Hemin Binding by *Porphyromonas gingivalis* strains is dependent on the presence of A-LPS

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SUMMARY.

Porphyromonas gingivalis is a Gram-negative black pigmenting anaerobe unable to synthesise haem (Fe (II)-protoporphyrin IX) or hemin (Fe(III)-protoporphyrin IX-Cl) which are important growth/virulence-factors, and must therefore derive them from the host *P. gingivalis* expresses several proteinaceous hemin -binding-sites which are important in the binding/ transport of haem/hemin from the host. *P. gingivalis* also synthesises several virulence factors, namely cysteine-proteases Arg- and Lys-gingipains and two lipopolysaccharides (LPS), O-LPS and A-LPS. The gingipains are required for the production of the black pigment, μ-oxo-bishaem ([Fe(III)PPIX]$_2$ O), which is derived from hemoglobin and deposited on the bacterial cell-surface leading to the characteristic black colonies when grown on blood agar. In this study we investigated the role of LPS in the deposition of μ-oxo-bishaem on the cell-surface. A *P. gingivalis* mutant defective in the biosynthesis of Arg-gingipains, namely *rgpA*/*rgpB* produces brown colonies on blood agar and mutants defective in Lys-gingipain (*kgp*) and LPS biosynthesis namely *porR*, *waaL*, *wzy* and *pg0129* (α-1, 3 -mannosyltransferase) produce non-pigmented colonies. However, only those mutants lacking A-LPS showed reduced hemin-binding when cells in suspension were incubated with hemin. Using native, de-*O*-phosphorylated and de-lipidated LPS from *P. gingivalis* W50 and *porR* strains, we demonstrated that hemin-binding to O-polysaccharide (PS) and to the lipid A moiety of LPS was reduced compared to hemin-binding to A-PS. We conclude that A-LPS in the outer-membrane of *P. gingivalis* serves as a scaffold/ anchor for the retention of μ-oxo-bishaem on the cell surface and pigmentation is dependent on the presence of A-LPS.

INTRODUCTION.

The black pigmenting anaerobe *Porphyromonas gingivalis* is a major pathogen in chronic adult periodontal disease Haffajee & Socransky (1994) and has recently been described as a "keystone pathogen" with wide-ranging effects critical for the development of dysbiosis and disease progression Hajishengalis & Lamont (2012). The main habitat of this organism is the human gingival crevice where nutrients are gained from gingival crevicular fluid- a plasma exudate. Hemin (Fe(III)-protoporphyrin IX-Cl) is an important requirement for growth of *P. gingivalis* (Gibbons & Macdonald, 1960; Shah et al., 1979) and since the organism is not able to synthesise protoporphyrin IX ring (Schifferle et al., 1996; Olczak et al., 2005) and does not contain any siderophores (Bramanti & Holt, 1991; Genco, 1995), the major source of haem (Fe(II)-protoporphyrin IX) is therefore the host.

Black pigmenting *Bacteroides* species have been shown to degrade plasma proteins involved in the transport and conservation of body iron**,** namely albumin, hemopexin, haptoglobin and transferrin to varying degrees, with *Bacteroides gingivalis* (*P. gingivalis*) being the most effective (Carlsson et al., 1984). The cysteine protease Lys-gingipain (Kgp) of *P. gingivalis* can cleave free hemoglobin (Lewis et al., 1999), haptoglobin, hemopexin and transferrin in human serum but was not able to degrade hemoglobin, or the β-chain of haptoglobin when these were present in a haptoglobin-hemoglobin complex in serum (Sroka et al., 2001).

P. gingivalis possesses additional outer membrane proteins which are important in the binding and transport of haem and which form part of the *hmu* haemin-uptake locus (Lewis et al., 2006), namely HmuY (Wojtowicz et al., 2009) and HBP35 protein has been described as an important hemin-binding protein (Shoji et al., 2010). Several putative TonB-dependent outer-membrane

receptors have been described including Tlr (Slakeski et al., 2000), IhtA (iron heme transport) (Dashper et al., 2000), HmuR (hemin utilization receptor) (Simpson et al., 2000; Olczak et al., 2005; Olczak et al., 2008) and HemR (hemin-regulated receptor) (Karunakaran et al., 1997). HmuR exhibited amino acid sequence homology to TonB-dependent receptors involved in heme, vitamin B_{12} or iron-siderophore transport in other bacteria (Simpson et al., 2000). A *P. gingivalis hmuR* isogenic mutant strain was shown to have impaired growth on hemin and hemoglobin as sole source of iron and showed decreased ability to bind hemin and hemoglobin. *E. coli* cells overexpressing *P. gingivalis* HmuR as well as purified recombinant HmuR were able to bind hemin, hemoglobin and serum-albumin-hemin complex (Simpson et al., 2000). *P. gingivalis* W50 produces several virulence factors including gingipain proteases and two LPSs, namely O-LPS (Paramonov et al., 2001) and A-LPS (Rangarajan et al., 2008).

In this study, we addressed the question whether the high abundance low-affinity hemin-binding site described by (Tompkins et al., 1997) may be one of the LPS of *P. gingivalis*. In order to test this hypothesis, we examined a variety of isogenic mutant strains of *P. gingivalis* lacking Arg-gingipains, Lys-gingipain and defective in the biosynthesis of O-LPS and A-LPS for their ability to pigment and to bind hemin not only to whole cells but also to LPS, de-phosphorylated LPS and de-lipidated LPS. *P. gingivalis porR* (PG1138) defective in A-LPS synthesis and *galE* (PG0347) mutant strains which synthesises a truncated O-PS repeating unit of O-LPS, have been described in greater detail in this manuscript. Shoji et al. (2002) described *porR* mutant strain in *P. gingivalis* ATCC33277 isolated by transposon and targeted mutagenesis and Gallagher et al. (2003) have referred to a *porR* mutant strain isolated by inactivation of PG1138 in *P. gingivalis* W50.

