The *Porphyromonas gingivalis* haemagglutinins HagB and HagC are major mediators of adhesion and biofilm formation.

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SUMMARY

Porphyromonas gingivalis is a bacterium associated with chronic periodontitis that possesses a family of genes encoding haemagglutinins required for haem acquisition. In this study we generated *ΔhagB* and *ΔhagC* mutants in strain W83 and demonstrate that both *hagB* and *hagC* are required for adherence to oral epithelial cells. Unexpectedly, a double *ΔhagB/ΔhagC* mutant had less severe adherence defects than either of the single mutants, but was found to exhibit deregulated expression of gingipain encoding genes, suggesting that a *ΔhagB/ΔhagC* mutant is only viable in populations of cells that exhibit increased expression of genes involved in haem acquisition. Disruption of *hagB* in the fimbriated strain ATCC33277 demonstrated that HagB is also required for stable attachment of fimbriated bacteria to oral epithelial cells. Mutants of *hagC* were also found to form defective single and multispecies biofilms that had reduced biomass relative to biofilms formed by the wild-type strain. This study highlights the hitherto unappreciated importance of these genes in oral colonisation and biofilm formation.

INTRODUCTION

Porphyromonas gingivalis is a Gram negative bacterium strongly associated with the progression of chronic periodontitis. *P. gingivalis* produces an array of virulence factors including several varieties of lipopolysaccharide and a family of proteases known as the gingipains, many of which play a role in the development and progression of periodontal disease (Lamont and Jenkinson, 2000; Yoshimura *et al.*, 2009; Darveau, 2010; Lamont and Hajishengallis, 2015). Rodent models of the disease indicate that *P. gingivalis* has a unique ability to promote a dysbiotic micro-flora in the sub-gingival crevice due to its ability to subvert local immunity and interfere with the inflammatory process (Hajishengallis *et al.*, 2011)

One large class of virulence factors that *P. gingivalis* produces are the haemagglutinins (Han *et al.*, 1996; Lee *et al.*, 1996; Shi *et al.*, 1999). 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 and 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 iron 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*, allowing the proteolytic gingipains to release the haem moiety from the haemoglobin molecule, which is then internalised by the HmuY/HmuR dual component uptake system (Lewis *et al.*, 2006; Olczak *et al.*, 2008).

P. gingivalis possess at least eight haemagglutinins and a subset of these eight are encoded by a group of five genes known commonly as the *hag* genes (*hagA* to *hagE*). The *hag* genes can be subdivided in to two distinct families; *hagA*, *hagD* and *hagE* which

possess >70% homology and the unrelated *hagA* and *hagB* family, that share 93% homology.

The genes *hagB* and *hagC* are located closely together on the chromosome and encode proteins with 98.6% amino acid sequence identity. Although *hagB* and *hagC* were first identified in 1995, their roles in host-cell interactions are only partially characterised (Progulske-Fox *et al.*, 1995; Lépine and Progulske-Fox, 1996; Lépine *et al.*, 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). Both *hagB* and *hagC* encode haemagglutination activity but they are not the sole mediators of this process in *P. gingivalis* (Lépine *et al.*, 1996). Heterologous expression studies carried out in *Escherichia coli* have shown that HagB is trafficked to the bacterial cell surface (Song *et al.*, 2005). HagB has also been shown to mediate adhesion to human coronary arterial endothelial cells (HCAECs), supporting the hypothesis that *P. gingivalis* may infect these tissues *in vivo* (Song *et al.*, 2005). HagB has also been shown to be highly immunogenic and has been proposed as a candidate vaccine antigen. Recombinant HagB is a TLR4 agonist and immunization with rHagB leads to the triggering of a rHagB-specific CD4$^+$ T cell responses and increased levels of anti-HagB serum IgG and salivary IgA (Dusek *et al.*, 1995; Zhang *et al.*, 2005; Gaddis *et al.*, 2009). Rats immunised with rHagB are protected from experimental *P. gingivalis* induced periodontitis, resulting in reduced levels of alveolar bone loss (Katz *et al.*, 1999). Interestingly, binding of antimicrobial peptides such as human-beta-defensin 3 (HβD3) attenuate pro-inflammatory immune responses to rHagB in human myeloid dendritic cell cultures (Pingel *et al.*, 2008; Borgwardt *et al.*, 2014).

Single mutants of *hagB* and *hagC* have been generated previously in *P. gingivalis* strain 381 (Lépine *et al.*, 1996). However, attempts to create a double *ΔhagB/ΔhagC* mutant in

this background were unsuccessful, leading the authors to conclude that these genes were essential for the viability of *P. gingivalis*. Since the role of *hagB* and *hagC* in host cell interactions has yet to be clearly elucidated, we undertook to inactivate the *hagB* and *hagC* loci in *P. gingivalis* strain W83, which is a robust, non-fimbriated strain of *P. gingivalis*. Mutant construction was also performed in strain ATCC33277 in order to assess the roles of these proteins in a fimbriated strain.

Our data strongly indicate a hitherto unappreciated role for HagB and HagC in mediating adherence to oral epithelial cells, even in fimbriated strains of the bacterium. HagC is also implicated in biofilm formation. Unexpectedly, a *ΔhagB/ΔhagC* mutant was found to be viable in strain W83, but only in a background where deregulated gingipain expression was observed. This result serves to reinforce the notion that HagB and HagC are essential for normal haem homeostasis in this bacterium and raises an important question for investigators involved in mutant construction in *P. gingivalis* due to the presence of variant subpopulations bacteria.

METHODS

Strains and culture conditions

The *P. gingivalis* strains used in this study are outlined in Table S1. All growth media and supplements were obtained from Sigma-Aldrich (Co. Wicklow, Ireland) unless stated otherwise. *P. gingivalis* strains were grown on Tryptic Soy Agar (TSA) supplemented with 5 g/l yeast extract, 0.50 g/l L-cysteine hydrochloride, 5.0 µg/ml haemin and 1.0 µg/ml menadione and 5% horse blood (Oxoid Limited, Basingstoke, UK). 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, 1.0 µg/ml tetracycline or 10 µg/ml chloramphenicol. For liquid cultures supplemented Tryptic Soy Broth (sTSB) containing identical supplements was used. Plasmid pJW1 was obtained from Prof. A. Progulske-Fox, University of Florida (Lépine *et al.*, 1996). The ATCC33277 *fimA* mutant was obtained from Hua Xie, School of Dentistry, Meharry Medical College, Nashville TN (Zheng *et al.*, 2011).

