The haem pigment of the oral anaerobes Prevotella nigrescens and Prevotella intermedia is composed of iron(III) protoporphyrin IX in the monomeric form

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INTRODUCTION

Haem (iron protoporphyrin IX) is an important virulence regulator in a number of pathogenic Gram-negative bacteria. However, the ability to produce and accumulate haem as a cell-surface pigment during growth on blood-containing media is restricted to a small number of bacterial species. These include Yersinia pestis, the causal agent of bubonic plague (Pendrak & Perry, 1997), Aeromonas salmonicida, the cause of furunculosis in salmonid fish (Hirst et al., 1994), and black-pigmenting anaerobes of the genera Prevotella and Porphyromonas. These black-pigmenting species are opportunistic pathogens involved in mixed anaerobe infections in the body (Finegold et al., 1993). They are found in higher numbers in patients with periodontal diseases (Rawlinson et al., 1991, 1993; Moore et al., 1983, 1987) and are considered to play a major role in the initiation and progression of these conditions.

It was established by Schwabacher et al. (1947) that the black pigmentation in this group of organisms was not melanin, but was due to the presence of iron protoporphyrin IX, and it was widely accepted that this iron porphyrin [which can account for up to 50% of the dry weight of the biomass during growth on blood agar (Rizza et al., 1968)] was accumulated only to serve as a cytochrome prosthetic group (Rizza et al., 1968; Shah et al., 1979). However, more recent studies using Mössbauer, Raman and UV-visible spectroscopy (Smalley et al., 1998, 2002; Withnall et al., 1999) have shown that the major haem component in the pigment of Porphyromonas gingivalis is the [Fe(III)PPIX]₂O complex of iron protoporphyrin IX [sometimes referred to as dimeric haem], but more correctly termed the μ-oxo bishaem complex or μ-oxo oligomer (Silver & Lukas, 1983). [Fe(III)PPIX]₂O is formed by P. gingivalis through the reaction of haemoglobin-derived iron(II) protoporphyrin IX molecules and O₂ (Smalley et al., 1998), which is a mechanism for mopping up oxygen to engender an anaerobic atmosphere conducive to the growth of the organism. Importantly, a bacterial cell-surface layer of [Fe(III)PPIX]₂O can act defensively by protecting cells of P. gingivalis against attack by hydrogen peroxide (Smalley et al., 2000) through its inherent ‘catalase’ activity (Jones et al., 1973). This feature, coupled with its propensity to aggregate on the cell surface (Smalley et al., 1998), has established the [Fe(III)PPIX]₂O complex as an important virulence factor for P. gingivalis. However, the exact nature and function of the iron porphyrin in pigmenting Prevotella species has not been established.

It is the presence of the [Fe(III)PPIX]₂O complex which
imparts the initial green colouration to colonies of *P. gingivalis*. The development of the black pigmentation results from a hyperchromic shift as the concentration of [Fe(II)PPIX]_2O associated with the colonies increases. The formation of [Fe(II)PPIX]_2O is encouraged by alkaline conditions (Silver & Lukas, 1983; Smalley et al., 2002). A slightly alkaline environment is conducive to the growth of *P. gingivalis* (McDermid et al., 1988) and it can generate such conditions due to utilization and metabolism of peptides and amino acids (Shah et al., 1976, 1987). However, we have observed that colonies of black-pigmenting strains of *Prevotella nigrescens* and *Prevotella intermedia* do not develop a green-black colouration on blood agar, but initially become dark orange-brown before the pigment blackens. In addition, suspensions of these cells in neutral pH buffers are orange-brown and not dark green. This is evidence that they accumulate haem in another form, most probably monomeric Fe(III)PPIX, which is orange-brown in concentrated solution at acid pH (Silver & Lukas, 1983). From this it is concluded that the mechanism of pigment formation by *Prevotella* species may be different from that employed by *P. gingivalis* which produces the [Fe(III)PPIX]_2O complex. Given the ability of ferrihaems to protect *P. gingivalis* against hydrogen peroxide through their inherent catalase activity (Smalley et al., 2000), it is vital that the exact nature of the iron porphyrin in the *Prevotella* pigment and the mechanism of its generation are elucidated. Accordingly, we have undertaken more detailed spectroscopic investigations of the pigments and report here that *Pr. intermedia* and *Pr. nigrescens* generate and accumulate iron(III) protoporphyrin IX in the monomeric form Fe(III)PPIX.OH (haematin). The chemical and biological implications of these findings are discussed.

