A combination of both arginine- and lysine-specific gingipain activity of *Porphyromonas gingivalis* is necessary for the generation of the μ -oxo bishaem-containing pigment from haemoglobin

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The black pigment of *Porphyromonas gingivalis* is composed of the μ -oxo bishaem complex of Fe(III) protoporphyrin IX (μ -oxo oligomer, dimeric haem), namely [Fe(III)PPIX]₂O. *P. gingivalis* W50 and Rgp (Arg-gingipain)- and Kgp (Lys-gingipain)-deficient mutants K1A, D7, E8 and W501 [Aduse-Opoku, Davies, Gallagher, Hashim, Evans, Rangarajan, Slaney and Curtis (2000) Microbiology 146, 1933–1940] were grown on horse blood/agar for 14 days and examined for the production of μ -oxo bishaem. μ -oxo Bishaem was detected by UV-visible, Mössbauer and Raman spectroscopies in wild-type W50 and in the black-pigmented RgpA- and RgpB-deficient mutants (W501 and D7 respectively), whereas no haem species were detected in the straw-coloured colonies of Kgp-deficient strain K1A. The dark brown pigment of the double RgpA/RgpB knockout mutant (E8) was not composed of μ -oxo bishaem, but of a high-spin monomeric Fe(III) protoporphyrin IX species (possibly a haem-albumin complex). *In vitro* incubation of oxyhaemoglobin with cells of the W50 strain and the RgpA- and RgpB-deficient mutants (W501 and D7)

resulted in the formation of μ -oxo bishaem via methaemoglobin as an intermediate. Although the Kgp-deficient strain K1A converted oxyhaemoglobin into methaemoglobin, this was not further degraded into μ -oxo bishaem. The double RgpA/RgpB knockout was also not capable of producing μ -oxo bishaem from oxyhaemoglobin, but instead generated a haemoglobin haemichrome. Inhibition of Arg-X protease activity of W50, W501, D7 and K1A with leupeptin, under conditions where Lys-X protease activity was unaffected, prevented the production of μ -oxo bishaem from oxyhaemoglobin, but resulted in the formation of a haemoglobin haemichrome. These results show that one or both of RgpA and RgpB gingipains, in addition to the lysine-specific gingipain, is necessary for the production of μ -oxo bishaem from haemoglobin by whole cells of *P. gingivalis*.

Key words: Arg-gingipain, haemoglobin, Lys-gingipain, μ -oxo bishaem, *Porphyromonas*, protease.

INTRODUCTION

Porphyromonas gingivalis is considered to be one of the principal aetiological agents in adult periodontitis [1,2]. The black pigment phenotype of this organism is attributed to the accumulation of the μ -oxo bishaem complex of Fe(III) protoporphyrin IX, [Fe(III)PPIX]₂O [3,4]. This haem complex, which is also termed the μ -oxo oligomer and commonly known as dimeric haem, is a structure composed of two Fe(III) protoporphyrin IX moieties bridged by an oxygen atom [5]. It is generated by *P. gingivalis* from both oxy- and deoxy-haemoglobin [4]. The μ -oxo bishaem species is green in solution and imparts the greenish coloration to colonies of *P. gingivalis* during the initial growth on blood/agar, whereas the black coloration of the pigment, which occurs after prolonged incubation, is a result of the increase in concentration of μ -oxo bishaem.

The μ -oxo bishaem complex is an important virulence factor for *P. gingivalis*, since its generation from haemoglobin serves to mop up oxygen and, in so doing, also prevents free monomeric haems from participating in the generation of reactive oxygen species [3]. In addition, μ -oxo bishaem has a greater propensity to aggregate compared with the monomeric Fe(III) protoporphyrin IX, and, through deposition on the cell surface, serves as a defensive layer to protect against ingress of oxygen and reactive oxidants. μ -oxo Bishaem also protects *P. gingivalis* against H₂O₂ [6] by

virtue of its inherent catalase activity [7,8]. Importantly, a cell-surface layer of μ -oxo bishaem can also protect *P. gingivalis* by acting as a sacrificial substrate during oxidative attack by H₂O₂ [6,9].

