-2.9424747	CRISPR-associated helicase Cas3 core	2196018	2193898	-
PGN_1623	-2.9317497	hypothetical protein	1817637	1814278	-
PGN_2013	-2.923472	cation efflux system protein	2256270	2253151	-
PGN_1104	-2.922812	chorismate synthase	1230992	1229886	-
PGN_1998	-2.8899735	hypothetical protein	2237028	2236357	-
PGN_0492	-2.879712	cation-transporting ATPase	541825	539618	-
PGN_0719	-2.8275413	ABC transporter permease protein	785159	782790	+
PGN_1917	-2.8210944	ABC transporter ATP-binding protein	2139147	2137393	-
PGN_2066	-2.8205346	ABC transporter ATP-binding protein	2318463	2316526	-
PGN_1046	-2.799664	DNA repair protein	1164981	1164289	+
PGN_0683	-2.7867493	TonB-linked receptor Tlr	743879	741759	-
PGN_1475	-2.760782	5'-methylthioadenosine/S- adenosylhomocysteine nucleosidase	1651509	1650823	-
PGN_0936	-2.756032	glycerate dehydrogenase	1043583	1042630	+
<i>mazG</i>	-2.7443623	nucleoside triphosphate pyrophosphohydrolase	447698	446913	+
PGN_1962	-2.7412323	hypothetical protein	2192571	2191513	-
PGN_1217	-2.7402767	hypothetical protein	1357895	1357785	+
PGN_1777	-2.7231344	bleomycin hydrolase	1998918	1997647	-
PGN_1234	-2.6844543	hypothetical protein	1380269	1378668	-
PGN_0445	-2.6547398	membrane fusion efflux protein	487315	486209	-
PGN_0084	-2.64699	DNA topoisomerase I	91156	89027	+
PGN_1227	-2.621258	TPR domain protein	1372175	1370001	+
PGN_1723	-2.5895814	hypothetical protein	1924163	1922976	+
PGN_1200	-2.582709	recombination factor protein RarA	1336127	1334823	+
PGN_1814	-2.572983	tRNA 2-selenouridine synthase	2039152	2038118	+
PGN_0005	-2.540678	hypothetical protein	5041	3887	-
PGN_1497	-2.5399236	iron-sulfur cluster binding protein	1674005	1673034	-
PGN_1974	-2.5278996	hypothetical protein	2211090	2210566	+
PGN_0836	-2.5273746	hypothetical protein	929554	926879	-
PGN_1744	-2.523222	hypothetical protein	1957500	1956817	-
Name	Normalised Ratio	Description	End	Start	Strand
PGN_1350	-2.5114216	hypothetical protein	1509077	1508085	-
PGN_1808	-2.508868	hypothetical protein	2031652	2030210	+
PGN_0484	-2.5018524	galactokinase	532437	531283	+
<i>fimC</i>	-2.482772	minor component FimC	193873	192485	+

PGN_1180	-2.469481	hypothetical protein	1313111	1312263	-
PGN_0504	-2.4622993	methylmalonyl-CoA decarboxylase beta subunit	551430	550279	+
PGN_0519	-2.4528226	competence protein	568076	566592	+
PGN_0334	-2.440459	hypothetical protein	366042	364744	+
PGN_0885	-2.432753	nitroimidazole resistance protein	981476	980940	-
PGN_1797	-2.430033	hypothetical protein	2019256	2018735	-
PGN_2033	-2.4195694	ATP-dependent RNA helicase	2278437	2276623	-
<i>fimE</i>	-2.406615	minor component FimE	197549	195897	+
PGN_0071	-2.388865	hypothetical protein	76942	76589	-
PGN_1017	-2.387608	hypothetical protein	1132352	1131963	+
PGN_0014	-2.3846263	hypothetical protein	20517	18271	+
PGN_1837	-2.381192	hypothetical protein	2067926	2067081	+
PGN_0520	-2.362276	shikimate kinase	568575	568015	-
PGN_0010	-2.312466	L-threonine-O-3-phosphate decarboxylase	14206	13178	+
PGN_0786	-2.2908467	hypothetical protein	859363	858011	-
PGN_1960	-2.288571	CRISPR-associated protein Cas1	2190889	2189873	-
PGN_1879	-2.2795934	hypothetical protein	2097292	2097131	+
PGN_1629	-2.2736477	hypothetical protein	1823141	1822068	-
PGN_1769	-2.261283	hypothetical protein	1985137	1984961	-
PGN_0006	-2.2510933	Na ⁺ -driven multidrug efflux pump	6473	5094	-
PGN_2074	-2.229063	hypothetical protein	2332404	2332150	-
PGN_0740	-2.2282743	hypothetical protein	808345	807914	+

7.4 Discussion:

The process of identifying gene function by traditional methods such as mutagenesis and phenotypic characterisation is a lengthy process. It is also costly if the work is carried out in a speculative fashion on genes of poorly understood or unknown function and may yield little worthwhile data. However, mutagenesis is essential in order for us to develop our understanding of virulence and other key bacterial processes such as metabolism. The creation of the transposon mutagenesis library used in this study allows researchers to approach mutagenesis in a global and quantitative fashion. Initial studies with this library identified genes that are essential for *in vitro* growth in *P. gingivalis*. It was found that 463 genes did not take up the transposon suggesting that they are essential for viability *in vitro* (Klein et al., 2012).

We adapted the technique used in the work of Klein *et al.*, (2012) to help pinpoint and identify genes involved in adherence, invasion and survival during *in vitro* cell based assays. By examining differences in the relative abundance of insertion mutants in input pools of bacteria and pools of bacteria following interaction with host cells we have firstly confirmed the roles of several well characterised genes in these processes (e.g. *serB*, *fimR*) and identified a number of genes of unknown function of interest for future work. By using the screening system we designed here, where many thousands of mutants can be assayed in parallel, we have identified a cohort of genes that are worthy of further investigation in these processes.

In order to confirm our hypothesis, that the technique could be used to identify genes involved in specific processes it was essential that we examine the data for genes whose function is already established. A substantial reduction in reads recovered in mutants with insertions in *serB* was observed (Fig 7.4 b).

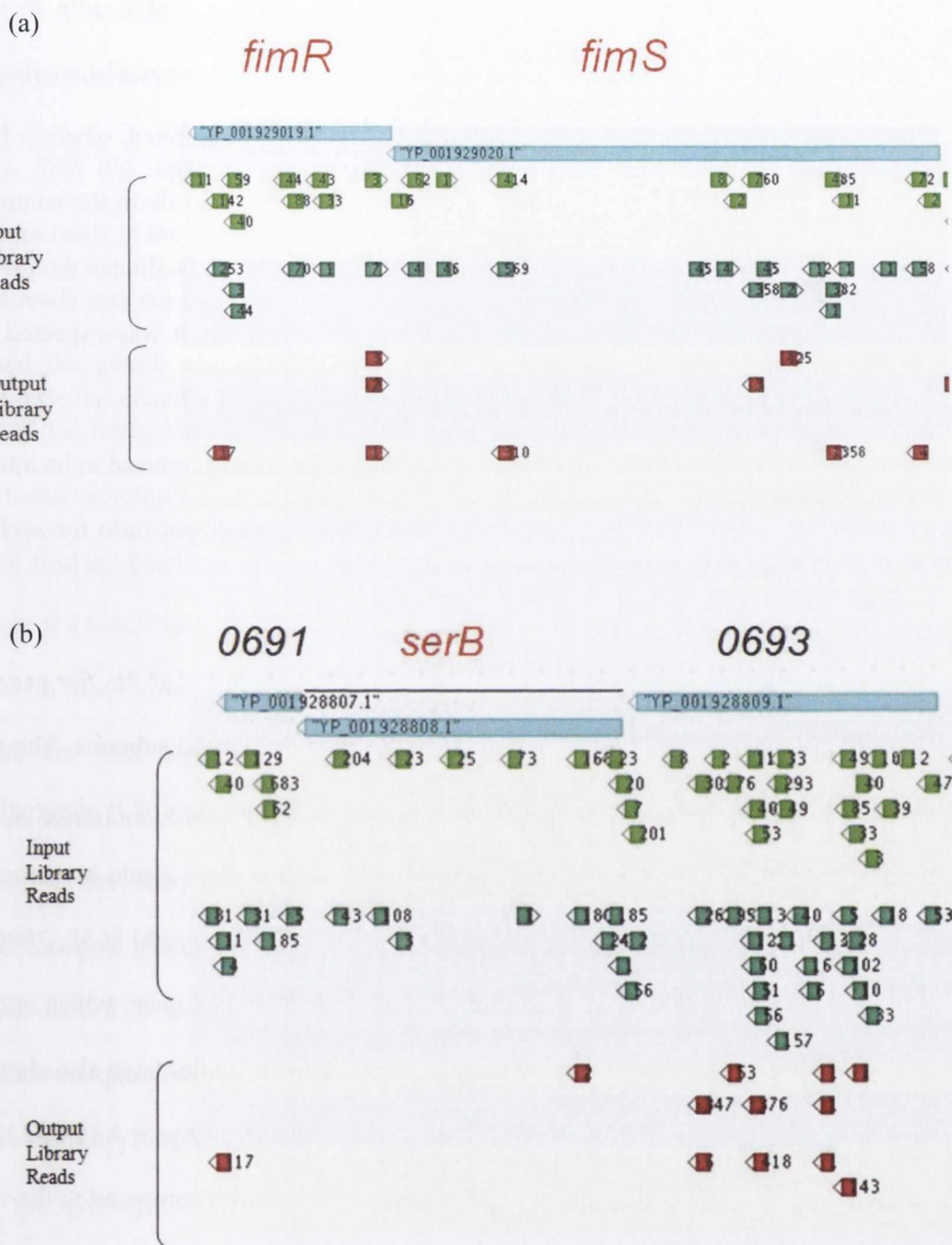


Figure 7.4: Diagram showing the location and abundance of insertions, generated using GenomeView. a) Shows the location and abundance of reads recovered for *fimR* and *fimS* in the input and output libraries. b) Shows the location and abundance of reads recovered for *serB* in the input and output libraries.

SerB is a key virulence factor of *P. gingivalis* and plays a central role in enabling dysbiosis. SerB plays an important part in internalisation and *P. gingivalis* survival within cells. When SerB comes into contact with epithelial cells it is secreted, where it has been shown to affect the cell signal transduction pathways that play a role in the control of the cytoskeleton architecture, by interfering with this architecture it facilitates the invasion of *P. gingivalis* into cells (Hasegawa et al., 2008). For this assay it was expected that we would see a reduction in *serB* reads in the output files. Without a functional *serB* gene due to the presence of the insertion the mutant, the bacterium is compromised in its interactions with host cells, and this is reflected in the normalised output/input ratio for *serB* (-3.76) (Table 7.1).

Significant reductions in abundance were observed for members of the *fim* gene family, including *fimR*, *fimC* and *fimE* (Fig 7.4a & 7.5) and other fimbrial subunits. The *fimR* and *fimS* gene encodes for proteins which play a role in the fimbriation of *P. gingivalis* and in biofilm formation. They form a two component system that plays a role in controlling the expression of *fimA* through a cascade regulation mechanism (Hayashi et al., 2000). FimR also plays a direct role in modulating the expression of the *mfal* gene, which encodes for the short fimbriae of *P. gingivalis* (Wu et al., 2007). Mutant studies have also shown a role for *fimS* in biofilm formation, as when *fimS* was inactivated in strain ATCC32277 it was found to have significantly reduced biofilm forming capabilities compared to the wild-type parental strain (Lo et al., 2010).

The *fimA* gene exhibited a reduction in abundance in the output samples but did not meet the 5-fold cut off for significance. The role of *fimA* has been extensively studied in *P. gingivalis*; it encodes for the adhesive subunit of the major fimbriae and is required for colonization of salivary coated surfaces and the colonization of dental plaque (Levesque et al., 2003, Malek et al., 1994). It is also a known adhesin for *P. gingivalis* and inactivation

studies have shown that in the absence of a functional *fimA* gene the ability of the organism to adhere to epithelial cells is significantly reduced (Njoroge et al., 1997).

Similarly, *fimS* did not exhibit a 5-fold reduction in output reads, with only a ~50% reduction in reads in the output samples. One reason that a more dramatic reduction in *fimS* and *fimA* reads was not observed could be due to the fact that not all insertions completely inactivated the genes, meaning that a functional protein is still expressed despite the insertion of the transposon. In the case of *fimA*, when the position of the Tn insertions are mapped using GenomeView, it can be seen that most of the sequence reads recovered from the output files are positioned at the 5' or 3' end of the gene and may not interfere with the translation of a functional FimA protein (Fig 7.4 a and 7.5). Similarly, a high proportion of insertions in *fimS* were recovered from the 5' end. Although Tn-Seq is a powerful technique, our data suggest that non-inactivating insertions may mask the effects of deleterious insertions, which may be a draw back in the use of Tn-Seq to study these types of processes. The assay also allowed for us to identify genes of poorly understood or unknown function which may play a role in adhesion, invasion and survival in cellular assays carried out *in vitro*. All of these genes had a 5 fold or greater reduction in their output reads compared to their input samples. One of these genes, PGN_0378, encodes for a putative exopolyphosphatase (PPX). Exopolyphosphatases are a group of enzymes which catalyse the hydrolysis of inorganic polyphosphate (poly-P). In many bacterial species including *Campylobacter jejuni*, poly-P is a key regulator of stress responses particularly the stringent response which is a regulatory mechanism in response to nutrient limitation (Jeon, 2014). These enzymes are also thought to play a role in virulence, in addition to their role in poly-P homeostasis. Mutant studies in *C. jejuni* showed that mutation of *ppx* genes resulted in defective biofilm formation, invasion, nutrient stress survival, invasion and intracellular survival (Malde et al., 2014).