**METHODS**

**Bacterial strains and culture conditions.** *Pr. intermedia* ATCC 25611 and *Pr. nigrescens* ATCC 25261, well-characterized strains that have been the subject of other growth studies (Takahashi & Schachtele, 1990; Takahashi et al., 1997; Takahashi & Yamada, 2000), were used for the majority of the work in this investigation. These and additional *Pr. nigrescens* strains T588 and HG403, *Pr. intermedia* strains HG404 and NY653, and *P. gingivalis* W50 were maintained anaerobically by routine weekly subculture on horse-blood agar. Cells were also grown anaerobically in liquid culture at 37°C in Schaedler Anaerobe Broth (Oxoid) in a Mark III Don Whitley cabinet as also grown anaerobically in liquid culture at 37°C. 5000 inoculate 50 ml starter batches of Schaedler Anaerobe broth, which were used to inoculate 50 ml starter batches of Schaedler Anaerobe broth, which were grown for 3 days. These were then used to inoculate 950 ml batches of anaerobically equilibrated Schaedler broth which were incubated with stirring for 3 days. These cultures were centrifuged at 5000 g for 45 min at 5°C and pelleted cells were washed in 0·14 M NaCl buffered either at pH 6·5 with 0·1 M Na2HPO4/NaH2PO4 or at pH 7·5 with 0·1 M Tris/HCl, pH 7·5, and recovered by centrifugation as above. The above procedures were used to minimize any carryover of haemoglobin or haem from the blood agar or Schaedler broth, respectively.

**Mössbauer spectroscopy of the haem-pigmented cells.** The bacterial samples for Mössbauer spectroscopy were prepared as described previously (Smalley et al., 1998) using the experimental set-up reported in full elsewhere (Hameed et al., 1982). Briefly, cells were cultured as a heavy confluent growth for 8 days on blood agar, after which time they were black with an orange-brown tint. The bacterial growth was gently scraped from the plate using a plastic loop and then pasted into an aluminium sample holder with a circular cavity, 10 mm diameter × 2 mm deep, for Mössbauer spectroscopy. The sample, which was held in place either side by a single layer of Scotch clear adhesive tape, was then freeze-dried. The spectrometer was operated in a saw tooth mode and the sample was maintained at 78 K. The source was 57Co in Rh and the spectrometer was calibrated with a 25 μm thick natural iron reference absorber; all isomer shifts are referred to this zero shift.

**Raman microscopy.** A Labram spectrometer configured with an Olympus BX40 microscope was used to obtain Raman spectra of the pigmented bacterial cells (Withnall et al., 1999), which were grown as above on blood agar for 8 days. These were spread onto glass microscope slides and freeze-dried. The exciting radiation of wavelength equal to 632·8 nm was provided by a helium-neon laser giving a power of 8 mW at the sample unless an attenuation filter was used. An ultra-long working distance achromatic microscope objective (Olympus) with a magnification of ×50 was used both to focus the incident laser light and to collect the back-scattered radiation. The Rayleigh scattered light was blocked by a holographic notch filter and the Stokes Raman scattered light was dispersed on to a 1 inch pixelated CCD chip (MPP1) by a holographic grating with 1800 grooves mm⁻¹. The chip of the CCD detector was maintained at a temperature of approximately −40°C by a Peltier cooler to minimize thermal noise.

**Iron(III) protoporphyrin IX preparations.** Reference solutions of Fe(III)PPIX.OH and Fe(III)PPIX.O were made from bovine haematin, Fe(III)PPIX.Cl. Haemin was first dissolved in 0·14 M NaCl, 0·1 M Tris (pH=9·8) at 1 mM, and the pH of this solution was reduced to 8 by addition of dilute HCl to yield a solution containing predominantly the [Fe(III)PPIX]_2O complex (Silver & Lukas, 1983). The stock solution was also diluted in 0·14 M NaCl buffered at pH 6·5 with 0·2 M NaHP04/NaH2PO4 to produce a solution comprising predominantly Fe(III)PPIX.OH (Silver & Lukas, 1983).

