Haemin-binding proteins of *Porphyromonas gingivalis* W50 grown in a chemostat under haemin-limitation

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*Porphyromonas gingivalis* W50 was grown in a chemostat at pH 7.3 under haemin-limitation and haemin-excess at a constant mean doubling time of 6.9 h. Outer membranes (OM) were extracted from whole cells using EDTA and compared by SDS-PAGE. Haemin-limited cells expressed novel outer membrane proteins (OMPs) of mol. mass 115, 113 and 19 kDa when samples were solubilized at 100 °C. A 46 kDa OMP was observed in haemin-excess cells but not in those from haemin-limited conditions. Tetramethylbenzidine (TMBZ) staining of gels, after OM solubilization at 20 °C, was used to detect haemin-binding proteins (HBPs). HBPs were observed only in OM from haemin-limited cells. The major HBP (mol. mass 32.4 kDa) corresponded to a similar sized Kenacid-blue-stained protein which was not observed in haemin-excess-derived OM. Haemin-limited cells and OM displayed a ladder-like series of Kenacid-blue-stained proteins. Lighter TMBZ-stained proteins of mol. mass 51, 53, 56 and 60 kDa, with mobilities corresponding to those of silver-stained LPS components, were observed in haemin-limited OM. No soluble HBPs were detected extracellularly. The greater number of HBPs expressed by cells grown under haemin-limitation may reflect an additional cell surface receptor system for haemin acquisition under low environmental levels of this essential cofactor.

Introduction

The availability of haemin in gingival crevicular fluid (GCF) may be significant for the growth and virulence of the periodontopathogen *Porphyromonas gingivalis*, which displays an absolute requirement for this porphyrin (Gibbons & MacDonald, 1960). Free haemin in GCF is probably derived from haemoglobin during periods of bleeding (Mukherjee, 1985). However, both haemin and haemoglobin are avidly complexed by the proteins haptoglobin, albumin and haemopexin (Eaton et al., 1980; Laurell & Gronvall, 1962; Payne, 1988), rendering the porphyrin unavailable to the resident microflora. Pathogenic Gram-negative organisms such as *Escherichia coli* (Griffiths et al., 1983), *Proteus mirabilis* and *Klebsiella pneumoniae* (Shand et al., 1985) have been shown to grow in the host under iron-restriction, responding by expressing phenotypic changes in outer-membrane proteins (OMPs). There is also recent evidence that *P. gingivalis* might grow at times under haemin-limitation in humans since OMPs only expressed by *P. gingivalis* when grown in vitro under haemin-limitation were recognized by sera from patients with severe progressive periodontal disease (Papaoannou et al., 1991). Haemin-restriction has been shown to attenuate the pathogenicity of *P. gingivalis* W50 in a mouse virulence model (McKee et al., 1986). Haemin-limited cells also exhibit reduced cellular protease activity and display lower cytotoxin production (Marsh et al., 1988).

It is not clear how *P. gingivalis* acquires haemin for growth. This species can degrade haem-containing host macromolecules (Carlsson et al., 1984), perhaps thereby releasing the porphyrin, but the mode of cellular uptake is not understood. The outer membrane (OM) can bind haemin (Grenier, 1991), while cells grown in batch or continuous culture in an excess of haemin display increased haemin binding compared to those grown under haemin-restriction (Carman et al., 1990; Smalley et al., 1991). Studies of *P. gingivalis* have shown that growth under haemin-limitation can lead to rapid changes in the profile of OMPs and to the induction of

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Abbreviations: EP, extracellular protein; HBP, haemin-binding protein; OM, outer membrane; OMP, outer-membrane protein; e-ACA, e-aminocaproic acid; NEM, N-ethylmaleimide; TMBZ, tetramethylbenzidine.