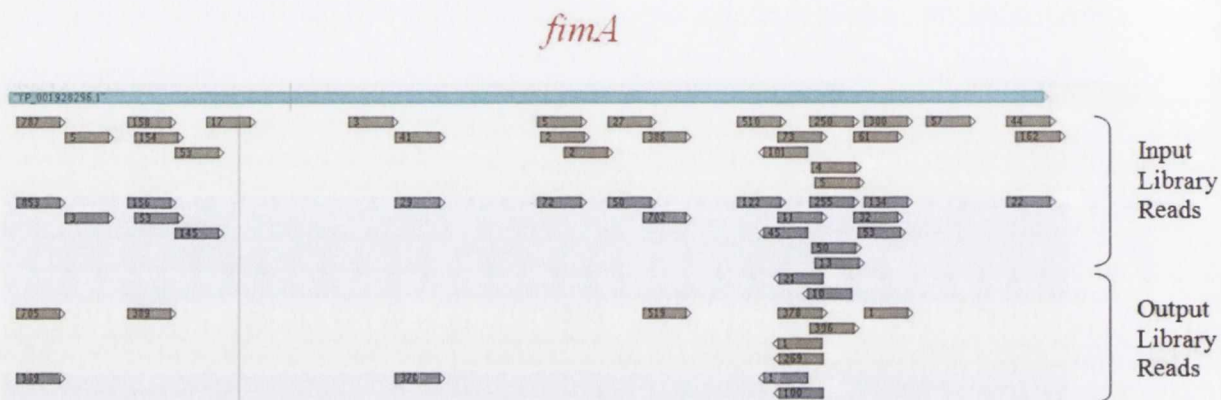
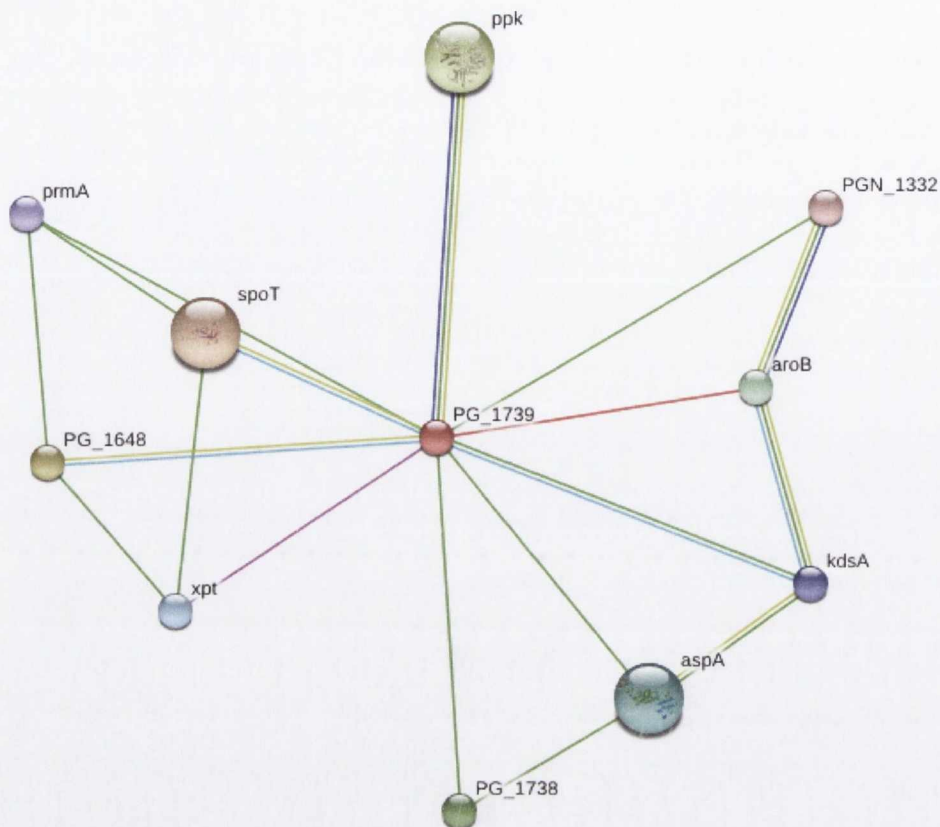


Figure 7.5: Diagram showing the input and output reads for the *fimA* gene. It can be seen clearly that the insertions in the output files are predominantly located at the 3'- and 5'- ends of the gene.

In *P. gingivalis*, although no studies have examined the role of exopolyphosphatases, a study has been carried out on a polyphosphate kinase (Ppk) which PGN_0378 is predicted to interact with (Fig 7.6). Mutations in Ppk were found to affect survivability and biofilm formation in *P. gingivalis* (Chen et al., 2002). Given this knowledge investigation of the function of PGN_0378 would be beneficial to our understanding of virulence and the bacterium as a whole.

PGN_1347 is a putative TonB dependent receptor exported protein; it clearly showed a decrease in output reads (Fig 7.7) and exhibited a normalised ratio of -4.67. TonB is important for transport of nutrient across the periplasm. In *P. gingivalis* it has been established that RagA and RagB which are outer membrane proteins associated with active transport of protein degradation products (Nagano et al., 2007) and virulence (Curtis et al., 1999) are also TonB dependent receptors. Analysis carried out in UniProt suggests that PGN_1347 may interact with RagA (Fig 7.8), as well as a number of other hypothetical proteins suggesting that PGN_1347 may have a similar function to RagA and or RagB. Similarly, PGN_1953 also encodes a putative TonB dependent outer membrane receptor and also exhibited a greatly reduced normalised ratio (-4.05).

PGN_1611 is a previously uncharacterized protein that contains leucine rich repeats (LRRs) and has homology to the internalin protein family found in *Listeria monocytogenes*.



Your Input:

- PG_1739 putative exopolyphosphatase (302 aa)
(*Porphyromonas gingivalis* 33277)

Predicted Functional Partners:

		Neighborhood	Gene Fusion	Cooccurrence	Coexpression	Experiments	Databases	Textmining	[Homology]	Score
spoT	GTP pyrophosphokinase; In eubacteria ppGpp (guanosine 3'-diphosphate 5'-diphosphate) is a medi [...]									0.960
PG_1648	GTP pyrophosphokinase (746 aa)									0.960
ppk	polyphosphate kinase; Catalyzes the reversible transfer of the terminal phosphate of ATP to for [...]									0.935
PG_1738	hypothetical protein (310 aa)									0.744
aroB	putative 3-dehydroquinase synthase (334 aa)									0.649
aspA	aspartate ammonia-lyase (475 aa)									0.621
xpt	xanthine phosphoribosyltransferase; Converts the preformed base xanthine, a product of nucleic [...]									0.520
kdsA	2-dehydro-3-deoxyphosphooctonate aldolase (272 aa)									0.495
prmA	ribosomal protein L11 methyltransferase; Methylates ribosomal protein L11 (By similarity) (311 aa)									0.480
PGN_1332	para-aminobenzoate synthase component I (325 aa)									0.472

Figure 7.6: Diagram outlining the potential interactions of PGN_0378, an exopolyphosphatase enzyme. An interaction with Ppk was observed among these interactions.

Your Input:

- PG_1006 putative TonB-dependent receptor exported protein (834 aa)
(*Porphyromonas gingivalis* 33277)

Predicted Functional Partners:

		Neighborhood	Gene Fusion	Cooccurrence	Coexpression	Experiments	Databases	Textmining	[Homology]	Score
PG_1005	hypothetical protein (246 aa)	•	•							0.802
PG_1007	putative transcriptional regulator (123 aa)	•								0.605
PG_1899	putative TonB-dependent receptor (794 aa)			•						0.566
PG_1010	probable ABC transporter ATP-binding protein (275 aa)	•								0.565
PG_0780	hypothetical protein (204 aa)			•						0.544
PG_1626	hypothetical protein (554 aa)			•						0.480
PG_0928	response regulator (518 aa)			•						0.479
PG_1008	hypothetical protein (252 aa)	•								0.470
PG_1009	hypothetical protein (284 aa)	•								0.470
ragA	receptor antigen A (1036 aa)			•				•		0.456

Figure 7.8: Diagram outlining the potential interactions of PGN_1347, a putative TonB dependent exported protein. An interaction with RagA was observed among these interactions.

Homologues to internalin have been found previously in *P. gingivalis* strain ATCC33277. PG0350 was found to be an InlJ type protein which is a cysteine-containing internalin protein (Sabet et al., 2005). Expression of an InlJ homologue was detected after contact with host epithelial cells (Chen et al., 2001b). However further investigation showed that a mutant of InlJ had no invasion related phenotype (Zhang et al., 2005b). Inactivation of the gene was found to reduce mono-species biofilm formation by *P. gingivalis*. However, the same mutation enhanced biofilm formation in the presence of *S. gordonii* suggesting that InlJ plays a nuanced role in biofilm formation by *P. gingivalis* (Capestany et al., 2006). Apart from the studies out-lined here, internalin homologues in *P. gingivalis* are a neglected group in terms of research. Given the importance of these proteins in other bacterial species such as *L. monocytogenes* (Pizarro-Cerda et al., 2012) establishing their role in *P. gingivalis* would be beneficial in understanding the bacterial as a whole.

The next step will be to inactivate these genes using mutagenesis based methods and to run the individual mutants through the adherence assay in order to provide proof of concept for this screening method. This is work that is currently ongoing in the laboratory and if successful will prove that this screening protocol can be used to identify a broad range of genes involved in specific processes using this methodology. This would allow for the rapid identification of targeted candidate genes for mutagenesis, these mutants could then be analysed under the specific relevant circumstances to fully establish their function.

Chapter 8

General Discussion

P. gingivalis plays a central role in the development of periodontitis. The pathogen's unique ability to alter the relationship between the host and the microbiota of the oral cavity triggers community wide effects which lead to the development of dysbiosis. *P. gingivalis* does not have to be present in large numbers in order for this to occur. Both of these traits have earned it the title of a "keystone" pathogen in relation to chronic forms of periodontitis (Hajishengallis and Lamont, 2012, Lamont and Hajishengallis, 2015).

In Chapter 1 the wealth of virulence factors that *P. gingivalis* possesses were described. Some of these virulence factors have been found to have wide reaching effects and evidence has been presented to suggest that they contribute to the systemic inflammation seen in some autoimmune diseases such as atherosclerosis (Saffi et al., 2015) and rheumatoid arthritis (Atanasova and Yilmaz, 2014).

The role of HagB and HagC in the pathogenicity of *P. gingivalis* has been postulated but remains to be fully elaborated on. It is well established that both genes are expressed during the infectious process under the control of different promoters (Lepine and Progulske-Fox, 1996), however whether expression varies between strains has yet to be clarified. *P. gingivalis* has extensive genomic rearrangements between strains which could lead to differential expression of these two genes amongst *P. gingivalis* strains (Naito et al., 2008).

It is important that the role of HagB and HagC in the virulence of *P. gingivalis* is elaborated on for a number of reasons. In order to understand how dysbiosis develops, we need to fully understand how *P. gingivalis* virulence factors contribute to the process either through their ability to orchestrate specific responses from the host and/or the microbial community and this includes establishing the role that HagB and HagC may play in the process. There is some evidence to suggest that HagB may contribute to the development of atherosclerosis by mediating adhesion to coronary arterial endothelial cells (Song et al.,

2005). In fact, the role of these genes in mediating interactions with HCAECs has been subjected to more intense investigation than their role in interactions with host cells and other bacteria in the oral cavity, a disparity that this study aimed to address.

It has previously been established that HagB is highly immunogenic and possesses strong potential as a vaccine antigen, which has been discussed in detail previously (Chapter 6). However a vaccine cannot be created until the effects of the protein being used have been fully studied and established *in vitro* and *in vivo*. In addition, evidence supporting the role of a vaccine antigen in adhesion and invasion *in vitro* would support its use as an effective vaccine antigen *in vivo* as antibodies would block colonisation by the bacteria. Several haemagglutinin proteins of viruses (influenza virus) and bacteria (*Bordetella pertussis*) have been successfully used as effective vaccines and neutralising antibodies against these proteins are postulated to be an important aspect of the protective response (Sato et al., 1984, Sridhar et al., 2015).