**pH measurements.** The haem-pigmented colonies from approximately half the area of a three-inch diameter blood agar plate after 8 days growth were gently scraped off and resuspended in 5 ml distilled water. The pH was measured using a standard Gelplas combination pH/reference probe (BDH).

**Preparation of haemoglobin.** Horse oxyhaemoglobin was prepared as a whole haemolysate as described previously (Smalley et al., 2002) by hypo-osmotic lysis of fresh erythrocytes in 1 mM Tris/HCl, pH 7·0. The haemolysate was centrifuged at 20 000 g for 20 min at 5°C to remove cell membranes and any remaining intact erythrocytes. The haemoglobin stock preparation, which was stroma-free as judged by SDS-PAGE on 10% polyacrylamide gels, was stored in 1 mM Tris/HCl, pH 7·0, at −80°C until needed. All molarities of haemoglobin are quoted on a haem basis.

**Incubation of non-viable bacterial cells with oxyhaemoglobin.** Non-viable liquid-culture-grown cells were used to examine the interactions with oxyhaemoglobin. These cells did not display any Soret band absorbance (data not shown) indicating the absence of haem or haemoglobin which may have interfered with the spectroscopic analysis. Immediately before use the haemoglobin stock solution was diluted to 16 μM in 0·14 M NaCl buffered at either pH 7·5 with 0·1 M Tris/HCl or pH 6·5 with 0·1 M Na2HPO4/NaH2PO4 and bubbled with oxygen at room temperature for approximately 20 min at a flow rate of 3 ml min⁻¹. The oxyhaemoglobin was then mixed with an equal volume of liquid-culture-grown cells suspended in the
above buffers at either pH 7.5 or 6.5. In the incubation mixture the final cell protein concentration was 125 μg ml⁻¹, whilst the molarity of the oxyhaemoglobin was 8 μM. The cell-haemoglobin mixtures were incubated in tightly capped tubes and at intervals 1 ml aliquots were removed and the UV-visible spectra recorded immediately. The spectra of parallel samples of bacterial cells incubated alone were also recorded and were used to correct for the background light scatter as described previously (Smalley et al., 2002).

**RESULTS**

After 8 days growth on blood agar, *P. gingivalis* W50 yielded green-black colonies which became jet-black after a further 7 days incubation. Suspensions of these black colonies in either 0.14 M NaCl/0.1 M Tris/HCl buffer, pH 7.5, or distilled water gave a dark green solution containing both soluble [Fe(III)PPIX]₂O and that bound to the cell surface (Smalley et al., 2002). In contrast, all strains of *Pr. intermedia* and *Pr. nigrescens* gave black colonies with an orange-brown tint after 8 days. Whilst these colonies also became jet-black after further incubation for 7 days, suspensions of these in distilled water or NaCl/Tris buffer, pH 7.5, gave a dark orange-brown rather than a dark green colouration. The haem pigment associated with the *Pr. intermedia* and *Pr. nigrescens* cells was strongly bound to the cell walls and a soluble extract could not be obtained in the above buffer or distilled water for the purposes of UV-visible spectroscopy. For this reason the haem species in the pigmented cells were subjected to Raman and Mössbauer spectroscopy to examine them in their native form.

Pigmented colonies of *Pr. nigrescens* ATCC 25261 and *Pr. intermedia* ATCC 25611 after growth for 8 days on blood agar were spread as thin films on glass microscope slides and examined by Raman microscopy using 632.8 nm excitation. The Raman spectra of the pigmented cells of both *Pr. intermedia* ATCC 25611 and *Pr. nigrescens* ATCC 25261 (Fig. 1a) were almost identical. These spectra showed three sharp features at 1549, 1580 and 1621 cm⁻¹ and at 338 and 370 cm⁻¹, which were also observed in the reference sample of Fe(III)PPIX.OH, but not in [Fe(III)PPIX]₂O (Fig. 1b). In contrast, the spectrum of the cells of *P. gingivalis* W50 showed a broad doublet in the 1500–1650 cm⁻¹ region with components at approximately 1570 and 1618 cm⁻¹, whilst the bands at 338 and 370 cm⁻¹ were not seen (Fig. 1a). Thus, the spectrum of *P. gingivalis* W50 was similar to that of a reference sample of [Fe(III)PPIX]₂O, whilst those of *Pr. intermedia* and *Pr. nigrescens* were similar to that of the Fe(III)PPIX.OH monomer.

