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SDS-PAGE of the outer-membrane (OM) proteins of *Haemophilus parainfluenzae* P205 grown under iron-sufficient conditions revealed three major proteins of 40, 37 and 13 kDa. In addition, growth under conditions of iron-restriction resulted in the expression of at least four iron-repressible OM proteins (IROMPs) of 72, 81, 88 and 90 kDa. OM proteins of 40 and 13 kDa were non-covalently associated with peptidoglycan and were resistant to digestion with trypsin. A 38 kDa peptidoglycan-associated protein, which was masked by the abundant 37 kDa protein, was also observed following tryptic digestion of whole cells or OMs. Neither the 37 kDa protein (which was heat-modifiable) nor the IROMPs were peptidoglycan-associated, and both were cleaved following treatment of whole cells with trypsin, indicating that they are exposed at the cell surface. A variety of IROMPs from five other *H. parainfluenzae* strains was also observed. In each strain, both the IROMPs and a major protein of 37 kDa were exposed at the cell surface.

INTRODUCTION

*Haemophilus parainfluenzae* is a facultatively anaerobic Gram-negative bacterium. On the basis of DNA homology (Pohl, 1980; Burbach *et al.*, 1986) this species forms a heterogeneous genetic cluster sharing certain biochemical characteristics and an absolute requirement for nicotinamide adenine dinucleotide (NAD). *H. parainfluenzae* is ubiquitous on all surfaces of the human mouth and pharynx, constituting about 60% of the total population of haemophili in the upper respiratory tract. It has also been isolated from the vaginal mucosa and the genito-urinary tract, and is present in over 20% of faecal samples (Kilian & Frederiksen, 1980). This bacterium is only occasionally responsible for clinical infections and exhibits a spectrum of diseases similar to *H. influenzae* including epiglottitis, otitis media, cellulitis, pneumonia, endocarditis, meningitis and bacteraemia (Albritton, 1982; Bruun *et al.*, 1984). However, in a survey of paediatric infections ascribed to *H. parainfluenzae*, Black *et al.* (1988) suggested that difficulty in identifying this organism may have resulted in misdiagnosis in the past. The increased reporting of *H. parainfluenzae* paediatric infections (Black *et al.*, 1988) requires that the pathogenic potential of this organism should not be overlooked.

The outer membrane (OM) composition and structure of this common human commensal have not been fully characterized. Examination of the OM proteins of *H. parainfluenzae* by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) has revealed a variety of patterns similar to those of other Gram-negative bacteria (Roberts *et al.*, 1986; Williams & Brown, 1986) in which at least two proteins in the 28–48 kDa range predominate (Osborn & Wu, 1980; Lugtenberg & van Alphen, 1983; Hancock, 1987). In addition, and in common with many

Abbreviations: BHI, brain heart infusion broth; IROMPs, iron-repressible outer-membrane proteins; OM, outer membrane.

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other Gram-negative bacteria, the organism responds to iron-restriction by inducing several high-molecular-mass iron-repressible OM proteins (IROMPs) in the 70–90 kDa range (Williams & Brown, 1986). The present study was undertaken to characterize further the properties (heat modifiability, peptidoglycan-association and cell-surface exposure) of the OM proteins of _H. parainfluenzae_ grown under iron-sufficient and iron-restricted conditions.

**METHODS**

_Bacterial strains._ _H. parainfluenzae_ NCTC 7857 and NCTC 10665 were obtained from the National Collection of Type Cultures, Colindale Avenue, London, UK; P205 was a clinical isolate from Dudley Road Hospital, Birmingham, UK (kindly donated by Dr L. Piddock). Strains HK115, HK116 and HK255 were kindly supplied by Professor M. Kilian, The Royal Dental College, Aarhus, Denmark; these strains were isolated from human saliva (HK115 and HK116) and the urethra (HK255).

_Media._ Bacteria were routinely grown overnight at 37 °C to stationary phase in brain heart infusion broth (BHI) supplemented with NAD (2 μg ml⁻¹). Iron-restriction was achieved, as described previously (Williams & Brown, 1986), by addition of the iron chelator desferrioxamine (Ciba-Geigy) to BHI at concentrations of 50 μg ml⁻¹ (strains NCTC 7857, NCTC 10665, P205, and HK255) or 100 μg ml⁻¹ (strains HK115 and HK116).