Here we aimed to create the first double $\Delta hagB/\Delta hagC$ mutant in *P. gingivalis* as well as a full panel of single and complemented mutants in order to fully understand how these genes contribute to the virulence of the organism as a whole. Using the same plasmid which created the previous $\Delta hagB$ mutants (Progulske-Fox et al., 1995) we created $\Delta hagB$ mutants in strain W83 (ECM100) and strain ATCC33277 ($\Delta hagB$ ATCC33277). In order to successfully create a $\Delta hagB/\Delta hagC$ mutant in any strain of *P. gingivalis* *hagC* has to first be fully removed from the genome; this is in order to prevent a double disruption event of both *hagB* and *hagC* that can occur when using plasmid pJW1 to interrupt *hagB* (Chapter 3, Fig 3.6).

We designed a *hagC* deletion cassette for both strain W83 and ATCC33277 in order to facilitate the creation of a double *hagB/hagC* mutant in both strains so that a fimbriated and non-fimbriated strain could be compared to one another. However, on transformation

only strain W83 yielded successful transformants despite multiple attempts in strain ATCC33277. This could be because *hagB* and *hagC* have differential roles and value in the individual strains of *P. gingivalis* as suggested by our data. We clearly demonstrate that *hagB* is not essential to either strain but that *hagC* may be essential to strain ATCC33277.

Due to the fact that *hagC* may be essential in strain ATCC33277 we were unable to create a Δ *hagB*/ Δ *hagC* mutant in this strain. However, we successfully generated this mutant in the faster growing, robust strain W83. This was achieved by first deleting *hagC* from the genome and subsequently interrupting *hagB* with plasmid pJW1. This prevented the double disruption event discussed previously and allowed us to confirm that in this strain neither of these genes are essential, a fact that is further supported by the ability of pJW1 to recombine with both genes and remove the majority of the locus from the genome of W83 (Chapter 3, Fig 3.6).

This is not the first time that variations in the ability of *P. gingivalis* strains to cope with the removal of these genes have been reported. In strain Pg381 it has been shown that unlike ATCC33277, single mutants of each gene can be created (Lepine et al., 1996). However to date no successful double mutant has been made in this strain. This further supports the theory that the essentiality of these genes varies depending on strain type. Strain W83 is robust enough to survive mutation of both of these genes whereas strain Pg381 can tolerate the mutation of either *hagB* or *hagC*, and strain ATCC33277 only tolerates mutation of *hagB*. This could be due to the previously mentioned genomic rearrangements in these strains, which could generate variation that influences bacterial survival post-transformation. Strain W83 is a faster growing strain *in vitro* compared to ATCC33277 and Pg381 and this may be due to more efficient mechanisms of haem uptake, resulting in a strain that may better tolerate mutagenesis of haemagglutinins.

The presence of variant sub-populations within a strain may also influence the outcome of mutagenic studies, as mutagenesis may only be possible in variant sub-populations that express characteristics required for survival in the absence of the gene in question. Proteomic differences between the strains may also influence outcome. Strain W83 is a non fimbriated strain of *P. gingivalis*, whereas both Pg381 and ATCC33277 possess fimbriae. It could be that fimbriated strains possess weaker cell-surface associated haemagglutinin activity, as *fimA* itself contributes to haemagglutination. While these strains can survive in the absence of the *fimA* gene, it appears that *hagB* and/or *hagC* may be essential for haem acquisition in these strains. In non-fimbriated strains it is possible that in the absence of fimbriae, other mediators of adhesion and haemagglutination are expressed to compensate, such as the gingipains (Chen and Duncan, 2004). This could be examined further by examining if *hagB* and *hagC* could be removed from a second non-fimbriated strain of *P.gingivalis*, such as strain W50.

All of the mutant strains displayed altered phenotypes compared to WT in a number of areas, including pigmentation, adhesion, invasion, biofilm formation and immunostimulation. In particular strain ECM233 and GMC100 gave unexpected results. The most apparent of these phenotypic differences was seen in pigmentation on solid media and all of the mutant strains displayed some degree of defect (Chapter 4 Fig 4.1). The single mutants including strain GMC100 were a light shade of brown/green while the strongest defect was seen in strain ECM233 which was cream in colour. The appearance of these mutants is similar to that described in gingipain and *hagA* mutants (Shi et al., 1999). Our $\Delta hagB/\Delta hagC$ double mutant produced colonies similar to those observed when a triple gingipain mutant was examined by Shi *et al* (1999) This highlights that *hagB* and *hagC* may be as important to the process of haemagglutination as members of the gingipain family. It also indicates that *hagB* and *hagC* are potentially co-expressed under the examined conditions and that they act together to facilitate haem acquisition. Our findings

highlight that in strain W83 *hagB* and *hagC* are of equal importance in haem acquisition to *rgpA*, *kgp* and *hagA*. The data also indicate that the capacity of *P. gingivalis* to acquire haem would have been significantly enhanced after the gene duplication event which created the *hagB/hagC* locus. This is likely to have been an important evolutionary event which would have coincided with the expansion of the gingipain family. The work presented here also firmly disputes the theory that *rgpA*, *kgp* and *hagA* are the sole mediators of haemagglutination in *P. gingivalis*, as this study demonstrates a substantial role for *hagB* and *hagC* in this process, which is backed by previous studies carried out in single mutant strains (Lepine et al., 1996).

Our qPCR data also exemplifies the importance of *hagB* and *hagC*. Our data shows that *rgpA* and *rgpB* are steadily expressed in wild-type W83 and the single *hag* mutants but not in strain ECM233 (Chapter 5, Fig 5.17). The single mutants retain a similar level of well controlled expression compared to WT however in the Δ *hagB*/ Δ *hagC* mutant gene expression becomes deregulated. Although we have only examined the expression of a few candidate genes it is likely that other changes in gene expression are present in this strain that might account for the aberrant increased adherence and the increased immunogenicity observed in strain ECM233. There are two possible explanations for the changes in gene expression and the apparent gain of function in adherence and immunogenicity. Firstly, this may be a direct response to the stress of haem starvation associated with deletion of both *hagB* and *hagC*. However complementation with *hagB* in GMC100, which restored pigment production and therefore haem acquisition, did not alleviate this expression pattern or the hyper-adherent phenotypes of the double *hagB/hagC* mutant. If this was a transient response to haem starvation, restoration of *hagB* to the genome would have reversed it, which suggests that in this case the observed phenotype may be the result of a stable, heritable modification in the strains themselves. Alternatively, the mutant may have been generated in a background where gingipain expression was deregulated. Such a

phenomenon has already been described where *rgpA* is one of the genes that exhibited variable expression in bi-stable populations of *P. gingivalis*. Conceivably, this could result in sub-populations of cells that might tolerate some mutations better than others and our $\Delta hagB/\Delta hagC$ mutant may have been generated in such a bistable sub-population. Whole genome transcript profiling of the mutants might possibly reveal if selection of a variant subpopulation has occurred during mutant construction.

Further studies were carried out to determine if *hagB* and *hagC* contribute to the ability of *P. gingivalis* to adhere to and invade an oral epithelial cell line, an aspect of their function which has been neglected. Two important discoveries were made during this part of the study, one in strain ATCC33277 and one in strain W83.

Strain W83 is reliant on non-fimbrial adhesins such as the gingipains for attachment (Kesavalu et al., 2003). The findings of this study showed that as with haemagglutination, *hagB* and *hagC* are central to the adherence process. Removal of either *hagB* or *hagC* had a significant impact on the ability of *P. gingivalis* strain W83 to adhere to epithelial cells which is either directly due to mutagenesis or is an indirect effect of the mutagenic process as discussed in chapter 5. Although the role of HagB in mediating adherence to HCAECs has been described, this is the first study to implicate these proteins in mediating adherence to oral cells. The second important discovery relates to strain ATCC33277, a fimbriated strain of *P. gingivalis*. In this study we compared the wild-type ATCC33277 strain to a *hagB* mutant, constructed in the current study and a previously studied *fimA* mutant (Chapter 5). Previous work carried out on the *fimA* mutant has highlighted that this protein makes a large contribution to the adherence capabilities of the bacterium (Njoroge et al., 1997). Here we found that removal of *hagB* from the ATCC33277 genome also had a substantial affect on the ability of the organism to adhere to H357 cells. When *hagB* was interrupted it was found that there was a ten-fold decrease in the ability of the strain to

adhere to H357 cells. Deletion of *fimA* also had a significant effect, confirming the work of previous studies carried out on this gene. These data indicate that the process of adherence in this strain is multi-factorial, involving both fimbrial and non-fimbrial adhesins. Adherence in this strain may be a two stage process with both FimA and HagB playing important roles (Fig 8.1). It is clear that FimA plays an essential role in mediating adherence to H357 cells, the data presented here highlights that HagB may play a role in stabilising this interaction. Alternatively the phenotype seen in the *hagB* mutant is due to an indirect effect caused by mutagenesis (Fig 8.2).

Given the affects seen in adhesion and invasion it was hypothesised that not only host cell interactions would be affected but that potentially interactions with other microbial species would be altered. For this reason biofilm studies were carried out which highlighted a role for the *hag* genes in both mono and multi-species biofilms. When grown in mono-species biofilms it was found that ECM200 and ECM233 had a significant defect in the ability to form single species biofilms when compared to the WT strain, with the most significant reduction been seen in strain ECM233, as emphasised by CFSE staining. Our data indicates that in the absence of both genes strain ECM233 has a reduced capacity to bind to itself, a fact which was previously unknown.

One of the most significant findings was the role of *hagC* in biofilm formation which is a novel finding. In a multi-species model the importance of *hagC* in maintaining a biofilm of *P. gingivalis*, *S. mitis*, *F. nucleatum* and *A. actinomycetemcomitans* was shown. There were no differences in the overall species composition of each of the biofilms (Chapter 5 Fig 5.11) however when the biomass of each of the biofilms was examined it was shown that multi-species biofilms incorporating strain ECM200 had a statistically significant reduction in biomass when compared to the other strains. It is difficult to postulate exactly why these biofilms exhibited such a reduced biomass, but this could indicate a role for

HagC in interspecies co-aggregation. HagC may also be involved in interspecies communication and could stimulate the production of adhesins or polysaccharides by other bacteria in the biofilm, although more extensive studies are required to determine this.

This study has highlighted several new important findings about the biology of *P. gingivalis*. Firstly the results of the work carried out here indicate that HagB and HagC are essential to the pathogenicity of *P. gingivalis*, with multiple roles. We clearly demonstrate a role for them in nutrient acquisition as haemagglutinins, adhesins and a previously unknown role in biofilm formation. The data also suggests that a number of our mutants have been selected out from a sub-population of *P. gingivalis* which has a more robust survival phenotype; this is particularly true in strain ECM233. The presence of these bi-stable populations in *P. gingivalis* will directly impact mutagenic studies. These sub-populations are naturally present prior to mutagenesis; however it may be that during the process of deleting or interrupting a gene that populations that are better able to survive the transformation process are selected in these experiments. These data indicate that it is essential that we characterise *P. gingivalis* populations in detail so that the pre-existing genetic traits of these sub-populations can be established prior to mutagenesis. This would allow us to identify if such selection has occurred and for the affects of bi-stability to be taken into account when analysing subsequent data. Efforts have been made previously to understand the dynamics of the *P. gingivalis* populations (Suwannakul et al., 2010) but further genetic analysis of wild type strains and any mutant strains that have been created in their background would allow for a deepening of this understanding.

Studies have indicated that *in vivo* there is also high intra-individual heterogeneity in the *P. gingivalis* population (Enersen, 2011), potentially highlighting the presence of bi-stable populations *in vivo* within the oral cavity, where certain genes will be expressed differently. This raises interesting questions about the immunogenicity of various virulence

factors and their potential as vaccine antigens (Jong and van der Reijden, 2010). A key trait of a vaccine antigen is that the molecule is always present in the pathogen in order to stimulate the immune response and to be targeted by T-cell and B-cell responses.

The presence of a bi-stable population needs to be taken into consideration during vaccine design as variation in expression of an antigen could mean that a small sub-population of bacteria without the antigen can still infect and trigger the development of disease. In the case of HagB, current data suggests that the gene is present in all strains and constitutively expressed, indicating that it might be a good vaccine candidate. One approach to determine whether immune responses to HagB are present in patients with chronic periodontitis would be to examine antibody levels in GCF and serum. It would be interesting to correlate these data with the presence of the microorganism, the clinical parameters of the infection and whether a strong antibody response was associated with reduced rates of recurrent infections.

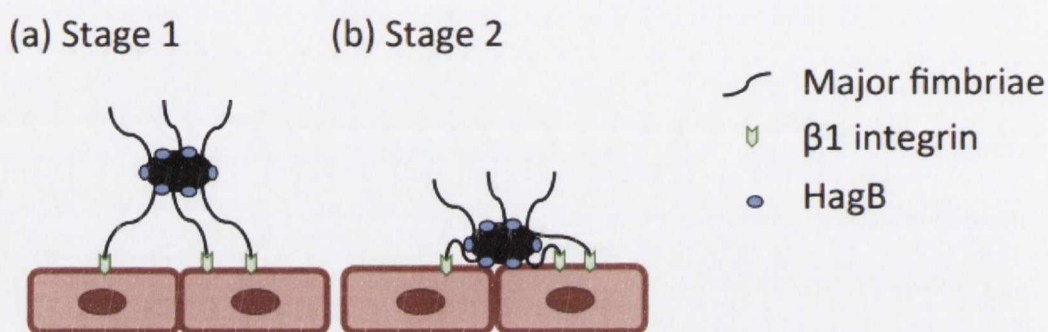


Figure 8.1: Diagram outlining a two stage model of adherence in *P. gingivalis*. (a) Stage 1 of the interactions is a long range interaction mediated by the FimA subunit of the major fimbriae interacting with $\beta 1$ -integrins present on the surface of epithelial cells. (b) Stage 2 occurs after the bacteria has moved closer to the cells and is a short range interaction mediated by HagB and an unknown receptor which acts to stabilise the interaction between the bacterium and the host cell surface.