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novel proteins (Bramanti & Holt, 1990; Papaioannou et al., 1991). The aim of the present study was to determine whether any of these OMPs were haemin-binding proteins. In this study, in order to avoid the growth-rate-related changes in OMPs observed by Bramanti & Holt (1990) in batch culture, OMS were extracted from cells cultured in a chemostat at a constant growth rate and environmental pH, under either haemin-excess or haemin-limited conditions.

Methods

Bacterial strain and growth conditions. P. gingivalis strain W50 was grown in a chemostat in BM medium as previously described (McKee et al., 1986), except that the haemin levels were fixed at 0·001 and 2·5 μg ml⁻¹ for haemin-limited and haemin-excess (presumptive peptidase limitation) conditions, respectively. The amount of haemin needed to be added to BM in order to achieve haemin limitation was lower than that reported in our previous studies (McKee et al., 1986; Marsh et al., 1988); this was probably due to higher levels of endogenous haemin in the particular batches of media. Free iron could be detected in culture filtrates (≥ 0·8 μg ml⁻¹), while the biomass of haemin-limited cultures could be increased by further additions of haemin, confirming that growth of cells was regulated by the porphyrin concentration in the medium. The dilution rate was 0·1 h⁻¹, giving a mean generation time of 6·9 h, and the pH was maintained automatically at 7·3 ± 0·1. Once ‘steady-state’ conditions had been established, cultures were harvested at 4 °C, and bacterial cells were sedimented by centrifugation at 10000 g for 30 min at 4 °C, washed once in 0·01 M-phosphate-buffered isotonic saline (PBS; 0·14 M- NaCl) and once in distilled water, and then freeze-dried. The cell-free supernatant liquor was then taken to 70% saturation by the addition of (NH₄)₂SO₄. The precipitate which formed was then centrifuged differentially as described by Smalley et al. (1989), to remove insoluble extracellular membrane vesicles and yield a soluble supernatant extracellular protein (EP) fraction. The materials used in this study were those generated by the chemostat runs described by Smalley et al. (1991), where details of cell yields are given.

Preparation of outer membranes. Freeze-dried whole cells (200 mg) were suspended in 60 ml 0·14 M-NaCl containing 10 mm-EDTA, pH 7·3, and incubated with stirring at 37 °C for 30 min (Smalley & Birss, 1987). This serves to dissociate the OM and to inhibit any possible cell-associated protease activity (Sorsa et al., 1987; Tsutsui et al., 1987; Yoshimura et al., 1984) during preparation. After being passed twice through a 25 gauge needle, the cells were pelleted by centrifugation (20000 g for 30 min, 4 °C) leaving the supernatant which contained the crude OM preparation. Residual EDTA and buffer salts were removed by dialysis against distilled water for 4 h at 4 °C and the OM fraction was then freeze-dried. In other experiments in which 2 mm-phenylmethanesulphonyl fluoride (PMSF), N-ethylmaleimide (NEM) and ε-aminocaproic acid (ε-ACA) were included as protease inhibitors, in addition to EDTA, no differences were observed in the SDS-PAGE profiles compared to OM extracted using EDTA as the only protease inhibitor. Moreover, no differences were seen in profiles obtained when outer membranes were extracted by incubation and shearing in 0·14 M-NaCl alone, indicating that little or no detectable proteolytic modification of the OM had occurred. The protein content of the OM from haemin-limited and haemin-excess grown cells was 14·7 and 10·5% (on a dry weight basis), respectively. Typical yields of OM protein from 200 mg of freeze-dried cells lay between 8 and 10 mg for both haemin-limited and haemin-excess cells.