The cysteine proteases Rgp (Arg-gingipain) and Kgp (Lys-gingipain) of *P. gingivalis*, which have specificity for Arg-X and Lys-X bonds respectively are the most widely studied virulence attributes of this organism [10]. They display a number of biological activities, including activation of coagulation factors, degradation of fibrinogen and fibrin, disruption of leucocyte functions and potentiation of the inflammatory response, which contribute to the pathogenesis of the periodontal disease [11]. Rgps are encoded by the homologous genes *rgpA* and *rgpB*, whereas Kgp is encoded by *kgp* [12]. Three translation products arise from the *rgpA* gene as a result of proteolytic processing and post-translational modifications. These include RgpA_{cat}, a catalytic domain alone; a membrane-associated form, mt-RgpA_{cat}, modified with polysaccharide decorations; and HrgpA, which is a stable non-covalent complex composed of the catalytic domain plus haemagglutinin/adhesin domains [13–15]. *rgpB*, however, encodes a product that is almost completely lacking in the haemagglutinin/adhesin domains, although post-translational polysaccharide decorations lead to the generation of a membrane-associated form, namely mt-RgpB [14]. The *kgp* gene codes for a polyprotein, which, after proteolytic processing, gives rise to non-covalent

Abbreviations used: Fe(III)PPIX.OH, Fe(III) protoporphyrin IX monomer; Kgp, Lys-gingipain; Rgp, Arg-gingipain.

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complexes comprising catalytic and haemagglutinin/adhesin domains [15].

The importance of gingipains in the process of haem acquisition from haemoglobin has been highlighted by the observation that strains lacking Kgp do not pigment black when grown on blood/agar [12,16]. This inability is predictable on the grounds that α and β globin chains of both human and horse haemoglobin each contain 11 lysine residues [17], and that efficient release of iron protoporphyrin IX may be possible by cleavage at these numerous sites. For native Kgp purified from the *P. gingivalis* strain W83, α - and β -chain cleavage products have been identified, indicating that lysine residues flanking the proximal histidine-haem co-ordination site are attacked [18]. In contrast, the α and β chains of human haemoglobin contain only three and four arginine residues respectively (the horse β chain possesses an additional arginine residue at position 116); thus, human haemoglobin would be less susceptible to complete degradation by Rgp enzymes alone. This has been confirmed experimentally through the demonstration that, whereas neither RgpA nor RgpB has the ability to degrade haemoglobin [19], purified Kgp can cause a complete degradation of the haem protein [18,19]. In addition, it has been observed that strains lacking both RgpA and RgpB also appear to produce colonies that pigment at the same rate as the wild-type strains [12,20]. Taken together, these results have led to the conclusion that Kgp alone is the most important protease in the haem pigmentation process.

We have taken a holistic approach by studying the whole *P. gingivalis* cell to determine the underlying chemical mechanisms of the pigment generation process. In our laboratory, a comparison of the wild-type pigmented strain W50 with the Kgp- and Rgp-deficient strains K1A, W501, D7 and E8 generated by Aduse-Opoku et al. [12] has revealed some anomalous and unexpected pigmentation behaviour on blood/agar. We have observed that, whereas the Kgp knockout mutant K1A predictably gives cream/straw-coloured colonies, the W50 strain and the RgpA- and RgpB-deficient strains (W501 and D7 respectively, derived from wild-type W50) yield green-black-pigmented colonies, indicating the presence of the $[\text{Fe(III)PIX}]_2\text{O}$ complex. In keeping with previous observations [12], the double *rgpArgpB* knockout mutant (E8) produces dark-brown-pigmented colonies on blood/agar; however, intriguingly, these do not develop the classic green-black or black coloration even after prolonged incubation. This proves that some haem-containing species other than the μ -oxo bishaem complex is generated from haemoglobin. In view of these observations, we undertook more detailed studies of pigments of these strains using Mössbauer, UV-visible and Raman spectroscopies to determine the exact nature of the haem species present and to determine the possible roles played by Rgp and Kgp enzymes of *P. gingivalis* in the production of μ -oxo bishaem from haemoglobin.

EXPERIMENTAL

Bacterial strains and growth conditions

The *P. gingivalis* strains W50, K1A (Kgp-deficient), D7 (RgpB-deficient), W501 (RgpA-deficient) and E8 (RgpA/RgpB-deficient) were kindly provided by Professor Michael Curtis (St. Bartholomew's and The Royal London School of Medicine and Dentistry, London, U.K.). The generation of these protease-deficient strains has been described previously [12]. All strains were maintained by routine subculture on horse blood/agar in an anaerobic atmosphere in a Don Whitley, Mark III anaerobic cabinet.

Bacterial growth on horse blood/agar and extraction of haem pigments

Lawn growths were cultured on horse blood/agar by heavy inoculation over the whole surface of the plates. These were incubated anaerobically and examined daily to monitor the colour of the growth. Cells, gently scraped from approx. one-third of each plate using a sterile plastic loop, were suspended in 2 ml of 0.14 M NaCl/0.1 M Tris/HCl (NaCl/Tris buffer, pH 7.5), and sonicated in an ultrasonic bath for 2 min to wash the pigment from the cells. The suspensions were centrifuged at 11 000 *g* for 10 min at 20 °C and the supernatant buffer extract was then diluted as appropriate in NaCl/Tris buffer, and the UV-visible spectra were recorded between 300 and 800 nm in an Ultrospec 2000 UV-visible scanning spectrophotometer (Amersham Biosciences) using 1 cm pathlength microcuvettes.