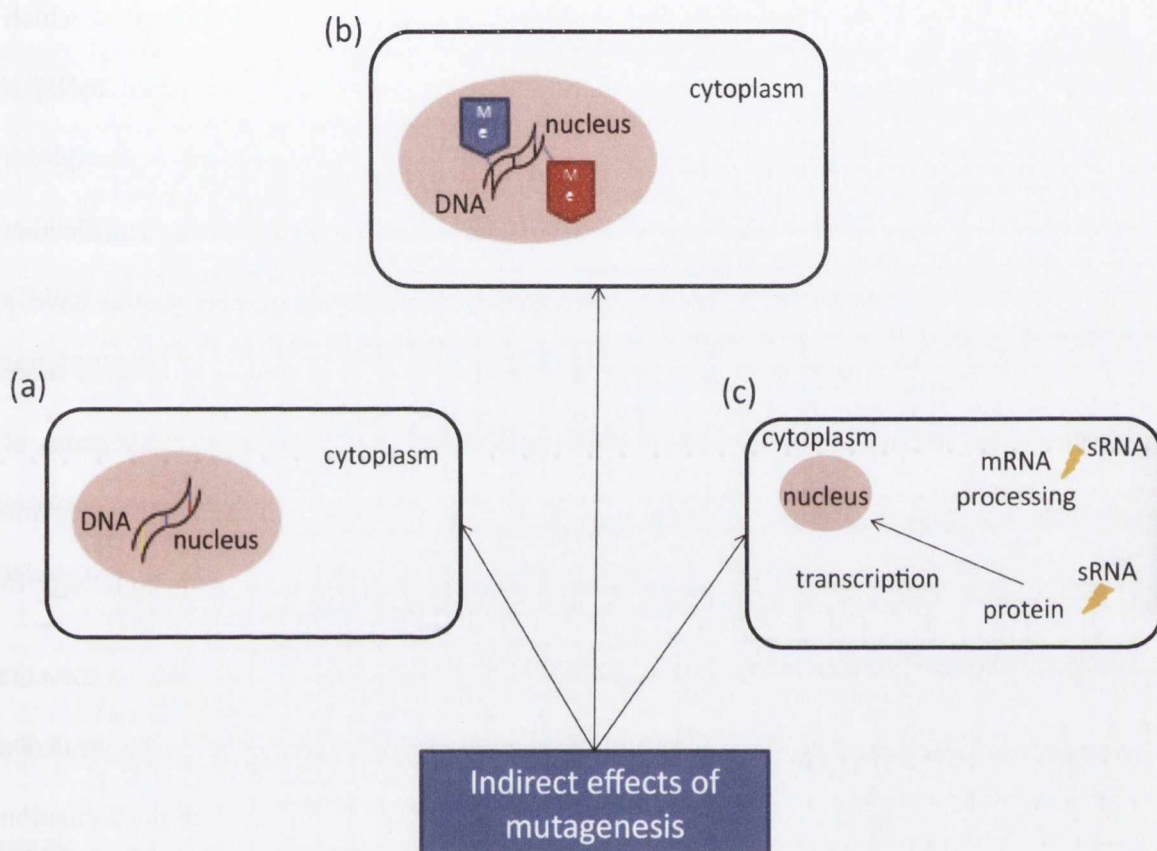


Figure 8.2: Diagram showing the indirect effects that mutagenesis may have. (a) The development of a compensatory secondary mutation (red) after the primary mutation is introduced (yellow). (b) Mutagenesis can affect the bacterial epi-genome leading to changes in bacterial epigenetics such as alterations to methylation patterns. (c) Mutagenesis may interfere with sRNA molecules and as such RNA regulatory. This will affect key processes where sRNAs play a role such as 1. mRNA processing and 2. sRNA protein binding involved in genomic transcription regulation.

Another aspect of this study was the development of a transposon mutagenesis library based screening process to help identify genes that play a role in the process of adherence, invasion and survival under certain conditions. We demonstrated the utility of this approach, as a number of expected genes showed a significant reduction in abundance in the output samples. We are currently creating mutant strains in genes of interest which showed a 5-fold or greater reduction in out-put sequencing reads compared to input sequencing reads. If successful it will serve to highlight how screening a transposon mutagenesis library through an assay or experimental set-up allows for rapid identification of genes which may be involved in the process being examined. This would have a significant experimental impact, as it would allow for mutagenic studies to become more precise, as well as allowing for our knowledge of poorly understood genes or genes of unknown function to develop. In our study we have identified several candidate genes that may be involved in stress responses, nutrient acquisition and a gene with homology to internalin that could play a role in cellular invasion.

Overall we have demonstrated a clear and concise role for *hagB* and *hagC* in the virulence of *P. gingivalis*. We have successfully identified a specific role for *hagC* in the formation of the multi-species biofilm. Our data suggests that bi-stable populations within *P. gingivalis* influence the outcome of mutagenesis and that this needs careful consideration when carrying out gene deletion experiments, as these sub-populations have stable heritable traits which will influence data. We also developed a rapid screening process which will allow for targeted mutagenesis studies in the future that can identify genes which are relevant to the process being investigated.

References:

- ABBAYYA, K., PUTHANAKAR, N. Y., NADUWINMANI, S. & CHIDAMBAR, Y. S. 2015. Association between Periodontitis and Alzheimer's Disease. *N Am J Med Sci*, 7, 241-6.
- AL-QUTUB, M. N., BRAHAM, P. H., KARIMI-NASER, L. M., LIU, X., GENCO, C. A. & DARVEAU, R. P. 2006. Hemin-dependent modulation of the lipid A structure of *Porphyromonas gingivalis* lipopolysaccharide. *Infect Immun*, 74, 4474-85.
- ALJEHANI, Y. A. 2014. Risk factors of periodontal disease: review of the literature. *Int J Dent*, 2014, 182513.
- AMANO, A., NAKAGAWA, I., OKAHASHI, N. & HAMADA, N. 2004. Variations of *Porphyromonas gingivalis* fimbriae in relation to microbial pathogenesis. *J Periodontal Res*, 39, 136-42.
- ANDRIAN, E., GRENIER, D. & ROUABHIA, M. 2004. *In vitro* models of tissue penetration and destruction by *Porphyromonas gingivalis*. *Infect Immun*, 72, 4689-98.
- ATANASOVA, K. R. & YILMAZ, O. 2014. Looking in the *Porphyromonas gingivalis* cabinet of curiosities: the microbium, the host and cancer association. *Mol Oral Microbiol*, 29, 55-66.
- BAINBRIDGE, B., VERMA, R. K., EASTMAN, C., YEHA, B., RIVERA, M., MOFFATT, C., BHATTACHARYYA, I., LAMONT, R. J. & KESAVALU, L. 2010. Role of *Porphyromonas gingivalis* phosphoserine phosphatase enzyme SerB in inflammation, immune response, and induction of alveolar bone resorption in rats. *Infect Immun*, 78, 4560-9.
- BANAS, J. A., BISWAS, S. & ZHU, M. 2011. Effects of DNA methylation on expression of virulence genes in *Streptococcus mutans*. *Appl Environ Microbiol*, 77, 7236-42.
- BELANGER, M., KOZAROV, E., SONG, H., WHITLOCK, J. & PROGULSKE-FOX, A. 2012. Both the unique and repeat regions of the *Porphyromonas gingivalis* hemagglutinin A are involved in adhesion and invasion of host cells. *Anaerobe*, 18, 128-34.
- BELANGER, M., RODRIGUES, P. & PROGULSKE-FOX, A. 2007. Genetic manipulation of *Porphyromonas gingivalis*. *Curr Protoc Microbiol*, Chapter 13, Unit13C 2.
- BIK, E. M., LONG, C. D., ARMITAGE, G. C., LOOMER, P., EMERSON, J., MONGODIN, E. F., NELSON, K. E., GILL, S. R., FRASER-LIGGETT, C. M. & RELMAN, D. A. 2010. Bacterial diversity in the oral cavity of 10 healthy individuals. *ISME J*, 4, 962-74.
- BLACK, G. V. 1884. *The formation of poisons by micro-organisms. A biological study of the germ theory of disease.* By G. V. Black, Philadelphia, P. Blakiston, son & co.
- BOISVERT, H. & DUNCAN, M. J. 2010. Translocation of *Porphyromonas gingivalis* gingipain adhesin peptide A44 to host mitochondria prevents apoptosis. *Infect Immun*, 78, 3616-24.
- BORGWARDT, D. S., MARTIN, A. D., VAN HEMERT, J. R., YANG, J., FISCHER, C. L., RECKER, E. N., NAIR, P. R., VIDVA, R., CHANDRASHEKARAIAH, S., PROGULSKE-FOX, A., DRAKE, D., CAVANAUGH, J. E., VALI, S., ZHANG, Y. & BROGDEN, K. A. 2014. Histatin 5 binds to *Porphyromonas gingivalis* hemagglutinin B (HagB) and alters HagB-induced chemokine responses. *Sci Rep*, 4, 3904.
- BOSTANCI, N. & BELIBASAKIS, G. N. 2012. *Porphyromonas gingivalis*: an invasive and evasive opportunistic oral pathogen. *FEMS Microbiol Lett*, 333, 1-9.
- BROWN, L. J., JOHNS, B. A. & WALL, T. P. 2002. The economics of periodontal diseases. *Periodontol 2000*, 29, 223-34.
- BRUNNER, J., SCHERES, N., EL IDRISSI, N. B., DENG, D. M., LAINE, M. L., VAN WINKELHOFF, A. J. & CRIELAARD, W. 2010a. The capsule of *Porphyromonas gingivalis* reduces the immune response of human gingival fibroblasts. *BMC Microbiol*, 10, 5.
- BRUNNER, J., WITTINK, F. R., JONKER, M. J., DE JONG, M., BREIT, T. M., LAINE, M. L., DE SOET, J. J. & CRIELAARD, W. 2010b. The core genome of the anaerobic oral pathogenic bacterium *Porphyromonas gingivalis*. *BMC Microbiol*, 10, 252.
- CAPESTANY, C. A., KUBONIWA, M., JUNG, I. Y., PARK, Y., TRIBBLE, G. D. & LAMONT, R. J. 2006. Role of the *Porphyromonas gingivalis* InlJ protein in homotypic and heterotypic biofilm development. *Infect Immun*, 74, 3002-5.

