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

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33 **SUMMARY.**

34 *Porphyromonas gingivalis* is a Gram-negative black pigmented anaerobe unable to synthesise
35 haem (Fe (II)-protoporphyrin IX) or hemin (Fe(III)-protoporphyrin IX-Cl) which are important
36 growth/virulence-factors, and must therefore derive them from the host. *P. gingivalis* expresses
37 several proteinaceous hemin-binding-sites which are important in the binding/ transport of
38 haem/hemin from the host. *P. gingivalis* also synthesises several virulence factors, namely
39 cysteine-proteases Arg- and Lys-gingipains and two lipopolysaccharides (LPS), O-LPS and A-
40 LPS. The gingipains are required for the production of the black pigment, μ -oxo-bishaem
41 ($[\text{Fe(III)PPIX}]_2\text{O}$), which is derived from hemoglobin and deposited on the bacterial cell-surface
42 leading to the characteristic black colonies when grown on blood agar. In this study we
43 investigated the role of LPS in the deposition of μ -oxo-bishaem on the cell-surface.

44 *A P. gingivalis* mutant defective in the biosynthesis of Arg-gingipains, namely *rgpA/rgpB*
45 produces brown colonies on blood agar and mutants defective in Lys-gingipain (*kgp*) and LPS
46 biosynthesis namely *porR*, *waaL*, *wzy* and *pg0129* (α -1, 3 -mannosyltransferase) produce non-
47 pigmented colonies. However, only those mutants lacking A-LPS showed reduced hemin-
48 binding when cells in suspension were incubated with hemin. Using native, de-O-
49 phosphorylated and de-lipidated LPS from *P. gingivalis* W50 and *porR* strains, we demonstrated
50 that hemin-binding to O-polysaccharide (PS) and to the lipid A moiety of LPS was reduced
51 compared to hemin-binding to A-PS. We conclude that A-LPS in the outer-membrane of *P.*
52 *gingivalis* serves as a scaffold/ anchor for the retention of μ -oxo-bishaem on the cell surface and
53 pigmentation is dependent on the presence of A-LPS.

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INTRODUCTION.

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

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

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

80 receptors have been described including Tlr (Slakeski et al., 2000), IhtA (iron heme transport)
81 (Dashper et al., 2000), HmuR (hemin utilization receptor) (Simpson et al., 2000; Olczak et al.,
82 2005; Olczak et al., 2008) and HemR (hemin-regulated receptor) (Karunakaran et al., 1997).
83 HmuR exhibited amino acid sequence homology to TonB-dependent receptors involved in heme,
84 vitamin B₁₂ or iron-siderophore transport in other bacteria (Simpson et al., 2000). A *P.*
85 *gingivalis* *hmuR* isogenic mutant strain was shown to have impaired growth on hemin and
86 hemoglobin as sole source of iron and showed decreased ability to bind hemin and hemoglobin.
87 *E. coli* cells overexpressing *P. gingivalis* HmuR as well as purified recombinant HmuR were able
88 to bind hemin, hemoglobin and serum-albumin-hemin complex (Simpson et al., 2000).
89 *P. gingivalis* W50 produces several virulence factors including gingipain proteases and two
90 LPSs, namely O-LPS (Paramonov et al., 2001) and A-LPS (Rangarajan et al., 2008).
91 In this study, we addressed the question whether the high abundance low-affinity hemin-binding
92 site described by (Tompkins et al., 1997) may be one of the LPS of *P. gingivalis*. In order to test
93 this hypothesis, we examined a variety of isogenic mutant strains of *P. gingivalis* lacking Arg-
94 gingipains, Lys-gingipain and defective in the biosynthesis of O-LPS and A-LPS for their ability
95 to pigment and to bind hemin not only to whole cells but also to LPS, de-phosphorylated LPS and
96 de-lipidated LPS. *P. gingivalis* *porR* (PG1138) defective in A-LPS synthesis and *galE* (PG0347)
97 mutant strains which synthesises a truncated O-PS repeating unit of O-LPS, have been described
98 in greater detail in this manuscript. Shoji et al. (2002) described *porR* mutant strain in *P.*
99 *gingivalis* ATCC33277 isolated by transposon and targeted mutagenesis and Gallagher et al.
100 (2003) have referred to a *porR* mutant strain isolated by inactivation of PG1138 in *P. gingivalis*
101 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* (Δ PG0347), 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.