Mutant construction

Plasmid pJW1, containing the *hagB* gene interrupted by the *ermF-ermAM* cassette was used to inactivate the *hagB* gene in wild-type strains W83 and ATCC33277 (Lépine *et al.*, 1996). Preparation of electrocompetent *P. gingivalis* and transformation was carried out as described previously (Belanger, 2007). Briefly, transforming DNA was pipetted into a sterile electroporation cuvette (0.2 cm gap) with electrocompetent cells (100 µl) and electroporated using a Bio-Rad Minipulser (Bio-Rad Laboratories Ltd. Hemel Hempstead, UK) with 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 then incubated

anaerobically at 37°C overnight before the culture was plated on sTSA containing 50 µg/ml gentamicin and 5 µg/ml erythromycin. To generate a deletion cassette for the *hagC* gene, the *tetQ* gene was amplified from plasmid pT-Cow using Expand High Fidelity DNA polymerase (Roche Life Sciences) and primers TetQF and TetQR (Table S2). The PCR amplicon was digested with *Bam*HI and *Spe*I enzymes and ligated to *Bam*HI/*Spe*I digested pBlueScript II to generate pBSTetQ. A 313 bp region adjacent to the 5' end of *hagC* (RAHC1) was amplified with HagCF1 and HagCR1 (Table S2), digested with enzymes *Xho*I and *Hin*dIII and ligated to *Xho*I/*Hin*dIII 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 (Table S2), 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. Plasmid phagCΔ was transformed in *P. gingivalis* W83 by electroporation and transformants were selected on sTSA containing 5 µg/ml tetracycline resulting in the generation of ECM200 (*ΔhagC*). In order to generate a *ΔhagB/ΔhagC* mutant, ECM200 was transformed with plasmid pJW1 to generate strain ECM233._The *hagB* gene was reintroduced into strain ECM233 to generate the complemented strain GMC100 by generating a PCR fusion between the promoter of the *P. gingivalis tnaA* gene (amplified with primers TnF1_EcoRI and TnR31; Table S2) and the gene encoding chloramphenicol acetyl transferase (*cat1*; amplified with CMF72   and   CMR_HindIII; Table S2) as described by Yoshida et al. (Yoshida *et al.*, 2009). This fusion product was digested with *Eco*RI and *Hin*dIII and ligated to pBluescript II containing the downstream sequences of the *tnaA* gene on a *Hin*dIII/*Xho*I fragment that were amplified using primers TnF4_HindIII   and   TnR6_XhoI   (Table   S2). A *Sac*II/*Not*I fragment containing the entire *hagB* gene plus flanking sequences was ligated upstream of this *tnaA-cat1* fusion. A

*Hin*dIII/*Xho*I fragment containing sequences downstream of *hagB* was amplified with primers Hag3F_HindIII and Hag3R_XhoI (Table S2) and ligated downstream of the *tnaA*-cat1 fragment to generate pHAGB-CAT. The entire construct was released from the plasmid backbone by digestion with *Sac*II and *Apa*I and used to transform ECM233. Transformants were selected on sTSB plates containing 10 µg/ml chloramphenicol.

Host cell interaction assays

Haemagglutination of sheep erythrocytes by *P. gingivalis* strains was assessed as previously described (Lépine *et al.*, 1996). The oral squamous cell carcinoma (OSCC) cell line H357 (kindly supplied by Dr Simon Whawell, University of Sheffield, UK) was cultured and maintained as described previously (Suwannakul *et al.*, 2010). For adherence assays, a 24 well plate (Cellstar, Greiner Bio-one GmBH, Austria) was seeded with 5×10^3 H357 cells/well and incubated for 48 h in 5 % CO_2 at 37°C (~80 % confluency). Bacteria were grown in sTSB for 24 h under anaerobic conditions. The optical density (OD) of the broths was adjusted to OD_{600} of 0.7 and diluted 1:50 to generate a suspension of 1×10^7 bacteria/ml. Approximately 1×10^7 bacteria were added to triplicate wells to yield a multiplicity of infection (M.O.I) of 100. Plates were spun at 800 $x\ g$ (Sorvall Legend T centrifuge) for 5 min and the infected cells were incubated for 90 min in 5% CO_2 at 37°C. The cells were washed with sterile PBS three times before being lysed in 1 ml sterile water for 20 min. The lysates were serially diluted tenfold and plated onto Columbia blood agar (CBA; Cruinn Diagnostics Ltd., Dublin 12, Ireland) and incubated anaerobically at 37°C for seven days before counting. The percentage of adherence was calculated as the number of bacteria recovered post epithelial cell lysis expressed as a percentage of the bacteria recovered from the original inoculum. For the antibiotic protection assay of cellular invasion, the assay was incubated in cell media containing 30 µg/ml metronidazole prior to epithelial cell lysis.

P. gingivalis biofilm studies

Biofilm formation was assessed in *P. gingivalis* W83 and its derivatives as described previously (Sherry *et al.*, 2013; Millhouse *et al.*, 2014). Briefly, bacteria grown in Schaedler anaerobic broth for 48 h were used to prepare a suspension of to 1×10^7 bacteria/ml in artificial saliva (AS), which was composed of porcine stomach mucins (0.25% w/v), sodium chloride (0.35 w/v), potassium chloride (0.02 w/v), calcium chloride dihydrate (0.02 w/v), yeast extract (0.2 w/v), lab lemco powder (0.1 w/v), proteose peptone (0.5 w/v) in ddH$_2$O. Urea was diluted in PBS (40% w/v) and added to a final concentration of 0.05% (v/v). Biofilms were prepared in 24 well plates (Corning, NY, USA) containing customised Thermanox™ coverslips (13 mm diameter, Fisher Scientific). A 1ml aliquot of bacteria was added to each well and biofilm growth was assessed over 3 days by either crystal violet staining or staining with carboxyfluorescein succinimidyl ester (CFSE) and fluorescent intensity measurement. Six independent biofilms were assessed by both methods. Briefly, to quantify biomass, biofilms were washed with PBS and allowed to air dry for 1 hour at 37°C. 100 μL of 0.05 (w/v) of crystal violet (CV) solution was added to each biofilm and incubated at room temperature for 30 minutes to allow for the uptake of the dye. Following incubation the CV solution was removed and biofilms were washed using ddH$_2$O to remove any excess dye. Next, 100 μL of 100% ethanol was added to samples to de-stain each biofilm and mixed thoroughly to ensure complete removal. Contents of each well were then transferred to a fresh 96 well flat-bottom microtitre plate for measurement. The biomass was quantified spectrophotometrically by reading absorbance at 570 nm in a microtitre plate reader (FLUOStar Omega, BMG Labtech, VA, USA). All absorbance values were blank corrected based upon the negative control where no biofilms were formed. Biofilms were stained with 2 μL /mL of the green fluorescent dye carboxyfluorescein succinimidyl ester

(CFSE) in 1mL PBS (Sigma-Aldrich, Dorset, UK) and incubated anaerobically for 30 min on a shaking platform. Following incubation the solution was removed and samples were washed with PBS in triplicate to remove and excess dye. Fluorescence was quantified using the fluorescence plate reader (FLUOStar Omega, BMG Labtech, VA, USA) using 485nm excitation and 520nm emission. All absorbance values were blank corrected based upon the negative control where no biofilm was present.

Multi-species biofilms containing of *Streptococcus mitis* NCTC12261, *Fusobacterium nucleatum* ATCC10596, *Actinobacteria actinomycetemcomitans* OSM1123 and *P. gingivalis* (both W83 and *hag* mutants) were established using similar methods (Sherry *et al.*, 2013; Millhouse *et al.*, 2014). Mature multi-species biofilm biomass was measured using crystal violet. Compositional analysis of the biofilms was assessed using qPCR, as described (Sherry *et al.*, 2013). Briefly, biofilms were sonicated in 1 mL of PBS for 10 min and DNA extracted using the MasterPure Gram Positive DNA Purificiation Kit (Epicentre®, Cambridge, UK), following manufacturers instructions. 1 μL of extracted DNA was added to a mastermix containing 12.5 μL SYBR® GreenERTM, 9.5 μL UV-treated RNase-free water and 1 μL of 10 μM forward/ reverse primers for each bacterial species. Pimers and reaction conditions were identical to those previously described (Sherry *et al.*, 2013).