The Mössbauer spectral data for the haem-containing pigments from the *Prevotella* strains are presented in Table 1. The data for the [Fe(III)PPIX]₂O-containing pigment obtained previously for *P. gingivalis* W50 (Smalley et al., 1998) and for reference samples of [Fe(III)PPIX]₂O and Fe(III)PPIX.OH are given for comparison. The Mössbauer spectrum for *Pr. nigrescens* is shown in Fig. 2 and is typical of monomeric Fe(III)PPIX, showing the asymmetry expected for iron(III) sites, where the upper levels of the Kramer’s doublets are thermally populated. This type of site is quite unlike that of [Fe(III)PPIX]₂O where the iron atoms are antiferromagnetically coupled, which facilitates the relaxation in the latter, and consequently the Mössbauer spectrum exhibits a symmetric doublet (Silver & Lukas, 1983). The spectrum for *Pr. intermedia* ATCC 25611 was similar to that for *Pr. nigrescens* strain ATCC 25261 (data not shown). In the cases of the two *Prevotella* strains reported here the Mössbauer parameters are similar to those of Fe(III)PPIX.OH (solid), although allowing for experimental error, these monomer sites may differ from those of the latter solid.

Evidence supporting the presence of monomeric iron(III) protoporphyrin IX as the major haem species within the pigment of both *Pr. nigrescens* and *Pr. intermedia* species was
obtained after treatment of 8-day-old blood-agar-grown cells with 0·14 M NaCl/0·1 M Tris, pH 10. At this high pH, the originally orange-brown-coloured suspensions of cells yielded a dark-green solution with UV-visible spectra similar to those of the μ-oxo bishaem (Smalley et al., 2002) in which the Soret band was centred on 365 and 385 nm (with the 385 nm peak having the higher extinction), and a low intensity Q band at ≈610 nm (data not shown). These observations are consistent with the conversion of the insoluble cell-associated iron(III) protoporphyrin IX monomer into soluble [Fe(III)PPIX]₂O in the presence of base (Silver & Lukas, 1983). Treatment of suspensions of the black 8-day-old colonies of \textit{Pr. nigrescens} strains T588 and HG403, and \textit{Pr. intermedia} strains T588 and HG404 and NY653 with Tris buffer at pH 10 also yielded dark green solutions, the spectra of which were identical to those obtained for \textit{Pr. intermedia} ATCC 25611 and \textit{Pr. nigrescens} ATCC 25261 (data not presented). This indicated that the pigment of these other strains had also been composed of iron(III) protoporphyrin IX monomers, prior to treatment with the pH 10 buffer.

pH measurements were made on the biomass of \textit{Pr. intermedia}, \textit{Pr. nigrescens} and \textit{P. gingivalis} taken from confluent growths on blood agar for 8 days. The growth was removed from approximately half the blood agar plate and suspended in approximately 5 ml distilled water. The pH of these suspensions was found to be 5·80 and 6·08 for \textit{Pr. nigrescens} and \textit{Pr. intermedia}, respectively. Samples of the control un-inoculated blood agar ground up in distilled water gave a pH of 7·3. In contrast, the pH of the green-black colonies of \textit{P. gingivalis} W50 was approximately 7·5.

In view of this pH depression of the cell biomass of both \textit{Pr. nigrescens} and \textit{Pr. intermedia} during growth on blood agar, the interaction of liquid-culture-grown cells with oxyhaemoglobin was investigated under slightly acid conditions at pH 6·5 using UV-visible spectroscopy (Fig. 3). This was carried out at pH 6·5 and not at pH 6·0 to obviate the risk of precipitating insoluble haem species. This allowed observation of any spectroscopic changes to the iron porphyrin during the incubation. Incubations were also carried out at pH 7·5 to allow comparison with the activity of \textit{P. gingivalis} (Smalley et al., 2002). After 24 h incubation with both \textit{Pr. nigrescens} and \textit{Pr. intermedia} at pH 6·5 the Soret band \(\lambda_{\text{max}}\) of oxyhaemoglobin decreased from 413±4 to 404±3 nm (Figs 3a and b). In addition, the 541 and 576 nm Q bands of oxyhaemoglobin were replaced by low intensity bands at 500 and 630 nm (insets, Figs 3a and b). These changes are indicative of aquomethaemoglobin formation at this pH (Keilin & Hartree, 1951; Antonini & Brunori, 1971). Further incubation resulted in the gradual decrease in the Soret band intensity (\(\lambda_{\text{max}}\) 404±3 nm) and the appearance, after 144 h, of a Soret absorbance below 400 nm which was characterized by wavelength maxima at 395 and 365 nm. This is consistent with the presence of monomeric iron(III) protoporphyrin IX in mixture with some [Fe(III)PPIX]₂O (Silver & Lukas, 1983). Aquomethaemoglobin (with a Soret peak at ≈403 nm) was also formed in the control incubations in the absence of bacterial cells (Fig. 3c).