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113

113 **MATERIALS and METHODS.**

114
115 **Materials.** A solution containing 30% acrylamide-N,N-methylenebisacrylamide (BIS) (37.5:1)
116 was obtained from Bio-Rad Laboratories (Hercules, CA, USA). Horseradish peroxide-labelled
117 mouse immunoglobulin was purchased from Dako A/S, High Wycombe, Buckinghamshire,
118 United Kingdom. All other chemicals were from VWR, Lutterworth, Leicestershire, United
119 Kingdom, or from Sigma-Aldrich Co. Ltd., Poole, Dorset, United Kingdom and were the purest
120 grades available. N α -acetyl-Lys-p-nitroanilide was obtained from Bachem Feinchemikalein AG
121 (Bubendorf, Switzerland). Hemin was obtained from Roche (East Sussex, United Kingdom).
122 Restriction and modification enzymes were purchased from New England BioLabs, and DNA
123 purification reagents were obtained from Qiagen.

124
125 **Bacterial strains and growth conditions.**

126 *P. gingivalis* W50 and mutant strains were grown either on blood agar plates containing 5%
127 defibrinated horse blood or in Brain Heart Infusion broth (Oxoid, Basingstoke, United Kingdom)
128 supplemented with hemin (5 μ g /ml) in an anaerobic atmosphere consisting of 80% N₂, 10% H₂
129 and 10% CO₂ (Aduse-Opoku et al., 1995). Clindamycin HCl and tetracycline HCl were added to
130 5 μ g /ml and 1 μ g /ml respectively, for selection of *ermF* and *tet Q* in *P. gingivalis*. Ampicillin
131 (Na⁺ salt; 100 μ g /ml) or erythromycin (300 μ g /ml) was added to the growth medium to select for
132 pUC-derived or *ermAM*-containing plasmids respectively, in *Escherichia coli*.

133 **Generation of *P. gingivalis* mutants.**

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

137 For the generation of *P. gingivalis* mutant strains *porR* and *galE*, chromosomal DNA from *P.*
138 *gingivalis* W50 was used as the template for amplification/ cloning purposes. The nomenclature
139 originally used by TIGR is used throughout the manuscript. The genes encoding UDP-Glucose-
140 4-epimerase *galE* and *porR* (Shoji et al., 2002; Gallagher et al., 2003; Paramonov et al., 2005;
141 Slaney et al., 2006) in *P.gingivalis* W50 were insertionally inactivated with *ermF-ermAM* by
142 allelic exchange following electro-transformation and described in detail in Supplemental
143 Material (Figure S1). **The primers used in this study are listed in Supplemental Methods.**

144 **Measurement of Enzyme activity.**

145 Arg-gingipain and Lys-gingipain activities in whole cultures and culture supernatants of *P.*
146 *gingivalis* and isogenic mutant strains were measured using N-benzoyl-DL-arginine-*p*-
147 nitroanilide (DL-BR*p*NA) and N- α -acetyl-L-lysine-*p*-nitroanilide (L-AcK*p*NA) respectively as
148 substrates, in spectrophotometric assays, as previously described (Rangarajan et al.,1997). **Units**
149 **of enzyme activity are expressed as change in absorbance at 405 nm per minute per O.D. _{600nm} at**
150 **30°C. Enzyme activities were usually measured in triplicate using batches of bacterial cultures**
151 **grown on different days. Student's tTest for paired samples was used and the data were**
152 **considered to be significant at a *p* value of < 0.05.**

153 **SDS-PAGE and SDS-Urea-PAGE.**

154 SDS-urea-PAGE of LPS was performed according to Inzana & Apicella (1999). Samples were
155 transferred onto nitrocellulose membranes and probed with MAb1B5, **which recognises the**
156 **epitope Man α 1-2-Man α 1-phosphate fragment in A-PS of A-LPS, as described previously (**
157 **Paramonov et al., 2005).** Silver staining of gels was performed using the Silver staining kit
158 (Sigma-Aldrich Co. Ltd.) according to the manufacturer's instructions.