102 *P. gingivalis galE* (PG0347) shares homology with *galE* of *E. coli* which encodes UDP-
103 galactose-4-epimerase responsible for the conversion of UDP-Glc to UDP-Gal. Galactose is a
104 component of the repeating unit of O-PS (Paramonov et al., 2001) and in *galE* (ΔPG0347), O-
105 LPS is still synthesised, but its repeating unit is shortened by one residue, namely Gal
106 (Unpublished data).
107 The results of hemin-binding to the mutant strains of *P. gingivalis* exhibit a consistent pattern
108 which suggests that the deposition of μ- oxobishaem on the cell surface of the *P. gingivalis*
109 strains appears to be related to the synthesis/presence of A-LPS in the outer leaflet of the outer-
110 membrane. We propose that the presence of A-LPS serves as a matrix for the deposition of μ-
111 oxo bishaem on the *P. gingivalis* cell surface.
112
113

MATERIALS and METHODS.

Materials. A solution containing 30% acrylamide-N,N-methylenebisacrylamide (BIS) (37.5:1) was obtained from Bio-Rad Laboratories (Hercules, CA, USA). Horseradish peroxide-labelled mouse immunoglobulin was purchased from Dako A/S, High Wycombe, Buckinghamshire, United Kingdom. All other chamicals were from VWR, Lutterworth, Leicestershire, United Kingdom, or from Sigma-Aldrich Co. Ltd., Poole, Dorset, United Kingdom and were the purest grades available. Nα-acetyl-Lys-*p*-nitroanilide was obtained from Bachem Feinchemikalein AG (Bubendorf, Switzerland). Hemin was obtained from Roche (East Sussex, United Kingdom). Restriction and modification enzymes were purchased from New England BioLabs, and DNA purification reagents were obtained from Qiagen.

Bacterial strains and growth conditions.

P. gingivalis W50 and mutant strains were grown either on blood agar plates containing 5% defibrinated horse blood or in Brain Heart Infusion broth (Oxoid, Basingstoke, United Kingdom) supplemented with hemin (5 μg /ml) in an anaerobic atmosphere consisting of 80% N_2, 10% H_2 and 10% CO_2 (Aduse-Opoku et al., 1995). Clindamycin HCl and tetracycline HCl were added to 5μg /ml and 1μg /ml respectively, for selection of *ermF* and *tet Q* in *P. gingivalis*. Ampicillin (Na^+ salt; 100μg /ml) or erythromycin (300μg /ml) was added to the growth medium to select for pUC-derived or *ermAM*-containing plasmids respectively, in *Escherichia coli*.

Generation of *P. gingivalis* mutants.

Purification and general manipulation of DNA, restriction mapping of plasmids and transformation of *E. coli* were as described (Sambrook et al., 1989; Aduse-Opoku et al., 1995). A list of *P. gingivalis* strains used in this study is shown in Table S1.

For the generation of *P. gingivalis* mutant strains *porR* and *galE*, chromosomal DNA from *P. gingivalis* W50 was used as the template for amplification/ cloning purposes. The nomenclature originally used by TIGR is used throughout the manuscript. The genes encoding UDP-Glucose-4-epimerase *galE* and *porR* (Shoji et al., 2002; Gallagher et al., 2003; Paramonov et al., 2005; Slaney et al., 2006) in *P.gingivalis* W50 were insertionally inactivated with *ermF-ermAM* by allelic exchange following electro-transformation and described in detail in Supplemental Material (Figure S1). The primers used in this study are listed in Supplemental Methods.

Measurement of Enzyme activity.

Arg-gingipain and Lys-gingipain activities in whole cultures and culture supernatants of *P. gingivalis* and isogenic mutant strains were measured using N-benzoyl-DL-arginine-*p*-nitroanilide (DL-BR*p*NA) and N-α-acetyl-L-lysine-*p*-nitroanilide (L-AcK*p*NA) respectively as substrates, in spectrophotometric assays, as previously described (Rangarajan et al.,1997). Units of enzyme activity are expressed as change in absorbance at 405 nm per minute per O.D. $_{600nm}$ at 30ºC. Enzyme activities were usually measured in triplicate using batches of bacterial cultures grown on different days. Student's tTest for paired samples was used and the data were considered to be significant at a p value of < 0.05.

SDS-PAGE and SDS-Urea-PAGE.

SDS-urea-PAGE of LPS was performed according to Inzana & Apicella (1999). Samples were transferred onto nitrocellulose membranes and probed with MAb1B5, which recognises the epitope Manα1-2-Manα1-phosphate fragment in A-PS of A-LPS, as described previously (Paramonov et al., 2005). Silver staining of gels was performed using the Silver staining kit (Sigma-Aldrich Co. Ltd.) according to the manufacturer's instructions.

Isolation of LPS and Lipid A.

LPS from *P. gingivalis* W50 and mutant strains for use in SDS-urea-PAGE experiments was prepared using an LPS extraction kit from Intron Biotechnology (South Korea).

LPS used in hemin binding studies was prepared as described in Paramonov et al. (2009). De-*O*-phosphorylated LPS samples used in hemin-binding experiments were prepared by dissolving LPS (10 to 15 mg) in 0.5 ml of 48% aqueous hydrofluoric acid (HF) at 4ºC and incubating at 4ºC for 16 h. Excess HF was removed by dialysis against distilled water (6000 to 8000 MWCO tubing) at 4ºC followed by freeze-drying.