Table 1. Mössbauer spectroscopic parameters obtained from authentic samples of Fe(III)PPIX.OH, [Fe(III)PPIX]₂O, and for pigmented cells of \textit{P. gingivalis}, \textit{Pr. intermedia} and \textit{Pr. nigrescens} grown anaerobically on horse-blood agar for 8 days

\begin{table}
\begin{center}
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{Compound} & \textbf{\(\delta\) mm s\(^{-1}\)} & \textbf{\(\Delta E_q\) mm s\(^{-1}\)} & \textbf{Reference} \\
\hline
Fe(III)PPIX.OH\(^*\) & 0·38 (3) & 0·67 (5) & Cornelius et al. (2003) \\
[Fe(III)PPIX]₂O\(^+\) & 0·39 (1) & 0·60 (1) & Silver & Lukas (1983) \\
[Fe(III)PPIX]₂O in pigment of \textit{P. gingivalis} W50 & 0·39 (1) & 0·59 (2) & Smalley et al. (1998) \\
\textit{Pr. intermedia} ATCC 25611 & 0·36 (3) & 0·63 (10) & This study \\
\textit{Pr. nigrescens} ATCC 25261 & 0·35 (3) & 0·53 (8) & This study \\
\hline
\end{tabular}
\end{center}
\textsuperscript{*Solid sample prepared in our laboratories.}
\textsuperscript{†Frozen solution at pH 8·0.}
\end{table}

Fig. 2. Mössbauer spectrum of the haem-pigmented cells of \textit{Pr. nigrescens} ATCC 25261 at 78 K. This spectrum shows an asymmetric doublet typical of a monomeric high spin iron(III) electronic environment. The cells were grown on horse-blood agar for 8 days. See text for details.
this band decreased with time and was accompanied by an increase in absorbance over the whole visible range of the spectrum, suggesting that there may have been some protein aggregation. The Soret bands produced as a result of incubation of haemoglobin with the bacterial cells were broad, indicating that the haems were present in an aggregated state. The higher ratio of $A_{365\text{nm}} : A_{395\text{nm}}$ observed for the Pr. nigrescens strain indicated a greater concentration of the monomeric species compared to [Fe(III)PPIX]$_2$O (Silver & Lukas, 1983). The digestion mixtures were centrifuged to sediment the bacterial cells and the spectra of the supernatant buffers were recorded. Based on the Soret band absorbances, approximately 87 and 89 % of the iron protoporphyrin IX generated by Pr. nigrescens and Pr. intermedia, respectively, was found to be associated with the cells. These data indicate the progressive loss of the intact aquomethaemoglobin and the initial production of some soluble μ-oxo bishaem, followed by its subsequent conversion into the monomeric iron(III) protoporphyrin IX species at the acidic pH.