Growth in liquid culture

For liquid cultures, the cells were grown in Schaedler anaerobe broth (Oxoid, Basingstoke, U.K.) for 3 days as described previously [4]. The cells were harvested by centrifugation at 5000 *g* for 45 min at 5 °C and washed twice with NaCl/Tris buffer (pH 7.5) to remove growth medium constituents. Total cell protein was determined by the Lowry method using BSA as the standard.

Enzyme assays

Arg-X protease activity of whole cells was measured spectrophotometrically using the synthetic substrate *N*- α -benzoyl-DL-arginine-*p*-nitroanilide (BAPNA; 0.8 mM) in NaCl/Tris buffer (pH 7.5) at 37 °C as described previously [4]. Lys-X protease activity was assessed using *N*- α -acetyl-L-lysine-*p*-nitroanilide (Bachem, St Helens, U.K.) at 0.4 mM in NaCl/Tris buffer (pH 7.5).

Interaction of whole cells with oxyhaemoglobin

We have demonstrated previously that the progress of μ -oxo bishaem formation by cells of *P. gingivalis* from both oxy- and deoxy-haemoglobin can be followed *in vitro* using UV-visible spectroscopy [4]. Accordingly, this method was used to examine the changes occurring in oxyhaemoglobin during incubation with cells in isolation. Oxygenated haemoglobin was prepared as described previously as a whole haemolysate from fresh horse erythrocytes [4], and its concentration was determined by measuring the molar absorption coefficients of the Soret and 576 nm Q bands. Bacterial cell-oxyhaemoglobin incubations were performed at 37 °C in NaCl/Tris buffer (pH 7.5) using cell suspensions standardized to an A_{400} 0.5 (approx. 60 μg of cell protein/ml) and an oxyhaemoglobin concentration of 4 μM in a total volume of 1 ml. Incubation was performed in sealed tubes to prevent evaporation, and the UV-visible spectra was recorded periodically. All molarities of haemoglobin are expressed on haemoglobin tetramer basis.

Preparation of hydroxymethaemoglobin and Fe(III) haemoglobin haemichrome

Hydroxymethaemoglobin was prepared by oxidation of a sample of oxyhaemoglobin in NaCl/Tris buffer (pH 7.5) in the presence of a slight molar excess of $\text{K}_3\text{Fe}(\text{CN})_6$ [21]. Fe(III) haemoglobin haemichrome was prepared from hydroxymethaemoglobin by incubation for 5 h at 20 °C in NaCl/Tris buffer (pH 7.5), containing

urea at a final molarity of 8 M [22]. The conversion of Fe(III) haemoglobin haemichrome into the haemichrome form was monitored by UV-visible spectroscopy.

Preparation of an Fe(III) protoporphyrin IX-albumin complex

A 0.1 mM solution of Fe(III) protoporphyrin IX in NaCl/Tris buffer (pH 7.0) was reacted with a 2:1 molar excess of BSA (Sigma Chemical) at 20 °C. This complex gave a Soret band absorbance at 404 nm, which is in keeping with the albumin-haem complex reported by Kamal and Behere [23].

UV-visible spectroscopy

Electronic spectra were recorded at room temperature (20 °C) using an Ultrospec 2000 UV-visible scanning spectrophotometer in quartz or plastic 1 ml semi-microcuvettes (Elkay UltraVu, Shrewsbury, MA, U.S.A.) with a 1 cm pathlength.

Mössbauer spectroscopy

Haem-pigmented bacterial cells for Mössbauer spectroscopy were grown as above from heavy inocula on horse blood/agar for 14 days. The biomass was scraped from the plates and transferred on to nylon Mössbauer sample holders (effective diameter, 17 mm), and freeze-dried. The freeze-dried cells were then compressed by applying gentle pressure with the aid of a g-clamp and then more of the bacterial biomass was introduced into the holder, which was again freeze-dried. This process was repeated to concentrate the haem-containing samples as a disc approx. 2–3 mm in depth, which contained approx. 400 mg dry weight of biomass. The Mössbauer spectrometer used a ^{57}Co source in an Rh matrix of strength approx. 50 mCi in the transmission mode, with the 14.4 keV γ rays being detected in an argon-filled proportional counter. The Doppler motion was driven by a double-ramp, constant acceleration waveform so that the folded spectra had a flat background, which was important when fitting weak signals on a noisy background. The spectrometer was calibrated using a 25 μm foil of α -iron at room temperature and the values of isomer shift are quoted relative to this. All spectra were recorded at 77 K.