De-lipidation of LPS samples was carried out by treatment with 1.5% aqueous acetic acid at 100ºC for 2 – 4 h in a heating block. Insoluble lipid A and traces of undegraded LPS were removed by ultracentrifugation at 30,000 g for 30 min at 10ºC. The water-soluble supernatant was lyophilized twice to remove all traces of acetic acid.

Hemin binding to whole cells of *P. gingivalis*.

P. gingivalis W50 and mutant strains were grown for 48 h and cells were harvested by centrifugation (13,300 x g) for 20 min at 4ºC in Eppendorf tubes. The cells were washed with ice-cold sterile PBS (3x1 ml) and stored at –70ºC until required. Frozen cells were thawed and washed twice with 1 ml of sterile PBS. The cells were resuspended in PBS to give an $O.D._{600nm}$ of 1.25. Cell suspensions (0.8 ml) in triplicate were mixed with 0.2 ml of hemin solution containing 5 µg or 10 µg hemin and incubated at 37ºC for 1 h. Control samples contained 0.8 ml of PBS mixed with 0.2 ml of hemin solution containing 5µg or 10 µg hemin (as above) for each set of experiments. The reaction mixture was centrifuged at 13,300 g for 20 min at 4ºC, the supernatant was transferred to 1 ml plastic disposable cuvettes and the O.D. at 400nm was measured. Concentration of hemin in the supernatant was calculated from standard curves for hemin. The hemin bound (µg/ O.D. 600nm of cells) was equal to the difference between the

values for the control samples (hemin solution with no added cells, zero binding) and the supernatant from the experimental samples (bound hemin). The standard deviation was calculated.

For statistical analysis, a Student's *t* test for paired values was used, and data were considered to be significant at a *p* value of < 0.05.

Binding of hemin to LPS.

Freeze-dried native LPS, de-*O*-phosphorylated LPS and de-lipidated LPS samples from *P. gingivalis* W50 and *porR* were dissolved/resuspended in 0.05M Tris-HCl, pH7.2 at a concentration of 1 mg/ml. Aliquots (50 µl) of LPS containing 50 µg was added to PBS (0.95 ml) containing 20 µg or 30 µg of hemin in duplicate in an Eppendorf tube and incubated at 37ºC with shaking. LPS-, de-*O*-phosphorylated LPS- and de-lipidated-LPS-hemin complexes were pelletted by high speed centrifugation (30,000 g) for 60 min at 14ºC (Cutler et al., 1996). The amount of unbound hemin in the supernatant was determined by measuring the O.D. at 400 nm and the concentration determined using a standard curve for hemin. The amount of hemin bound was calculated as the difference between the total hemin added to the reaction mixture and the amount present in the supernatant. The mean of two separate determinations ± standard error of the mean was calculated (http://www.upscale.utoronto.ca/PVB/Harrison/ErrorAnalysis/.

RESULTS.

The *P. gingivalis* mutant strains used in this study have been described elsewhere (Table S1). These include strains in which the genes encoding the proteases Rgps (*rgpA*/*rgpB*) and Kgp, (*kgp*) have been inactivated, leading to loss of Arg-gingipains and Lys-gingipain respectively (Aduse-Opoku et al., 2000), both of which have been strongly implicated in hemin acquisition by *P. gingivalis* (Smalley et al., 2007).

Inactivation of PG1051(*waaL* ,O-antigen ligase) (Rangarajan et al., 2008; Paramonov et al., 2009), PG1142 (*wzy*, O-antigen polymerase) (Paramonov et al., 2009) and PG0129 (α-1,3-mannosyl transferase (Paramonov et al., 2015) lead to defects in LPS synthesis and have been described in detail elsewhere. In this manuscript, we have also studied *porR* where there is no A-LPS synthesis and *galE* which synthesises O-LPS, where the O-PS repeating unit is shortened by a residue of Gal, in greater detail.

PorR is a putative transaminase and is homologous to RfbE orthologue of *P. gingivalis* and belongs to the DegT Clusters of Orthologous Groups (COGs), the prototype of which is DegT of *Geobacillus* (*Bacillus*) *stearothermophilus* (Takagi et al., 1990) which is involved in a range of biochemical functions including glycan synthesis, regulation of extracellular enzymes, altered control of sporulation, abnormal cell division and loss of flagella (Takagi et al., 1990). Proteins homologous to PorR have been found in several microorganisms involved in the biosynthesis of sugars present in capsular polysaccharide and aminoglycosides. In *P. gingivalis*, the inactivation of *porR* leads to pleiotropic effects involving loss of pigmentation, lack of synthesis of A-LPS (Paramonov et al., 2005), processing of other proteins including fimbriae, and major alteration to the surface of the cell without perceptible effect on O-LPS (Shoji et al., 2002; Gallagher et al., 2003; Paramonov et al., 2005; Slaney et al., 2006). In addition, the Rgp isoforms namely

HRgpA and RgpB which do not acquire the MAb1B5 reactive glycan are present in the *porR* mutant strain whereas the isoforms which usually contain the MAb 1B5 cross-reactive epitope, namely $RgpA_{cat}$ and mt-Rgps (Paramonov et al., 2005) are not synthesised. However, the synthesis of O-LPS is not affected in the *porR* mutant strain and ^{1}H-NMR spectroscopy of the O-PS isolated from O-LPS of this strain showed an identical ^{1}H-NMR spectrum to that of O-PS from the *P. gingivalis* W50 parent strain (Paramonov et al., 2005). Biologically, these effects translate to cell fragility, loss of recognition by antibodies of the periodontal patients' sera, and an enhanced complement mediated killing as a result of the inability to synthesise A-LPS (Gallagher et al., 2003; Shoji et al., 2002; Paramonov et al., 2005; Slaney et al., 2006).