Tetramethylbenzidine (TMBZ) staining of haemin-binding proteins (HBPs). HBPs were detected in polyacrylamide gels by staining with TMBZ (Sigma), a colourless chromogen which turns blue in areas of haem-associated peroxidase activity (Holland et al., 1974; Sugard et al., 1989). After incubation with exogenous haemin (see below), OM and cell samples were solubilized at 20 °C for 1 h in Laemmli sample application buffer and electrophoresed as described above. In some instances, the protein loading was doubled (relative to that for Kenacid blue staining) to facilitate visualization of TMBZ-stained bands. Separated proteins in the gels were fixed in sodium acetate (0·25 M, pH 5·0)/methanol/water (6:3:1, by vol.) for 1 h at 4 °C and then immersed in TMBZ (6·3 mm in 20 ml methanol) mixed with 70 ml sodium acetate and 10 ml water for 30 min in the dark at 4 °C. HBPs were revealed by addition of H₂O₂ (30 mm final concentration). Gels were then washed and stored in 0·25 M-sodium acetate/propan-2-ol (8:2, v/v).

SDS-PAGE. This was performed according to the method of Laemmli (1970) employing 7 or 10% (w/v) polyacrylamide slab gels with 3% stacking gels. OMs and cells were solubilized in Laemmli sample application buffer containing 1% SDS, 2 M-urea and 30 mm-dithiothreitol (DTT) at either 20 °C or 100 °C for 5 min. Sample solubilization at 20 °C for 1 h was performed in application buffer containing protease inhibitors (2 mm-PMSF, -NEM and -ε-ACA). Proteins were stained with Kenacid blue (0·1%, w/v, in 50% methanol/7% acetic acid/43% water). Gels were diffusion-diaestained in the above solvent and finally stored in distilled water prior to examination and photography.

The exact migration distances of Kenacid-blue- and TMBZ-stained bands were measured from densitometric scans of gels using a 2202 Ultrioscan laser densitometer and 2220 recording integrator-plotter (Pharmacia-LKB). Molecular masses were determined by reference to plots of log₁₀ mol. mass versus relative mobility for the concomitantly electrophoresed protein standard mixture SDS-7 (Sigma) comprising bovine albumin (68 kDa); ovalbumin (45 kDa); glyceraldehyde-3-phosphate dehydrogenase (36 kDa); carbonic anhydrase (29 kDa); trypsinogen (24 kDa); trypsin inhibitor (20 kDa); and bovine α-lactalbumin (14 kDa).

Haemin binding. For experiments involving TMBZ-staining, OM and cell samples were incubated with exogenous haemin in 100 mm-Tris-buffered 140 mm-NaCl, pH 7·4, as previously described (Smalley et al., 1991). The buffer also contained 2 mm-PMSF, -NEM and -ε-ACA to protect against any possible protease activity. After exposure to haemin, the samples were pelleted (50000 g for 1 h at 4 °C) and washed in the above buffer, re-centrifuged as above to remove residual unbound haemin, and solubilized directly in Laemmli sample buffer prior to electrophoresis.

Lipopolysaccharide (LPS). This was detected in polyacrylamide gels by the silver stain method of Tsui & Frasch (1982).

Protein estimations. The protein content of cellular and extracellular fractions was measured using the Lowry method with bovine serum albumin as standard.

Results

SDS-PAGE polypeptide profiles

(i) Outer membrane. At least 20 polypeptides in the molecular mass range 115 to 16 kDa were observed in the OM fractions when solubilized at 100 °C for 5 min and stained with Kenacid blue (Fig. 1, tracks 1 and 2).
Fig. 1. SDS-PAGE protein profiles on Kenacid-blue-stained 10% gels of OM derived from P. gingivalis W50 cells grown in a chemostat under haemin-limitation or haem-in-excess conditions. Track 1, haemin-limited OM; track 2, haemin-excess OM. Samples were solubilized by heating at 100 °C for 5 min in Laemmli application buffer; 100 μg protein was loaded on each track.