Raman spectroscopy

Raman spectra were obtained as described previously [4,24,25] using a LabRam Raman spectrometer equipped with an Olympus BX40 microscope and a Peltier-cooled CCD (charge-coupled-device) detector. Excitation at wavelength 514.5 nm was provided by an argon ion laser giving approx. 2 mW of laser power at the sample. The laser light was focused on the sample using an Olympus ultra-long working distance microscope objective having a magnification of $\times 50$, which also collected the backscattered light. The Rayleigh scattered light was blocked by a holographic notch filter, and the Raman scattered light was dispersed into its component wavelengths by a 1800 grooves/mm holographic grating in the spectrograph and then imaged on to the 1 inch pixelated chip (MPP1) of the CCD detector.

RESULTS

UV-visible spectra of the pigments from cells grown on blood/agar

Incubation of cells of the parent strain *P. gingivalis* W50 as a lawn growth on horse blood/agar gave a green-black coloration

Figure 1 UV-Visible spectra of the haem pigments washed from *P. gingivalis* wild-type W50 and protease-deficient mutants grown on blood/agar

(a) The wild-type strain W50 and strains D7 (RgpB-deficient) and W501 (RgpA-deficient) after growth on blood/agar for 14 days. (b) Reference sample of μ -oxo bishaem (40 μM , on a haem monomer basis) with a small amount of haematin present (approx. 10% of total). (c) E8 strain (both RgpA- and RgpB-deficient) after growth on blood/agar for 9 days. (d) Reference samples of an Fe(III) haemichrome (3.75 μM ;) and an Fe(II) haemochrome (—). The haemochrome was produced by treatment of the haemichrome with 10 mM $\text{Na}_2\text{S}_2\text{O}_4$. The inset in (d) shows the Q band regions of the haemichrome and haemochrome spectra with an expanded ordinate scale. All spectra were recorded at room temperature in NaCl/Tris buffer (pH 7.5).

after 8 days, which became a jet-black pigmentation after 14 days. Extraction of the pigment into NaCl/Tris buffer (pH 7.5) produced a dark green solution, the UV-visible spectrum of which was characterized by a broad Soret band having λ_{max} values centred at 385 and 365 nm (Figure 1a). This spectrum was similar to the one published previously for the haem pigment of *P. gingivalis* [3,4] and also to that of an authentic sample of μ -oxo bishaem in mixture with a smaller amount (approx. 10% of the total) of the monomeric Fe(III) protoporphyrin IX [haematin, Fe(III) PPIX.OH] [5] (Figure 1b). During the same time period of incubation, the RgpA- and RgpB-deficient strains (W501 and D7 respectively) also produced jet-black-pigmented colonies. When extracted into NaCl/Tris buffer (pH 7.5), the colonies of both these strains, similar to W50, gave dark green solutions, the spectra of which were characterized by broad Soret bands with λ_{max} values centred at both 385 and 365 nm, indicating the presence of $[\text{Fe(III)PPIX}]_2\text{O}$ in mixture with some monomeric Fe(III)PPIX.OH (see Figure 1a). In contrast, after 14 days incubation, the Kgp-deficient strain K1A produced straw-coloured colonies that did not yield any soluble pigment.

During the same time period in which wild-type W50 pigmented black, colonies of the double *rgpArgpB* knockout mutant E8 became brown. During the initial phase of pigmentation, suspension of the colonies in NaCl/Tris buffer (pH 7.5) for 7–9 days, followed by centrifugation, yielded solutions which were brown and not dark green. UV-visible spectroscopy revealed the presence of a Soret band with a λ_{max} value of 408 nm and with weak Q bands positioned at approx. 540 and at 575 nm (Figure 1c). Absorbance bands were not observed at 429 and 555 nm or at 500 and 631 nm, indicating the absence of deoxyhaemoglobin and methaemoglobin respectively [21,26]. However, the