Pigmentation and hemolysis of *P. gingivalis* strains.

P. gingivalis W50, *rgpA/rgpB* and *galE* form brown or black pigmented colonies on blood agar plates (Fig. 1), whereas colonies of *kgp*, *porR*, *waaL*, *wzy* and *pg0129* are non-pigmented. Also

[Fig. 1 here…Colonies of *P. gingivalis*…]

shown is the *P. gingivalis* mutant strain *wbpB* which has been described in detail by Slaney et al. (2006) and Shoji et al. (2014) and gives non-pigmented colonies on blood agar plates.

Arg- and Lys-gingipain activities in *P. gingivalis* strains.

Arg-gingipain and Lys-gingipain activities in whole cultures and culture supernatants of *P. gingivalis* W50 and isogenic mutant strains were measured after either 24 h or 48 h and the results are shown in Fig. 2A. The Arg-gingipain (Rgp) activities present in the *P. gingivalis* strains vary widely. Total Rgp activity (100%) and activities present in cell-associated and secreted forms in *P. gingivalis* W50 and *kgp* mutant strain are similar (~80% and ~20%,

respectively) after 24 h of growth as expected. *P. gingivalis rgpA/rgpB* mutant strain contains no Rgp activity, as expected. However, mutant strains *galE*, *porR*, *waaL*, *wzy* and *pg0129* in which LPS synthesis is affected, contain lower levels of Rgp activity compared to the parent W50 strain (p values < 0.0003). In addition, in mutant strains *porR*, *waaL*, *wzy* and *pg0129*, almost all the enzyme activity (~90% to 100%) is secreted into the supernatant after 24 h of growth compared to the parent W50 strain in which only ~20% of Rgp activity is shed into the supernatant. Although *galE* contains approximately 50% of total Rgp activity compared to W50 parent strain after 48 h of growth, the distribution of enzyme activity between cell-associated and secreted forms was similar to that of the parent W50 strain : ~30% and ~20% of Rgp activity in cell-associated and supernatant forms in *galE* compared to ~60% and ~40% of Rgp activity in cell-associated and supernatant forms in *P. gingivalis* W50.

[Fig. 2. here…Rgp and Kgp activities of *P. gingivalis*….]

Similarly, the Lys-gingipain (Kgp) activities (Fig. 2B) in whole cultures of the *P. gingivalis* mutant strains also show wide variation. *P. gingivalis* W50 contains the highest amount of Kgp activity. As expected, *kgp* shows no detectable Kgp activity. The amount of Kgp activity in cell-associated and culture supernatants also show wide variation (Fig. 2B). However, in *P. gingivalis* mutant strains, namely *porR*, *waaL*, *wzy* and *pg0129* almost all the Kgp activity is present in the culture supernatant after 24 h of growth which is very similar to that observed for Rgp activity. Although the Rgp and Kgp activities of the *P. gingivalis* mutant strains show great variation, these results highlight the properties of the isogenic mutant strains *porR*, *waaL*, *wzy* and *pg0129*, where almost all the Rgp and Kgp activities are released into the culture supernatant

after 24 h to 48 h of growth indicating the lack of tethering/anchoring molecules on the cell surface of these strains which would otherwise enable these enzymes from being shed into the culture medium. Since the mutant strains *porR*, *waaL*, *wzy* and *pg0129* are defective in LPS biosynthesis, the inability to retain the gingipains on the cell-surface could be a direct result of this deficiency.

Cross-streaking Experiments.

P. gingivalis W50 was initially streaked on a blood agar plate and following the formation of a zone of hemolysis (3 days), the cells were removed with a swab containing clindamycin to suppress regrowth of the wild type strain and the plates were cross-streaked with *P. gingivalis* mutant strains (Fig. 3). Although *rgpA*/*rgpB* and *kgp* give brown and non-pigmenting colonies when grown on blood agar plates due to the lack of Rgps and Kgp respectively, they do pigment when cross-streaked on plates on which *P. gingivalis* W50 has been previously grown and caused hemolysis (Fig. 3). This suggests that *rgpA*/*rgpB* and *kgp* have the ability to pigment if supplied with externally added hemin. However, cross-streaking of *P. gingivalis porR*, *waaL*, *wzy* and *pg0129* strains on BHI plates as above did not cause the deposition of hemin/black pigment on the surfaces of these cells (Fig. 3). This indicates that the mutant strains are unable to harness any available hemin in the environment and retain it on their cell surface.

[Fig. 3 here…Cross-streaking of *P. gingivalis*…]

Analysis of LPS.

SDS-urea PAGE followed by silver staining of LPS purified from *P. gingivalis* W50 and mutant strains *rgpA/rgpB*, *kgp*, *porR* and *galE* show the characteristic laddering pattern (Fig. 4A). However, in *porR* and *galE*, the O-LPS shows a higher intensity of bands in the core-, core-plus one repeating unit and core-plus two repeating units (Fig. 4A). In the *P. gingivalis galE* mutant strain, the O-PS repeating unit, [→3)-α-D-Gal*p*-(1→6)-α-D-Glc*p*-(1→4)-α-L-Rha*p*-(1→3)-β-D-GalNAc*p*-(1→] is shortened by one Gal residue (Unpublished data). The SDS-urea-PAGE of LPS from *P. gingivalis waaL*, *wzy* and *pg0129* mutant strains have been described elsewhere (Rangarajan et al., 2008; Paramonov et al., 2009; Paramonov et al., 2015) and are not shown here.

