Transmissible *Burkholderia cepacia* genovar IIIa strains bind and convert monomeric iron(III) protoporphyrin IX into the \( \mu \)-oxo oligomeric form

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*Burkholderia cepacia* isolates of genovar III are highly transmissible amongst patients with cystic fibrosis (CF) and express a 97 kDa putative haem-binding protein (HBP) [Smalley, J. W., Charalabous, P., Birss, A. J. & Hart, C. A. (2001). *Clin Diagn Lab Immunol* 8, 509–514]. An investigation of the interactions of iron(III) protoporphyrin IX with epidemic and non-epidemic strains of *B. cepacia* to determine the role of the above protein in haem acquisition and binding is reported herein. Spectrophotometric titrations of cell suspensions of genovar IIIa strains BC7 and C5424 with iron(III) protoporphyrin IX, at pH 7.0, resulted in the depletion of Fe(III)PPIX.OH monomers and formation of the \( \mu \)-oxo oligomeric species, [Fe(III)PPIX]_2O. Difference spectroscopy indicated a continuous conversion of the monomeric iron(III) protoporphyrin IX into \( \mu \)-oxo oligomers.

Incubations with Fe(III)PPIX.OH monomers at pH 6.5 also showed that cells could shift the equilibrium to generate the \( \mu \)-oxo oligomeric form. Genomovar I strains ATCC 25416 and LMG 17997 were unable to mediate this conversion. SDS-PAGE of genovar IIIa strains exposed to Fe(III)PPIX.OH at pH 6.5 followed by tetramethylbenzidine/H_2O_2 staining revealed, in addition to the 97 kDa HBP, two proteins of 77 and 149 kDa located in the outer membrane which bound Fe(III)PPIX.OH monomers. These proteins were absent from the genomovar I strains. Genomovar IIIa strains BC7 and C5424 showed increased cellular binding of [Fe(III)PPIX]_2O, and as a consequence, displayed increased catalase activities compared to cells of the genomovar I isolates. It is concluded that, in addition to the putative 97 kDa HBP, *B. cepacia* genovar IIIa strains express two outer-membrane proteins which function to bind and convert Fe(III)PPIX.OH monomers into the \( \mu \)-oxo oligomeric form, [Fe(III)PPIX]_2O. The ability to perform this conversion at both neutral and slightly acidic pHs may enable epidemic strains to withstand attack from neutrophil-derived H_2O_2 in the inflamed CF lung.
protoporphyrin IX is not restricted to HBP-positive genovar IIIa isolates (Smalley et al., 2001). Moreover, it is thus not known how, or in what form iron protoporphyrin IX reacts with the cell surface. In an attempt to clarify these issues we have examined in more detail the interaction of HBP-positive and HBP-negative strains with both the μ-oxo oligomeric and the monomeric forms of iron(III) protoporphyrin IX. We report here the ability of genovar IIIa strains of \textit{B. cepacia} to convert Fe(III) PPIX.OH monomers into the μ-oxo oligomers, [Fe(III) PPIX]O, and the presence of hitherto unidentified iron(III) protoporphyrin IX monomer-binding outer-membrane proteins in these isolates. The microbiological and biochemical significances of these findings are discussed.

**METHODS**

**Bacterial strains and growth conditions.** Two \textit{B. cepacia} genovar IIIa strains, C5424 and BC7, expressing the putative 97 kDa outer-membrane HBP and two HBP-negative \textit{B. cepacia} genovar I strains, LMG 17997 and ATCC 25416, were chosen as isolates from the previously studied panel of organisms (Smalley et al., 2001). The bacteria were maintained by routine transfer on horse-blood agar, on Columbia agar (Oxoid) and on M9 Minimal Salts Medium agar (Sigma) containing 0.5% (w/v) glucose. We have found that Columbia agar contains low levels of iron protoporphyrin IX (Smalley et al., 2001) and therefore haem–cell interaction studies were performed on cells subcultured at least three times on M9 Minimal Salts Medium agar to deplete any exogenous haem derived from growth on blood or Columbia agar. Cells were then cultured as lawn growths on M9 agar for 3 days and harvested by gentle scraping with sterile plastic loops and collected into 0-14 M NaCl buffered at pH 7-5 with 0-1 M Tris/HCl (NaCl/Tris, pH 7-5), or at pH 6-5 with 0-2 M NaHPO4/NaH2PO4 (NaCl/phosphate, pH 6-5). Homogeneous cell suspensions were made by treatment for 5 min in a bath sonifier. Cell numbers were enumerated using a Neubauer counting chamber. For the haem–cell interaction studies the cells were standardized by dilution in either NaCl/Tris, pH 7-5, or in NaCl/phosphate, pH 6-5, to give 10⁶ cells ml⁻¹. Cells were also grown on \textit{Burkholderia cepacia} Medium Agar (Oxoid), on which all the strains produced pink colonies, indicating the generation of an alkaline growth end point.

**Preparation of iron(III) protoporphyrin IX solutions.** Iron(III) protoporphyrin IX was prepared as either the monomer, Fe(III) PPIX.OH (haematin), or the μ-oxo oligomer, [Fe(III)PPIX]O, as follows. Bovine haemin [Fe(III)PPIX.Cl] was dissolved in 0-14 M NaCl in 0-1 M Tris, at pH 9-8, to give a 1 mM stock solution. The pH was then adjusted to pH 7-5 by slow drop-wise addition of dilute HCl. This was further diluted with 0-14 M NaCl, 0-1 M Tris/HCl, at pH 7-0, to give a 100 μM solution containing a mixture of both the μ-oxo oligomer, or in NaCl/phosphate at pH 6-5 to yield a solution comprising predominantly the monomeric iron(III) protoporphyrin IX species (Silver & Lukas, 1983; Miller et al., 1987). The UV-visible spectra of these solutions were then recorded to confirm the presence of the monomeric and μ-oxo oligomeric forms of iron(III) protoporphyrin IX, which can be seen by the presence of prominent Soret bands at 365 and 385 nm, respectively (Silver & Lukas, 1983). The solutions were used immediately and no longer than 1 h after preparation.