Soret band position at 408 nm and the presence of weak Q bands at 540 and 575 nm, with a shallow trough at 560 nm, indicate the presence of an Fe(III) haemoglobin haemichrome in a mixture with some oxyhaemoglobin [27]. Addition of $\text{Na}_2\text{S}_2\text{O}_4$ (10 mM final molarity) to the solution of the E8 pigment resulted in a Soret band having a λ_{max} value of approx. 417 nm (results not shown). This behaviour is consistent with the reduction of the Fe(III) species to an Fe(II) haemochrome [26], as observed for the reference sample of Fe(III) haemoglobin haemichrome, produced by treatment of methaemoglobin with 8 M urea (Figure 1d). Unlike the strains W50, W501 and D7, further incubation of the brown colonies of the E8 strain up to 14 days did not result in green-black pigmentation, indicating that μ -oxo bishaem was not formed. Moreover, the brown pigment was extremely adherent and could not be removed by washing with NaCl/Tris buffer at either pH 7.5 or 10. Since solution spectra could not be obtained, the haem-pigmented cells were examined by Mössbauer spectroscopy along with the pigmented cells of strains W501 and D7 (see below).

Mössbauer spectroscopy

Mössbauer spectra of the haem-pigmented bacterial cells of strains W501, D7 and E8 were obtained after they were concentrated by compressing the freeze-dried biomass into the nylon sample holders. In addition, since the iron in the samples was natural and not enriched in ^{57}Fe , long counting times (of the order of 100 h) were necessary to resolve the signal from background noise. As shown in Figures 2(A) and 2(B), these Mössbauer spectra were composed of slightly asymmetrical doublets, which were fitted with two singlet components whose centroid position, width and area were allowed to vary. No further components were required for good fits. The fitted parameters are presented in Table 1. The spectra of the haem pigments of strains W501 and D7 were similar (Figures 2A and 2A), and the Mössbauer parameters were similar to those previously obtained for wild-type W50 (Table 1) and for an authentic sample of μ -oxo bishaem [5]. The reasons why the spectra are nearly symmetrical [although they arise from high-spin Fe(III) electronic sites] have been described previously [5]. In contrast, however, the Mössbauer spectrum of the pigmented cells of the E8 strain (Figure 2C) was not typical of μ -oxo bishaem; here, the absorption lines are highly asymmetrical and this is typical of a high-spin monomeric, five co-ordinate Fe(III) protoporphyrin IX species [5].

Raman spectroscopy

Raman spectra of cells of the wild-type strain W50 (Figure 3a), strains of the RgpB- and RgpA-deficient strains D7 and W501 (Figures 3b and 3c respectively) and the double RgpA/RgpB knockout strain E8 (Figure 3d) were recorded after growth on horse blood/agar for 14 days. As seen in Figure 3(a), the 1500–1650 cm^{-1} region of the Raman spectrum of *P. gingivalis* W50 showed a broad doublet with components at approx. 1570 and 1621 cm^{-1} , as did the Raman spectra of strains D7 and W501 (Figures 3b and 3c). All three spectra showed similarities in the 1300–1400 cm^{-1} region, including a Raman band at 1370 cm^{-1} . This band is known to be an oxidation state marker and its location at 1370 cm^{-1} is indicative of iron in oxidation state +3 [28]. The Raman spectrum of the brown pigment of strain E8 was less strong compared with pigments of the other three strains and, thus, was not sufficiently intense to show clearly the 1370 cm^{-1} oxidation state marker. This may be attributed either to the lower concentration of Fe(III) protoporphyrin

Figure 2 Mössbauer spectra from the freeze-dried biomass of haem-pigmented cells of (A) W501, (B) D7 and (C) E8 strains grown on blood/agar for 14 days

The spectra were recorded at 77 K.

Table 1 Mössbauer parameters for the haem-pigmented cells of *P. gingivalis* W50 and protease-deficient mutants, grown for 14 days on horse blood/agar

Sample	Isomer shift δ (mm/s)	Quadrupole splitting ΔE_Q (mm/s)	Reference
W50 wild-type	0.39 ± 0.01	0.59 ± 0.02	[3,23]
W501 <i>rgpA</i> -deficient	0.40 ± 0.01	0.57 ± 0.01	The present study
D7 <i>rgpB</i> -deficient	0.40 ± 0.01	0.55 ± 0.01	The present study
E8	0.47 ± 0.03	0.67 ± 0.03	The present study

IX or to the fact that the bands were less enhanced by the resonance Raman effect when compared with μ -oxo bishaem. We have shown previously that the Raman spectrum of the

Figure 3 Raman spectra over the range 1800–500 cm^{-1} obtained from cells of (a) wild-type W50, (b) D7, (c) W501 and (d) E8 after growth on blood/agar for 14 days

All spectra were obtained using laser excitation at 514.5 nm.

pigment of *P. gingivalis* W50 corresponds to that of an authentic sample of μ -oxo bishaem [24,25]. From these results, it can be concluded that cells of the D7 and W501 strains are also pigmented with μ -oxo bishaem. However, the Raman spectrum from the cells of the double RgpARgpB knockout strain E8 is very different from the Raman spectra obtained from cells of W50, D7 and W501, showing weak Raman bands at 919 and 1156 cm^{-1} and a weak doublet with components at 1493 and 1522 cm^{-1} . Although we cannot, at present, identify the brown pigment on the cells of the E8 strain from the Raman spectrum alone, it can be concluded that they were not pigmented with μ -oxo bishaem. No haem-related Raman bands were observed in the blood/agar-grown cells of the Kgp mutant strain K1A (results not shown).