SDS-urea-PAGE of LPS from *P. gingivalis* W50 and mutant strains followed by silver staining indicate that all these strains synthesise O-LPS. SDS-urea-PAGE of LPS followed by Western blotting vs MAb 1B5 which recognises the epitope Manα1-2-Manα1-phosphate fragment in A-PS of A-LPS (Paramonov et al., 2005) show that W50, *rgpA/rgpB*, *kgp* and *galE* also synthesise A-LPS (Fig. 4B) as indicated by the laddering pattern and immunoreactivity with MAb 1B5. However, *porR* synthesises only O-LPS and A-LPS is absent as shown by the lack of cross-reactivity with MAb 1B5 (Fig. 4B).

[Fig. 4 here…SDS-Urea-PAGE of LPS….]

Hemin Binding.

Hemin Binding by whole cells.

Hemin binding by whole cells of *P. gingivalis* W50, *rgpA/rgpB*, *kgp*, *galE*, *porR*, *waaL*, *wzy* and *pg0129* was measured as described in the Methods Section and the results obtained are shown in

Fig. 5. *P. gingivalis* W50 and mutant strains *rgpA/rgpB* and *galE* which pigmented brown and black on blood agar plates respectively (Fig. 1) showed hemin binding values (5.6 μg/O.D.$_{600nm}$, 6.8 μg/O.D. $_{600nm}$ and 5.9 μg/O.D.$_{600nm}$ respectively), at the highest concentration (10 μg/ ml) of hemin used in the binding experiments. Although *kgp* was non-pigmenting on blood agar plates due to the absence of Kgp, it shows hemin binding (6.1 μg/O.D.$_{600nm}$) when supplied with hemin (also observed when *kgp* is cross-streaked on blood agar plates on which *P. gingivalis* W50 was previously grown (Fig. 3). *P. gingivalis* mutant strains *porR*, *waaL*, *wzy* and *pg0129* which were non-pigmenting on blood agar plates, were able to bind between ~2.5 and 3.7 μg of hemin /O.D.$_{600nm}$ of cells which is approximately 45% to 65% of hemin bound by the parent W50 strain. Thus, *P. gingivalis* mutant strains which do not synthesise A-LPS show reduced hemin binding.

[Fig. 5 here…Hemin binding by *P. gingivalis*…]

Hemin binding by LPS.

Hemin binding to LPS isolated from *P. gingivalis* W50 and mutant strain *porR* grown in BHI were measured at two different concentrations of hemin, namely 20 μg/ml and 30 μg/ml and are shown in Fig. 6. At 30 μg/ml of added hemin, there is a slightly higher amount of hemin bound by all the LPS (Fig. 6) compared to the amounts bound at 20μg/ml of hemin. Henceforth, all the values for hemin binding to LPS will only refer to those obtained at the higher concentration of hemin used in the experiment, namely 30 μg/ml. LPS from *P. gingivalis* W50 is able to bind hemin at ~ 10.3 μg/50 μg of LPS.

[Fig. 6 here…Binding of hemin by LPS]

However, LPS from *porR* which is devoid of A-LPS (Paramonov et al., 2005) was able to bind 3.6 μg hemin/50 μg of LPS which is considerably lower than that of LPS from the parent W50 strain.

In order to determine the extent of hemin binding to the lipid A portion of the LPS molecule, the LPS was delipidated and the binding of hemin to the resulting polysaccharide (PS) was measured. In addition to removal of lipid A, delipidation of LPS causes the destruction of A-PS (Paramonov et al., 2005) whereas O-PS is largely unaffected (Paramonov et al., 2001). The binding of hemin by de-lipidated LPS (Fig. 6) from *P. gingivalis* W50 is reduced to ~ 2.9 μg /50 μg of LPS and hemin binding by de-lipidated *porR* LPS is reduced to 2.5 μg hemin /50 μg of LPS. These results enable us to apportion the extent of hemin binding by A-LPS, O-LPS and lipid A. Since *porR* LPS contains only O-LPS, the hemin bound (~3.6 μg/50 μg of LPS) must be due to O-LPS. De-lipidation of *porR* LPS reduces the hemin bound to ~ 2.5 μg/50 μg of LPS suggesting that ~1.1 μg/50 μg of hemin bound to de-lipidated LPS must be due to binding to lipid A and the remaining ~2.5 μg of hemin bound to 50 μg of LPS must be due to binding to the O-PS component of O-LPS. The hemin binding to de-lipidated LPS from *P. gingivalis* W50 and *porR* strains is remarkably similar (2.9 μg vs 2.5 μg hemin bound to 50 μg of de-lipidated LPS respectively) whereas hemin bound to native LPS differs greatly, 10.3 μg vs 3.6 μg hemin bound to W50 and *porR* LPS respectively. Thus, it can be deduced that ~ 6.3 μg of hemin bound/50 μg of native LPS in *P. gingivalis* W50 must be due to binding to A-LPS.

We also investigated the binding of hemin to de-*O*-phosphorylated LPS derived from the parent W50 and *porR* mutant strain. De-*O*-phosphorylation of O-LPS and A-LPS does not cause de-polymerisation of the PS chains, but results in loss of phosphoethanolamine in O-PS (Paramonov

et al., 2001) and in the loss of the cross-reacting epitope Manα1-2Manα1-phosphate in A-LPS (Paramonov et al., 2005; Rangarajan et al., 2008). The results (Fig. 6) show the binding of hemin is reduced in de-*O*-phosphorylated LPS from the parent and *porR* mutant strain to ~3.3 μg and 1.9 μg respectively. Thus, the reduction in binding of hemin caused by de-*O*-phosphorylation of LPS from *P. gingivalis* W50 compared to that for LPS from *porR* mutant strain suggests that negatively-charged A-LPS may have an important role to play in the binding/deposition of hemin on the cell surface of *P. gingivalis*.