The haemin-limited OM displayed two unique polypeptides of $R_f$ 0.10 and 0.77 (arrowed) on a 10% gel. The faster migrating component was 19 kDa. Because of the non-linearity of the relationship between $R_f$ and log mol. mass above 68 kDa in this 10% gel system, these samples were electrophoresed on a 7% gel, where the slower migrating of the two novel polypeptides was resolved as a doublet with peptides of mol. mass 115 and 113 kDa (results not shown). No components with molecular masses greater than this doublet were observed on 7% gels under these electrophoretic conditions. The haemin-excess OM contained an additional protein of 46 kDa.

SDS-PAGE of OMs treated with sample application buffer at 20 °C revealed a reduced number and intensity of Kenacid-blue-stained bands (Fig. 2) compared to those solubilized at 100 °C (Fig. 1). This finding was in keeping with the observations of Bramanti & Holt (1990), and necessitated solubilization of larger amounts of sample. Despite this, a faint ladder-like series of polypeptides was observed in the OM derived from haemin-limited cells (track 3), but not from those grown under conditions of haemin-excess (track 4). This pattern was similar to that described for the OM of strain W50 grown in haemin-restricted batch culture (Bramanti & Holt, 1990), which was attributed by Kennell & Holt (1990) to the association of peptides with LPS components. A high molecular mass component of $R_f$ ≈ 0.02, which was resolved by densitometry as a doublet (Fig. 2, track 5), and a 32 kDa protein were observed in haemin-limited OM but not in OM from...
haemin-excess cells. When the haemin-limited OM sample was electrophoresed on a 7% gel (results not shown), the high molecular mass component was found to migrate as a tight doublet with a mean mol. mass of 147 kDa.

(ii) Cells. The ladder-like pattern of Kenacid-blue-stained bands was seen more clearly in haemin-limited whole cells than in the OM, when solubilized at 20 °C (Fig. 3, tracks 4 and 5). A high molecular mass doublet, of the same Rf as that in the OM, and a band at 32 kDa were also observed in haemin-limited cells. Faint Kenacid blue staining was seen at the position of the doublet in haemin-excess cells.

Identification of haemin-binding proteins by TMBZ staining

(i) Outer membrane. TMBZ staining was observed only in OM preparations derived from haemin-limited cells of P. gingivalis W50 (Fig. 2, track 2). The major positive staining component was observed as a band of Rf 0·54 (mol. mass 32 kDa), which corresponded to a protein of similar mobility in the parallel Kenacid-blue-stained gel (Fig. 2, track 3). Determinations from nine separate TMBZ-stained gels gave a mol. mass for this HBP as 32·4 kDa (±0·7 kDa). A protein was also observed in the same region of the gel for the haemin-excess OM (track 4), but this component had an Rf of 0·55. A series of lighter TMBZ-stained bands of mol. mass 51, 53, 56 and 60 kDa was also seen in the haemin-limited OM (Fig. 2, track 2). These bands appeared to correspond in mobility to certain of the LPS components present in haemin-limited cells (Fig. 3, track 6). Identical TMBZ-staining patterns were obtained when the protease inhibitor cocktail was omitted during both exposure to haemin and solubilization at 20 °C. No TMBZ-staining was observed without prior exposure to haemin or when OM was solubilized at 100 °C after exposure to haemin (results not shown). This precluded identification of the 115, 113 and 19 kDa OMPs (expressed under haemin-limitation and observed after sample solubilization at 100 °C; Fig. 1) as HBPs.

(ii) Cells. Following incubation with exogenous haemin, whole cells originating from haemin-limited conditions (solubilized at 20 °C for 1 h) displayed diffuse TMBZ-positive bands in the 30 and 50 kDa regions, and a high molecular mass doublet with an Rf of 0·02 (Fig. 3, tracks 2a and 2b, arrowed). A TMBZ-stained high molecular mass band was also observed in haemin-excess cells (Fig. 3, track 1). This was not observed as a doublet as in the haemin-limited cells, and there was little corresponding Kenacid blue staining (Fig. 3, track 3). For haemin-limited cells solubilized at 100 °C, neither TMBZ-staining nor the ladder-like arrangement of peptides was observed (results not shown).