_Isolation of outer membranes._ OM proteins were isolated as described before (Williams & Brown, 1986) using 2% (w/v) sodium N-lauroyl sarcosine (Sarkosyl) to remove cytoplasmic membrane material from envelopes prepared by sonication of whole cells.

_SDS-PAGE._ OM proteins were separated on SDS-polyacrylamide gels (10 μg of protein per lane). Following electrophoresis, gels were fixed and stained with Coomassie Brilliant Blue R250 (Sigma) in 50% (v/v) methanol, 10% (v/v) glacial acetic acid in water, as described by Williams et al. (1984).

_Peptidoglycan-associated OM proteins._ The presence of these proteins was determined by solubilizing OM preparations in a buffer containing 2% (w/v) SDS, 10% (w/v) glycerol and 10 mM-Tris/HCl, pH 7.4, at 30, 45, 50 or 60 °C for 30 min as described by Darveau et al. (1983). The pellet obtained after centrifugation at 38000 g for 60 min was either (1) incubated in SDS-PAGE sample buffer at 100 °C for 5 min prior to electrophoresis or (2) incubated in the same buffer containing 0.5 M- NaCl for 30 min at 30 °C to release the proteins from peptidoglycan. The supernatant obtained after centrifugation was then incubated with SDS-PAGE sample buffer at 37 or 60 °C for 30 min or 100 °C for 5 min prior to electrophoresis.

_Heat modifiability of the OM proteins._ This was assessed by incubating OM preparations in sample buffer at 37 or 60 °C for 30 min or 100 °C for 10 min prior to SDS-PAGE.

_Trypsin sensitivity of OM proteins._ This was determined by incubation of whole cells (resuspended to OD₆⁰₀ 1.0, 1 cm path length; LKB 4049 spectrophotometer) with trypsin (1 mg ml⁻¹) prior to isolation of OM proteins or by incubation of OM preparations with trypsin (100 μg trypsin per μg protein). Digestion was carried out in 10 mM-Tris/HCl pH 8.0 at 37 °C. Exposure times were varied from 30 min to 48 h.

**RESULTS**

_OM protein profiles of _H. parainfluenzae._*

The OM protein profiles of all six _H. parainfluenzae_ strains grown under iron-sufficient and under iron-restricted conditions are shown in Figs 1(a) and 1(b) respectively. Strain P205 produced three major proteins of molecular mass 40, 37 and 13 kDa. In addition, growth of the organism under conditions of iron-restriction resulted in the expression of at least four IROMPs of 72, 81, 88 and 90 kDa, of which the 81 kDa and 72 kDa proteins were the most strongly induced (Fig. 1b, lane A). On lower percentage acrylamide gels, the 81 kDa protein was resolved into two bands which were reported as having molecular masses of 74 and 76 kDa respectively (Williams & Brown, 1986). However, the molecular masses of the P205 OM proteins reported previously (Williams & Brown, 1986) were calculated from SDS-PAGE molecular mass marker proteins. In the present paper they have been re-assigned by comparison with the OM proteins of _Escherichia coli_ K12 (data not shown).

_H. parainfluenzae_ strains NCTC 10665 and HK255 showed essentially similar IROMP patterns to that of P205 (Fig. 1b, lanes B and C). This IROMP profile is similar to that of _E. coli_ K12 which, when grown under conditions of iron deprivation, expresses two major IROMPs of 74 kDa and 81 kDa (Neilands, 1982; Griffiths, 1987). The three remaining strains had different patterns; in addition, strains HK115 and HK116 only expressed the IROMPs (Fig. 1b, lanes E and F respectively) in media containing 100 μg desferrioxamine ml⁻¹, a concentration which significantly reduced growth.
Peptidoglycan-associated proteins

By using SDS to perform differential solubilization of the OM of *H. parainfluenzae* P205, at least three proteins, of molecular masses 13, 38 and 40 kDa, remained with the insoluble peptidoglycan fraction after solubilization with 2% (w/v) SDS at 30 °C (Fig. 2, lane B). These proteins were released from the peptidoglycan by heating in 2% (w/v) SDS at temperatures of 45, 50 or 60 °C or following incubation at 30 °C in the same buffer containing 0.5 M-NaCl (Fig. 2,
Fig. 2. SDS-PAGE (12.5%, w/v, acrylamide) of the peptidoglycan-associated OM proteins of \textit{H. parainfluenzae} P205 grown under iron-restricted conditions. (M) Molecular weight marker proteins; (A) OM proteins; (B) proteins remaining with the peptidoglycan after incubation of OMS in 2% (w/v) SDS at 30 °C; (C) proteins released from peptidoglycan after incubation in 2% (w/v) SDS containing 0.5 M- NaCl as described in Methods.