DISCUSSION.

The hemin binding properties of *P. gingivalis* have been a major area of study and interest for several years. Iron utilisation systems in *P. gingivalis* are quite complex and several proteins have been implicated in hemin release from the host, to its transport and deposition on the bacterial cell surface. *P. gingivalis* virulence is closely associated with the ability of the organism to pigment namely to the deposition of the μ-oxo bishaem on the cell surface. The requirement of the dimeric Arg-gingipain (Rgp), HRgpA, and Lys-gingipain (Kgp) in the release of haem groups from hemoglobin and the formation of the μ-oxo bishaem complex is very well characterised (Smalley et al., 2004). However, the cell-surface molecules required for retention of μ-oxo-bishaem and pigmentation have not been fully elucidated.

Haem-starved *P. gingivalis* ATCC33277 and WT40 expressed two binding sites for hemin, a low abundance high affinity site (1000 to 1500 sites/cell) of K_d between 3.6×10^{-11} and 9.6×10^{-11} M and a high abundance low-affinity site (1.9×10^5 to 6.3×10^5 sites/cell) where the estimated K_d ranged between 2.6×10^{-7} and 6.5×10^{-8} M (Tompkins et al., 1997). Treatment with N-bromosuccinimide inactivated hemin binding by both sites in *P. gingivalis*, whereas pronase treatment caused only a limited reduction in hemin binding probably because only one of the two sites was sensitive to pronase. Tompkins et al. (1997) concluded that the higher-affinity site was probably exposed on the surface of *P. gingivalis* and sensitive to pronase whereas the lower affinity-site may be sequestered within the outer membrane especially if it functioned to store hemin.

The black haem-pigment deposited on the cell surface of *P. gingivalis* and which serves as an iron source for this organism, is composed of μ-oxo bishaem $[Fe(III)PPIX]_2O$, and the multidomain cysteine proteases Arg gingipains and Lys gingipain acting in concert have been

393 shown to be important in the production of μ-oxo bishaem from oxyhemoglobin (Smalley et al.,
394 2007). HRgpA, the dimeric isoform of RgpA, promotes the formation of methemoglobin from
395 oxyhemoglobin which is degraded by Kgp (Lewis et al., 1999) to form the black pigment μ-oxo-
396 bishaem. Thus, both Arg and Lys gingipains are required for the production of the black pigment
397 in *P. gingivalis*.

398 In the absence of Rgps, no μ-oxo bishaem is produced although the double knockout *P.*
399 *gingivalis rgpA/rgpB* strain which lacks Rgps gave brown-coloured colonies even after prolonged
400 incubation on blood agar plates. The brown pigment contained an Fe(III) haemoglobin-
401 haemichrome complex as the major haem-containing species (Smalley et al., 2004). The haem
402 from the complex was transferred to albumin after prolonged incubation of cells with
403 oxyhemoglobin in the presence of albumin and this was tightly bound to the cell surface in the *P.*
404 *gingivalis* (*rgpA/rgpB*) strain.

405 *P. gingivalis* W50 does not pigment when grown in liquid broth cultures with added
406 hemin (5mg/L), but gives black-pigmented colonies when grown on blood agar plates due to the
407 deposition of μ-oxo-bishaem, derived from haemoglobin, on the cell surface. This difference
408 may suggest that the source of hemin is critical for the pigmentation process. Here, we have
409 shown that *P. gingivalis* mutants *rgpA/rgpB* and *kgp* do not normally pigment, but produce black
410 pigmented colonies when cross-streaked on plates on which *P. gingivalis* W50 was previously
411 grown and caused hemolysis. These observations show that the ability to retain the pigment on
412 the *P. gingivalis* cell-surface can be uncoupled from the ability to release haem from hemoglobin
413 (with the concomitant formation of μ–oxo bishaem) by the combined action of Rgps and Kgp.
414 This behaviour mirrors the ability of the cells of *rgpA/rgpB* and *kgp* mutant strains to bind
415 externally added hemin to the same extent as the parent W50 strain when hemin-binding was

measured in liquid suspensions (Fig. 5). Therefore, we propose that the ability of the bacterial cells to bind hemin may parallel the retention of μ-oxo-bis-haem on the cell surface when the strains are grown on blood agar plates. The inability of *P. gingivalis* mutant strains *porR*, *waaL*, *wzy* and *pg0129* to produce black pigmented colonies in cross-streaking experiments is supported by the reduced binding of hemin to cells of these strains. The major difference between the *P. gingivalis* mutant strains which have the ability to acquire μ-oxo-bishaem (*rgpA*/*rgpB* and *kgp*) on cross-streaking and those mutant strains which lack this property (*porR*, *waaL*, *wzy* and *pg0129*) is the production of A-LPS by pigmenting strains.

This suggests that the *P. gingivalis* cell surface must contain a molecule which provides a scaffold/matrix for the deposition and retention of any hemin or pigment that is produced/acquired by the organism. Fig. 7 shows a simplified diagram of the pigmentation characteristics and the types of LPS synthesised by the *P. gingivalis* strains used in this study.

[Fig. 7 here…Structure of LPS and role…]

Transmission electron microscopy of *P. gingivalis porR* (Slaney et al., 2006; Paramonov et al., 2005) and *waaL* mutant strains (which lack A-LPS) (Rangarajan et al., 2008) show that their extracellular surface layers are of reduced thickness compared to the W50 parent and *rgpA*/*rgpB* mutant strains (which do synthesis A-LPS) and the cells appear more fragile based on the rate of decrease of the culture optical density in stationary phase (Shoji et al., 2002; Paramonov et al., 2005; Rangarajan et al., 2008). Shoji et al. (2002) suggested that strains which were unable to synthesise A-LPS probably lack a tethering/anchoring molecule(s) on their cell surface which retain gingipains and this could explain the release of Arg- and Lys-gingipains

into the culture supernatants in *P. gingivalis porR*, *waaL*, *wzy* and *pg0129* mutant strains which are defective in the LPS-biosynthetic pathway.