Interaction of liquid culture-grown cells with oxyhaemoglobin

As observed previously [4], incubation of oxyhaemoglobin with cells of the W50 strain resulted in its conversion into methaemoglobin as evidenced by the change in the λ_{max} value of the Soret band from 413.7 to 408.6 nm, the decrease in the intensity of the 541 and 576 nm Q bands and the appearance of broad bands at 500 and 631 nm (results not shown). After 24 h, the solution became green in colour and the spectrum was characterized by a Soret band having a λ_{max} value of 393 nm and a weaker Q band at approx. 608 nm (Figure 4a). These results are consistent with the formation of μ -oxo bishaem in almost pure form [5] from methaemoglobin [4]. Incubation of oxyhaemoglobin with cells of the *rgpA* and *rgpB* mutant strains W501 and D7 respectively also resulted in the initial generation of methaemoglobin, which was followed by a decrease in intensity of the Soret band, indicating the loss of the intact haem protein (results not shown) and, after 48 h, generation of spectra characteristic of μ -oxo bishaem ($\lambda_{\text{max}} = 394$ nm and a weak Q band at approx. 608 nm; [4]) (Figures 4b and 4c). In contrast, incubation of oxyhaemoglobin with cells of the Kgp-deficient strain K1A resulted in an initial shift in the λ_{max} value of the Soret band from 413.7 to 408 nm and a decrease in the 576 and 541 nm Q band intensities, indicating the oxidation of oxyhaemoglobin to methaemoglobin (results not shown). However, after 48 h, the λ_{max} value of the Soret band had decreased to approx. 404 nm, but no distinct peaks could be seen in the visible region (Figure 4d).

For the double *rgpArgpB* knockout mutant E8, incubation with oxyhaemoglobin also resulted in an initial decrease in the

Figure 4 UV-Visible spectra showing the results of incubating horse oxyhaemoglobin for 24 h with liquid culture-grown cells of (a) wild-type W50, (b) W501, (c) D7, (d) K1A and (e) and (f) E8

(f) The same incubation mixture as in (e), after treatment with 10 mM $\text{Na}_2\text{S}_2\text{O}_4$. The increase in absorbance (shown on the ordinate) below 400 nm is due to the presence of $\text{Na}_2\text{S}_2\text{O}_4$. The inset in (f) shows the ordinate expansion of the Q band region. The concentration of cell protein was 60 $\mu\text{g/ml}$, and the starting concentration of oxyhaemoglobin was 4 μM . Incubations were performed in NaCl/Tris buffer (pH 7.5).

λ_{max} value of the Soret band and a decrease in the 541 and 576 nm Q bands, indicating the oxidation of oxyhaemoglobin to methaemoglobin. However, after 24 h, the spectrum was characterized by a Soret band at 408 nm and a Q band at 531 nm (Figure 4e). In addition, the trough at 560 nm became shallower, and there was no prominent absorbance band at 630 nm. These features are consistent with the formation of an Fe(III) haemoglobin haemichrome [26,29] as the major haem species in the digestion mixture. This spectrum remained unchanged after incubation for up to 96 h. The identity of this haem species as a haemoglobin haemichrome was confirmed after treatment of the sample with sodium dithionite (final concentration, 10 mM). This yielded an Fe(II) haemochromic spectrum with a Soret band having a λ_{max} value at 420 nm (Figure 4f) and two Q bands in the visible region at 526 and 555 nm (Figure 4f, inset); this result was similar to that obtained for the Fe(II) haemoglobin haemochromic reference sample (Figure 1d). After centrifugation of a 72 h incubation mixture of E8 cells with oxyhaemoglobin at 11 500 g for 10 min at 20 °C to pellet the bacterial cells, the concentration of the haemoglobin haemichrome remaining in solution was determined from the Soret absorbance. This revealed that approx. 70 % of the haemichrome generated by the cells was insoluble and had become cell-associated. When E8 cells were incubated for 72 h with a mixture of oxyhaemoglobin and bovine albumin with the concentrations of these two proteins at the