**Reaction of whole cells with iron(III) protoporphyrin IX species**

1. **Spectrophotometric titrations of cells with iron(III) protoporphyrin IX.** Suspensions of cells (1 ml) standardized to an OD₅₅₀ of 0-5 in NaCl/Tris, pH 7-0, or in NaCl/phosphate, pH 6-5, were used. For each haem–cell titration four 1 cm path length semi-micro optical cuvettes (A–D) were set up as follows: A and B contained 1 ml of the above cell suspension, and C and D contained 1 ml of appropriate buffer. To cuvettes A and C, 10 μl iron(III) protoporphyrin IX solutions (100 μM on a haem monomer basis) at pH 6-5 or 7-0, were added. These titrations were carried out at pH 7-0 to examine the interactions of cells with a mixture of both Fe(III)PPIX.OH monomers and μ-oxo oligomers, and at pH 6-5 to examine the interaction with Fe(III)PPIX.OH monomers in the absence of μ-oxo oligomers (Silver & Lukas, 1983; Miller et al., 1987). Cuvettes B and D received 10 μl aliquots each of the appropriate buffer. The contents of the cuvettes were mixed and the UV-visible spectra recorded immediately. Further sequential 10 μl additions of either iron porphyrin or buffer were made and spectra were recorded immediately after mixing. The spectra for cuvettes A and C were corrected by subtraction of spectra of the cells alone (cuvette B) or buffer (cuvette D). Difference spectra were obtained by subtracting the corrected spectrum obtained for the haem–cell interaction from that of the corrected haem spectrum i.e. (A–B)–(C–D). Where appropriate, the overall absorbance differences (ΔA) in each spectrum between the minimum and maximum points were plotted versus the concentration of iron porphyrin added to the cell suspension.

2. **Time-course of interaction of cells with iron(III) protoporphyrin IX solutions.** Cell suspensions standardized as above were mixed with 25 nmol Fe(III)PPIX.OH in a total volume of 1 ml NaCl/phosphate, pH 6-5, at 20°C and the spectrum was recorded immediately, and then at 10 min intervals. These spectra were corrected for the background absorbance due to the presence of cells in suspension.

3. **Exposure of whole cells to iron(III) protoporphyrin IX and identification of haem-binding components.** For these experiments cells were firstly grown on M9 Minimal Salts Medium agar and suspensions of these (0-5×10⁶ in 0-5 ml) were incubated at 37°C for 30 min with an equal volume of concentrations of either Fe(III)PPIX.OH or [Fe(III)PPIX]O (0-160 μM on a haem monomer basis) in 0-14 M NaCl buffered either at pH 6-5 or at pH 7-5, respectively. The incubation mixtures were centrifuged at 13 000 g for 5 min, and the pelleted cells were washed three times in the original assay buffer to remove any residual unbound iron porphyrin. The cell pellets were resuspended in 1 ml of the above buffers and solubilized at 37°C in non-reducing application buffer for subsequent SDS-PAGE and staining with tetramethylbenzidine/H₂O₂ for detection of haem protein-associated peroxidase activity as described previously (Smalley et al., 2001). The equivalent of 8×10⁵ solubilized cells were loaded per track. Following SDS-PAGE and tetramethylbenzidine/H₂O₂ staining, the gels were counter-stained for protein with 0-1% (w/v) Coomassie blue in 50% (v/v) methanol, 7% (v/v) acetic acid and 43% H₂O₂, and diffusion destained in the above solvent mixture, to allow a precise identification of the tetramethylbenzidine/H₂O₂-positive polypeptides.

**Iron(III) protoporphyrin binding assays.** Iron protoporphyrin IX binding to whole cells was performed at pH 7-5 to examine the binding of the μ-oxo oligomer which exists as the predominant ferrihaem species at this pH (Silver & Lukas, 1983; Miller et al., 1987). For these experiments, as with the cell–haem titrations, cells grown on M9 Minimal Salts Medium agar were used since this medium contains no endogenous haem which could have interfered with the iron(III) protoporphyrin–cell binding interactions. Cells were suspended in NaCl/Tris, pH 7-5, standardized to 10⁶ ml⁻¹ and aliquots (0-5 ml) were mixed with 0-5 ml NaCl/Tris buffer (as above) containing iron(III) protoporphyrin IX to give a range of concentrations between 0 and 240 nmol ml⁻¹ (on a haem monomer basis). These were incubated at 37°C for 30 min by end-over-end mixing. The
incubation mixtures were centrifuged at 11 000 g for 10 min at 20°C and the pelleted cells were washed and recentrifuged three times in the same buffer to remove any unbound residual iron porphyrin. The cell-bound iron(III) protoporphyrin IX was converted to the iron(II) form by the addition of freshly prepared Na₂S₂O₄ (10 mM final molarity) and assayed as the pyridine-haemochrome as described previously (Smalley et al., 2001). The amounts bound to the cells were expressed on a haem monomer basis.

Outer membrane extraction. The outer membrane fraction was isolated from cells grown on M9 Minimal Salts Medium agar using the EDTA-shearing method as described previously (Smalley et al., 2001).