Analysis of gene expression by quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Strains were grown anaerobically on sTSA for four days at 37°C. Cells were flash frozen in liquid nitrogen in 50 ml polystyrene Falcon tubes. Frozen cells were ground using a Mikro-Dismembranator S series (Sartorius Mechatronics) mechanical grinder for 2 min at 1500 r.p.m. Bacterial RNA was isolated from this material using the RNeasy kit (Qiagen)

according to the manufacturer's instuctions. To remove genomic DNA, TURBO DNase reagent (Ambion, Life technologies, USA) was used according to the manufacturer's instructions. First strand synthesis of cDNA was performed using the Superscript III First strand synthesis system for real time PCR (Life Technologies) using 100 ng of total RNA and 50 µM Random hexamers (Promega). Quantitative PCR was carried out on selected genes using previously described primers listed in Table S3 (Hiratsuka *et al.*, 2008; Dou *et al.*, 2010). The amplification efficiency of each primer set was assessed and compared to the internal control primers for the 16s gene using a standard curve of Ct values plotted versus template concentration. The R^2 value for each primer set was calculated and primer sets with values > 0.98 were selected for use in experiments. Reactions were carried out in MicroAmp Fast Optical 96-well reaction plates (Applied Biosystems) in 15 µl reactions using 1X Fast SYBR Green PCR Master Mix (Applied Biosytems), 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. Gene expression was expressed relative to the expression of the 16s gene using the ΔCt method (Schmittgen and Livak, 2008).

RESULTS

Disruption of *hagB* and *hagC* in *P. gingivalis* W83

To disrupt the *hagB* gene in *P. gingivalis* W83, we employed the previously described *ermF-ermAM* selectable disruption cassette from plasmid pJW1 (Lépine *et al.*, 1996). Disruption of *hagB* was confirmed in strain ECM100 by PCR using primers specific for the *ermF-ermAM* cassette and the chromosomal flanking regions of *hagB* (Fig. S1). To create a double *ΔhagB/ΔhagC* mutant in *P. gingivalis* W83, a two-step disruption strategy was employed. A *tetQ* selectable disruption cassette was used to delete the entire *hagC* gene from strain W83, resulting in strain ECM200. Disruption of *hagC* in ECM200 was confirmed by PCR using primers specific for the *tetQ* gene and the chromosomal flanking regions of *hagC* (Fig. S1). The *ΔhagC* strain ECM200 was then used in a subsequent transformation with plasmid pJW1 to interrupt the *hagB* gene, thus generating strain ECM233 (*ΔhagB/ΔhagC*). PCR and Southern blot analysis was used to confirm mutagenesis of both genes in strain ECM233 (Fig. S1). The entire *ΔhagB/ΔhagC* locus was also amplified by PCR from strain ECM233 and sequenced which confirmed the disruptions (data not shown). Plasmid pJW1 was also used to create a single *ΔhagB* mutant in strain ATCC33277. PCR was used to confirm the integration of the *ermF-ermAM* cassette into the chromosomal *hagB* locus in ATCC33277, resulting in strain ECM300. Attempts to generate a double *ΔhagB/ΔhagC* mutant in strain ATCC33277 were unsuccessful (data not shown).

Mutagenesis of *hagB* and *hagC* affects pigmentation and haemagglutination in *P. gingivalis* W83.

After four days growth on blood agar plates (sTSA) we observed major differences in pigmentation between wild-type W83 and the Δ*hagB*/Δ*hagC* mutant ECM233. The wild-type strain W83 exhibited typical black, shiny colonies whereas ECM233 produced colonies that were white to cream in colour and were significantly smaller compared to strain W83, indicating a slower growth rate (Fig. 1a). The single *hagB* and *hagC* mutants ECM100 and ECM200 exhibited intermediate phenotypes and produced pale brown colonies by comparison (Fig. 1b). After further incubation for another seven days it was found that all mutant strains had darkened in their pigmentation, with ECM100 and ECM200 exhibiting darker pigmentation compared to ECM233 (data not shown). However, all mutant strains remained a dark brown colour and never exhibited the typical black pigmentation exhibited by W83. Restoration of *hagB* to strain ECM233 (strain GMC100) resulted in partial restoration of WT pigmentation similar to that exhibited by ECM200 (Fig. 1b).

We also analysed the capacity of each strain to acquire pigment in liquid media. Each strain was cultured in haemin free sTSB for 4 days until the cell culture pellets were devoid of pigment (Fig 1c). The bacteria were then restored to sTSB containing 10 μM haemoglobin. After 24 h growth in haemoglobin, wild-type W83 exhibited the greatest level of pigment accumulation with cell pellets that were a deep brown colour, while strains ECM100, ECM200 and ECM233 were defective in pigment accumulation (Fig. 1c). After 48 h incubation, all strains had darkened further with strain ECM100, ECM200 and ECM233 showing regions of deep brown pigmentation (Fig. 1c). The *hagB* complemented *ΔhagB/ΔhagC* mutant strain, GMC100, exhibited greater levels of pigment acquisition in sTSB than the *ΔhagB/ΔhagC* mutant ECM233.

Haemagglutination assays were also carried out to determine if there were differences in the ability of wild-type W83 and mutant strains to agglutinate red blood cells (Fig 1d). Cultures of wild-type W83 could agglutinate sheep erythrocytes at a titre that was three 2-fold dilutions lower than the double mutant ECM233 (Fig. 1d). The single *hagB* and *hagC* mutants ECM100 and ECM200 exhibited intermediate phenotypes, with haemagglutination titres 1 to 2 dilutions higher than wild-type (Fig. 1d).

Deletion of *hagB* and *hagC* effects adhesion and invasion of oral epithelial cells

The single mutants of *hagB* (ECM100) and *hagC* (ECM200) generated in the non-fimbriated strain W83 exhibited a statistically significant reduction in their capacity to adhere to the oral epithelial H357 cells compared to the wild-type strain W83 (Fig. 2a). Next, we assessed whether a double mutation in both *hagB* and *hagC* had an additive effect on adherence in strain W83. Unexpectedly, the *ΔhagB/ΔhagC* mutant ECM233 exhibited levels of adherence that were greater than either of the single *hagB* or *hagC* mutants and were not statistically significantly different to wild-type W83 (Fig. 2a). In order to confirm this unexpected finding, we examined a second, independent double *ΔhagB/ΔhagC* mutant, strain DKO3, and found that it exhibited an adherence phenotype that was indistinguishable from the initial mutant ECM233 (Fig. 2b). Interestingly, when *hagB* was restored to strain ECM233 in strain GMC100, this resulted in a hyper adherent phenotype when compared to the wild-type strain (Fig. 2b). ANOVA with Dunnet's multiple comparison showed statistically significant differences between W83 adherence and strains ECM100, ECM200 and GMC100 (p-value < 0.05).

We next analysed the capability of these mutants to invade the H357 cell line using an antibiotic protection assay (Fig 2c). Following 24 h incubation, strains ECM100 and

ECM200 showed reduced levels of invasion compared to the wild-type strain, although these differences were not significant when normalised for adherence (data not shown). However, GMC100 exhibited significantly lower levels of invasion relative to wild-type, despite the fact that the mutant was hyper-adherent (p-value <0.05).