Grenier (1991) reported that the lipid A component of LPS mediated the binding of uncomplexed hemin by *P. gingivalis*. Since hemin is a lipophilic molecule, it would be expected to bind to Lipid A/LPS. *Escherichia coli* which does not require exogenous haem when grown in iron-replete conditions was shown to bind as much uncomplexed hemin as *P. intermedia*. This effect was inhibited by albumin which indicated that when haem is provided in the free form, most of it binds to the bacterium with an affinity lower than that for albumin. However, Tompkins et al. (1997) concluded that most Gram-negative bacteria would exhibit similar non-specific hemin binding and that the LPS-mediated hemin binding is probably not biologically relevant because of the low affinity of the interaction and the presence of large amounts of host plasma proteins which function to counter the lipophilic disposition of hemin. Tompkins et al. (1997) showed that treatment of *P. gingivalis* cells with pronase caused a slight reduction in, but did not eliminate, hemin-binding and the authors suggested that this was probably due to the pronase-sensitive hemin-binding sites not being exposed on the surface of the cell and therefore not digested by pronase treatment. However, it seems more plausible that it is the presence of A-LPS (which is not sensitive to pronase treatment) on the surface of *P. gingivalis* which acts as a site for the deposition/binding of hemin.

Studies on hemin binding to whole cells of *P. gingivalis* W50 and mutant strains and hemin binding to native LPS and de-lipidated LPS from *P. gingivalis* W50 and *porR* strains show that absence of A-LPS causes a reduction in hemin binding. Thus, absence of A-LPS in the extracellular surface of *P. gingivalis* eliminates or reduces a scaffold /anchoring mechanism not only for retention of Arg- and Lys-gingipains but also for the deposition of μ-oxo bishaem

462 pigment or hemin derived from the environment and highlights the importance of A- LPS in the
463 virulence of this organism.

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Legends to Figures.

Figure 1. Pigmentation of *P. gingivalis* strains on blood agar plates.

P. gingivalis W50 and mutant strains were grown on blood agar plates for seven days.

Figure 2. Arg-gingipain and Lys-gingipain activities in *P. gingivalis* strains.

P. gingivalis W50 and isogenic mutant strains were grown in BHI broth for 24 h or 48 h. Arg-X and Lys-X activities were measured using substrates DL-BR*p*NA and L-AcK*p*NA respectively as described in Methods. Enzyme activities are expressed as % activity relative to that of the parent *P. gingivalis* W50 strain (Absorbance $_{405nm}$ units/ min. / O.D. $_{600nm}$). Black bars represent cell-associated activities and grey bars represent enzyme activities in the culture supernatants.

Figure 3. Cross-streaking of *P. gingivalis kgp* mutant strain on blood agar.

(A). *P. gingivalis* W50 on blood agar, *kgp* on blood agar, *P. gingivalis* W50 was initially streaked on a blood agar plate and following the formation of a zone of hemolysis (3 days), the cells were removed with a swab containing clindamycin to suppress regrowth of the wild type strain and the plates were cross-streaked with *kgp*. Note pigmentation of the *kgp* mutant cells takes place only on the zone of hemolysis produced by the parent strain. (B). Blood agar plates were initially streaked with W50 as in (A). Plates were cross-streaked with r*gpA*/*rgpB*, *kgp*, *porR*, *pg0129*, *waaL* and *wzy*. Pigmentation of *kgp* takes place on the zone of hemolysis whereas the other strains do not pigment even after 6 days of growth.

Figure 4 . SDS-urea-PAGE and silver staining of LPS from *P. gingivalis* W50 and mutant strains (A). Western blotting vs MAb 1B5 of whole cell extracts of *P. gingivalis* W50 and mutant strains (B).

637 LPS purified as described in Methods were subjected to SDS-urea-PAGE and silver staining.
638 The *P. gingivalis* strains used in the isolation of LPS is indicated below the lanes. The control
639 sample in (B) is the phenol extract of *P. gingivalis* W50 cells containing predominantly LPS.

640 **Figure 5. Hemin binding by whole cells of *P. gingivalis* W50 and isogenic mutant strains.**
641 *P. gingivalis* W50 and isogenic mutant strains were grown in BHI broth for 48 h. Details of
642 hemin binding are as described in Methods. The amount of hemin bound by the cells (μg hemin
643 bound / O.D. cells $_{600nm}$) at the added concentrations of hemin of 5 μg and 10 μg are shown. The
644 characteristics of the *P. gingivalis* strains are indicated below the figure. Statistical analyses (*p*
645 values by Student's ttest) of the amount of hemin bound by W50 and *P. gingivalis* mutant strains
646 are also indicated. *p* values for hemin-binding by strains *rgpA/rgpB*, *kgp* and *galE* compared to
647 W50 were > 0.05 whereas *p* values for hemin-binding by strains *porR*, *waaL*, *wzy* and *pg0129*
648 compared to W50 were < 0.0003.

649 **Figure 6. Hemin binding to LPS, de-lipidated LPS and de-O-phosphorylated LPS derived**
650 **from *P. gingivalis* strains.**
651 The hemin binding studies were performed as described in Methods. Black bars = LPS and
652 derivatives from *P. gingivalis* W50 and grey bars = LPS and derivatives from *porR* mutant strain.
653 Inset: Hemin binding to Native LPS, lipid A, O-PS and A-PS derived from *P. gingivalis* and
654 *porR* mutant strains.