Catalase assays. Catalase activity was determined using the UV absorbance method of Beers & Sizer (1952) as described previously (Smalley et al., 2000). Cell suspensions (0·5·10⁹ in 1 ml) were exposed to iron(III) protoporphyrin IX monomers or μ-oxo oligomers (60 nmol ml⁻¹, on a monomer basis) at either pH 6·5 or 7·5, respectively, for 30 min at 37°C as above, and washed repeatedly to remove any unbound iron porphyrin. Suspensions of these cells (0·5·10⁹ ml⁻¹) carrying bound iron(III) protoporphyrin IX were mixed with H₂O₂ (10 mM final molarity) in the appropriate buffer and the UV absorbance at 240 nm monitored over the first 60 s of the reaction at 20°C. The activities of the cells with bound iron porphyrin were corrected for endogenous background catalase activity. The rates of H₂O₂ destruction were calculated by regression analysis from the initial linear decrease in A₂₄₀ using GraphPad Prism.

Spectrophotometry. UV-visible spectra were recorded in an Ultrospec 2000 scanning spectrophotometer (Pharmacia Biotech) in quartz or plastic 1 ml semi-micro cuvettes (Elkay UltraVu) with a 1 cm path length.

RESULTS

Interactions of bacterial cells with iron(III) protoporphyrin IX

Titrations of iron(III) protoporphyrin IX with whole cells were carried out initially at pH 7·0 to determine the interaction with the ferrihaem species at a pH physiologically pertinent to that of the surface liquid layer in the lung (Jayaraman et al., 2001). At this pH iron(III) protoporphyrin IX solutions contain a mixture of both the monomeric and μ-oxo oligomeric forms (Silver & Lukas, 1983; Miller et al., 1987). Sequential 1 nmol additions of the Fe(III)PPIX to the buffer yielded a series of spectra (Fig. 1a) with a broad Soret band centred on two λmax values of 365 and 385 nm, which is characteristic of the presence of a mixture of both the Fe(III)PPIX.OH monomer and the μ-oxo oligomer, [Fe(III)PPIX]₂O (Silver & Lukas, 1983; Miller et al., 1987). These spectra were maintained throughout the course of the sequential additions of the control iron(III) porphyrin. However, upon addition of the mixture of Fe(III)PPIX.OH monomer and [Fe(III)PPIX]₂O to the cell suspension of B. cepacia genovar IIIa strain BC7, there was an immediate decrease in the absorbance in the region of 365 nm and an increase in that at 385 nm (Fig. 1a). The higher A₃₈₅ to A₃₆₅ ratio was observed for all subsequent additions of the iron porphyrin to the bacterial cells. In addition, expansion of the ordinate scale in the visible region of the spectra for the titrations of strain BC7 with iron porphyrin, revealed a low intensity band at approximately 610 nm which is characteristic of the presence of the μ-oxo oligomer (Silver & Lukas, 1983) (data not shown). These data indicated that there had been a shift in the equilibrium between the monomeric and the μ-oxo oligomeric forms such that [Fe(III)PPIX]₂O became the dominant species present. A similar result was obtained for strain C5424 (data not shown). In contrast, the ratios of

Fig. 1. Titrations of cells of B. cepacia with a mixture of iron(III) protoporphyrin IX monomers and μ-oxo oligomers at pH 7·0. Iron porphyrin was added sequentially in 1 nmol amounts to cell suspensions in 0·14 M NaCl, 0·1 M Tris/HCl, pH 7·0. (a) B. cepacia genovar IIIa strain BC7; (b) B. cepacia genovar I strain LMG 17997; (c) iron protoporphyrin IX control. See text for details.
A<sub>365</sub> to A<sub>385</sub> in the spectra of the *B. cepacia* genomovar I strain LMG 17997 (Fig. 1c) were similar to those of the control ferrihaem spectra, showing that the cells were unable to alter the concentrations of the monomeric and μ-oxo oligomeric species present in solution. A similar result was observed for strain ATCC 25416 (data not shown). However, when cells of BC7 and C5424 grown on Columbia agar were titrated with iron(III) protoporphyrin IX under the above conditions, the magnitude of the decrease in A<sub>365</sub> and the increase in A<sub>385</sub> was much decreased compared to the counterparts cultured on M9 Minimal Salts Medium agar (data not shown). We attribute this to the presence of iron porphyrin molecules on the cell surfaces [as a result of growth in Columbia agar which contains haem (Smalley et al., 2001)] which would interfere with the interaction of the cells with any exogenously added haem ligand during the titrations.