Due to the unusual adherence phenotypes observed in the *ΔhagB/ΔhagC* double mutant ECM233 and its *hagB* complemented derivative GMC100, we hypothesised that deletion of both *hag* genes resulted in activation of accessory genes involved in the processes of adhesion and haem acquisition that compensated for the loss of both *hagB* and *hagC*. We analysed the expression of a number of genes including *hagA, rgpA, rgpB* and *kgp* by qRT-PCR. In the case of *rgpA, rgpB* and *kgp* it was found that the double mutant ECM233 exhibited higher levels of gene expression compared to wild-type W83 or the single *hag* mutants (Fig 2d). In particular, expression of *rgpA* and *rgpB* in ECM233 were highly deregulated and statistical significance was difficult to determine. Interestingly, there was far less variance in the gene expression data generated from wild-type W83 and the two single mutants, indicating a greater degree of stability in the transcriptomes of these strains (Fig 2d). Strain GMC100 also showed elevated levels of *rgpA* and *rgpB*, but in contrast exhibited reduced expression *hagA* and *kgp* relative to wild-type W83 (p-value 0.0422).

Deletion of *hagB* in the fimbriated strain ATCC33277 affects adherence

In order to determine if HagB played any role in the adherence of fimbriated strains of *P. gingivalis*, we also generated a *hagB* mutant in strain ATCC33277 and compared its relative contribution to adherence with *fimA*. Strain ATCC33277 was found to exhibit higher levels of adherence to the H357 cells than strain W83, as would be expected for a

fimbriated strain of the bacterium (Fig. 3). 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. 3). It was found that mutation of *hagB* also significantly affected the ability of ATCC33277 cells to adhere to H357 cells (Fig. 3). This mutation resulted in an approximately 10-fold reduction in the adherence of strain ATCC33277 from ~10 % of the inoculum to approximately ~1 % recovered with the *hagB* mutant (Fig. 3).

Biofilm formation in *P. gingivalis* W83 requires *hagC*

The ability of *P. gingivalis* W83 and mutant derivatives to form single species biofilms was analysed over the course of three days using both crystal violet staining and CFSE staining to assess biofilm biomasss (Fig. 4). Following 3 days of biofilm growth significant differences in biofilm biomass could be detected. When crystal violet was used to measure biomass it was found that strains ECM200 (*ΔhagC)* and ECM233 (*ΔhagB/ΔhagC)* formed significantly lower levels of biofilm compared to wild-type W83 (p-values < 0.001; Fig. 4a). Using CFSE fluorescence to measure biomass it was similarly shown 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. 4b). Fluorescent microscopic analysis of CFSE stained biofilms confirmed the reduced levels of biofilm in the mutant strains relative to wild-type W83 (Fig. 4c). The role of HagB and HagC in forming multi-species biofilms containing *S. mitis, F. nucleatum, A. actinomycetemcomitans* and *P. gingivalis* was also assessed. Mature biofilm biomass was measured using crystal violet. Significant differences were observed between the biomass of biofilms containing *P. gingivalis* wild-type and those containing *P. gingivalis ΔhagC*

mutant ECM200 (p<0.001; Fig 5a). The abundance of each species within the biofilm was also examined by qRT-PCR (Fig 5b). It was found that there were no significant differences in the overall abundance of each species present.

DISCUSSION

The *hagB* and *hagC* genes of *P. gingivalis* encode two highly homologous haemagglutinins. Conservation of these two homologous genes in close proximity in the highly plastic genome of *P. gingivalis* suggests that possession of both genes is required for maximal fitness. The importance of these two genes to the growth and survival of *P. gingivalis* is also underlined by the failure of previous attempts to generate a double Δ*hagB*/Δ*hagC* mutant in *P. gingivalis* 381 (Lépine *et al.*, 1996). Our goal in this study was to reassess the role of these genes in the growth and fitness of *P. gingivalis* as well as for the first time fully assess their role in adherence to oral epithelial cells and other oral bacteria. We chose W83 as the main background strain for mutant construction as this is a robust, fast growing strain that may better tolerate defects in haemagglutination and because this strain is non-fimbriated and would facilitate the characterisation of these putative non-fimbrial adhesins. We used a sequential disruption strategy that first eliminated the entire *hagC* gene followed by targeted mutagenesis of *hagB*. The double disruption was confirmed by a combination of PCR, Southern hybridisation and direct sequence analysis of the locus, all of which confirmed that ECM233 was a *bone fide* Δ*hagB*/Δ*hagC* mutant.

Deletion of both genes resulted in a striking absence of black pigmentation and a reduction in colony size relative to wild-type, reminiscent of the phenotype of a gingipain triple mutant (Shi *et al.*, 1999). This finding suggests that the *hagB/C* genes are at least as

important as the gingipains in the process of erythrocyte capture for haem liberation. Colonies of the ΔhagB/ΔhagC mutant did darken over time however, indicating that accessory erythrocyte capture mechanisms were still active.

Previous studies have shown that heterologously expressed HagB mediates adherence to human coronary arterial endothelial cells (HCAECs; Song *et al.*, 2005). Here for the first time we demonstrate a role for these genes in mediating adherence to oral epithelial cells. Individual *hagB* or *hagC* mutants in strain W83 exhibited ~60% reduction in adhesion in our model. These data suggest that either both proteins are co-expressed at the cell surface, or that subpopulations expressing both proteins exist in strain W83. Due to the high homology of these proteins, it is difficult to discriminate between them by biochemical methods, however we can detect mRNA of both genes in broth culture (data not shown).

Strain ECM233, the mutant strain of W83 that has both *hagB* and *hagC* mutated in its genome, produced some unexpected results. It was expected that an additive defect in adhesion would be observed in this strain. Instead it was found that the level of adherence in strain ECM233 was comparable to that of the WT parent strain. Independent *ΔhagB/ΔhagC* double mutants had identical adhesive phenotypes indicating that this finding was not the result of a random gain of function mutation. Complementation of ECM233 with *hagB* produced a hyper adherent strain that was compromised in terms of its invasion capabilities. It was expected that restoration of *hagB* to the genome of ECM233 would generate a strain with similar adherence characteristics of strain ECM200 (ΔhagC) as complementation with *hagB* restored pigmentation to the levels of the ΔhagC mutant. This finding led us to hypothesise that genes involved in adhesion were induced or overexpressed in ECM233 to compensate for the loss of *hagB* and *hagC*, and perhaps this overexpression was required for viability of the mutant. Previously reported attempts to generate a double *ΔhagB/ΔhagC* mutant in *P. gingivalis* 381 were unsuccessful,

suggesting that this combination of mutations is very stressful for the bacterium. Analysis of gene expression in strain ECM233 indicates that expression of the gingipains *rgpA*, *rgpB* and *kgp* is highly deregulated. The data suggest that ECM233 expresses compensatory mechanisms to counteract the loss of *hagB* and *hagC*. Complementation with *hagB* thus resulted in the hyper-adherent strain GMC100. Our current hypothesis is that the double *ΔhagB/ΔhagC* deletion was only viable in a subpopulation of cells in the W83 background where expression of these genes was deregulated. Support for this hypothesis comes from the work of Suwannakul et al. (2010) who have shown that *P. gingivalis* populations are heterogeneous and consist of subpopulations of cells that differ in the expression of genes including *rgpB*, *hmuR* and s*odB*. In the current study, it is likely that the double *ΔhagB/ΔhagC* mutation was only viable in cells belonging to such an adherent sub-population and that these cells would have been selected during the construction of strain ECM233. It is not know whether all strains of *P. gingivalis* exhibit this "bi-stable population" structure or if all strains exhibit variability in the same subsets of genes. The absence of this phenomenon in some strains of *P. gingivalis* may explain why attempts to generate a *ΔhagB/ΔhagC* mutant were not successful in strains 381 (Lépine *et al.*, 1996) and ATCC33277 (this study). Similarly, it has also been reported that *ragA* mutants are viable in some strains of *P. gingivalis* (WPH35) and not others, including ATCC33277 (Klein *et al.*, 2012).