- CHAMPAIBOON, C., POOLGESORN, M., WISITRASAMEEWONG, W., SA-ARD-IAM, N., RERKYEN, P. & MAHANONDA, R. 2014. Differential inflammasome activation by *Porphyromonas gingivalis* and cholesterol crystals in human macrophages and coronary artery endothelial cells. *Atherosclerosis*, 235, 38-44.
- CHEN, T., DONG, H., TANG, Y. P., DALLAS, M. M., MALAMY, M. H. & DUNCAN, M. J. 2000. Identification and cloning of genes from *Porphyromonas gingivalis* after mutagenesis with a modified Tn4400 transposon from *Bacteroides fragilis*. *Infect Immun*, 68, 420-3.
- CHEN, T. & DUNCAN, M. J. 2004. Gingipain adhesin domains mediate *Porphyromonas gingivalis* adherence to epithelial cells. *Microb Pathog*, 36, 205-9.
- CHEN, T., HOSOGI, Y., NISHIKAWA, K., ABBEY, K., FLEISCHMANN, R. D., WALLING, J. & DUNCAN, M. J. 2004. Comparative whole-genome analysis of virulent and avirulent strains of *Porphyromonas gingivalis*. *J Bacteriol*, 186, 5473-9.
- CHEN, T., NAKAYAMA, K., BELLIVEAU, L. & DUNCAN, M. J. 2001a. *Porphyromonas gingivalis* gingipains and adhesion to epithelial cells. *Infect Immun*, 69, 3048-56.
- CHEN, W., LAIDIG, K. E., PARK, Y., PARK, K., YATES, J. R., 3RD, LAMONT, R. J. & HACKETT, M. 2001b. Searching the *Porphyromonas gingivalis* genome with peptide fragmentation mass spectra. *Analyst*, 126, 52-7.
- CHEN, W., PALMER, R. J. & KURAMITSU, H. K. 2002. Role of polyphosphate kinase in biofilm formation by *Porphyromonas gingivalis*. *Infect Immun*, 70, 4708-15.
- COLOMBO, A. V., SILVA, C. M., HAFFAJEE, A. & COLOMBO, A. P. 2006. Identification of oral bacteria associated with crevicular epithelial cells from chronic periodontitis lesions. *J Med Microbiol*, 55, 609-15.
- CURTIS, M. A., HANLEY, S. A. & ADUSE-OPOKU, J. 1999. The *rag* locus of *Porphyromonas* : a novel pathogenicity island. *J Periodontal Res*, 34, 400-5.
- CURTIS, M. A., PERCIVAL, R. S., DEVINE, D., DARVEAU, R. P., COATS, S. R., RANGARAJAN, M., TARELLI, E. & MARSH, P. D. 2011. Temperature-dependent modulation of *Porphyromonas gingivalis* lipid A structure and interaction with the innate host defenses. *Infect Immun*, 79, 1187-93.
- DARVEAU, R. P. 2010. Periodontitis: a polymicrobial disruption of host homeostasis. *Nat Rev Microbiol*, 8, 481-490.
- DARVEAU, R. P., BELTON, C. M., REIFE, R. A. & LAMONT, R. J. 1998. Local chemokine paralysis, a novel pathogenic mechanism for *Porphyromonas gingivalis*. *Infect Immun*, 66, 1660-5.
- DARVEAU, R. P., HAJISHENGALLIS, G. & CURTIS, M. A. 2012. *Porphyromonas gingivalis* as a Potential Community Activist for Disease. *J Dent Res*.
- DASHPER, S. G., ANG, C. S., VEITH, P. D., MITCHELL, H. L., LO, A. W., SEERS, C. A., WALSH, K. A., SLAKESKI, N., CHEN, D., LISSEL, J. P., BUTLER, C. A., O'BRIEN-SIMPSON, N. M., BARR, I. G. & REYNOLDS, E. C. 2009. Response of *Porphyromonas gingivalis* to heme limitation in continuous culture. *J Bacteriol*, 191, 1044-55.
- DEWHIRST, F. E., CHEN, T., IZARD, J., PASTER, B. J., TANNER, A. C., YU, W. H., LAKSHMANAN, A. & WADE, W. G. 2010. The human oral microbiome. *J Bacteriol*, 192, 5002-17.
- DIAZ, P. I. & ROGERS, A. H. 2004. The effect of oxygen on the growth and physiology of *Porphyromonas gingivalis*. *Oral Microbiol Immunol*, 19, 88-94.
- DIERICKX, K., PAUWELS, M., LAINE, M. L., VAN ELDERE, J., CASSIMAN, J. J., VAN WINKELHOFF, A. J., VAN STEENBERGHE, D. & QUIRYNEN, M. 2003. Adhesion of *Porphyromonas gingivalis* serotypes to pocket epithelium. *J Periodontol*, 74, 844-8.
- DIYA, Z., LILI, C., SHENGLAI, L., ZHIYUAN, G. & JIE, Y. 2008. Lipopolysaccharide (LPS) of *Porphyromonas gingivalis* induces IL-1 β , TNF- α and IL-6 production by THP-1 cells in a way different from that of *Escherichia coli* LPS. *Innate Immun*, 14, 99-107.
- DOCAMPO, R. & MORENO, S. N. 1990. The metabolism and mode of action of gentian violet. *Drug Metab Rev*, 22, 161-78.
- DORN, B. R., DUNN, W. A., JR. & PROGULSKE-FOX, A. 1999. Invasion of human coronary artery cells by periodontal pathogens. *Infect Immun*, 67, 5792-8.

- DOU, Y., ARUNI, W., LUO, T., ROY, F., WANG, C. & FLETCHER, H. M. 2014. Involvement of PG2212 zinc finger protein in the regulation of oxidative stress resistance in *Porphyromonas gingivalis* W83. *J Bacteriol*, 196, 4057-70.
- DOU, Y., OSBOURNE, D., MCKENZIE, R. & FLETCHER, H. M. 2010. Involvement of extracytoplasmic function sigma factors in virulence regulation in *Porphyromonas gingivalis* W83. *FEMS Microbiol Lett*, 312, 24-32.
- DURAN-PINEDO, A. E., CHEN, T., TELES, R., STARR, J. R., WANG, X., KRISHNAN, K. & FRIAS-LOPEZ, J. 2014. Community-wide transcriptome of the oral microbiome in subjects with and without periodontitis. *ISME J*, 8, 1659-72.
- DUSEK, D. M., PROGULSKE-FOX, A. & BROWN, T. A. 1994. Systemic and mucosal immune responses in mice orally immunized with avirulent *Salmonella typhimurium* expressing a cloned *Porphyromonas gingivalis* hemagglutinin. *Infect Immun*, 62, 1652-7.
- EBERHARD, J., JEPSEN, S., JERVOE-STORM, P. M., NEEDLEMAN, I. & WORTHINGTON, H. V. 2008a. Full-mouth disinfection for the treatment of adult chronic periodontitis. *Cochrane Database Syst Rev*, CD004622.
- EBERHARD, J., JERVOE-STORM, P. M., NEEDLEMAN, I., WORTHINGTON, H. & JEPSEN, S. 2008b. Full-mouth treatment concepts for chronic periodontitis: a systematic review. *J Clin Periodontol*, 35, 591-604.
- ENERSEN, M. 2011. *Porphyromonas gingivalis*: a clonal pathogen?: Diversities in housekeeping genes and the major fimbriae gene. *J Oral Microbiol*, 3.
- ENERSEN, M., NAKANO, K. & AMANO, A. 2013. *Porphyromonas gingivalis* fimbriae. *J Oral Microbiol*, 5.
- FOEY, A. D. & CREAN, S. 2013. Macrophage subset sensitivity to endotoxin tolerisation by *Porphyromonas gingivalis*. *PLoS One*, 8, e67955.
- FRIEDEN, T. R. 2013. CDC Health Disparities and Inequalities Report - United States, 2013. Foreword. *MMWR Surveill Summ*, 62 Suppl 3, 1-2.
- GADDIS, D. E., MICHALEK, S. M. & KATZ, J. 2009. Requirement of TLR4 and CD14 in dendritic cell activation by Hemagglutinin B from *Porphyromonas gingivalis*. *Mol Immunol*, 46, 2493-504.
- GADDIS, D. E., MICHALEK, S. M. & KATZ, J. 2011. TLR4 signaling via MyD88 and TRIF differentially shape the CD4+ T cell response to *Porphyromonas gingivalis* hemagglutinin B. *J Immunol*, 186, 5772-83.
- GAO, J. L., NGUYEN, K. A. & HUNTER, N. 2010. Characterization of a hemophore-like protein from *Porphyromonas gingivalis*. *J Biol Chem*, 285, 40028-38.
- GAO, L., XU, Y., MENG, S., WU, Y., HUANG, H., SU, R. & ZHAO, L. 2012. Identification of the Putative Specific Pathogenic Genes of *Porphyromonas gingivalis* with Type II Fimbriae. *DNA Cell Biol*.
- GAWRONSKI, J. D., WONG, S. M., GIANNOUKOS, G., WARD, D. V. & AKERLEY, B. J. 2009. Tracking insertion mutants within libraries by deep sequencing and a genome-wide screen for Haemophilus genes required in the lung. *Proc Natl Acad Sci U S A*, 106, 16422-7.
- GEMMELL, E., YAMAZAKI, K. & SEYMOUR, G. J. 2007. The role of T cells in periodontal disease: homeostasis and autoimmunity. *Periodontol 2000*, 43, 14-40.
- GHAZ-JAHANIAN, M. A., KHODAPARASTAN, F., BERENJIAN, A. & JAFARIZADEH-MALMIRI, H. 2013. Influence of small RNAs on biofilm formation process in bacteria. *Mol Biotechnol*, 55, 288-97.
- GOUIN, E., WELCH, M. D. & COSSART, P. 2005. Actin-based motility of intracellular pathogens. *Curr Opin Microbiol*, 8, 35-45.
- GRAZIANO, T. S., CLOSS, P., POPPI, T., FRANCO, G. C., CORTELLI, J. R., GROPPPO, F. C. & COGO, K. 2014. Catecholamines promote the expression of virulence and oxidative stress genes in *Porphyromonas gingivalis*. *J Periodontal Res*, 49, 660-9.
- GRENIER, D. 2013. *Porphyromonas gingivalis* Outer Membrane Vesicles Mediate Coaggregation and Piggybacking of *Treponema denticola* and *Lachnoanaerobaculum saburreum*. *Int J Dent*, 2013, 305476.

- GRIFFEN, A. L., BEALL, C. J., CAMPBELL, J. H., FIRESTONE, N. D., KUMAR, P. S., YANG, Z. K., PODAR, M. & LEYS, E. J. 2012. Distinct and complex bacterial profiles in human periodontitis and health revealed by 16S pyrosequencing. *ISME J*, 6, 1176-85.
- GU, Y., LEE, H. M., SORSA, T., SALMINEN, A., RYAN, M. E., SLEPIAN, M. J. & GOLUB, L. M. 2011. Non-antibacterial tetracyclines modulate mediators of periodontitis and atherosclerotic cardiovascular disease: A mechanistic link between local and systemic inflammation. *Pharmacol Res*.
- HAJISHENGALLIS, G. 2010. Complement and periodontitis. *Biochem Pharmacol*, 80, 1992-2001.
- HAJISHENGALLIS, G. 2015. Periodontitis: from microbial immune subversion to systemic inflammation. *Nat Rev Immunol*, 15, 30-44.
- HAJISHENGALLIS, G. & LAMONT, R. J. 2012. Beyond the red complex and into more complexity: the polymicrobial synergy and dysbiosis (PSD) model of periodontal disease etiology. *Mol Oral Microbiol*, 27, 409-19.
- HAJISHENGALLIS, G., LIANG, S., PAYNE, M. A., HASHIM, A., JOTWANI, R., ESKAN, M. A., MCINTOSH, M. L., ALSAM, A., KIRKWOOD, K. L., LAMBRIS, J. D., DARVEAU, R. P. & CURTIS, M. A. 2011. Low-Abundance Biofilm Species Orchestrates Inflammatory Periodontal Disease through the Commensal Microbiota and Complement. *Cell Host Microbe*, 10, 497-506.
- HAJISHENGALLIS, G. & MOUTSOPOULOS, N. M. 2014. Etiology of leukocyte adhesion deficiency-associated periodontitis revisited: not a raging infection but a raging inflammatory response. *Expert Rev Clin Immunol*, 10, 973-5.
- HALL, B. G. 1988. Adaptive evolution that requires multiple spontaneous mutations. I. Mutations involving an insertion sequence. *Genetics*, 120, 887-97.
- HALL, B. G. 1990. Spontaneous point mutations that occur more often when advantageous than when neutral. *Genetics*, 126, 5-16.
- HANS, M. & HANS, V. M. 2011. Toll-like receptors and their dual role in periodontitis: a review. *J Oral Sci*, 53, 263-71.
- HARVEY, L. E., KOHLGRAF, K. G., MEHALICK, L. A., RAINA, M., RECKER, E. N., RADHAKRISHNAN, S., PRASAD, S. A., VIDVA, R., PROGULSKE-FOX, A., CAVANAUGH, J. E., VALI, S. & BROGDEN, K. A. 2013. Defensin DEFB103 bidirectionally regulates chemokine and cytokine responses to a pro-inflammatory stimulus. *Sci Rep*, 3, 1232.
- HASEGAWA, Y., TRIBBLE, G. D., BAKER, H. V., MANS, J. J., HANDFIELD, M. & LAMONT, R. J. 2008. Role of *Porphyromonas gingivalis* SerB in gingival epithelial cell cytoskeletal remodeling and cytokine production. *Infect Immun*, 76, 2420-7.
- HASTURK, H., JONES, V. L., ANDRY, C. & KANTARCI, A. 2007. 1-Tetradecanol complex reduces progression of *Porphyromonas gingivalis*-induced experimental periodontitis in rabbits. *J Periodontol*, 78, 924-32.
- HAYASHI, J., NISHIKAWA, K., HIRANO, R., NOGUCHI, T. & YOSHIMURA, F. 2000. Identification of a two-component signal transduction system involved in fimbriation of *Porphyromonas gingivalis*. *Microbiol Immunol*, 44, 279-82.
- HAYES, F. 2003. Transposon-based strategies for microbial functional genomics and proteomics. *Annu Rev Genet*, 37, 3-29.
- HEITHOFF, D. M., SINSHEIMER, R. L., LOW, D. A. & MAHAN, M. J. 1999. An essential role for DNA adenine methylation in bacterial virulence. *Science*, 284, 967-70.
- HIRATSUKA, K., HAYAKAWA, M., KIYAMA-KISHIKAWA, M., SASAKI, Y., HIRAI, T. & ABIKO, Y. 2008. Role of the hemin-binding protein 35 (HBP35) of *Porphyromonas gingivalis* in coaggregation. *Microb Pathog*, 44, 320-8.
- HOBDELL, M. H., OLIVEIRA, E. R., BAUTISTA, R., MYBURGH, N. G., LALLOO, R., NARENDHAN, S. & JOHNSON, N. W. 2003. Oral diseases and socio-economic status (SES). *Br Dent J*, 194, 91-6; discussion 88.
- HOLT, S. C., EBERSOLE, J., FELTON, J., BRUNSVOLD, M. & KORNMAN, K. S. 1988. Implantation of *Bacteroides gingivalis* in nonhuman primates initiates progression of periodontitis. *Science*, 239, 55-7.

