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

JOHN W. SMALLEY,¹* ANDREW J. BIRSS,¹ AILSA S. MCKEE² and PHILIP D. MARSH²

¹Department of Clinical Dental Sciences, University of Liverpool, PO Box 147, Liverpool L69 3BX, UK

²Pathology Division, PHLS Centre for Applied Microbiology and Research, Porton Down, Salisbury SP4 0JG, UK

(Received 16 December 1992; revised 16 March 1993; accepted 21 April 1993)

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.*, 1982; 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 (Papaioannou *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

* Author for correspondence. Tel. (051) 706 5251; fax (051) 706 5807.

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

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-limitation and haemin-excess (presumptive peptide 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 $\pm$ 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 solid (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; Stugard *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 50 mM-dithiothreitol (DTT) at either 20 °C for 1 h or at 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-destained 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 Ultrosan 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, recentrifuged 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 Tsai & 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 haemin-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. Electrophoresis with lower loadings confirmed that the major peptide in both the haemin-limited and haemin-excess OM had a mol. mass of 50 kDa.

SDS-PAGE of OM 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

Fig. 2. SDS-PAGE on 10% gels of OMPs of *P. gingivalis* W50 solubilized at 20 °C. Tracks 1 and 4, haemin-excess OM; tracks 2 and 3, haemin-limited OM. In tracks 1 and 2, OM samples were exposed to exogenous haemin prior to electrophoresis, and the gel stained with TMBZ to reveal HBPs. In tracks 3 and 4, OM samples were stained with Kenacid blue, and densitometric scans made from colour transparencies of the original gel. Protein loading (measured by the Lowry method) was 250 µg protein per track. Track 5, densitometric scan (threefold abscissa expansion) of the top part of a less-exposed photographic transparency of track 3, showing the presence of the doublet which was calculated as 147 kDa after electrophoresis on a 7% gel.

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 $R_F \approx 0.02$, 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 R_F 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 R_F 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 ($\pm$ SD of 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 R_F 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 R_F 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

Fig. 3. SDS-PAGE on 10% gels of whole cells solubilized at 20 °C. Tracks 1 and 3, Haemin-excess cells; tracks 2(a and b) and 4, Haemin-limited cells. Sample loadings were 650 to 700 µg protein in tracks 1 and 2(a), and 320 to 350 µg protein in tracks 2(b), 3 and 4. In tracks 1, 2(a) and 2(b), cells were exposed to exogenous haemin prior to electrophoresis, and stained with TMBZ to reveal haemin-binding proteins. In tracks 3 and 4, proteins were stained with Kenacid blue. Track 5, densitometric scan of Kenacid-blue-stained haemin-limited cells (as in track 4), performed with an ordinate expansion fourfold that used in Fig. 2, to show the ladder-like series of components. Track 6, densitometric scan of whole cells (40 µg dried weight) from haemin-limited conditions, stained by the silver method of Tsai & Frasch (1982) to show the ladder-like arrangement of LPS components. LPS bands with mobilities corresponding to the TMBZ-stained proteins of mol. mass 51 to 60 kDa observed in haemin-limited OM are indicated (arrows).

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 $\approx 300 \mu\text{g}$ 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 & Hansen, 1991; Thomas *et al.*, 1976). A major TMBZ-stained protein of 32 kDa, and minor components of 51, 53, 56 and 60 kDa were definitely identified in the OM (when samples were solubilized at 20 °C) as HBPs expressed uniquely under haemin-limitation. It is not known whether the 115, 113 or 19 kDa proteins are HBPs, as TMBZ-staining was abrogated after sample solubilization at 100 °C, conditions under which these were detected by SDS-PAGE. Although cytochromes can be stained with TMBZ in polyacrylamide gels (Daldal *et al.*, 1986; Thomas *et al.*, 1976), the proteins described in this study required prior exposure to exogenous haemin to yield a positive TMBZ-staining reaction. This is the first description of such components in cells of *P. gingivalis*, 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

- BRAMANTI, T. E. & HOLT, S. C. (1990). Iron-regulated outer membrane proteins in the periodontopathic bacterium, *Bacteroides gingivalis*. *Biochemical and Biophysical Research Communications* **166**, 1146–1154.
- CARLSSON, J., HOFLING, J. F. & SUNDQVIST, G. K. (1984). Degradation of albumin, haemopexin, haptoglobin and transferrin, by black-pigmented *Bacteroides* species. *Journal of Medical Microbiology* **18**, 39–46.
- CARMAN, R., RAMAKRISHNAN, M. D. & HARPER, F. H. (1990). Hemin levels in culture medium of *Porphyromonas (Bacteroides) gingivalis* regulate both hemin binding and trypsin-like protease production. *Infection and Immunity* **58**, 4016–4019.
- COULTON, J. W. & PANG, J. C. S. (1983). Transport of hemin by *Haemophilus influenzae* type b. *Current Microbiology* **9**, 93–98.
- DALDAL, F., CHENG, S., APPLEBAUM, J., DAVIDSON, E. & PRINCE, R. C. (1986). Cytochrome *c*₂ is not essential for photosynthetic growth of *Rhodospseudomonas capsulata*. *Proceedings of the National Academy of Sciences of the United States of America* **83**, 2012–2016.