Difference spectra derived from these data are shown in Fig. 2(a, b). Those for the genomovar IIIa strain BC7 were characterized by a wavelength minimum at ~345 nm and a maximum at 417 nm. However, no distinct maxima and minima were observed for LMG 17997, indicating the lack of any specific interaction of the iron porphyrin and the cells of this genomovar I strain. The overall absorbance difference in each spectrum (ΔA) between the minimum and maximum points at 345 and 417 nm, respectively, was plotted versus the concentration of iron porphyrin added to the cell suspension of the genomovar IIIa strain BC7 and revealed a slight curve with no discernable plateau (Fig. 2a; inset). This indicated that saturation binding had not occurred and that sequential addition of the mixture of both Fe(III)PPIX.OH monomers and [Fe(III)PPIX]O had resulted in the production of the dimeric species as a continuous process. In view of the depletion of the monomeric iron(III) protoporphyrin IX during incubation with the genomovar IIIa cells at pH 7-0, the above experiment was repeated to specifically evaluate the interaction of the cells with the monomeric species in the absence of the μ-oxo oligomer. Accordingly, this was performed at pH 6-5 where the predominant species in solution is the Fe(III)PPIX.OH monomer (Silver & Lukas, 1983; Miller et al., 1987). Sequential additions of Fe(III)PPIX.OH to the genomovar I strain LMG 17997 did not result in any significant change in the shape of the spectra over the range 1-40 nmol (Fig. 3a) and these spectra were almost identical to those obtained for control Fe(III)PPIX.OH monomers (Fig. 3b). Titrations of cells of genomovar I strain ATCC 25416 revealed that this organism was also unable to induce a shift in the monomer–μ-oxo oligomer equilibrium (data not shown). In contrast, initial additions of monomer to genomovar IIIa strain BC7 resulted in a marked reduction in A<sub>365</sub> and an increase in A<sub>385</sub> (Fig. 3c), indicating an increase in the concentration of the μ-oxo oligomeric species. After addition of ~25 nmol of iron porphyrin, the spectra were characterized by a discernable hypsochromic shift of the Soret band towards 365 nm and a reduction in the A<sub>385</sub> to A<sub>365</sub> ratio, indicating that the binding system was saturated and that the monomer was the dominant ferrihaem species present. Genomovar IIIa strain C5424 was also able to mediate a shift in the equilibrium from Fe(III)PPIX.OH to [Fe(III)PPIX]O at pH 6-5 (data not presented). Difference spectra derived from these data showed a distinct minimum at 350 nm for the genomovar IIIa strain BC7 (Fig. 4a), whereas that for genomovar I strain LMG 17997 showed a minimum at 365 nm, although the magnitude of the overall absorbance changes was much less than that for strain BC7 as shown in Fig. 4(a). Plots of ΔA versus iron(III) protoporphyrin IX concentration for strain BC7 yielded a curve which plateaued at approximately 30 nmol (Fig. 4a, inset), confirming that saturation binding had occurred.
The difference spectra for genomovar I strain LMG 17997 indicated that there had been some interaction of the cells with the iron(III) protoporphyrin IX monomers. A plot of $\Delta A$ versus amount of iron porphyrin added showed that the cells of the genomovar I strain had become saturated with much lower levels of Fe(III)PPIX.OH (Fig. 4b, inset).

**Fig. 3.** Spectra obtained from titrations of bacterial cells with sequential 1 nmol additions of iron(III) protoporphyrin IX monomers at pH 6.5. The experimental set up was the same as in Fig. 1 except that the solution was buffered with 0.2 M Na$_2$HPO$_4$/NaH$_2$PO$_4$ and the iron porphyrin concentration ranged from 1 to 40 nmol. (a) B. cepacia genomovar I strain LMG 17997; (b) control iron(III) protoporphyrin IX; (c) B. cepacia genomovar IIIa strain BC7.

**Fig. 4.** Difference spectra derived from the data shown in Fig. 3 for titration of bacterial cells of B. cepacia genomovar IIIa strain BC7 (a) and B. cepacia genomovar I strain LMG 17997 (b) with iron(III) protoporphyrin IX monomers at pH 6.5. The insets show plots of the overall absorbance changes ($\Delta A$) between the minimum and maximum points (arrowed) in each spectrum versus the amount of haem added. For the sake of clarity not all the spectra are displayed.

**Time-course of the interaction of cells of genomovar IIIa strains BC7 and C5424 with Fe(III)PPIX.OH monomers**

The ability of the genomovar IIIa strains BC7 and C5424 to mediate conversion of monomeric iron protoporphyrin IX in solution into the $\mu$-oxo oligomeric form at an acid pH (which would normally maintain the iron porphyrin in the monomeric form) was confirmed by incubating the cells with Fe(III)PPIX.OH over a 1 h time period. This yielded spectra in which the Soret band, initially centred on 365 nm, was shifted to 385 nm (Fig. 5), indicating
the depletion of the monomer and an increase in the concentration of the \( \mu \)-oxo oligomeric form. In addition, there was a time-dependent decrease in the intensity of the Soret band, showing that the haems had become aggregated. This effect was more pronounced in strain BC7 compared to C5424.

**Assessment of the cellular binding affinities for iron(III) protoporphyrin IX**

The assays were performed at pH 7–5 to assess the binding of iron(III) protoporphyrin IX in the \( \mu \)-oxo oligomeric form. In agreement with our previous findings (Smalley et al., 2001) both the HBP-positive and HBP-negative strains bound \([\text{Fe(III)}\text{PPIX}]_2\text{O}\) (Fig. 6). The plots of bound versus free iron porphyrin showed saturation binding curves, although the two genomovar I strains, ATCC 25461 and LMG 17997, were saturated at lower ligand concentrations than the genomovar IIIa isolates. Scatchard transformation of these data (bound/free versus bound ligand) revealed straight line plots for all the strains, indicating a monophasic binding. The lower binding maxima for the genomovar I strains were confirmed by linear regression analysis of the Scatchard plots and by analysis of the bound versus free ligand data using GraphPad Prism assuming a one-site binding model (Table 1).