655 **Figure 7. Structure of *P. gingivalis* LPS and role of A-LPS in pigmentation.**
656 The *P. gingivalis* W50 parent strain synthesise two LPS: O-LPS and A-LPS and is black-
657 pigmented on blood agar (filled circle). Inactivation of Wzy (O-antigen polymerase) leads to a
658 core-plus-one repeating unit structure for both LPS. Inactivation of either WaaL (O-antigen
659 ligase) or PG0129 (mannosyl-transferase) leads to the absence of A-PS and O-PS. In all three

660 cases, the mutants lose the ability to pigment (open circles). Inactivation of GalE affects the
661 synthesis of the O-PS but not A-PS and pigmentation is unaffected. Inactivation of PorR
662 abolishes the synthesis of A-PS but not O-PS and this mutant fails to pigment.
663

SUPPLEMENTAL.

METHODS.

Generation of *P. gingivalis* mutant strains *porR* and *galE*.

Chromosomal DNA from *P.gingivalis* W50 was used as the template for amplification/ cloning purposes. The nomenclature originally used by TIGR is used throughout. The genes encoding UDP-Glucose-4-epimerase *galE* (PG0347) and *porR* (PG1138 (Shoji et al., 2002; Gallagher et al., 2003; Paramonov et al., 2005; Slaney et al., 2006) in *P.gingivalis* W50 were insertionally inactivated with *ermF-ermAM* by allelic exchange following electro-transformation.

Primer pairs incorporating NotI sites (in bold), PorRF1:

*atatat**gcggccgc***TTGCGGAAGATTTGGCAG and PorRR1:

*atatat**gcggccgc***GGGATGGAGAGAACAGTTCG were used to amplify *porR*(PG1138).

GalEF1: *atatat**gcggccgc***GGCATCAACGATCCATACG and GalER1:

*atata**tgcggccgc***GAGTACGTACAGGAGTTGCTGG were used to amplify *galE* in PCR master mix (Extensor mix Reddy Load PCR master mix (Buffer 2, Thermoscientific)) as previously described (Aduse-Opoku et al., 2006). The amplicons were cloned at the NotI site of pUC18not and inserts of *porR* (937 bp) and *galE* (2545 bp) were further manipulated to ligate a 2.1 kb *erm* cassette (Fletcher et al., 1995) at the unique NcoI and BamHI-EcoRV sites respectively. NotI-restricted plasmids were used to electrotransform 6h grown cells of *P.gingivalis* W50 to clindamycin resistance. PCR was performed on purified chromosomal DNA from six separate isolates of each mutant strain. The original primers were used to amplify the region *porR*::*erm* and *galE*::*erm* to show correct insertion of the *erm* cassette. One strain from each was chosen and designated as either *porR* or *galE* (Fig. S1).

Gallagher et al. (2003) have referred to a *porR* mutant strain isolated by inactivation of PG1138 in *P. gingivalis* W50 which is described in greater detail in this manuscript.

Description of PorR.

PorR is a putative transaminase and is homologous to RfbE orthologue of *P. gingivalis* and belongs to the DegT Clusters of Orthologous Groups (COGs), the prototype of which is DegT of *Geobacillus* (*Bacillus*) *stearothermophilus* (Takagi et al., 1990) which is involved in a range of biochemical functions including glycan synthesis, regulation of extracellular enzymes, altered control of sporulation, abnormal cell division and loss of flagella (Takagi et al., 1990). Proteins homologous to PorR have been found in several microorganisms involved in the biosynthesis of sugars present in capsular polysaccharide and aminoglycosides. In *Vibrio cholerae* O1 and *E. coli* O157, *rfbE* encodes perosamine synthetase (Bilge et al., 1996; Albermann & Piepersburg, 2001) and the *rfbE* orthologue *per* in *Caulobacter crescentus* also encodes a perosamine synthetase (Awram & Smit, 2001). In *P. gingivalis*, the inactivation of *porR* leads to pleiotropic effects involving pigmentation, lack of synthesis of A-LPS (Paramonov et al., 2005), processing of other proteins including fimbriae, and major alteration to the surface of the cell without perceptible effect on O-LPS (Shoji et al., 2002; Gallagher et al., 2003; Paramonov et al., 2005; Slaney et al., 2006). In addition, the Rgp isoforms namely HRgpA and RgpB which do not acquire the MAb1B5 reactive glycan are present in the *porR* mutant strain whereas the isoforms which usually contain the MAb 1B5 cross-reactive epitope, namely $RgpA_{cat}$ and mt-Rgps (Paramonov et al., 2005) are not synthesised. However, the synthesis of O-LPS is not affected in the *porR* mutant strain and ^{1}H-NMR spectroscopy of the O-PS isolated from O-LPS of this strain showed an identical ^{1}H-NMR spectrum to that of O-PS from the *P. gingivalis* W50 parent strain (Paramonov et al., 2005). Biologically, these effects translate to cell fragility, loss of recognition

by antibodies of the periodontal patients' sera, and an enhanced complement mediated killing as a result of the inability to synthesise A-LPS (Gallagher et al., 2003; Shoji et al., 2002; Paramonov et al., 2005; Slaney et al., 2006).

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784 Table S1. List of Strains used in this study.

Figure S1.

Organisation of the *porR* (PG1138) and *galE* (PG0347) loci in *P. gingivalis* W50. The location of the *erm* cassette at the NcoI (Nc) site in *porR* and BamHI (B)-EcoRV (Ev) sites in *galE* are shown. Relative positions of primers used in initial cloning of PCR products are indicated below each locus. The black arrows correspond to the directions of open reading frames.