Strain GMC100 did exhibit some altered gene expression patterns compared to its parent EMC233, with lower levels of *kgp* and *hagA* expression. It is possible that restoration of *hagB* removes some of the selective pressure to maintain expression of these genes, resulting in an altered pattern of transcription. This may be linked to the non-invasive phenotype exhibited by this strain, although further studies are required to determine this.

Unexpectedly, we also observed that HagB makes a major contribution to adherence in the fimbriated strain ATCC33277. Our data confirm the importance of *fimA* in mediating adhesion to oral epithelial cells, as adherence of the *fimA* mutant to H357 cells drops to ~0.16 % of the inoculum compared to 10% in the wild-type strain. However, we also demonstrate that removal of *hagB* from the ATCC33277 genome has 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 mediating stable adhesion, even in fimbriated strains. These data suggest that adhesion to the H357 cell line is a mutifactorial process involving fimbrial and non-fimbrial adhesins. Although the data confirm that FimA plays an essential role in mediating adherence, it also indicates that HagB is required to stabilise this interaction. Alternatively, adherence may be a two-stage process where fimbriae are required for initial long-range interactions and outer membrane adhesins such as HagB mediate stable, short-range interactions (Fig. 6). Both stages may be required for wild-type levels of adherence observed in strain ATCC33277 and disruption of either stage could result in the severe defects in adhesion observed in the current study.

Our data also supports a role for the *hag* genes in biofilm formation. When mono-species biofilm formation was assessed either by crystal violet or CFSE staining, it was found that there was a statistically significant difference in the ability of strains ECM200 and ECM233 to form biofilms compared to the WT parent strain. Mutants that lack the *hagC* gene were most affected in this assay, suggesting that *hagC* plays an important role in this process. The *hagC* gene is known to be induced in low haem environments, and the internal environment of the biofilm may favour *hagC* expression. The multispecies biofilm model data also support a role for *hagC* in biofilm formation. While there was no significant difference in the overall composition of each of the biofilms, when the total

biomass of each of the mature multi-species biofilms was assessed it was found that biofilms containing the *hagC* mutant ECM200 had a statistically significant reduction within the overall biomass compared to other *P. gingivalis* strains within the other biofilms. This reduction in biomass is unexpected given the relatively low abundance of *P. gingivalis* in the biofilm, however it may indicate that co-aggregation with HagC expressing *P. gingivalis* has important implications for the other species in terms of stimulating quorum sensing or exopolysaccharide production.

These data indicate that HagB and HagC are crucial haemagglutinins required for the normal physiology and growth of *P. gingivalis*. HagB has been proposed as a candidate vaccine antigen and the data presented in this study would support this proposal as a protective antibody response could potentially inhibit colonisation and subgingival biofilm formation, thereby reducing the potential for dysbiosis in the gingival crevice.

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Figure Legends

Figure 1. Analysis of pigment production and haemagglutination in *ΔhagB* and *ΔhagC* mutants. (a) Colony pigmentation in wild-type W83 and *ΔhagB/ΔhagC* mutant ECM233 on sTSA. (b) Colony pigmentation in the single *ΔhagB* and *ΔhagC* mutants ECM100 and ECM200 compared to the *ΔhagB/ΔhagC* mutant ECM233 and its *hagB* complemented derivative GMC100. (c) Pigment production in liquid sTSB cultures of the indicated *P. gingivalis* strains. Top row (haemin starved) shows pigment of bacterial pellets following 4 days growth in haem-free sTSB. Rows below show pellet pigmentation following resuspension of the bacterial culture in sTSB containing 10 μM haemoglobin and incubation for 24 (middle row) and 48 h (bottom row). (d) Haemagglutination patterns exhibited by the indicated strains following incubation with sheep erythrocytes. Rows 1-7 (top to bottom) contain 2-fold dilutions of the indicated bacterial strains, in triplicate. Bottom row contains a negative control.

Figure 2. (a-c) Interaction of *P. gingivalis* wild-type and *ΔhagB* and *ΔhagC* mutant strains with the oral epithelial H357 cell-line. Stars indicate data significantly different from wild-type W83 (p-value < 0.05) following ANOVA with Dunnet's multiple comparsion. (a) Adherence (as % of inoculated bacteria) of wild-type W83, ECM100 (*ΔhagB*), ECM200 (*ΔhagC*) and ECM233 (*ΔhagB/ΔhagC*) to H357 cells following 90 min incubation. (b) Adherence of ECM233 (*ΔhagB/ΔhagC*) relative to DKO3 (*ΔhagB/ΔhagC*) and GMC100 (*hagB*::*cat*/*ΔhagC)*. (c) Levels of bacterial invasion of H357 cells following 24 h incubation assessed using the antibiotic protection assay. (d) Expression levels of *rgpA, rgpB, kgp* and *hagA* in the indicated *P. gingivalis* strains determined by qRT-PCR following growth on sTSA. Expression level is expressed

relative to the 16s gene in the same culture. Expression of *kgp* and *hagA* were significantly lower in strain GMC100 relative to wild-type W83 (* *t*-test p-value of 0.0422 and *** 0.0003, respectively).

Figure 3. Adherence (expressed as % of inoculated bacteria) of *P. gingivalis* wild-type ATCC33277, a *ΔhagB* mutant and a *ΔfimA* mutant derivative to the H357 cell line following 90 min incubation. Stars indicate data significantly different from wild-type W83 (p-value < 0.05) following ANOVA with Dunnet's multiple comparsion.

Figure 4. Single-species biofilm formation by the indicated strains determined by crystal violet staining (a) or staining with CFSE and measurement of fluorescent intensity measured (b). Data are mean ± SEM of six independent biofilms per group. * p<0.05, *** p<0.001. (c) Single species biofilms (3 day) of *P. gingivalis* wild-type, *ΔhagB* (ECM100), *ΔhagC* (ECM200) and *ΔhagB/ΔhagC* (ECM233) stained with CFSE and imaged using fluorescent microscopy.

Figure 5. Multi-species biofilms were grown containing *S. mitis, F. nucleatum, A. actinomycetemcomitans* and the *P. gingivalis* strains. (a) Mature biofilm biomass was measured using crystal violet. Significant differences were observed between the biomass of biofilms containing *P. gingivalis* wild-type and those containing the *P. gingivalis hagC* mutant (*** p<0.001). Data are mean ± SEM of 3 independent biofilms per group. (b) The composition 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.