- EATON, J. W., BRANDT, P., MAHONEY, J. R. & LEE, J. T. (1982). Haptoglobin: a natural bacteriostat. *Science* **215**, 691–693.
- GIBBONS, R. J. & MACDONALD, J. B. (1960). Hemin and vitamin K compounds as required factors for the cultivation of certain strains of *Bacteroides melaninogenicus*. *Journal of Bacteriology* **80**, 164–170.
- GRENIER, D. (1991). Hemin-binding property of *Porphyromonas gingivalis* outer membranes. *FEMS Microbiology Letters* **77**, 45–50.
- GRIFFITHS, E., STEVENSON, P. & JOYCE, P. (1983). Pathogenic *Escherichia coli* express new outer membrane proteins when growing *in vivo*. *FEMS Microbiology Letters* **16**, 95–99.
- HANSON, M. S. & HANSEN, E. J. (1991). Molecular cloning, partial purification, and characterization of a haemin-binding lipoprotein from *Haemophilus influenzae* type b. *Molecular Microbiology* **5**, 267–278.
- HOLLAND, V. R., SAUNDERS, B. C., ROSE, F. L. & WALPOLE, A. L. (1974). A safer substitute for benzidine in the detection of blood. *Tetrahedron* **30**, 3299–3302.
- KENNEL, W. & HOLT, S. C. (1990). Comparative studies of the outer membranes of *Bacteroides gingivalis*, strains ATCC 33277, W50, W83 and 381. *Oral Microbiology and Immunology* **5**, 121–130.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- LAURELL, C. B. & GRONVALL, C. (1962). Haptoglobins. *Advances in Clinical Chemistry* **5**, 135–172.
- McKEE, A. S., McDERMID, A. S., BASKERVILLE, A., DOWSETT, A. B., ELLWOOD, D. C. & MARSH, P. D. (1986). Effect of hemin on the physiology and virulence of *Bacteroides gingivalis* W50. *Infection and Immunity* **52**, 349–355.
- MARSH, P. D., McKEE, A. S. & McDERMID, A. S. (1988). Effect of haemin on enzyme activity and cytotoxin production by *Bacteroides gingivalis* W50. *FEMS Microbiology Letters* **55**, 87–92.
- MUKHERJEE, S. (1985). The role of crevicular iron in periodontal disease. *Journal of Periodontology* **56** (suppl.), 22–27.
- OTTO, B. R., VERWEIJ-VAN VUGHT, A. M. J. J., VAN DOORN, J. & MACLAREN, D. M. (1988). Outer membrane proteins of *Bacteroides fragilis* and *Bacteroides vulgatus* in relation to iron uptake and virulence. *Microbial Pathogenicity* **4**, 279–287.
- OTTO, B. R., SPARRIUS, M., VERWEIJ-VAN VUGHT, A. M. J. J. & MACLAREN, D. M. (1990). Iron-regulated outer membrane protein of *Bacteroides fragilis* involved in heme uptake. *Infection and Immunity* **58**, 3954–3958.
- PAPAIANOOU, S., MARSH, P. D. & IVANYI, L. (1991). The immunogenicity of outer membrane proteins of haemin-depleted *Porphyromonas (Bacteroides) gingivalis* W50 in periodontal disease. *Oral Microbiology and Immunology* **6**, 327–331.
- PAYNE, S. M. (1988). Iron and virulence in the family Enterobacteriaceae. *CRC Critical Reviews in Microbiology* **16**, 81–111.
- SIHANG, G. H., ANWAR, H., KADURNAMUWA, J., BROWN, M. R. W., SILVERMAN, S. H. & MELLING, J. (1985). *In vivo* evidence that bacteria in urinary tract infection grow under iron-restricted conditions. *Infection and Immunity* **48**, 35–39.
- SORSA, T., UUTTO, V.-J., SUOMALAINEN, K., TURTO, H. & LINDY, S. (1987). A trypsin-like protease from *Bacteroides gingivalis*: partial purification and characterization. *Journal of Periodontal Research* **22**, 375–380.
- SMALLEY, J. W. & BIRSS, A. J. (1987). Trypsin-like enzyme activity of the extracellular vesicles of *Bacteroides gingivalis* W50. *Journal of General Microbiology* **133**, 2883–2894.
- SMALLEY, J. W., BIRSS, A. J., KAY, H. M., McKEE, A. S. & MARSH, P. D. (1989). The distribution of trypsin-like enzyme activity in cultures of a virulent and an avirulent strain of *B. gingivalis* W50. *Oral Microbiology and Immunology* **4**, 178–181.
- SMALLEY, J. W., BIRSS, A. J., McKEE, A. S. & MARSH, P. D. (1991). Haemin-restriction influences haemin-binding, haemagglutination and protease activity of cells and extracellular membrane vesicles of *Porphyromonas gingivalis* W50. *FEMS Microbiology Letters* **90**, 63–68.
- STUGARD, C. E., DASKALEROS, P. A. & PAYNE, S. M. (1989). A 101-kilodalton heme-binding protein associated with Congo red binding and virulence of *Shigella flexneri* and enteroinvasive *Escherichia coli* strains. *Infection and Immunity* **57**, 3534–3539.
- THOMAS, P. E., RYAN, D. & LEVIN, W. (1976). An improved staining procedure for the detection of peroxidase activity of cytochrome P-450 on sodium dodecyl sulphate polyacrylamide gels. *Analytical Biochemistry* **75**, 168–176.
- TSAL, C. M. & FRASCH, C. A. (1982). A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Analytical Biochemistry* **119**, 115–119.
- TSUTSUI, H., KINOCHI, T., WAKANO, Y. & OHNISHI, Y. (1987). Purification and characterization of a protease from *Bacteroides gingivalis* 381. *Infection and Immunity* **55**, 420–427.
- YOSHIMURA, F., NISHIKATA, M., SUZUKI, T., HOOVER, C. I. & NEWBRUN, E. (1984). Characterization of a trypsin-like protease from the bacterium *Bacteroides gingivalis* isolated from human dental plaque. *Archives of Oral Biology* **29**, 559–564.