(iii) Extracellular protein. In an attempt to detect HBPs released into the culture medium, EP from haemin-limited and haemin-excess cultures (at a concentration of
8 mg protein ml\(^{-1}\)) were exposed to haemin and examined on a 10% gel. No TMBZ-staining was obtained even when large amounts of EP (equivalent to a protein load of \(\approx 300\) µg per track) were solubilized (results not shown).

**Discussion**

This study has confirmed that *P. gingivalis* W50 expresses novel OMPs when cultured at a constant growth rate in the chemostat under haemin-limitation (Papaioannou et al., 1991). These results are similar to other recent findings which have demonstrated the expression of three new proteins (mol. mass 9, 60 and 80 kDa) and the amplification of nine others (mol. mass 77, 71, 62, 58, 57, 56, 50, 39 and 34 kDa) in the OM of *P. gingivalis* under haemin-restriction (Bramanti & Holt, 1990). In this latter study, cells were grown in batch culture and transferred successively to media increasingly haemin-depleted, so that some of these variations in expression may have been due to the inevitable concomitant large reductions in growth rate. Our study revealed that only three novel OMPs of mol. mass 115, 113 and 19 kDa (detected when samples were solubilized at 100 °C) were expressed by cells grown at a constant doubling time under haemin-limitation. The possible role of these components as binding proteins in the acquisition of haemin was studied using TMBZ to detect haem-protein complexes (Hanson (Kennel1 trim.), 1991). These results are similar to other recent studies which have demonstrated the expression of three novel proteins and lipid A, the haemin-binding component of *P. gingivalis* LPS (Grenier, 1991), although their location in the OM is unknown.

Increased levels of a 52 kDa surface OMP have been detected by extrinsic radio-iodination in batch-grown haemin-restricted cells (Bramanti & Holt, 1990). This was observed in the region of the LPS-associated peptides (Kennell & Holt, 1990), which displayed increased Kenacid blue staining under haemin-restriction (Bramanti & Holt, 1990). We also observed this phenomenon, which was seen more clearly in haemin-limited whole cells. The TMBZ-positive bands of mol. mass 51, 53, 56 and 60 kDa had mobilities corresponding to LPS components as revealed by silver staining. An increase in such proteins under haemin-limitation might indicate elevated levels of LPS and may, in view of the present findings and those of Grenier (1991), point to enhanced haemin binding. However, this does not appear to be the case, as haemin-excess OM (and cells and extracellular vesicles) bind quantitatively more haemin than do their haemin-limited counterparts (Smalley et al., 1991), despite the latter displaying more haemin–protein complexes. These proteins might not be involved in the quantitative binding of haemin, therefore, but may represent a multiple and/or higher affinity receptor/binding system for haemin uptake or processing that is only induced under low environmental levels of haemin. HBPs did not appear to be secreted extracellularly as soluble components since the EP fraction contained no TMBZ-staining proteins, supporting the suggestion that extracellular scavenging of haemin might be performed by extracellular vesicles (Smalley et al., 1991).

There is evidence from studies of other bacteria that OMPs function to transport haem across the cell wall. *Bacteroides fragilis* (Otto et al., 1988, 1990) and *Haemophilus influenzae* (Coulton & Pang, 1983) express a novel 44 kDa OMP under iron-restriction which appears to function as a transmembrane transport for haem. At present, the exact function of the greater number of HBPs expressed by *P. gingivalis* under haemin-limitation is not known. It is difficult to reconcile lower levels of haemin binding by cells and OMs which display additional haemin-binding proteins. Perhaps the requirement for haemin by cells under conditions of haemin-excess might be satisfied largely by LPS-mediated haemin-binding (Grenier, 1991). Determination of the relative haemin-binding affinities of these proteins and lipid A, the haemin-binding component of *P. gingivalis* LPS (Grenier, 1991), might resolve this apparent ambiguity.

**References**


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