159 **Isolation of LPS and Lipid A.**

160 LPS from *P. gingivalis* W50 and mutant strains for use in SDS-urea-PAGE experiments was
161 prepared using an LPS extraction kit from Intron Biotechnology (South Korea).
162 LPS used in hemin binding studies was prepared as described in Paramonov et al. (2009). De-
163 phosphorylated LPS samples used in hemin-binding experiments were prepared by dissolving
164 LPS (10 to 15 mg) in 0.5 ml of 48% aqueous hydrofluoric acid (HF) at 4°C and incubating at 4°C
165 for 16 h. Excess HF was removed by dialysis against distilled water (6000 to 8000 MWCO
166 tubing) at 4°C followed by freeze-drying.
167 De-lipidation of LPS samples was carried out by treatment with 1.5% aqueous acetic acid at
168 100°C for 2 – 4 h in a heating block. Insoluble lipid A and traces of undegraded LPS were
169 removed by ultracentrifugation at 30,000 g for 30 min at 10°C. The water-soluble supernatant
170 was lyophilized twice to remove all traces of acetic acid.

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

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

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

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

188 **Binding of hemin to LPS.**

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

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202
203 **RESULTS.**

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

209 Inactivation of PG1051(*waaL* ,O-antigen ligase) (Rangarajan et al., 2008; Paramonov et al.,
210 2009), PG1142 (*wzy*, O-antigen polymerase) (Paramonov et al., 2009) and PG0129 (α -1,3-
211 mannosyl transferase (Paramonov et al., 2015) lead to defects in LPS synthesis and have been
212 described in detail elsewhere. In this manuscript, we have also studied *porR* where there is no
213 A-LPS synthesis and *galE* which synthesises O-LPS, where the O-PS repeating unit is shortened
214 by a residue of Gal, in greater detail.

215 **PorR is a putative transaminase and is homologous to RfbE orthologue of *P. gingivalis* and**
216 **belongs to the DegT Clusters of Orthologous Groups (COGs), the prototype of which is DegT of**
217 ***Geobacillus (Bacillus) stearothermophilus* (Takagi et al., 1990) which is involved in a range of**
218 **biochemical functions including glycan synthesis, regulation of extracellular enzymes, altered**
219 **control of sporulation, abnormal cell division and loss of flagella (Takagi et al., 1990). Proteins**
220 **homologous to PorR have been found in several microorganisms involved in the biosynthesis of**
221 **sugars present in capsular polysaccharide and aminoglycosides. In *P. gingivalis*, the inactivation**
222 **of *porR* leads to pleiotropic effects involving loss of pigmentation, lack of synthesis of A-LPS**
223 **(Paramonov et al., 2005), processing of other proteins including fimbriae, and major alteration to**
224 **the surface of the cell without perceptible effect on O-LPS (Shoji et al., 2002; Gallagher et al.,**
225 **2003; Paramonov et al., 2005; Slaney et al., 2006). In addition, the Rgp isoforms namely**

226 HRgpA and RgpB which do not acquire the MAAb1B5 reactive glycan are present in the *porR*
227 mutant strain whereas the isoforms which usually contain the MAAb 1B5 cross-reactive epitope,
228 namely RgpA_{cat} and mt-Rgps (Paramonov et al., 2005) are not synthesised. However, the
229 synthesis of O-LPS is not affected in the *porR* mutant strain and ¹H-NMR spectroscopy of the O-
230 PS isolated from O-LPS of this strain showed an identical ¹H-NMR spectrum to that of O-PS
231 from the *P. gingivalis* W50 parent strain (Paramonov et al., 2005). Biologically, these effects
232 translate to cell fragility, loss of recognition by antibodies of the periodontal patients' sera, and
233 an enhanced complement mediated killing as a result of the inability to synthesise A-LPS
234 (Gallagher et al., 2003; Shoji et al., 2002; Paramonov et al., 2005; Slaney et al., 2006).