**Visualization of cellular ferrihaem-binding components after exposure to monomeric and dimeric iron(III) protoporphyrin IX**

When genomovar IIIa strains BC7 and C5424 were exposed to \( \mu \)-oxo oligomers at pH 7–5, two other weak tetramethyl-benzidine/H\(_2\)O\(_2\)-stained bands in addition to the putative 97 kDa HBP were seen after sample solubilization at 37°C and electrophoresis under non-reducing conditions. These were only observed after exposure of the cells to the highest concentrations of \([\text{Fe(III)}\text{PPIX}]_2\text{O}\) (Fig. 7a). Counter staining with Coomassie blue showed these TMB-positive bands to be major proteins with molecular masses of 77 and 149 kDa. However, when the genomovar IIIa cells were exposed to \(\text{Fe(III)}\text{PPIX.}\text{OH}\) monomers at pH 6–5, there was a dose-dependent increase in TMB staining of the 77 and 149 kDa proteins (Fig. 7b). These proteins were not stained with tetramethylbenzidine/H\(_2\)O\(_2\) without prior exposure to iron(III) protoporphyrin IX monomers. In contrast, no TMB staining was seen for the genomovar I strains LMG 17997 and ATCC 25416 even after exposure of the cells to the highest concentrations of either \(\text{Fe(III)}\text{PPIX.}\text{OH}\) monomers or \([\text{Fe(III)}\text{PPIX}]_2\text{O}\) (data not shown). Moreover, peptides with the above molecular masses were not observed for the genomovar I strains after Coomassie blue staining (data not shown). Further studies employing genomovar IIIa strain C5424 revealed that 77 and 149 kDa proteins were present in the EDTA-extracted outer-membrane preparation and that they were only stained with tetramethylbenzidine/H\(_2\)O\(_2\) after exposure to \(\text{Fe(III)}\text{PPIX.}\text{OH}\) at pH 6–5 (data not shown). The electrophoretic mobilities of the 149 and 77 kDa proteins were unchanged after sample solubilization in the presence of 50 mM dithiothreitol or by heating at 100°C for 5 min (data not shown).

**Catalase activities of cells exposed to \([\text{Fe(III)}\text{PPIX}]_2\text{O}\) and \(\text{Fe(III)}\text{PPIX.}\text{OH}\)**

Catalase activities were measured in cells grown on M9 Minimal Salts Medium agar after exposure to and binding of iron(III) protoporphyrin IX monomers and \( \mu \)-oxo oligomers. These were corrected for any intrinsic background catalase activity. In general the two genomovar IIIa isolates showed between 5- and 10-fold greater levels of catalase activity than the cells of the two genomovar I strains (Table 2). In addition, the activities of the cells exposed to monomers were approximately double those exposed to the \( \mu \)-oxo oligomer.

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**Fig. 5.** Changes in the iron(III) protoporphyrin IX spectra during incubation of *B. cepacia* genomovar IIIa strains BC7 and C5424 with a fixed amount of \(\text{Fe(III)}\text{PPIX.}\text{OH}\) monomer (25 nmol) in 1 ml 0·14 M NaCl, 0·2 M Na\(_2\)HPO\(_4\)/NaH\(_2\)PO\(_4\), at pH 6·5. The arrows at 365 and 385 nm denote absorbances due to the presence of \(\text{Fe(III)}\text{PPIX.}\text{OH}\) monomers and \( \mu \)-oxo oligomers, respectively.
DISCUSSION

We have previously reported the presence of a putative 97 kDa HBP in the outer membranes of *B. cepacia* genomovar IIIa isolates (Smalley et al., 2001). It is conventionally perceived that such proteins function solely as cell-surface receptors for capture and internalization of haem molecules from which elemental iron may be excised (Wandersman & Stojiljkovic, 2000). However, ferrihaem molecules, i.e. the \([\text{Fe(III)PPIX}_2\text{O}]\) complex and \(\text{Fe(III)PPIX.OH}\) monomers, can destroy hydrogen peroxide by virtue of their inherent catalase activity (Brown et al., 1970; Jones et al., 1973) and on this basis, it can be predicted that cell-surface-bound ferrihaems would act defensively against this oxidant. Indeed, it has been demonstrated recently that cells of *Porphyromonas gingivalis* bearing a surface layer of \([\text{Fe(III)PPIX}_2\text{O}]\) can endure \(\text{H}_2\text{O}_2\) (Smalley et al., 2000). Haem-containing and carrying molecules, including haemoglobin, haemopexin (Barnard et al., 1993; Liem et al., 1975) and myeloperoxidase released from neutrophils represent a source of haem in the infected CF lung during inflammation. The ability to pick up iron(III) protoporphyrin IX molecules with the ability to destroy hydrogen peroxide would be an advantage to bacterial cells colonizing the CF lung and surviving neutrophil attack. Free haems, however, also represent a threat to both host cells and micro-organisms as they are able to participate in oxygen-radical reactions leading to breakdown of lipids, DNA and proteins (Gutteridge & Smith, 1988). Therefore, the ability to pick up and immobilize haems either by rendering them insoluble through cell-surface deposition and aggregation as in pigment formation in *Porphyromonas gingivalis* (Smalley et al., 1998, 2002), or by binding to specific cell-surface haem receptors, is a way of avoiding these potentially damaging reactions. In an attempt to elucidate the role of the putative 97 kDa HBP of genomovar IIIa isolates in the process of haem acquisition we investigated the interaction of such cells with iron(III) protoporphyrin IX.