Incubation of oxyhaemoglobin with Pr. intermedia and Pr. nigrescens cells at pH 7.5 resulted in the generation (after 144 h) of a 395 nm absorbing Soret band with a shoulder at approximately 365 nm (Figs 4a and b). A weak Q band was also observed centred around 610 nm (insets, Fig. 4a and b, arrowed). As above, control incubations at pH 7.5 showed a decrease with time in the intensity of the methaemoglobin Soret band and an increase in the absorbance over the visible range of the spectrum (Fig. 4c), indicating that there may have been some protein aggregation. The following experiments were performed with CO to confirm the chemical identity of the component responsible for the Soret absorbance at 395 nm arising from incubation of haemoglobin with bacterial cells (spectral data not shown). The Soret band was unchanged in either wavelength or intensity when bubbled with CO for 5 min at a flow rate of approximately 3 ml min$^{-1}$, whereas it would have reacted if the haem species had contained Fe(II). However, treatment of the sample with 10 mM Na$_2$S$_2$O$_4$ in the absence of CO yielded a Soret band with $\lambda_{\text{max}}$ at 413-2 nm, which was due to the reduction of the haem iron from Fe(III) to Fe(II). Bubbling with CO in the presence of 10 mM Na$_2$S$_2$O$_4$ resulted in a change of the Soret band $\lambda_{\text{max}}$ to 419-2 nm, which was due to the formation of an iron(II) protoporphyrin IX carbonyl complex. The above observations are consistent with the generation from oxyhaemoglobin of [Fe(III)PPIX]$_2$O in solution along with a small amount of Fe(III)PPIX.OH monomer (Silver & Lukas, 1983; Smalley et al., 2002). Generally, the rate of haemoglobin degradation to yield free haems (based on the reduction of the haemoglobin Soret band absorbances) was much lower than the activity of P. gingivalis under the same conditions (Smalley et al., 2002).

**DISCUSSION**

In this paper we have used Mössbauer-, Raman- and UV-visible spectroscopy to re-evaluate the nature of the haem species in the pigment of Pr. intermedia and Pr. nigrescens. Taken together the results from these methods indicate that the haem pigment of these two species is composed of the monomeric form of iron(III) protoporphyrin IX [haematin; Fe(III)PPIX.OH]. This is in contrast to the haem pigment produced by P. gingivalis which is composed predominantly...
of [Fe(III)PPIX]_2O (Smalley et al., 1998, 2002; Withnall et al., 1999). The above findings explain the tinctorial differences between colonies of the above bacterial species when grown on blood agar viz., the green-black colour of *P. gingivalis* due to [Fe(III)PPIX]_2O and the orange-brown-tinted black colonies of both *Pr. nigrescens* and *Pr. intermedia* resulting from the presence of iron(III) protoporphyrin IX in the monomeric form. UV-visible spectral examination of the haem pigments of other strains of both *Pr. nigrescens* and *Pr. intermedia* grown on blood agar also showed the presence of the monomeric iron(III) protoporphyrin IX species. Thus, the colonies of *Pr. intermedia* and *Pr. nigrescens* growing on blood agar initially become tinted dark orange-brown, but the black colour results from a hyperchromic shift in the spectra due to the increased concentration of the monomeric iron(III) protoporphyrin IX, in a fashion analogous to the accumulation of the μ-oxo bishaem by *P. gingivalis*.

The pH of the cell biomass of both *Pr. nigrescens* and *Pr. intermedia* during growth on blood agar decreased to approximately 6 after 8 days. This is consistent with reports of acid production during growth in liquid culture and with the saccharolytic nature of these organisms (Shah et al., 1976, 1987; Takahashi & Yamada, 2000). In contrast, *P. gingivalis* produces an alkaline terminal growth pH as a result of peptide and amino acid metabolism (Shah et al., 1976, 1987) and also has a growth optimum around pH 8 (McDermid et al., 1988). This difference in the terminal growth pH is crucial in explaining the haem pigmentation behaviour of *Pr. intermedia* and *Pr. nigrescens*. In view of this, the interaction of bacterial cells with oxyhaemoglobin was investigated at pH 6-5. This resulted first in formation of aquomethaemoglobin which was gradually replaced by a broad Soret band with two overlapping peaks of λ_max _395 and 365 nm. The interaction of *Pr. intermedia* and *Pr. nigrescens* with oxyhaemoglobin was also examined at pH 7-5 to partially mimic the alkaline conditions in the diseased periodontal pocket (Bickel & Cimasoni, 1985) and to compare this with the activity of *P. gingivalis* (Smalley et al., 2002). Slightly alkaline pHs are not only conducive to the growth of *P. gingivalis* (McDermid et al., 1988), but also to formation of [Fe(III)PPIX]_2O from the monomeric species (Silver & Lukas, 1983). At pH 7-5 *P. gingivalis* rapidly generates [Fe(III)PPIX]_2O from both oxy- and deoxyhaemoglobin (Smalley et al., 2002), and under the same conditions in this study, both *Pr. nigrescens* and *Pr. intermedia* also yielded a major Soret band identified as [Fe(III)PPIX]_2O (Silver & Lukas, 1983; Smalley et al., 2002).