235 **Pigmentation and hemolysis of *P. gingivalis* strains.**

236 *P. gingivalis* W50, *rgpA/rgpB* and *galE* form brown or black pigmented colonies on blood agar
237 plates (Fig. 1), whereas colonies of *kgp*, *porR*, *waaL*, *wzy* and *pg0129* are non-pigmented. Also
238

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

240
241 shown is the *P. gingivalis* mutant strain *wbpB* which has been described in detail by Slaney et al.
242 (2006) and Shoji et al. (2014) and gives non-pigmented colonies on blood agar plates.

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

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

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

260

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

262

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

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

277 **Cross-streaking Experiments.**

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

289

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

291

292

293 **Analysis of LPS.**

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

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

310

311 [Fig. 4 here...SDS-Urea-PAGE of LPS...]

312

313 **Hemin Binding.**

314 **Hemin Binding by whole cells.**

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

317 Fig. 5. *P. gingivalis* W50 and mutant strains *rgpA/rgpB* and *galE* which pigmented brown and
318 black on blood agar plates respectively (Fig. 1) showed hemin binding values (5.6 $\mu\text{g}/\text{O.D.}_{600\text{nm}}$,
319 6.8 $\mu\text{g}/\text{O.D.}_{600\text{nm}}$ and 5.9 $\mu\text{g}/\text{O.D.}_{600\text{nm}}$ respectively), at the highest concentration (10 $\mu\text{g}/\text{ml}$) of
320 hemin used in the binding experiments. Although *kgp* was non-pigmenting on blood agar plates
321 due to the absence of Kgp, it shows hemin binding (6.1 $\mu\text{g}/\text{O.D.}_{600\text{nm}}$) when supplied with hemin
322 (also observed when *kgp* is cross-streaked on blood agar plates on which *P. gingivalis* W50 was
323 previously grown (Fig. 3). *P. gingivalis* mutant strains *porR*, *waaL*, *wzy* and *pg0129* which were
324 non-pigmenting on blood agar plates, were able to bind between ~ 2.5 and 3.7 μg of hemin
325 $/\text{O.D.}_{600\text{nm}}$ of cells which is approximately 45% to 65% of hemin bound by the parent W50 strain.
326 Thus, *P. gingivalis* mutant strains which do not synthesise A-LPS show reduced hemin binding.

327

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

329

330 **Hemin binding by LPS.**

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

338

339 [Fig. 6 here...Binding of hemin by LPS]

340
341 However, LPS from *porR* which is devoid of A-LPS (Paramonov et al., 2005) was able to bind
342 3.6 μg hemin/50 μg of LPS which is considerably lower than that of LPS from the parent W50
343 strain.

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

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

363 et al., 2001) and in the loss of the cross-reacting epitope Man α 1-2Man α 1-phosphate in A-LPS
364 (Paramonov et al., 2005; Rangarajan et al., 2008). The results (Fig. 6) show the binding of
365 hemin is reduced in de-*O*-phosphorylated LPS from the parent and *porR* mutant strain to ~3.3 μ g
366 and 1.9 μ g respectively. Thus, the reduction in binding of hemin caused by de-*O*-
367 phosphorylation of LPS from *P. gingivalis* W50 compared to that for LPS from *porR* mutant
368 strain suggests that negatively-charged A-LPS may have an important role to play in the
369 binding/deposition of hemin on the cell surface of *P. gingivalis*.
370

370 **DISCUSSION.**

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

380 Haem-starved *P. gingivalis* ATCC33277 and WT40 expressed two binding sites for
381 hemin, a low abundance high affinity site (1000 to 1500 sites/cell) of K_d between 3.6×10^{-11} and
382 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
383 estimated K_d ranged between 2.6×10^{-7} and 6.5×10^{-8} M (Tompkins et al., 1997). Treatment
384 with N-bromosuccinimide inactivated hemin binding by both sites in *P. gingivalis*, whereas
385 pronase treatment caused only a limited reduction in hemin binding probably because only one of
386 the two sites was sensitive to pronase. Tompkins et al. (1997) concluded that the higher-affinity
387 site was probably exposed on the surface of *P. gingivalis* and sensitive to pronase whereas the
388 lower affinity-site may be sequestered within the outer membrane especially if it functioned to
389 store hemin.