To understand the behaviour of iron(III) protoporphyrin IX it is necessary to appreciate that these molecules exist in both monomeric and \(\mu\)-oxo oligomeric forms. In solution,

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**Table 1.** Binding maxima (\(B_{\text{max}}\)) and affinity constant (\(K_a\)) values for binding of \([\text{Fe(III)PPIX}_2\text{O}]\) to cells of *B. cepacia* genomovars I and IIIa

<table>
<thead>
<tr>
<th>Strain/genomovar</th>
<th>(B_{\text{max}}) (nmol)*</th>
<th>(K_a) (M(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC7/IIIa</td>
<td>61.7</td>
<td>1.22 \times 10^4</td>
</tr>
<tr>
<td>C5424/IIIa</td>
<td>37.8</td>
<td>2.8 \times 10^4</td>
</tr>
<tr>
<td>LMG 17997/I</td>
<td>10.2</td>
<td>6.25 \times 10^4</td>
</tr>
<tr>
<td>ATCC 25416/I</td>
<td>26.3</td>
<td>2.94 \times 10^4</td>
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</tbody>
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*\(B_{\text{max}}\) was determined through binding analysis using GraphPad Prism assuming a one-site binding model. Suspensions of cells grown on M9 Minimal Salts Medium agar (0.5 \times 10^9 ml\(^{-1}\)) were exposed to a range of concentrations of \([\text{Fe(III)PPIX}_2\text{O}]\) at pH 7.5 and 37°C and the cell-bound haem was assayed by the pyridine-haemochrome method (see text for details).
the formation of the [Fe(III)PPIX]₂O complex from Fe(III) PPIX.OH monomers is promoted in the presence of base, and this complex can dissociate in the presence of protons according to the equation:

\[
[\text{Fe(III)PPIX}]_2\text{O (µ-oxo oligomer)} + \text{H}_2\text{O} \rightarrow 2\text{Fe(III)PPIX.OH (monomer)}
\]

This equilibrium is dependent not only upon the pH of the environment, but also on the concentrations of these two haem species in solution (Silver & Lukas, 1983; Miller et al., 1987). In addition, ferrihaems have a great propensity to aggregate due to weak π-bonding interactions which promotes their segregation from solution due to hydrophobic effects. This has important consequences for the interactions of these molecules with the bacterial surface.

The pH of the surface liquid layer in the normal lung is reported to be around 6-9 (Jayaraman et al., 2001). For this reason, the interaction of bacterial cells with iron(III) protoporphyrin IX was initially studied at pH 7-0. At

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**Table 2.** Catalase activity of *B. cepacia* genomovar IIIa and I strains after binding iron(III) protoporphyrin IX monomers and µ-oxo oligomers

Catalase activity was assayed at 20°C by the UV absorbance method of Beers & Sizer (1952) and is expressed as μmol H₂O₂ degraded min⁻¹ per 0.5 × 10⁸ bacterial cells ml⁻¹. Cells were exposed to iron(III) protoporphyrin IX in 0-14 M NaCl buffered at pH 6-5 or 7-5, respectively, and the assays were carried out at these pHs to maintain the iron(III) protoporphyrin IX in monomeric and µ-oxo oligomeric forms, respectively. The activities were calculated by regression analysis from the initial rates of decrease in A₂₄₀ and are corrected for any intrinsic background catalase of cells not exposed to iron porphyrin.

<table>
<thead>
<tr>
<th>Strain/genovar</th>
<th>Monomer</th>
<th>µ-oxo oligomer</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC7/IIIa F646</td>
<td>3.59 ± 0.24</td>
<td>1.91 ± 0.054</td>
</tr>
<tr>
<td>C5424/IIIa F647</td>
<td>2.51 ± 0.22</td>
<td>1.22 ± 0.10</td>
</tr>
<tr>
<td>LMG 17997/I F640</td>
<td>0.19 ± 0.025</td>
<td>0.15 ± 0.024</td>
</tr>
<tr>
<td>ATCC 25416/I F768</td>
<td>0.63 ± 0.12</td>
<td>0.30 ± 0.024</td>
</tr>
</tbody>
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**Fig. 7.** SDS-PAGE and tetramethylbenzidine/H₂O₂ staining of cells of *B. cepacia* genomovars IIIa to reveal HBPs after binding of (a) µ-oxo oligomers, [Fe(III)PPIX]₂O, at pH 7-5, or (b) Fe(III)PPIX.OH monomers at pH 6-5. The cells were exposed to a range iron(III) protoporphyrin IX concentrations (10-160 nmol ml⁻¹ on a monomer basis) for 30 min at 37°C and unbound iron porphyrin was removed from the cells by washing three times in the appropriate buffer. The cells were solubilized in non-reducing sample application buffer and electrophoresed on 10% acrylamide gels. Each track was loaded with the equivalent of ~8 × 10⁷ solubilized cells. After staining with tetramethylbenzidine/H₂O₂ (TMB) the gels were counterstained for protein with Coomassie blue (CB).
neutral pH, free ferrihaems will be present as a mixture of both the monomeric and mu-oxo oligomeric forms as a result of the pH-dependent equilibrium. Under these conditions B. cepacia genomovar IIIa strains mediated the depletion of the monomer in solution and the formation of [Fe(III)PPIX]_2O. This effect was not observed for the genomovar I strains. To our knowledge the ability to mediate this transformation is a novel phenomenon and has not been described in any other biological systems, although the [Fe(III)PPIX]_2O complex, which is the major haem species in the green-black pigment of P. gingivalis (Smalley et al., 1998), is generated as a result of degradation of both oxy- and deoxyhaemoglobin (Smalley et al., 2002). Thus, the pH of the liquid surface layer of the lung in healthy and disease will have a major influence on the behaviour of any free ferrihaems. It is noteworthy that endobronchial pHs of between 6.58 and 6.62 have been recorded in individuals with chronic lung disease and Gram-negative pneumonia (Bodem et al., 1983). In view of this, experiments were also conducted at pH 6.5 to permit the interaction of cells with Fe(III)PPIX.OH monomers in the absence of the mu-oxo oligomeric form. Under these conditions the genomovar IIIa strains were also able to achieve the transformation of Fe(III)PPIX.OH into [Fe(III)PPIX]_2O. These findings are important since they show that genomovar IIIa strains would be able to generate the mu-oxo oligomer from any free iron(III) protoporphyrin IX monomers both in the healthy lung (at neutral pH) and under the slightly lower pH conditions which may prevail during chronic lung inflammation. These observations may explain why epidemic genomovar IIIa strains are frequently found able to superinfect lungs already colonized by other species, including Pseudomonas aeruginosa and other B. cepacia genomovars (Hart & Winstanley, 2002; Mahenthiralingam et al., 2001) where, in the presence of chronic inflammation, the pH is likely to be lower than neutral (Bodem et al., 1983).