Figure 6. A simple, 2-stage model of adherence in *P. gingivalis*. (a) Stage 1 of the adherence process involves long-range interactions mediated by the *FimA* subunit of the major fimbriae and β1-integrins of epithelial cells. (b) Stage 2 involves close range
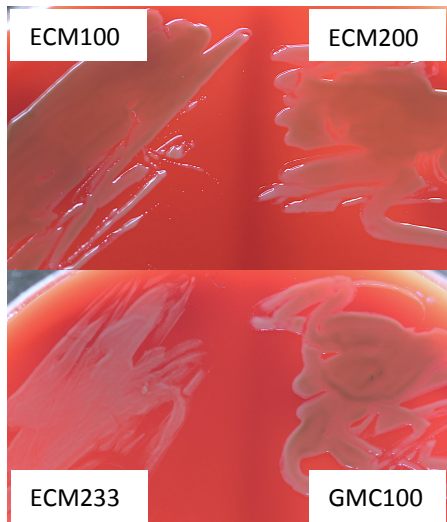
interactions mediated by HagB and an unknown receptor that stabilises the interaction of the bacterium with the host cell surface.
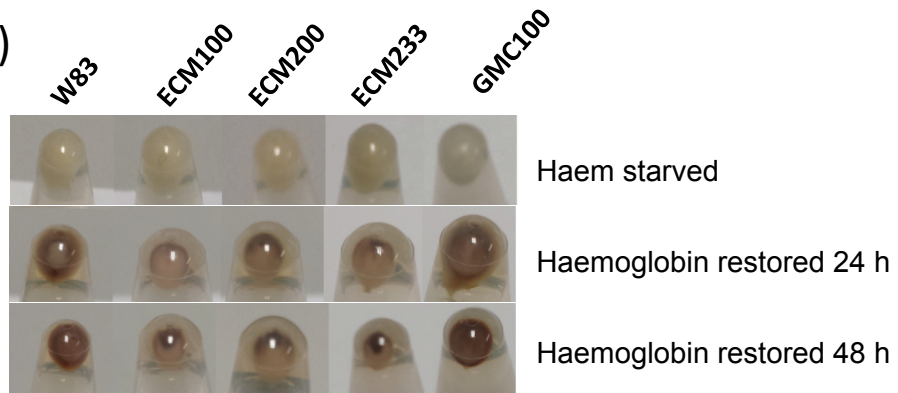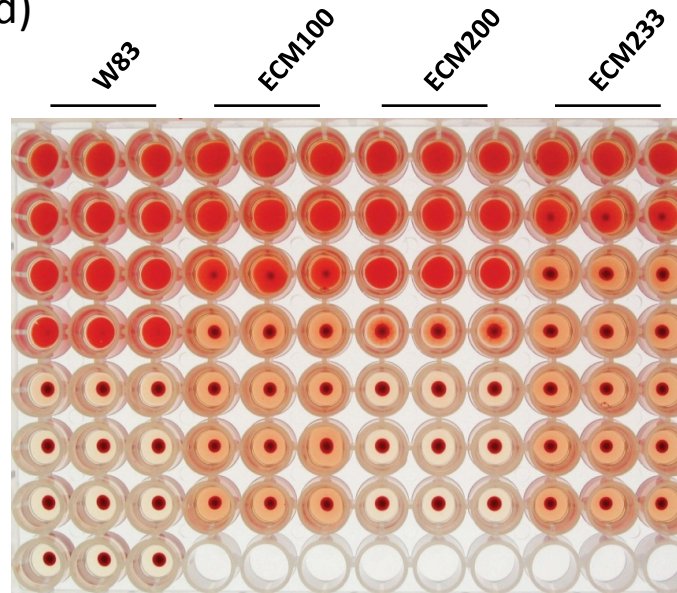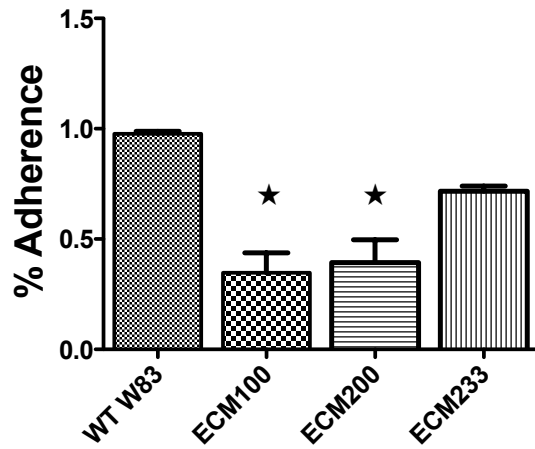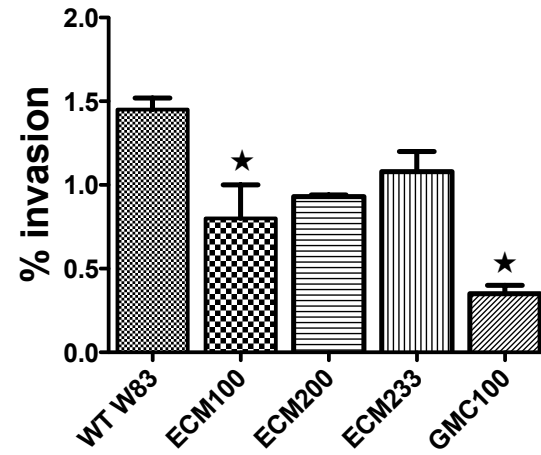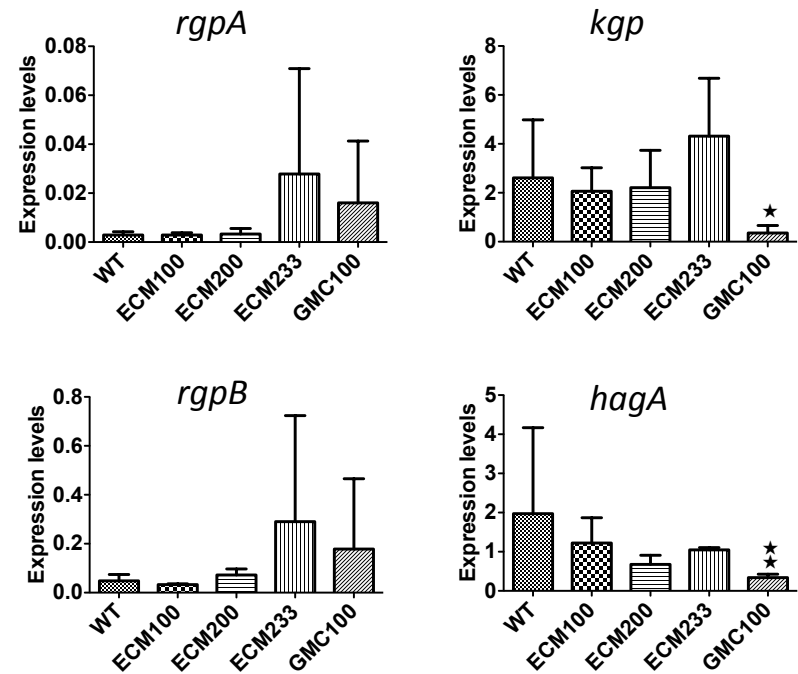
Fig. 1

(a)

(b)

(c)

(d)

Fig. 2

Fig. 3

Fig. 4

(a)

(b)

(c)

ECM100   ECM200   ECM233   WT W83
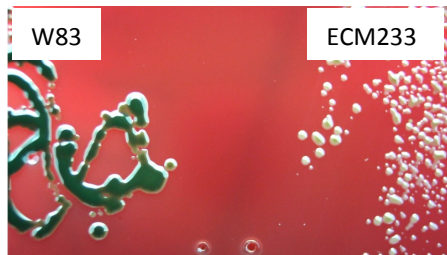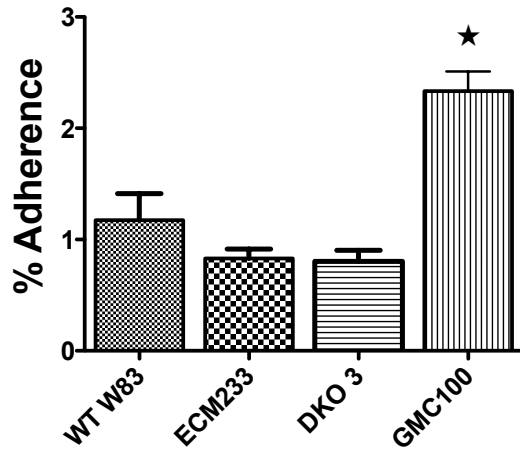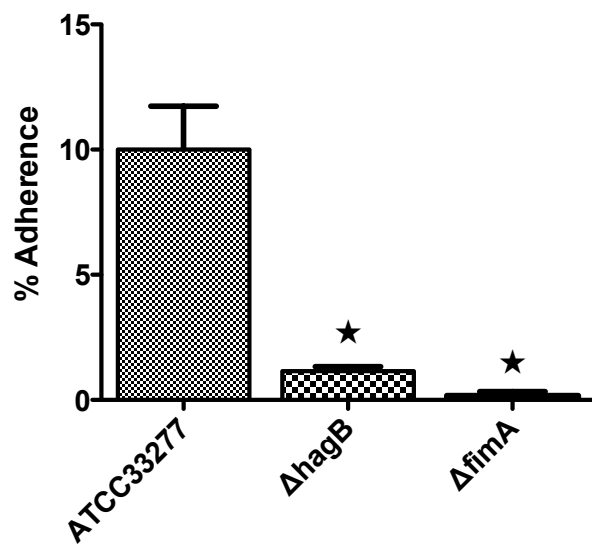
Fig. 5