390 The black haem-pigment deposited on the cell surface of *P. gingivalis* and which serves
391 as an iron source for this organism, is composed of μ -oxo bishaem $[\text{Fe(III)PPIX}]_2\text{O}$, and the
392 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

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

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

428

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

430

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

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

441 Grenier (1991) reported that the lipid A component of LPS mediated the binding of
442 uncomplexed hemin by *P. gingivalis*. Since hemin is a lipophilic molecule, it would be expected
443 to bind to Lipid A/LPS. *Escherichia coli* which does not require exogenous haem when grown
444 in iron-replete conditions was shown to bind as much uncomplexed hemin as *P. intermedia*.
445 This effect was inhibited by albumin which indicated that when haem is provided in the free
446 form, most of it binds to the bacterium with an affinity lower than that for albumin. However,
447 Tompkins et al. (1997) concluded that most Gram-negative bacteria would exhibit similar non-
448 specific hemin binding and that the LPS-mediated hemin binding is probably not biologically
449 relevant because of the low affinity of the interaction and the presence of large amounts of host
450 plasma proteins which function to counter the lipophilic disposition of hemin. Tompkins et al.
451 (1997) showed that treatment of *P. gingivalis* cells with pronase caused a slight reduction in, but
452 did not eliminate, hemin-binding and the authors suggested that this was probably due to the
453 pronase-sensitive hemin-binding sites not being exposed on the surface of the cell and therefore
454 not digested by pronase treatment. However, it seems more plausible that it is the presence of A-
455 LPS (which is not sensitive to pronase treatment) on the surface of *P. gingivalis* which acts as a
456 site for the deposition/binding of hemin.

457 Studies on hemin binding to whole cells of *P. gingivalis* W50 and mutant strains and
458 hemin binding to native LPS and de-lipidated LPS from *P. gingivalis* W50 and *porR* strains show
459 that absence of A-LPS causes a reduction in hemin binding. Thus, absence of A-LPS in the
460 extracellular surface of *P. gingivalis* eliminates or reduces a scaffold /anchoring mechanism not
461 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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- 616

616 **Legends to Figures.**

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

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

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

620 *P. gingivalis* W50 and isogenic mutant strains were grown in BHI broth for 24 h or 48 h. Arg-X

621 and Lys-X activities were measured using substrates DL-BRpNA and L-AcKpNA respectively as

622 described in Methods. Enzyme activities are expressed as % activity relative to that of the parent

623 *P. gingivalis* W50 strain (Absorbance_{405nm} units/ min. / O.D._{600nm}). Black bars represent cell-

624 associated activities and grey bars represent enzyme activities in the culture supernatants.

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

626 (A). *P. gingivalis* W50 on blood agar, *kgp* on blood agar, *P. gingivalis* W50 was initially

627 streaked on a blood agar plate and following the formation of a zone of hemolysis (3 days), the

628 cells were removed with a swab containing clindamycin to suppress regrowth of the wild type

629 strain and the plates were cross-streaked with *kgp*. Note pigmentation of the *kgp* mutant cells

630 takes place only on the zone of hemolysis produced by the parent strain. (B). Blood agar plates

631 were initially streaked with W50 as in (A). Plates were cross-streaked with *rgpA/rgpB*, *kgp*,

632 *porR*, *pg0129*, *waaL* and *wzy*. Pigmentation of *kgp* takes place on the zone of hemolysis

633 whereas the other strains do not pigment even after 6 days of growth.