Figure 5 UV-Visible spectrum showing the results of incubating cells of *P. gingivalis* W50 with oxyhaemoglobin for 24 h in the presence of the Arg-X protease inhibitor leupeptin (2 mM)

The inset shows the ordinate expansion of the Q band region. Experimental incubation conditions were the same as described for Figure 4.

same stoichiometric ratio as found in whole blood (molar ratio of albumin to haemoglobin tetramer, approx. 2.6:1), a spectrum was obtained with a Soret band at 404 nm and not at 408 nm. Prominent bands were not observed at 500 and 630 nm, indicating the absence of methaemoglobin. These results, together with the Soret band position at 408 nm, indicated the presence of an Fe(III) protoporphyrin IX–albumin complex and not an Fe(III) haemoglobin haemichrome.

Assay for Arg-X and Lys-X protease activity

The Arg-X and Lys-X protease activities of liquid culture-grown cells were measured using the hydrolyses of *N*- α -benzoyl-DL-arginine-*p*-nitroanilide and *N*- α -acetyl-L-lysine-*p*-nitroanilide respectively. In keeping with the results of Aduse-Opoku et al. [12], the Arg-X activities of cells of the RgpA- and RgpB-deficient strains D7 and W501 were approx. 50% of that of the wild-type strain W50 (results not shown). In addition, K1A showed an Arg-X activity similar to that of W50, whereas the double *RgpARgpB* knockout strain E8 did not. Addition of leupeptin completely inhibited Arg-X activity of cells of strains W50, D7, W501 and K1A, whereas it had no effect on Lys-X activity (results not shown).

Effects of leupeptin on gingipain activity and on the production of μ -oxo bishaem from oxyhaemoglobin

In agreement with Aduse-Opoku et al. [12], the presence of 2 mM leupeptin completely inhibited the Arg-X protease activity of strains W50, W501, D7 and K1A, whereas it had no effect on the Lys-X protease activity (results not shown). Whereas the W50 strain generated μ -oxo bishaem from oxyhaemoglobin after 24 h, incubations performed in the presence of leupeptin resulted in the formation of a component with a Soret band having a λ_{max} value of 409 nm and a 533 nm Q band (Figure 5), indicating that a haemoglobin haemichrome had formed. This was stable even after incubation for 72 h, and did not result in the formation of μ -oxo bishaem. No bands were seen at 500 or 631 nm, indicating the absence of methaemoglobin. A haemoglobin haemichrome also resulted when cells of strains W501, D7 and K1A were incubated with oxyhaemoglobin in the presence of leupeptin to

inhibit the residual Arg-X protease activity (results not shown). Incubation of control oxyhaemoglobin in the absence or presence of leupeptin resulted in the generation of methaemoglobin, but a haemoglobin haemichrome was not detected (results not shown).

DISCUSSION

By studying the pigmentation process as mediated by the whole cell, we have shown here, using UV-visible, Mössbauer and Raman spectroscopies, that the wild-type strain W50 (intact in its full complement of both Arg-X and Lys-X protease activities) and W501 and D7 (lacking in RgpA and RgpB respectively) can produce μ -oxo bishaem during growth on blood/agar or during incubation with isolated oxyhaemoglobin. In addition, we have shown that K1A (lacking in Kgp), which develops straw-coloured colonies, does not produce μ -oxo bishaem. In contrast, cells of the E8 strain, which lack both Rgps but possess intact Lys-X protease activity, do not produce the μ -oxo bishaem complex. During initial growth on blood/agar, the E8 strain, unlike W50, D7 and W501, did not become green-black, but gave brown-coloured colonies. Instead, these contained an Fe(III) haemoglobin haemichrome as the major haem-containing species. Prolonged incubation of E8 on blood/agar failed to yield the μ -oxo bishaem complex. However, Mössbauer spectroscopy revealed a five co-ordinate, high-spin Fe(III) monomeric species as the major haem component of the E8 pigment. This pigment was extremely adherent on to the cells, unlike that from strains W50, W501 and D7, and could not be removed by prolonged washing with 0.14 M NaCl at pH 7.5. Moreover, treatment of the cells with 0.14 M NaCl at pH 10 (which would otherwise have converted any free Fe(III)PPIX into μ -oxo bishaem as a result of the pH-dependent equilibrium between the two forms [5], yielding a green solution) did not release any pigment. This indicated that the Fe(III) monomeric species was tightly bound to the cell surface or was complexed in some insoluble form on the cell surface.