- IMAMURA, T., BANBULA, A., PEREIRA, P. J., TRAVIS, J. & POTEMPA, J. 2001a. Activation of human prothrombin by arginine-specific cysteine proteinases (Gingipains R) from *porphyromonas gingivalis*. *J Biol Chem*, 276, 18984-91.
- IMAMURA, T., TANASE, S., HAMAMOTO, T., POTEMPA, J. & TRAVIS, J. 2001b. Activation of blood coagulation factor IX by gingipains R, arginine-specific cysteine proteinases from *Porphyromonas gingivalis*. *Biochem J*, 353, 325-31.
- INABA, H., NAKANO, K., KATO, T., NOMURA, R., KAWAI, S., KUBONIWA, M., ISHIHARA, K., OOSHIMA, T. & AMANO, A. 2008. Heterogenic virulence and related factors among clinical isolates of *Porphyromonas gingivalis* with type II fimbriae. *Oral Microbiol Immunol*, 23, 29-35.
- ISODA, R., SIMANSKI, S. P., PATHANGEY, L., STONE, A. E. & BROWN, T. A. 2007. Expression of a *Porphyromonas gingivalis* hemagglutinin on the surface of a Salmonella vaccine vector. *Vaccine*, 25, 117-26.
- JAUREGUI, C. E., WANG, Q., WRIGHT, C. J., TAKEUCHI, H., URIARTE, S. M. & LAMONT, R. J. 2013. Suppression of T-cell chemokines by *Porphyromonas gingivalis*. *Infect Immun*, 81, 2288-95.
- JENKINSON, H. F. 2011. Beyond the oral microbiome. *Environ Microbiol*, 13, 3077-87.
- JEON, B. 2014. A tangle of poly-phosphate in *Campylobacter*. *Virulence*, 5, 449-50.
- JEONG, E., KIM, K., KIM, J. H., CHA, G. S., KIM, S. J., KANG, H. S. & CHOI, J. 2015. *Porphyromonas gingivalis* HSP60 peptides have distinct roles in the development of atherosclerosis. *Mol Immunol*, 63, 489-96.
- JONG, R. A. & VAN DER REIJDEN, W. A. 2010. Feasibility and therapeutic strategies of vaccines against *Porphyromonas gingivalis*. *Expert Rev Vaccines*, 9, 193-208.
- JUSKO, M., POTEMPA, J., KARIM, A. Y., KSIAZEK, M., RIESBECK, K., GARRED, P., EICK, S. & BLOM, A. M. 2012. A metalloproteinase karilysin present in the majority of *Tannerella forsythia* isolates inhibits all pathways of the complement system. *J Immunol*, 188, 2338-49.
- KATO, A., IMAI, K., OCHIAI, K. & OGATA, Y. 2014. Prevalence and quantitative analysis of Epstein-Barr virus DNA and *Porphyromonas gingivalis* associated with Japanese chronic periodontitis patients. *Clin Oral Investig*.
- KATZ, J., BLACK, K. P. & MICHALEK, S. M. 1999. Host responses to recombinant hemagglutinin B of *Porphyromonas gingivalis* in an experimental rat model. *Infect Immun*, 67, 4352-9.
- KESAVALU, L., HOLT, S. C. & EBERSOLE, J. L. 2003. In vitro environmental regulation of *Porphyromonas gingivalis* growth and virulence. *Oral Microbiol Immunol*, 18, 226-33.
- KESAVALU, L., SATHISHKUMAR, S., BAKTHAVATCHALU, V., MATTHEWS, C., DAWSON, D., STEFFEN, M. & EBERSOLE, J. L. 2007. Rat model of polymicrobial infection, immunity, and alveolar bone resorption in periodontal disease. *Infect Immun*, 75, 1704-12.
- KHALAF, H. & BENGTSSON, T. 2012. Altered T-cell responses by the periodontal pathogen *Porphyromonas gingivalis*. *PLoS One*, 7, e45192.
- KIYAMA-KISHIKAWA, M., HIRATSUKA, K. & ABIKO, Y. 2005. Gene expression profiling and characterization under hemin limitation in *Porphyromonas gingivalis*. *J Oral Sci*, 47, 191-7.
- KLEIN, B. A., TENORIO, E. L., LAZINSKI, D. W., CAMILLI, A., DUNCAN, M. J. & HU, L. T. 2012. Identification of essential genes of the periodontal pathogen *Porphyromonas gingivalis*. *BMC Genomics*, 13, 578.
- KONTANI, M., KIMURA, S., NAKAGAWA, I. & HAMADA, S. 1997. Adherence of *Porphyromonas gingivalis* to matrix proteins via a fimbrial cryptic receptor exposed by its own arginine-specific protease. *Mol Microbiol*, 24, 1179-87.
- KOZAROV, E. V., DORN, B. R., SHELBURNE, C. E., DUNN, W. A., JR. & PROGULSKE-FOX, A. 2005. Human atherosclerotic plaque contains viable invasive *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*. *Arterioscler Thromb Vasc Biol*, 25, e17-8.
- KOZIEL, J., MYDEL, P. & POTEMPA, J. 2014. The link between periodontal disease and rheumatoid arthritis: an updated review. *Curr Rheumatol Rep*, 16, 408.

- KUBONIWA, M., HASEGAWA, Y., MAO, S., SHIZUKUISHI, S., AMANO, A., LAMONT, R. J. & YILMAZ, O. 2008. *P. gingivalis* accelerates gingival epithelial cell progression through the cell cycle. *Microbes Infect*, 10, 122-8.
- KUBONIWA, M. & LAMONT, R. J. 2010. Subgingival biofilm formation. *Periodontol 2000*, 52, 38-52.
- KUSUMOTO, Y., HIRANO, H., SAITOH, K., YAMADA, S., TAKEDACHI, M., NOZAKI, T., OZAWA, Y., NAKAHIRA, Y., SAHO, T., OGO, H., SHIMABUKURO, Y., OKADA, H. & MURAKAMI, S. 2004. Human gingival epithelial cells produce chemotactic factors interleukin-8 and monocyte chemoattractant protein-1 after stimulation with *Porphyromonas gingivalis* via toll-like receptor 2. *J Periodontol*, 75, 370-9.
- LAINE, M. L. & VAN WINKELHOFF, A. J. 1998. Virulence of six capsular serotypes of *Porphyromonas gingivalis* in a mouse model. *Oral Microbiol Immunol*, 13, 322-5.
- LAMONT, R. J., CHAN, A., BELTON, C. M., IZUTSU, K. T., VASEL, D. & WEINBERG, A. 1995. *Porphyromonas gingivalis* invasion of gingival epithelial cells. *Infect Immun*, 63, 3878-85.
- LAMONT, R. J. & HAJISHENGALLIS, G. 2015. Polymicrobial synergy and dysbiosis in inflammatory disease. *Trends Mol Med*, 21, 172-83.
- LAMONT, R. J. & JENKINSON, H. F. 1998. Life below the gum line: pathogenic mechanisms of *Porphyromonas gingivalis*. *Microbiol Mol Biol Rev*, 62, 1244-63.
- LAMONT, R. J. & JENKINSON, H. F. 2000. Subgingival colonization by *Porphyromonas gingivalis*. *Oral Microbiol Immunol*, 15, 341-9.
- LAMPE, D. J., AKERLEY, B. J., RUBIN, E. J., MEKALANOS, J. J. & ROBERTSON, H. M. 1999. Hyperactive transposase mutants of the Himar1 mariner transposon. *Proc Natl Acad Sci U S A*, 96, 11428-33.
- LAMPE, D. J., CHURCHILL, M. E. & ROBERTSON, H. M. 1996. A purified mariner transposase is sufficient to mediate transposition in vitro. *EMBO J*, 15, 5470-9.
- LEE, S. W., HILLMAN, J. D. & PROGULSKE-FOX, A. 1996. The hemagglutinin genes *hagB* and *hagC* of *Porphyromonas gingivalis* are transcribed in vivo as shown by use of a new expression vector. *Infect Immun*, 64, 4802-10.
- LEPINE, G., ELLEN, R. P. & PROGULSKE-FOX, A. 1996. Construction and preliminary characterization of three hemagglutinin mutants of *Porphyromonas gingivalis*. *Infect Immun*, 64, 1467-72.
- LEPINE, G. & PROGULSKE-FOX, A. 1996. Duplication and differential expression of hemagglutinin genes in *Porphyromonas gingivalis*. *Oral Microbiol Immunol*, 11, 65-78.
- LEVESQUE, C., LAMOTHE, J. & FRENETTE, M. 2003. Coaggregation of *Streptococcus salivarius* with periodontopathogens: evidence for involvement of fimbriae in the interaction with *Prevotella intermedia*. *Oral Microbiol Immunol*, 18, 333-7.
- LI, N. & COLLYER, C. A. 2011. Gingipains from *Porphyromonas gingivalis* - Complex domain structures confer diverse functions. *Eur J Microbiol Immunol (Bp)*, 1, 41-58.
- LIU, B., FALLER, L. L., KLITGORD, N., MAZUMDAR, V., GHODSI, M., SOMMER, D. D., GIBBONS, T. R., TREANGEN, T. J., CHANG, Y. C., LI, S., STINE, O. C., HASTURK, H., KASIF, S., SEGRE, D., POP, M. & AMAR, S. 2012. Deep sequencing of the oral microbiome reveals signatures of periodontal disease. *PLoS One*, 7, e37919.
- LIU, J., WANG, Y. & OUYANG, X. 2014. Beyond toll-like receptors: *Porphyromonas gingivalis* induces IL-6, IL-8, and VCAM-1 expression through NOD-mediated NF-kappaB and ERK signaling pathways in periodontal fibroblasts. *Inflammation*, 37, 522-33.
- LIU, L., WEN, X., HE, H., SHI, J. & JI, C. 2003. Species-specific DNA probe for the detection of *Porphyromonas gingivalis* from adult Chinese periodontal patients and healthy subjects. *J Periodontol*, 74, 1000-6.
- LO, A., SEERS, C., DASHPER, S., BUTLER, C., WALKER, G., WALSH, K., CATMULL, D., HOFFMANN, B., CLEAL, S., LISSEL, P., BOYCE, J. & REYNOLDS, E. 2010. FimR and FimS: biofilm formation and gene expression in *Porphyromonas gingivalis*. *J Bacteriol*, 192, 1332-43.
- LOESCHE, W. J. 1976. Chemotherapy of dental plaque infections. *Oral Sci Rev*, 9, 65-107.
- LOESCHE, W. J. 1986a. The identification of bacteria associated with periodontal disease and dental caries by enzymatic methods. *Oral Microbiol Immunol*, 1, 65-72.