(a)

(b)

Fig. 6

(a) Stage 1   (b) Stage 2

Major fimbriae

β1 integrin

HagB

Supplementary Information

The *Porphyromonas gingivalis* haemagglutinins HagB and HagC are major mediators of adhesion and biofilm formation.

Eimear Connolly, Emma Millhouse, Rebecca Doyle, Shauna Culshaw, Gordon Ramage and Gary P. Moran.

Contents:

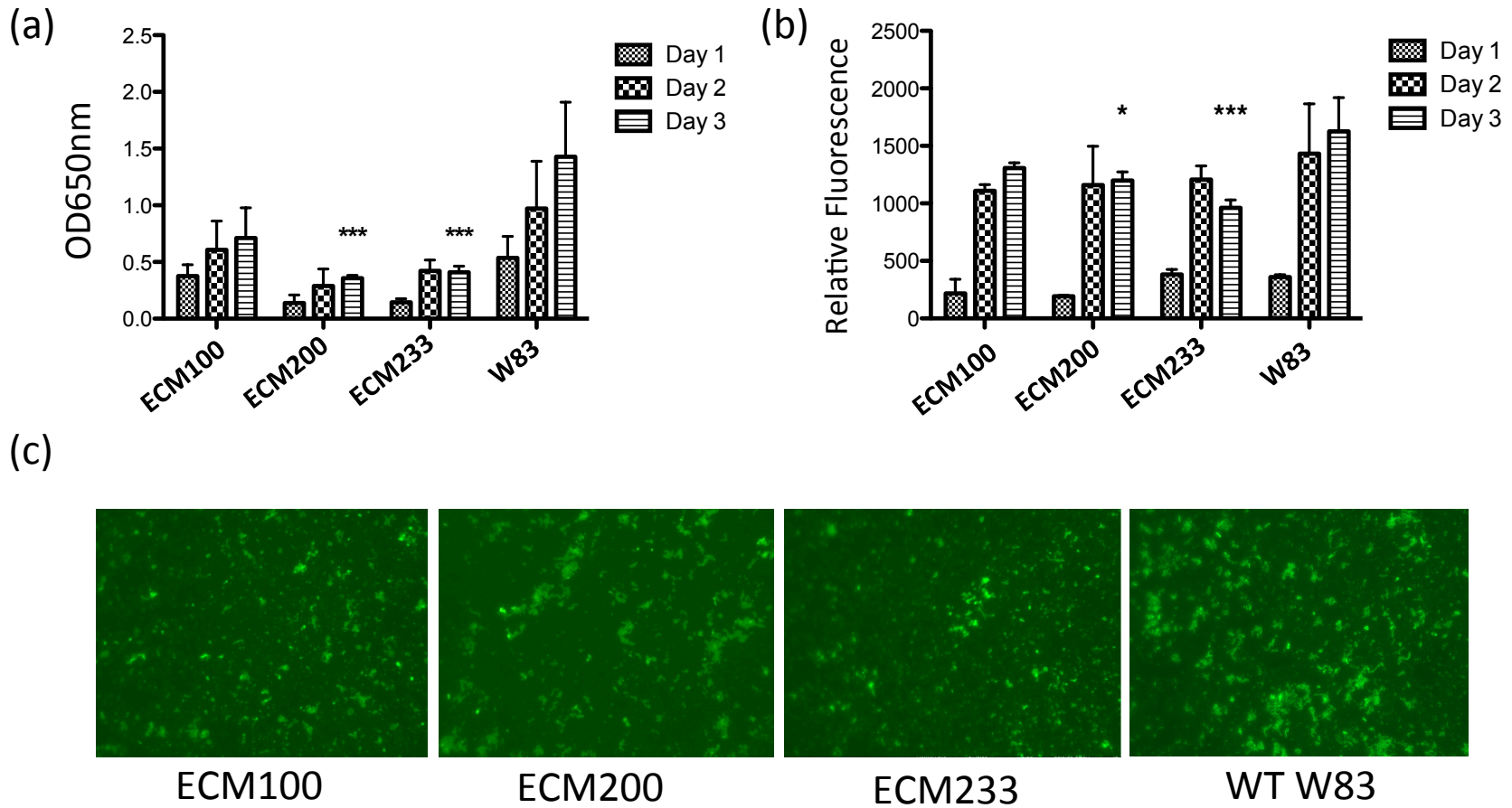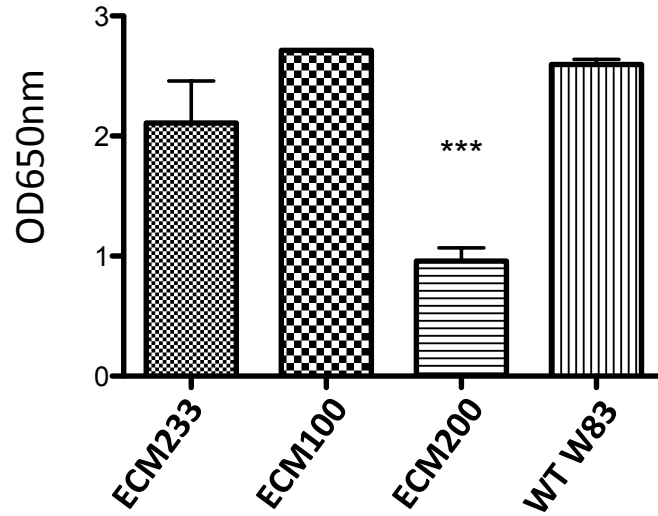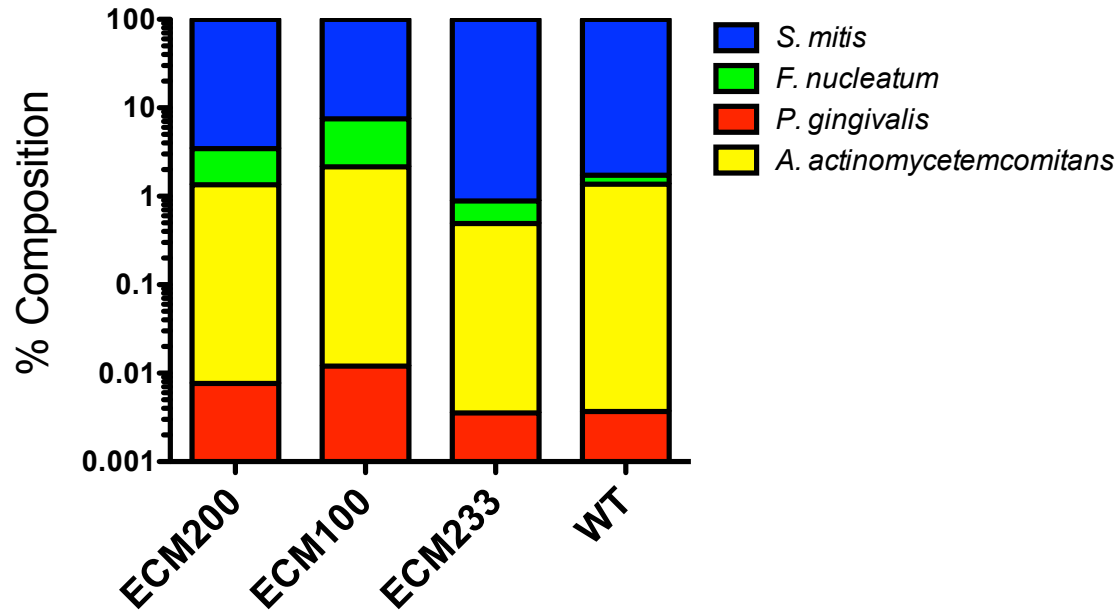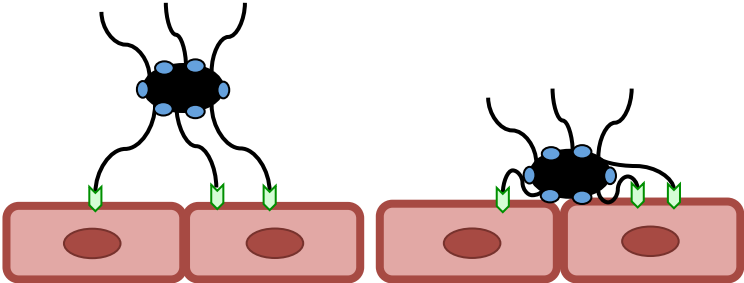**Table S1.** *P. gingivalis* strains used in this study.