Unlike *P. gingivalis* however, both *Pr. intermedia* and *Pr. nigrescens* degraded haemoglobin to liberate free haems at a much lower rate. We are unsure why this is the case, but it may simply reflect differences in protease activity as *Prevotella* species generally display lower levels compared to *P. gingivalis* (Carlsson et al., 1984; Jansen et al., 1994).

Overall, the present findings indicate that the two *Prevotella* species progressively degrade haemoglobin to initially generate [Fe(III)PPIX]_2O. At the lower pH of 6-5, the monomeric iron(III) protoporphyrin IX species would then arise as a result of the pH-dependent equilibrium between the monomeric and dimeric forms (Silver & Lukas, 1983). We propose that [Fe(III)PPIX]_2O would be generated from oxyhaemoglobin via the reaction of proteolytically freed Fe(II) haems with ligated dioxygen as previously described for *P. gingivalis* (Smalley et al., 1998, 2002) according to the equation:

$$4\text{Fe(II)PPIX} + O_2 \rightarrow 2\text{[Fe(III)PPIX]}_2O$$  (1)

The stoichiometry of this reaction is only satisfied when the haemoglobin is 25% oxygen-loaded, and it is noteworthy that O_2 concentrations yielding this level of saturation are found in the diseased gingival crevice and periodontal pocket (Mettraux et al., 1984). In addition, Fe(III)PPIX.OH would also be formed directly from aquomethaemoglobin since the ‘acid’ or ‘aquomet’ form of haemoglobin always retains a hydroxyl as the sixth co-ordinate ligand (Antonini & Brunori, 1971). Digestion of aquomethaemoglobin would liberate these haems as Fe(III)PPIX.OH monomers.

![Fig. 4. UV-visible spectra resulting from incubation of horse oxyhaemoglobin with cells of (a) *Pr. nigrescens* ATCC 25261 and (b) *Pr. intermedia* ATCC 25611 for 144 h at pH 7-5. Panel (c) shows the results of incubation of oxyhaemoglobin in the absence of bacterial cells. The methaemoglobin Soret band (404 nm) is arrowed. The incubation conditions were as described in the legend to Fig. 3 except that the solutions were buffered with 0-1 M Tris/HCl.](image-url)
The acid environment engendered by *Prevotella* species, which can be as low as pH 5 especially during growth in the presence of fermentable carbohydrate (Shah et al., 1976; Takahashi & Yamada, 2000), has two important consequences in addition to maintaining Fe(III)PPIX.OH in solution in monomeric form and dissociating any [Fe(III)PPIX]_2O into Fe(III)PPIX.OH monomers. First, pH depression would result in protonation of the haem carboxyl side chains, drastically reducing solubility and encouraging deposition of an insoluble layer on and around the cell surface. This accords with the difficulty in producing a soluble haem extract from the pigmented cells at near-neutral pH, but not at pH 10. Second, iron porphyrin molecules would be removed from engaging in solution chemistry which would otherwise give rise to the generation of reactive oxidant species. A layer of insoluble Fe(III)PPIX.OH monomers would also serve as a barrier to further prevent ingress of oxygen and reactive oxidants to the cell surface.

The presence of iron(III) protoporphyrin IX in monomeric form has major implications for the defensive quality of the *Prevotella* pigments. This arises from the fact that monomeric ferrihaem molecules are considerably more catalytic than [Fe(III)PPIX]_2O in degrading hydrogen peroxide (Jones et al., 1973). This is important since although *Prevotellae* are associated with periodontal lesions, they are also found at gingivitis sites and in supra-gingival plaque (Rawlinson et al., 1991, 1993) where the environmental pH can fall as low as 5-5 as a result of carbohydrate fermentation (Schachttele & Jensen, 1982). Under such conditions the pigment would aid survival during attack by H_2O_2 generated in the acid conditions engendered by the *Prevotella* in pigmentation behaviour of *Pr. nigrescens* expresses specific cell-surface proteins to bind haemoglobin, which is then used as a source of iron (Guan et al., 2002). Whilst binding and degradation of haemoglobin has been demonstrated for whole cells of other *Prevotella* species (Zwickel et al., 1992; Leung et al., 1998), further studies are needed to establish which protease(s) are responsible for haemoglobin breakdown.

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