- LOESCHE, W. J. 1986b. Role of *Streptococcus mutans* in human dental decay. *Microbiol Rev*, 50, 353-80.
- LOVE, R. M., MCMILLAN, M. D., PARK, Y. & JENKINSON, H. F. 2000. Coinvasion of dentinal tubules by *Porphyromonas gingivalis* and *Streptococcus gordonii* depends upon binding specificity of streptococcal antigen I/II adhesin. *Infect Immun*, 68, 1359-65.
- MAEKAWA, T., KRAUSS, J. L., ABE, T., JOTWANI, R., TRIANTAFILOU, M., TRIANTAFILOU, K., HASHIM, A., HOCH, S., CURTIS, M. A., NUSSBAUM, G., LAMBRIS, J. D. & HAJISHENGALLIS, G. 2014. *Porphyromonas gingivalis* Manipulates Complement and TLR Signaling to Uncouple Bacterial Clearance from Inflammation and Promote Dysbiosis. *Cell Host Microbe*, 15, 768-78.
- MALDE, A., GANGAIAH, D., CHANDRASHEKHAR, K., PINA-MIMBELA, R., TORRELLES, J. B. & RAJASHEKARA, G. 2014. Functional characterization of exopolyphosphatase/guanosine pentaphosphate phosphohydrolase (PPX/GPPA) of *Campylobacter jejuni*. *Virulence*, 5, 521-33.
- MALEK, R., FISHER, J. G., CALECA, A., STINSON, M., VAN OSS, C. J., LEE, J. Y., CHO, M. I., GENCO, R. J., EVANS, R. T. & DYER, D. W. 1994. Inactivation of the *Porphyromonas gingivalis* *fimA* gene blocks periodontal damage in gnotobiotic rats. *J Bacteriol*, 176, 1052-9.
- MALEY, A. M. & ARBISER, J. L. 2013. Gentian violet: a 19th century drug re-emerges in the 21st century. *Exp Dermatol*, 22, 775-80.
- MARINUS, M. G. & CASADESUS, J. 2009. Roles of DNA adenine methylation in host-pathogen interactions: mismatch repair, transcriptional regulation, and more. *FEMS Microbiol Rev*, 33, 488-503.
- MARSH, P. D. 1994. Microbial ecology of dental plaque and its significance in health and disease. *Adv Dent Res*, 8, 263-71.
- MAURER, M. & VON STEBUT, E. 2004. Macrophage inflammatory protein-1. *Int J Biochem Cell Biol*, 36, 1882-6.
- MEURIC, V., GRACIEUX, P., TAMANAI-SHACOORI, Z., PEREZ-CHAPARRO, J. & BONNAURE-MALLET, M. 2008. Expression patterns of genes induced by oxidative stress in *Porphyromonas gingivalis*. *Oral Microbiol Immunol*, 23, 308-14.
- MEURIC, V., MARTIN, B., GUYODO, H., ROUILLON, A., TAMANAI-SHACOORI, Z., BARLOY-HUBLER, F. & BONNAURE-MALLET, M. 2013. *Treponema denticola* improves adhesive capacities of *Porphyromonas gingivalis*. *Mol Oral Microbiol*, 28, 40-53.
- MILLHOUSE, E., JOSE, A., SHERRY, L., LAPPIN, D. F., PATEL, N., MIDDLETON, A. M., PRATTEN, J., CULSHAW, S. & RAMAGE, G. 2014. Development of an *in vitro* periodontal biofilm model for assessing antimicrobial and host modulatory effects of bioactive molecules. *BMC Oral Health*, 14, 80.
- MILNER, P., BATTEN, J. E. & CURTIS, M. A. 1996. Development of a simple chemically defined medium for *Porphyromonas gingivalis*: requirement for alpha-ketoglutarate. *FEMS Microbiol Lett*, 140, 125-30.
- MOLZEN, T. E., BURGHOUT, P., BOOTSMA, H. J., BRANDT, C. T., VAN DER GAAST-DE JONGH, C. E., ELEVELD, M. J., VERBEEK, M. M., FRIMODT-MOLLER, N., OSTERGAARD, C. & HERMANS, P. W. 2011. Genome-wide identification of *Streptococcus pneumoniae* genes essential for bacterial replication during experimental meningitis. *Infect Immun*, 79, 288-97.
- MORRIS, J. J., LENSKI, R. E. & ZINSER, E. R. 2012. The Black Queen Hypothesis: evolution of dependencies through adaptive gene loss. *MBio*, 3.
- MOUTSOPOULOS, N. M., KLING, H. M., ANGELOV, N., JIN, W., PALMER, R. J., NARES, S., OSORIO, M. & WAHL, S. M. 2012. *Porphyromonas gingivalis* promotes Th17 inducing pathways in chronic periodontitis. *J Autoimmun*, 39, 294-303.
- MUTHIAH, A. S., ARUNI, W., ROBLES, A. G., DOU, Y., ROY, F. & FLETCHER, H. M. 2013. In *Porphyromonas gingivalis* *VimF* is involved in gingipain maturation through the transfer of galactose. *PLoS One*, 8, e63367.

- NAGANO, K., MURAKAMI, Y., NISHIKAWA, K., SAKAKIBARA, J., SHIMOZATO, K. & YOSHIMURA, F. 2007. Characterization of *RagA* and *RagB* in *Porphyromonas gingivalis*: study using gene-deletion mutants. *J Med Microbiol*, 56, 1536-48.
- NAITO, M., HIRAKAWA, H., YAMASHITA, A., OHARA, N., SHOJI, M., YUKITAKE, H., NAKAYAMA, K., TOH, H., YOSHIMURA, F., KUHARA, S., HATTORI, M. & HAYASHI, T. 2008. Determination of the genome sequence of *Porphyromonas gingivalis* strain ATCC 33277 and genomic comparison with strain W83 revealed extensive genome rearrangements in *P. gingivalis*. *DNA Res*, 15, 215-25.
- NAKAO, R., TAKASHIBA, S., KOSONO, S., YOSHIDA, M., WATANABE, H., OHNISHI, M. & SENPUKU, H. 2014. Effect of *Porphyromonas gingivalis* outer membrane vesicles on gingipain-mediated detachment of cultured oral epithelial cells and immune responses. *Microbes Infect*, 16, 6-16.
- NISHIKAWA, K. & DUNCAN, M. J. 2010. Histidine kinase-mediated production and autoassembly of *Porphyromonas gingivalis* fimbriae. *J Bacteriol*, 192, 1975-87.
- NISHIKAWA, K., YOSHIMURA, F. & DUNCAN, M. J. 2004. A regulation cascade controls expression of *Porphyromonas gingivalis* fimbriae via the FimR response regulator. *Mol Microbiol*, 54, 546-60.
- NJOROGE, T., GENCO, R. J., SOJAR, H. T., HAMADA, N. & GENCO, C. A. 1997. A role for fimbriae in *Porphyromonas gingivalis* invasion of oral epithelial cells. *Infect Immun*, 65, 1980-4.
- NM, O. B.-S., VEITH, P. D., DASHPER, S. G. & REYNOLDS, E. C. 2003. *Porphyromonas gingivalis* gingipains: the molecular teeth of a microbial vampire. *Curr Protein Pept Sci*, 4, 409-26.
- OLCZAK, T., SIMPSON, W., LIU, X. & GENCO, C. A. 2005. Iron and heme utilization in *Porphyromonas gingivalis*. *FEMS Microbiol Rev*, 29, 119-44.
- OLCZAK, T., SROKA, A., POTEPA, J. & OLCZAK, M. 2008. *Porphyromonas gingivalis* HmuY and HmuR: further characterization of a novel mechanism of heme utilization. *Arch Microbiol*, 189, 197-210.
- ORALGEN. 2009a. *hagD* [Online]. Available: http://www.oralgen.org/cgi-bin/gene_id_search.cgi?dbname=pgin&gene_id=PG1768 [Accessed 09\07 2015].
- ORALGEN. 2009b. *hagE* [Online]. Available: http://www.oralgen.org/cgi-bin/gene_id_search.cgi?dbname=pgin&gene_id=PG1768 [Accessed].
- OZTEKIN, G., BASER, U., KUCUKCOSKUN, M., TANRIKULU-KUCUK, S., ADEMOGLU, E., ISIK, G., OZKAN, G., YALCIN, F. & KIYAN, E. 2014. The association between periodontal disease and chronic obstructive pulmonary disease: a case control study. *COPD*, 11, 424-30.
- PAGE, R. C., LANTZ, M. S., DARVEAU, R., JEFFCOAT, M., MANCL, L., HOUSTON, L., BRAHAM, P. & PERSSON, G. R. 2007. Immunization of *Macaca fascicularis* against experimental periodontitis using a vaccine containing cysteine proteases purified from *Porphyromonas gingivalis*. *Oral Microbiol Immunol*, 22, 162-8.
- PATHIRANA, R. D., O'BRIEN-SIMPSON, N. M., VISVANATHAN, K., HAMILTON, J. A. & REYNOLDS, E. C. 2007. Flow cytometric analysis of adherence of *Porphyromonas gingivalis* to oral epithelial cells. *Infect Immun*, 75, 2484-92.
- PERCIVAL, R. S., MARSH, P. D., DEVINE, D. A., RANGARAJAN, M., ADUSE-OPOKU, J., SHEPHERD, P. & CURTIS, M. A. 1999. Effect of temperature on growth, hemagglutination, and protease activity of *Porphyromonas gingivalis*. *Infect Immun*, 67, 1917-21.
- PINGEL, L. C., KOHLGRAF, K. G., HANSEN, C. J., EASTMAN, C. G., DIETRICH, D. E., BURNELL, K. K., SRIKANTHA, R. N., XIAO, X., BELANGER, M., PROGULSKE-FOX, A., CAVANAUGH, J. E., GUTHMILLER, J. M., JOHNSON, G. K., JOLY, S., KURAGO, Z. B., DAWSON, D. V. & BROGDEN, K. A. 2008. Human beta-defensin 3 binds to hemagglutinin B (rHagB), a non-fimbrial adhesin from *Porphyromonas gingivalis*, and attenuates a pro-inflammatory cytokine response. *Immunol Cell Biol*, 86, 643-9.
- PIZARRO-CERDA, J., KUHBACHER, A. & COSSART, P. 2012. Entry of *Listeria monocytogenes* in mammalian epithelial cells: an updated view. *Cold Spring Harb Perspect Med*, 2.
- POPADIAK, K., POTEPA, J., RIESBECK, K. & BLOM, A. M. 2007. Biphasic effect of gingipains from *Porphyromonas gingivalis* on the human complement system. *J Immunol*, 178, 7242-50.

- POTEMPA, M., POTEMPA, J., KANTYKA, T., NGUYEN, K. A., WAWRZONEK, K., MANANDHAR, S. P., POPADIAK, K., RIESBECK, K., EICK, S. & BLOM, A. M. 2009. Interpain A, a cysteine proteinase from *Prevotella intermedia*, inhibits complement by degrading complement factor C3. *PLoS Pathog*, 5, e1000316.
- POTEMPA, M., POTEMPA, J., OKROJ, M., POPADIAK, K., EICK, S., NGUYEN, K. A., RIESBECK, K. & BLOM, A. M. 2008. Binding of complement inhibitor C4b-binding protein contributes to serum resistance of *Porphyromonas gingivalis*. *J Immunol*, 181, 5537-44.
- PROGULSKE-FOX, A., TUMWASORN, S., LEPINE, G., WHITLOCK, J., SAVETT, D., FERRETTI, J. J. & BANAS, J. A. 1995. The cloning, expression and sequence analysis of a second *Porphyromonas gingivalis* gene that codes for a protein involved in hemagglutination. *Oral Microbiol Immunol*, 10, 311-8.
- RICKLIN, D., HAJISHENGALLIS, G., YANG, K. & LAMBRIS, J. D. 2010. Complement: a key system for immune surveillance and homeostasis. *Nat Immunol*, 11, 785-97.
- ROSIER, B. T., DE JAGER, M., ZAURA, E. & KROM, B. P. 2014. Historical and contemporary hypotheses on the development of oral diseases: are we there yet? *Front Cell Infect Microbiol*, 4, 92.
- SABET, C., LECUIT, M., CABANES, D., COSSART, P. & BIERNE, H. 2005. LPXTG protein InlJ, a newly identified internalin involved in *Listeria monocytogenes* virulence. *Infect Immun*, 73, 6912-22.
- SAFFI, M. A., FURTADO, M. V., POLANCZYK, C. A., MONTENEGRO, M. M., RIBEIRO, I. W., KAMPITS, C., HAAS, A. N., ROSING, C. K. & RABELO-SILVA, E. R. 2015. Relationship between vascular endothelium and periodontal disease in atherosclerotic lesions: Review article. *World J Cardiol*, 7, 26-30.
- SANCHEZ-ROMERO, M. A., COTA, I. & CASADESUS, J. 2015. DNA methylation in bacteria: from the methyl group to the methylome. *Curr Opin Microbiol*, 25, 9-16.
- SANCHEZ, G. A., ACQUIER, A. B., DE COUTO, A., BUSCH, L. & MENDEZ, C. F. 2015. Association between *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* in subgingival plaque and clinical parameters, in Argentine patients with aggressive periodontitis. *Microb Pathog*, 82, 31-6.
- SATO, Y., KIMURA, M. & FUKUMI, H. 1984. Development of a pertussis component vaccine in Japan. *Lancet*, 1, 122-6.
- SCHULZ ZUR WIESCH, P., ENGELSTADTER, J. & BONHOEFFER, S. 2010. Compensation of fitness costs and reversibility of antibiotic resistance mutations. *Antimicrob Agents Chemother*, 54, 2085-95.
- SCOTT, C. F., WHITAKER, E. J., HAMMOND, B. F. & COLMAN, R. W. 1993. Purification and characterization of a potent 70-kDa thiol lysyl-proteinase (Lys-gingivain) from *Porphyromonas gingivalis* that cleaves kininogens and fibrinogen. *J Biol Chem*, 268, 7935-42.
- SCOTT, J. C., KLEIN, B. A., DURAN-PINEDO, A., HU, L. & DUNCAN, M. J. 2013. A Two-Component System Regulates Hemin Acquisition in *Porphyromonas gingivalis*. *PLoS One*, 8, e73351.
- SHERRY, L., MILLHOUSE, E., LAPPIN, D. F., MURRAY, C., CULSHAW, S., NILE, C. J. & RAMAGE, G. 2013. Investigating the biological properties of carbohydrate derived fulvic acid (CHD-FA) as a potential novel therapy for the management of oral biofilm infections. *BMC Oral Health*, 13, 47.
- SHI, Y., RATNAYAKE, D. B., OKAMOTO, K., ABE, N., YAMAMOTO, K. & NAKAYAMA, K. 1999. Genetic analyses of proteolysis, hemoglobin binding, and hemagglutination of *Porphyromonas gingivalis*. Construction of mutants with a combination of *rgpA*, *rgpB*, *kgp*, and *hagA*. *J Biol Chem*, 274, 17955-60.
- SHIN, J. E., BAEK, K. J., CHOI, Y. S. & CHOI, Y. 2013. A periodontal pathogen *Treponema denticola* hijacks the *Fusobacterium nucleatum*-driven host response. *Immunol Cell Biol*, 91, 503-10.
- SIMPSON, W., OLCZAK, T. & GENCO, C. A. 2000. Characterization and expression of HmuR, a TonB-dependent hemoglobin receptor of *Porphyromonas gingivalis*. *J Bacteriol*, 182, 5737-48.