Strain	Genotype	Parent strain	Reference
P. gingivalis W83	*Wild-type*	-	
ECM100	*ΔhagB::ErmF*	W83	This study
ECM200	*ΔhagC::TetQ*	W83	This study
ECM233	*ΔhagB::ErmF, ΔhagC::TetQ*	ECM200	This study
DKO3	*ΔhagB::ErmF, ΔhagC::TetQ*	W83	This study
GMC100	*hagB::cat, ΔhagC::TetQ*	ECM233	This study
P. gingivalis ATCC33277	Wild-type	-	
(*ΔhagB*) ATCC33277	*ΔhagB::ErmF*	ATCC33277	This study
(ΔfimA) ATCC33277	*ΔfimA::ErmF*	ATCC33277	Zheng & Xie

Table S2 PCR primers used in this study

Primer	Sequence (cut sites underlined)	Enzyme Site
TnF1_EcoRI	GCG<u>GAATTC</u>CGAAAAGTCCTTGTCGTGA	*Eco*RI
TnR31	TGTTCTTTACGATGCCATTGGGATATATCAACGGTGGTAT ATCCAGTGATTTTTTTCTCCATCATTTTAATGCGGTAGGAT TC	-
CMF72	CAAAATCACAGTTACAATCCTATGGAATTACCTTTTTCAG AATCCTACCGCATTAAAATGATGGAGAAAAAAATCACTG GA	-
CMR_HindIII	GC<u>AAGCTT</u>TACGCCCCGCCCTGCCACTC	*Hin*dIII
TnF4_HindIII	GC<u>AAGCTT</u>TCACTTCACTGTTGAGCTGG	*Hin*dIII
TnR6_XhoI	CG*<u>CTCGAG</u>*ATCGGTATTCTTGGATTGGTA	*Xho*I
TetQF	GGG<u>GGATCC</u>GGGGGGGAAATACTAAATGC	*Bam*HI
TetQR	GGG<u>ACTAGT</u>AGCTCGTCTATTTTTTTATT	*Spe*I
Hag3F_HindIII	GC<u>AAGCTT</u>TCCCCCCGTGCCGTCCTGCCGGCCGCA	*Hin*dIII
Hag3R_XhoI	CG<u>CTCGAG</u>GCAAGAAACAAGCAAATCTG	*Xho*I
HagCF1	GGG<u>CTCGAG</u>ACCTGCAGAGTATGTA	*Xho*I
HagCR1	GGG<u>AAGCTT</u>TTTGAATTAAGGGGTTAAGTC	*Hin*dIII
HC2_F	GGG<u>TCTAGA</u>AAGGAGGCAGCAGTAGCGGCG	*Xba*I
HC2_R	GGGG<u>CGGCCGC</u>TTCACGGATTATCCCTTTTA	*Not*I

DKOBFP	CAGGAAGCCGTTGTCACG	-
DKOBRP	ATCTCTGGCAACAACATTGGGATAG	-
DKOCFP	GCCGATCTTCGAAATTCCAAG	-
DKOCRP	AGAGCGTTCACCGATTAT	-

Table S3 Primers used in qRT-PCR experiments

Primer	Sequence (5'-3')	Reference
16S rRNA F	AGGCAGCTTGCCATACTGCG	(Dou et al., 2010)
16S rRNA R	ACTGTTAGCAACTACCGATGT	(Dou et al., 2010)
HagA F	GTGGTAGAGGCCGGTAAGACTTATCA	(Dou et al., 2010)
HagA R	CGTCACCTGTATGAGTAGCAGTG	(Dou et al., 2010)
Kgp F	AATACGCCGGTTCGTATGCT	(Hiratsuka et al., 2008)
Kgp R	TGTCACCAACCAAAGCCAAG	(Hiratsuka et al., 2008)
RgpA F	TCAAGAACAATCTCGGCCTGACCG	(Hiratsuka et al., 2008)
RgpA R	AGCACGCTTACCCTTACAGTAGTT	(Hiratsuka et al., 2008)
RgpB F	TCGCTGATGAAACGAACTTGACG	(Hiratsuka et al., 2008)
RgpB R	TTCGAATACCATGCGGTTCTTAGC	(Hiratsuka et al., 2008)

Figure S1.

(a)

(b)

(c)

Legend: Confirmation of the genotypes of strains ECM100, ECM200 and ECM233.
(a) Cartoon showing the structure of the *hagB-hagC* locus and the position of primers used to identify the insertions of the *ermF-ermAM* and *tetQ* cassettes into the *hagB* and *hagC* loci, respectively. Primer sequences are listed in Table S2.
(b) Gel electrophoresis of PCR product generated with the indicated primers. Lane 1 contains a 1 Kb DNA ladder; lane 2, a no template control reaction; lane 3, amplimers generated from strain W83 (wild type) and lane 4, amplimers generated from strain ECM233 (*ΔhagB/ΔhagC*). Amplimers in lanes 4 show a shift in size indicating the insertion of the selectable marker.
(c) Southern hybridisation of *BglII* digested genomic DNA from the indicated strains with a digoxigenin-labelled probe (Roche) with 100% homology to *hagB* and *hagC*. Both genes can be detected in W83. The *hagC* hybridising fragment is lost in ECM200 *(ΔhagC*) and in ECM233 *(ΔhagB/ΔhagC)* the *hagB* hybridising fragment exhibits a shift due to insertion of the *ermF-ermAM* cassette.