Difference spectra obtained from titrations of iron(III) protoporphyrin IX with cells of the genomovar IIIa strains at pH 7.0 revealed that there was a continuous formation of [Fe(III)PPIX]_2O from Fe(III)PPIX.OH monomers. Moreover, the alkaline growth end point generated by all the strains in this study (as demonstrated by the production of pink colonies on Burkholderia cepacia Medium) is significant since this would favour mu-oxo oligomer formation from any free Fe(III)PPIX.OH (Silver & Lukas, 1983) and encourage haem aggregation on the cell surface. For these reasons it was felt pertinent to assess the cellular binding capacity for iron(III) protoporphyrin IX in the mu-oxo oligomeric form. The genomovar IIIa strains BC7 and C5424 displayed greater binding capacities for [Fe(III)PPIX]_2O than the genomovar I isolates (LMG 17997 and ATCC 25416), and Scatchard plots indicated monophasic binding. The apparent affinity constants were of the order 10^4 M^-1. Since we were unable to demonstrate a bathochromic shift of the Soret band during the titrations of cells at the lowest concentrations of added iron(III) protoporphyrin IX at pH 7.0 (other than an increase in A_385 indicative of [Fe(III)PPIX]_2O formation) we suggest that these curves represent binding to the cell surface via aggregation, rather than to some specific receptor for the mu-oxo oligomer. This is supported by our observation that the Soret bands resulting from incubation of cells of genomovar IIIa strains BC7 and C5424 with a fixed concentration of iron(III) protoporphyrin IX were both decreased in intensity and broadened, features indicative of aggregation. Aggregation of newly generated [Fe(III)PPIX]_2O and its subsequent removal from solution and deposition on the cell surface would result in a further shift in the equilibrium towards production of the mu-oxo oligomeric species from any monomers remaining in solution and encourage further deposition of mu-oxo oligomer molecules on the cell surface. It is thus noteworthy that genomovar IIIa strains BC7 and C5424 yield green-brown colonies when grown on blood agar and that incubation of oxyhaemoglobin with these strains results in the generation of a Soret band absorbing component with features of the [Fe(III)PPIX]_2O complex (Smalley et al., 2002; unpublished data). Although we cannot rule out the possibility that these isolates produce pyoverdin-like molecules, our observations support the contention that B. cepacia can generate and bind [Fe(III)PPIX]_2O during growth on blood-containing media. However, it is not yet clear as to whether other cell-surface components bind [Fe(III)PPIX]_2O. Thus, the ability to mediate the transformation of the Fe(III)PPIX.OH monomers into [Fe(III)PPIX]_2O is a novel phenomenon associated with genomovar IIIa isolates and constitutes a potential virulence determinant in this group of organisms.

Tetramethylbenzidine/H_2O_2 staining of cellular components on SDS-polyacrylamide gels after binding of iron(III) protoporphyrin IX has been used to identify cell-surface HBPs (Lee, 1992; Mazoy & Lemos, 1996; Smalley et al., 1993; Stugard et al., 1989). When genomovar IIIa strains were exposed to increasing concentrations of iron(III) protoporphyrin IX we observed the dose-dependent binding of iron(III) protoporphyrin IX monomers to two outer-membrane proteins of 77 and 149 kDa, which were not found in the genomovar I strains even after exposure to the highest concentrations of either Fe(III)PPIX.OH or [Fe(III)PPIX]_2O. In contrast to the 97 kDa putative HBP, the 77 and 149 kDa proteins were only stained after prior exposure to iron(III) protoporphyrin IX. We attribute the low degree of tetramethylbenzidine/H_2O_2 staining at pH 7.5 to the binding of some Fe(III)PPIX.OH monomers which would be present in the mu-oxo oligomer haem solution as a result of the equilibrium between the two forms. Thus, the fact that 77 and 149 kDa proteins were stained at 6.5 and not at 7.5 indicates that these components preferentially pick up monomers rather than mu-oxo oligomers. Since Fe(III)PPIX.OH monomers are more catalytic than the mu-oxo oligomers in their ability to destroy hydrogen peroxide (Brown et al., 1970; Jones et al., 1973), it is likely that Fe(III)PPIX.OH molecules bound to
the cell surface at pH 6.5 would more efficiently protect against H₂O₂ than μ-oxo oligomers. However, it should be noted that the μ-oxo oligomer (Brown et al., 1970) may be preferentially oxidized and partially decomposed compared to the monomer during exposure to H₂O₂ (Grinberg et al., 1999) and thus constitute a cell-surface layer which can act as a sacrificial barrier against this oxidant as reported for P. gingivalis (Smalley et al., 2000).