634 **Figure 4 . SDS-urea-PAGE and silver staining of LPS from *P. gingivalis* W50 and mutant**

635 **strains (A). Western blotting vs MAb 1B5 of whole cell extracts of *P. gingivalis* W50 and**

636 **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 *t* test) 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

663 **SUPPLEMENTAL.**664 **METHODS.**

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

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

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

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

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

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

675 *atatat**gcggccgc**GAGTACGTACAGGAGTTGCTGG* were used to amplify *galE* in PCR master

676 mix (Extensor mix Reddy Load PCR master mix (Buffer 2, ThermoScientific)) as previously

677 described (Aduse-Opoku et al., 2006). The amplicons were cloned at the NotI site of pUC18not

678 and inserts of *porR* (937 bp) and *galE* (2545 bp) were further manipulated to ligate a 2.1 kb *erm*

679 cassette (Fletcher et al., 1995) at the unique NcoI and BamHI-EcoRV sites respectively. NotI-

680 restricted plasmids were used to electrotransform 6h grown cells of *P.gingivalis* W50 to

681 clindamycin resistance. PCR was performed on purified chromosomal DNA from six separate

682 isolates of each mutant strain. The original primers were used to amplify the region *porR::erm*

683 and *galE::erm* to show correct insertion of the *erm* cassette. One strain from each was chosen

684 and designated as either *porR* or *galE* (Fig. S1).

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

687 **Description of PorR.**

688 PorR is a putative transaminase and is homologous to RfbE orthologue of *P. gingivalis* and
689 belongs to the DegT Clusters of Orthologous Groups (COGs), the prototype of which is DegT of
690 *Geobacillus (Bacillus) stearothermophilus* (Takagi et al., 1990) which is involved in a range of
691 biochemical functions including glycan synthesis, regulation of extracellular enzymes, altered
692 control of sporulation, abnormal cell division and loss of flagella (Takagi et al., 1990). Proteins
693 homologous to PorR have been found in several microorganisms involved in the biosynthesis of
694 sugars present in capsular polysaccharide and aminoglycosides. In *Vibrio cholerae* O1 and *E.*
695 *coli* O157, *rfbE* encodes perosamine synthetase (Bilge et al., 1996; Albermann & Piepersburg,
696 2001) and the *rfbE* orthologue *per* in *Caulobacter crescentus* also encodes a perosamine
697 synthetase (Awram & Smit, 2001). In *P. gingivalis*, the inactivation of *porR* leads to pleiotropic
698 effects involving pigmentation, lack of synthesis of A-LPS (Paramonov et al., 2005), processing
699 of other proteins including fimbriae, and major alteration to the surface of the cell without
700 perceptible effect on O-LPS (Shoji et al., 2002; Gallagher et al., 2003; Paramonov et al., 2005;
701 Slaney et al., 2006). In addition, the Rgp isoforms namely HRgpA and RgpB which do not
702 acquire the MAb1B5 reactive glycan are present in the *porR* mutant strain whereas the isoforms
703 which usually contain the MAb 1B5 cross-reactive epitope, namely RgpA_{cat} and mt-Rgps
704 (Paramonov et al., 2005) are not synthesised. However, the synthesis of O-LPS is not affected in
705 the *porR* mutant strain and ¹H-NMR spectroscopy of the O-PS isolated from O-LPS of this strain
706 showed an identical ¹H-NMR spectrum to that of O-PS from the *P. gingivalis* W50 parent strain
707 (Paramonov et al., 2005). Biologically, these effects translate to cell fragility, loss of recognition

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

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

785 **Figure S1.**

786

787 Organisation of the *porR* (PG1138) and *galE* (PG0347) loci in *P. gingivalis* W50.

788 The location of the *erm* cassette at the NcoI (Nc) site in *porR* and BamHI (B)-EcoRV (Ev) sites

789 in *galE* are shown. Relative positions of primers used in initial cloning of PCR products are

790 indicated below each locus. The black arrows correspond to the directions of open reading

791 frames.

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