Fig. 3. SDS-PAGE (7.5%, w/v, acrylamide) showing the monomeric and oligomeric forms of the peptidoglycan-associated proteins of \textit{H. parainfluenzae} P205. Proteins released from the peptidoglycan by 2% (w/v) SDS containing 0.5 M-NaCl were incubated in SDS-PAGE sample buffer at (A) 100 °C for 5 min; (B) 37 °C for 30 min.

This suggests that the OM of \textit{H. parainfluenzae} P205 contains at least three proteins which are tightly but non-covalently bound to peptidoglycan. Incubation of the peptidoglycan-associated proteins (released in the presence of 0.5 M-NaCl at 30 °C) in SDS-PAGE sample buffer at 37 °C prior to loading the gel revealed the presence of a broad, poorly staining band (Fig. 3, lane B) with a mean molecular mass of approximately 65 kDa. This broad band is probably an aggregated or oligomeric form of the 40 and 38 kDa OM proteins and was not observed after incubation of the sample at 60 °C or 100 °C. Neither the abundant 37 kDa OM protein (which masks the 38 kDa peptidoglycan-associated protein) nor the IROMPs were peptidoglycan-associated.

\textit{Heat modifiability of the OM proteins}

Solubilization of the OMs of \textit{H. parainfluenzae} P205 at increasing temperatures revealed the presence of a major heat-modifiable protein with an apparent molecular mass of 25 kDa at 37 °C (Fig. 4, lane A) but of 37 kDa at 100 °C (Fig. 4, lane C). In addition, a minor heat-modifiable protein of about 15 kDa at 37 °C increased to 20 kDa at 60 °C and at 100 °C (Fig. 4) The IROMPs were unaffected by the temperature of solubilization.

\textit{Trypsin sensitivity of the OM proteins}

An indication of the surface exposure of the \textit{H. parainfluenzae} OM proteins was obtained by incubation of both whole cells and OMs in the presence of the proteolytic enzyme trypsin. Fig. 5 reveals that all the IROMPs, and the heat-modifiable 37 kDa protein, were cleaved to yield major fragments of 50 kDa and 23 kDa respectively following incubation of whole cells with trypsin for 30 min prior to the preparation of OMs. Incubation with trypsin for up to 48 h yielded
comparable results. The 40, 38 and 13 kDa peptidoglycan-associated proteins were, however, insensitive to this enzyme. Similar results were obtained by incubating isolated OMs with trypsin although a different pattern of cleavage was observed in that an additional band appeared at 10 kDa and no 50 kDa fragment was apparent (data not shown). Thus in *H. parainfluenzae* P205, the IROMPs and 37 kDa heat-modifiable OM protein are exposed at the outer surface of the OM. These experiments were also repeated on isolated OMs of the remaining five strains. The IROMPs and the major heat-modifiable proteins of NCTC 10665, HK255, HK115 and HK116 were cleaved by trypsin. However, whilst the heat-modifiable protein of strain NCTC 7857 was cleaved by trypsin, the IROMPs were unaffected. The IROMPs of this strain may be resistant to trypsin because: (a) they have no trypsin cleavage sites, or (b) the cleavage sites are not exposed at the membrane surface, or (c) access of trypsin to these IROMPs is prevented by other OM macromolecules such as LPS. The IROMPs of this strain could, however, be digested following incubation with staphylococcal V8 protease (data not shown).