We have previously shown that the 97 kDa putative HBP appears to pick up both Fe(III)PPIX.OH monomers and μ-oxo oligomers (Smalley et al., 2001). However, importantly, we found in this study that, compared to the 149 and 77 kDa proteins, the 97 kDa putative HBP was stained even in the absence of any exogenously added haem and was not stained using the peroxidase substrate tetramethylbenzidine in a dose-dependent fashion after exposure of the cells to iron protoporphyrin IX. We conclude from this that this protein may contain an endogenously synthesized haem-like prosthetic group and that it may not have a true haem-binding function.

Although Fe(III)PPIX.OH monomers react spontaneously in solution to give the μ-oxo oligomeric species (Silver & Lukas, 1983; Miller et al., 1987) (through the reverse of reaction 1), we propose that the 149 and 77 kDa proteins act together or separately as templates to facilitate the conversion of the Fe(III)PPIX.OH into Fe(III)PPIX₂O. The continuous production would only occur if the 149 and 77 kDa protein receptor sites for the monomer were not saturated with the newly formed [Fe(III)PPIX]₂O. It is not clear what bonding interactions take place between the ferrihaem monomers and these outer-membrane proteins, but we propose the following mechanism to account for the formation of [Fe(III)PPIX]₂O from Fe(III)PPIX.OH as a continuous process. This is based upon the known behaviour of iron porphyrins which form μ-oxo oligomers (Silver & Lukas, 1983; Miller et al., 1987). First, monomers may initially become bonded to the NH₂ or COOH groups of these proteins either via the haem iron (by replacement of the weakly axially bonded H₂O in the sixth co-ordinate position), or via a peripheral substituent on the iron porphyrin (e.g. the methyl, vinyl or carboxylate groups). Formation of the Fe-O-Fe bond of the [Fe(III)PPIX]₂O complex could then occur through the reaction of the bound monomer with another Fe(III)PPIX.OH molecule (via the hydroxyl groups; Silver & Lukas, 1983), itself also bonded to the protein, or free in solution. Bonding of the iron protoporphyrin IX monomers to the protein(s) would facilitate this process since it would encourage electron withdrawal at the periphery of the porphyrin ring, a phenomenon which is known to encourage formation of the μ-oxo-bridged structure (Miller et al., 1987). This would weaken any peripheral or axial bonds and force the release of the μ-oxo oligomer from the protein(s). This reaction would be encouraged at a slightly alkaline pH and aggregation of the newly formed and released [Fe(III)PPIX]₂O molecules would then be possible.

The inability of the 77 and 149 kDa proteins to bind [Fe(III)PPIX]₂O molecules (as evidenced by the lack of TMB/H₂O₂ staining at pH 7.5) indicates that the μ-oxo oligomeric molecules may not be retained on the protein(s) once they have been formed. Indeed, this is supported by the spectroscopic data which show that [Fe(III)PPIX]₂O molecules, once generated, remain (initially at least) in solution. The above data also suggest that the 77 and 149 kDa proteins may not just represent a surface seed for the deposition of the μ-oxo oligomers on the cell surface, although we cannot rule out the possibility that some other component may also be responsible for mediating [Fe(III)PPIX]₂O formation and cell-surface binding. Importantly, however, aggregation of the μ-oxo oligomers would facilitate the shift in the equilibrium towards the production of more of the μ-oxo oligomeric species from the monomer due to its removal from solution.

When cell-ferrihaem titrations were conducted with Fe(III)PPIX monomers in the absence of [Fe(III)PPIX]₂O, genovar IIIa strain BC7 mediated a shift in the equilibrium towards the production of [Fe(III)PPIX]₂O, although this effect was not as pronounced as at the higher pH. Moreover, at pH 6.5 there was evidence from the difference spectra that the cells became saturated over the same concentration range studied. At this pH the equilibrium would favour the existence of the monomeric form and it is likely that under these conditions the 149 and 77 kDa proteins would remain occupied by Fe(III) PPIX.OH molecules. As the μ-oxo oligomer has a greater propensity to aggregate than the monomeric form, binding and conversion of Fe(III)PPIX.OH into [Fe(III)PPIX]₂O would provide an advantage by forming a protective barrier against ingress of reactive oxidant species to the cell surface. In addition, bound μ-oxo oligomers would be able to serve defensively by destroying peroxide through their inherent catalase activity. It has been reported previously that strains of B. cepacia display catalase activity (Gessner & Mortensen, 1990; Chester, 1979; Lefebvre & Valvano, 2001). However, it was found that the catalase activities of the two genovar IIIa isolates were between 5- and 10-fold greater than those of the two genovar I strains after exposure and binding of the monomer and μ-oxo oligomer (after correction for intrinsic cellular catalase activity). In addition, the catalase activities of cells of both genovar IIIa and I strains after exposure to iron(III) protoporphyrin IX monomers were double those of cells carrying bound μ-oxo oligomers. This finding is predictable on the basis that the catalase activity of the monomer is higher than the μ-oxo oligomer (Jones et al., 1973; Brown et al., 1970). The increased catalase activity displayed by the genovar IIIa isolates as a result of binding of ferrihaems would provide an advantage over other genovar strains to endure fluxes of H₂O₂ released from neutrophils. Such iron porphyrin binding behaviour may aid colonization and establishment of infections of the CF lung caused by this important group of respiratory pathogens. Examination of the SDS-PAGE profiles of the
strains described in our previous paper (Smalley et al., 2001) revealed that proteins with molecular masses of 77 and 149 kDa were only present in genomovar IIIa strains. Given the role of these cell-surface proteins in picking up haems, it is possible that they may be targeted specifically to inhibit cellular haem-binding activity. Alternatively, as they appear to be prominent components on the bacterial surface, they might be used as suitable candidates for vaccine development.

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