A clue to the nature of this haem-containing species was obtained when E8 cells were incubated with isolated oxyhaemoglobin in the presence of albumin. Under these conditions, a haemoglobin haemichrome was not formed, but in its place, a haem–albumin complex was detected. The parameters of the Mössbauer spectrum of the E8 pigment are very different from those of an Fe(III) haemoglobin haemichrome, but are consistent with the spin and oxidation state of albumin-bound haem [30]. Taken together, these findings suggest that Arg-X protease-deficient cells initially generate a haemoglobin haemichrome from which the haem is subsequently transferred to albumin after prolonged incubation. Presence of the haem as a cell-surface haem–albumin complex might explain the relative insolubility and persistently adherent nature of the pigment. In this regard, we have shown that haem–albumin complexing increases the avidity of albumin binding to cells of *P. gingivalis* compared with the non-haem-complexed protein [31]. Furthermore, such complexes are less susceptible to proteolytic digestion by this organism [32].

Participation of Arg-X protease activity in the generation of the μ -oxo bishaem complex from oxyhaemoglobin is also demonstrated by the observation that a haemoglobin haemichrome was formed when W50, W501 and D7 were incubated with oxyhaemoglobin under conditions where Arg-X-protease activity, but not Lys-X-protease activity, was totally inhibited by the presence of leupeptin. Thus, importantly, the concerted action of both Kgp and one or both of the Rgps is required for the cell to process efficiently haemoglobin into μ -oxo bishaem. It is not clear why a haemoglobin haemichrome results when oxyhaemoglobin is incubated with cells in which Arg-X protease activity is inhibited

or absent. We conclude that the absence of Rgp activity unmasks some hitherto unidentified bacterial cell–haemoglobin interaction, allowing the above conversion to take place.

The question arises, however, as to the function of the Rgps in the generation of μ -oxo bishaem by the whole cell, given that (1) isolated Kgp, and not Rgp, is responsible for the degradation of haemoglobin [18,19], and (2) in the present study, cells possessing intact Kgp activity (at levels comparable with wild-type W50), but deficient in Rgp, are not capable of generating the $[\text{Fe(III)PPIX}]_2\text{O}$ complex either during growth on blood containing media or from isolated haemoglobin. Thus, although Rgps may not effect complete globin chain degradation, they may be essential for priming the protein by cleaving Arg-X sites, at positions 31 and 92 in the α chain and at positions 30, 40 and 104 in the human β chain (and at position 116 in horse haemoglobin) [17], to make an attack by Kgp more facile.

An alternative explanation for the inability of the double Rgp mutant E8 to generate μ -oxo bishaem may stem from the decrease in adhesin domain-mediated haemoglobin binding as a result of the absence of cell-surface Rgp molecules. However, Shi et al. [20] have shown that mutants lacking either *kpg* (strain KDP129) or both *rgpA* and *rgpB* (strains KDP112 and KDP133) do not have decreased cellular haemoglobin binding activity, whereas this ability is only abrogated in triple mutants of *rgpA*, *rgpB* and *kpg*. We are also aware of reports that Rgps may be involved in post-translational processing of Kgp to generate and activate the mature form of the enzyme [33–35] and Kgp may also play a reciprocal role in Rgp processing [36]. Thus the inability of the double *rgpArgpB* mutant to generate μ -oxo bishaem is probably related indirectly to the lack of the mature active form of Kgp. However, in agreement with findings of Aduse-Opoku et al. [12], we found no decrease in Kgp activity either in the double *rgpArgpB* knockout mutant E8 or in the individual single *rgpA* and *rgpB* knockout mutants W501 and D7. Our findings are also in accordance with the results of Okamoto et al. [16], who found that the loss of function of *kpg* did not affect the Arg-X activity in the ATCC strain 33277. In addition, we found that the level of Arg-X protease activity of the Kgp-deficient strain K1A was also unaffected, and was similar to that of the wild-type strain W50. An analysis of the interactions of haemoglobin with the individual purified catalytic, adhesin and haemoglobin-binding domains of Rgp and Kgp may shed more light on the possible roles they play in processing the haemoglobin macromolecule.

We have shown recently [24] that the haem pigments of both *Prevotella nigrescens* and *Prevotella intermedia* are composed of Fe(III) protoporphyrin IX monomers, Fe(III)PPIX.OH, and not the μ -oxo bishaem complex. These pigments, as a result of the presence of the Fe(III)PPIX.OH, are brown and not green-black. In view of this and the present findings, care must be taken in describing the phenotype of black-pigmenting anaerobes, which should not be attempted until the haem species has been analysed. The above findings should be taken into consideration for development of strategies designed to attenuate the virulence of *P. gingivalis* by preventing production of the defensive μ -oxo bishaem-containing pigment.

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