- SIMS, T. J., SCHIFFERLE, R. E., ALI, R. W., SKAUG, N. & PAGE, R. C. 2001. Immunoglobulin G response of periodontitis patients to *Porphyromonas gingivalis* capsular carbohydrate and lipopolysaccharide antigens. *Oral Microbiol Immunol*, 16, 193-201.
- SLOCUM, C., COATS, S. R., HUA, N., KRAMER, C., PAPADOPOULOS, G., WEINBERG, E. O., GUDINO, C. V., HAMILTON, J. A., DARVEAU, R. P. & GENCO, C. A. 2014. Distinct lipid a moieties contribute to pathogen-induced site-specific vascular inflammation. *PLoS Pathog*, 10, e1004215.
- SMALLEY, J. W., BIRSS, A. J., WITHNALL, R. & SILVER, J. 2002. Interactions of *Porphyromonas gingivalis* with oxyhaemoglobin and deoxyhaemoglobin. *Biochem J*, 362, 239-45.
- SMALLEY, J. W., BYRNE, D. P., BIRSS, A. J., WOJTOWICZ, H., SROKA, A., POTEPA, J. & OLCZAK, T. 2011. HmuY Haemophore and Gingipain Proteases Constitute a Unique Syntrophic System of Haem Acquisition by *Porphyromonas gingivalis*. *PLoS One*, 6, e17182.
- SMALLEY, J. W., THOMAS, M. F., BIRSS, A. J., WITHNALL, R. & SILVER, J. 2004. A combination of both arginine- and lysine-specific gingipain activity of *Porphyromonas gingivalis* is necessary for the generation of the micro-oxo bishaem-containing pigment from haemoglobin. *Biochem J*, 379, 833-40.
- SOCRANSKY, S. S., HAFFAJEE, A. D., CUGINI, M. A., SMITH, C. & KENT, R. L., JR. 1998. Microbial complexes in subgingival plaque. *J Clin Periodontol*, 25, 134-44.
- SONG, H., BELANGER, M., WHITLOCK, J., KOZAROV, E. & PROGULSKE-FOX, A. 2005. Hemagglutinin B is involved in the adherence of *Porphyromonas gingivalis* to human coronary artery endothelial cells. *Infect Immun*, 73, 7267-73.
- SOUTO, G. R., QUEIROZ-JUNIOR, C. M., COSTA, F. O. & MESQUITA, R. A. 2014. Smoking effect on chemokines of the human chronic periodontitis. *Immunobiology*, 219, 633-6.
- SRIDHAR, S., BROKSTAD, K. A. & COX, R. J. 2015. Influenza Vaccination Strategies: Comparing Inactivated and Live Attenuated Influenza Vaccines. *Vaccines (Basel)*, 3, 373-89.
- STAHL, M. & STINTZI, A. 2011. Identification of essential genes in *C. jejuni* genome highlights hyper-variable plasticity regions. *Funct Integr Genomics*, 11, 241-57.
- SUNDQVIST, G., FIGDOR, D., HANSTROM, L., SORLIN, S. & SANDSTROM, G. 1991. Phagocytosis and virulence of different strains of *Porphyromonas gingivalis*. *Scand J Dent Res*, 99, 117-29.
- SUWANNAKUL, S., STAFFORD, G. P., WHAWELL, S. A. & DOUGLAS, C. W. 2010. Identification of bistable populations of *Porphyromonas gingivalis* that differ in epithelial cell invasion. *Microbiology*, 156, 3052-64.
- TAKADA, K. & HIRASAWA, M. 1998. Tn4351-generated non-haemolytic and/or non-pigmented mutants of *Porphyromonas gingivalis*. *Microbios*, 95, 35-44.
- TAKEDA, K. & AKIRA, S. 2005. Toll-like receptors in innate immunity. *Int Immunol*, 17, 1-14.
- TAKEUCHI, H., HIRANO, T., WHITMORE, S. E., MORISAKI, I., AMANO, A. & LAMONT, R. J. 2013. The serine phosphatase SerB of *Porphyromonas gingivalis* suppresses IL-8 production by dephosphorylation of NF-kappaB RelA/p65. *PLoS Pathog*, 9, e1003326.
- THEILADE, E. 1986. The non-specific theory in microbial etiology of inflammatory periodontal diseases. *J Clin Periodontol*, 13, 905-11.
- TRIBBLE, G. D., KERR, J. E. & WANG, B. Y. 2013. Genetic diversity in the oral pathogen *Porphyromonas gingivalis*: molecular mechanisms and biological consequences. *Future Microbiol*, 8, 607-20.
- TRIBBLE, G. D., MAO, S., JAMES, C. E. & LAMONT, R. J. 2006. A *Porphyromonas gingivalis* haloacid dehalogenase family phosphatase interacts with human phosphoproteins and is important for invasion. *Proc Natl Acad Sci U S A*, 103, 11027-32.
- TYMKIW, K. D., THUNELL, D. H., JOHNSON, G. K., JOLY, S., BURNELL, K. K., CAVANAUGH, J. E., BROGDEN, K. A. & GUTHMILLER, J. M. 2011. Influence of smoking on gingival crevicular fluid cytokines in severe chronic periodontitis. *J Clin Periodontol*, 38, 219-28.
- VAN HEMERT, J. R., RECKER, E. N., DIETRICH, D., PROGULSKE-FOX, A., KURAGO, Z. B., WALTERS, K. S., CAVANAUGH, J. E. & BROGDEN, K. A. 2012. Human beta-defensin-3 alters, but does not inhibit, the binding of *Porphyromonas gingivalis* haemagglutinin B to the surface of human dendritic cells. *Int J Antimicrob Agents*.

- VAN OPIJNEN, T. & CAMILLI, A. 2013. Transposon insertion sequencing: a new tool for systems-level analysis of microorganisms. *Nat Rev Microbiol*, 11, 435-42.
- VANTERPOOL, E., ROY, F. & FLETCHER, H. M. 2004. The *vimE* gene downstream of *vimA* is independently expressed and is involved in modulating proteolytic activity in *Porphyromonas gingivalis* W83. *Infect Immun*, 72, 5555-64.
- VEITH, P. D., CHEN, Y. Y., GORASIA, D. G., CHEN, D., GLEW, M. D., O'BRIEN-SIMPSON, N. M., CECIL, J. D., HOLDEN, J. A. & REYNOLDS, E. C. 2014. *Porphyromonas gingivalis* Outer Membrane Vesicles Exclusively Contain Outer Membrane and Periplasmic Proteins and Carry a Cargo Enriched with Virulence Factors. *J Proteome Res*.
- VILLARINO ROMERO, R., OSICKA, R. & SEBO, P. 2014. Filamentous hemagglutinin of *Bordetella pertussis*: a key adhesin with immunomodulatory properties? *Future Microbiol*, 9, 1339-60.
- WANG, M., KRAUSS, J. L., DOMON, H., HOSUR, K. B., LIANG, S., MAGOTTI, P., TRIANTAFILOU, M., TRIANTAFILOU, K., LAMBRIS, J. D. & HAJISHENGALLIS, G. 2010. Microbial hijacking of complement-toll-like receptor crosstalk. *Sci Signal*, 3, ra11.
- WATANABE, K., YAMAJI, Y. & UMEMOTO, T. 1992. Correlation between cell-adherent activity and surface structure in *Porphyromonas gingivalis*. *Oral Microbiol Immunol*, 7, 357-63.
- WATT, R. & SHEIHAM, A. 1999. Inequalities in oral health: a review of the evidence and recommendations for action. *Br Dent J*, 187, 6-12.
- WHO. 2003. World Health Organisation. The World Oral Health Report
- WRIGHT, C. J., XUE, P., HIRANO, T., LIU, C., WHITMORE, S. E., HACKETT, M. & LAMONT, R. J. 2014. Characterization of a bacterial tyrosine kinase in *Porphyromonas gingivalis* involved in polymicrobial synergy. *Microbiologyopen*.
- WU, J., LIN, X. & XIE, H. 2007. *Porphyromonas gingivalis* short fimbriae are regulated by a FimS/FimR two-component system. *FEMS Microbiol Lett*, 271, 214-21.
- XIE, H., CAI, S. & LAMONT, R. J. 1997. Environmental regulation of fimbrial gene expression in *Porphyromonas gingivalis*. *Infect Immun*, 65, 2265-71.
- YANAMANDRA, S. S., SARRAFEE, S. S., ANAYA-BERGMAN, C., JONES, K. & LEWIS, J. P. 2012. Role of the *Porphyromonas gingivalis* extracytoplasmic function sigma factor, SigH. *Mol Oral Microbiol*, 27, 202-19.
- YANG, Q. B., MARTIN, M., MICHALEK, S. M. & KATZ, J. 2002. Mechanisms of monophosphoryl lipid A augmentation of host responses to recombinant HagB from *Porphyromonas gingivalis*. *Infect Immun*, 70, 3557-65.
- YEE, M., KIM, A., ALPAGOT, T., DUZGUNES, N. & KONOPKA, K. 2012. *Porphyromonas gingivalis* stimulates IL-18 secretion in human monocytic THP-1 cells. *Microbes Infect*, 14, 684-9.
- YILMAZ, O. 2008. The chronicles of *Porphyromonas gingivalis*: the microbium, the human oral epithelium and their interplay. *Microbiology*, 154, 2897-903.
- YILMAZ, O., JUNGAS, T., VERBEKE, P. & OJCIUS, D. M. 2004. Activation of the phosphatidylinositol 3-kinase/Akt pathway contributes to survival of primary epithelial cells infected with the periodontal pathogen *Porphyromonas gingivalis*. *Infect Immun*, 72, 3743-51.
- YILMAZ, O., VERBEKE, P., LAMONT, R. J. & OJCIUS, D. M. 2006. Intercellular spreading of *Porphyromonas gingivalis* infection in primary gingival epithelial cells. *Infect Immun*, 74, 703-10.
- YILMAZ, O., WATANABE, K. & LAMONT, R. J. 2002. Involvement of integrins in fimbriae-mediated binding and invasion by *Porphyromonas gingivalis*. *Cell Microbiol*, 4, 305-14.
- YILMAZ, O., YAO, L., MAEDA, K., ROSE, T. M., LEWIS, E. L., DUMAN, M., LAMONT, R. J. & OJCIUS, D. M. 2008. ATP scavenging by the intracellular pathogen *Porphyromonas gingivalis* inhibits P2X7-mediated host-cell apoptosis. *Cell Microbiol*, 10, 863-75.
- YILMAZ, O., YOUNG, P. A., LAMONT, R. J. & KENNY, G. E. 2003. Gingival epithelial cell signalling and cytoskeletal responses to *Porphyromonas gingivalis* invasion. *Microbiology*, 149, 2417-26.
- YOSHIMURA, F., MURAKAMI, Y., NISHIKAWA, K., HASEGAWA, Y. & KAWAMINAMI, S. 2009. Surface components of *Porphyromonas gingivalis*. *J Periodontal Res*, 44, 1-12.

- YUAN, L., RODRIGUES, P. H., BELANGER, M., DUNN, W., JR. & PROGULSKE-FOX, A. 2007. The *Porphyromonas gingivalis* clpB gene is involved in cellular invasion in vitro and virulence in vivo. *FEMS Immunol Med Microbiol*, 51, 388-98.
- YUZAWA, S., KURITA-OCHIAI, T., HASHIZUME, T., KOBAYASHI, R., ABIKO, Y. & YAMAMOTO, M. 2012. Sublingual vaccination with fusion protein consisting of the functional domain of hemagglutinin A of *Porphyromonas gingivalis* and *Escherichia coli* maltose-binding protein elicits protective immunity in the oral cavity. *FEMS Immunol Med Microbiol*, 64, 265-72.
- ZAMBON, J. J., GROSSI, S. G., MACHTEI, E. E., HO, A. W., DUNFORD, R. & GENCO, R. J. 1996. Cigarette smoking increases the risk for subgingival infection with periodontal pathogens. *J Periodontol*, 67, 1050-4.
- ZENG, X. T., TU, M. L., LIU, D. Y., ZHENG, D., ZHANG, J. & LENG, W. 2012. Periodontal disease and risk of chronic obstructive pulmonary disease: a meta-analysis of observational studies. *PLoS One*, 7, e46508.
- ZENOBIA, C., HASTURK, H., NGUYEN, D., VAN DYKE, T. E., KANTARCI, A. & DARVEAU, R. P. 2014. *Porphyromonas gingivalis* lipid A phosphatase activity is critical for colonization and increasing the commensal load in the rabbit ligature model. *Infect Immun*, 82, 650-9.
- ZHANG, P., MARTIN, M., MICHALEK, S. M. & KATZ, J. 2005a. Role of mitogen-activated protein kinases and NF-kappaB in the regulation of proinflammatory and anti-inflammatory cytokines by *Porphyromonas gingivalis* hemagglutinin B. *Infect Immun*, 73, 3990-8.
- ZHANG, Y., WANG, T., CHEN, W., YILMAZ, O., PARK, Y., JUNG, I. Y., HACKETT, M. & LAMONT, R. J. 2005b. Differential protein expression by *Porphyromonas gingivalis* in response to secreted epithelial cell components. *Proteomics*, 5, 198-211.