**DISCUSSION**

The OM of Gram-negative bacteria generally contains at least three classes of abundant OM proteins: the porin (or transmembrane pore) proteins (e.g. OmpC and OmpF in *E. coli* K12), a heat-modifiable protein (designated OmpA in *E. coli* K12) and the lipoproteins (Osborn & Wu, 1980; Lugtenberg & van Alphen, 1983; Hancock, 1987). Amongst the enteric bacteria, the transmembrane pore proteins, which have molecular masses in the 28–44 kDa range, share several characteristics. These include a strong noncovalent association with peptidoglycan and
retention of their native oligomeric form even in the presence of 2\% (w/v) SDS at 60 °C, which solubilizes all other OM proteins except the lipoproteins. These proteins can be released from the peptidoglycan by incubation in 2\% (w/v) SDS at temperatures of 70 °C or higher or by incubation in a solution of high ionic strength containing SDS (Lugtenberg & van Alphen, 1983; Hancock, 1987). Growth of *H. parainfluenzae* P205 in BHI revealed at least three major OM proteins of molecular masses 13, 37 and 40 kDa. SDS-PAGE of the insoluble fraction obtained after incubation of isolated OM with 2\% (w/v) SDS at 30 °C showed the presence of three peptidoglycan-associated proteins, of 13, 38 and 40 kDa. These proteins were released following incubation in 2\% (w/v) SDS and 0.5 M-NaCl at 30 °C or by incubation in 2\% (w/v) SDS at higher temperatures. The peptidoglycan-associated OM proteins of *H. parainfluenzae* are therefore associated more weakly than those of the enteric bacteria, but are much more strongly bound than those of *H. influenzae* (van Alphen *et al.*, 1983) and *H. pleuropneumoniae* (Rycroft & Taylor, 1987). Incubation of the cell envelopes of either non-typable or type b *H. influenzae* strains in 2\% (w/v) SDS at 30 °C results in the release of all OM proteins (van Alphen *et al.*, 1983).

In *E. coli* and *Salmonella typhimurium*, the porin proteins are present in, and function as, oligomers (usually trimers) in the OM (Hancock, 1987). They have been observed to migrate as oligomers on SDS-PAGE when solubilized in SDS at temperatures below 70 °C. In contrast, the 40 kDa porin of *H. influenzae* is dissociated by SDS even at room temperature although cross-linking studies have indicated that it probably exists as a trimer within the OM (Vachon *et al.*, 1988). Incubation of the *H. parainfluenzae* peptidoglycan-associated proteins at 37 °C prior to electrophoresis revealed the presence of a broad poorly staining band of about 65 kDa which is probably a mixture of oligomeric forms of the 38 and 40 kDa peptidoglycan-associated proteins. Thus *H. parainfluenzae* P205 has at least two proteins of molecular masses 38 and 40 kDa which probably function as pore proteins.

In addition to the peptidoglycan-associated proteins, the OM of *H. parainfluenzae* contained a major heat-modifiable protein of 37 kDa which was not associated with peptidoglycan and was susceptible to trypsin. These properties are characteristic of the *E. coli* OmpA protein, antibodies raised against which show cross-reactivity with similar proteins in the OM of other enteric bacteria (Behr *et al.*, 1980) and also *H. influenzae* (van Alphen *et al.*, 1983). The 37 kDa heat-modifiable protein of *H. parainfluenzae*, however, differs from that of *E. coli* in that it is susceptible to proteolytic cleavage both in whole cells and in isolated membranes. In contrast, the *E. coli* OmpA protein is only susceptible to trypsin in isolated cell envelope fractions in which the periplasmic face of the protein has been made accessible (Osborn & Wu, 1980).

Apart from the 37 kDa heat-modifiable protein, the only other protease-susceptible, surface-exposed *H. parainfluenzae* OM proteins were those induced when the organism was grown under iron-restricted conditions. For many bacterial species, such IROMPs function as components of high-affinity iron-sequestering systems (Griffiths, 1987). Bacterial IROMPs may function as receptors for low-molecular-mass iron chelators (siderophores) or via a direct interaction at the bacterial surface with vertebrate host iron-binding glycoproteins such as transferrin and lactoferrin. The function of the *H. parainfluenzae* IROMPs is not known although we have recently shown that this organism produces siderophores (D. Morton & P. Williams, unpublished observation). The IROMPs may therefore function as receptors for siderophores produced by *H. parainfluenzae* or by other micro-organisms inhabiting the same ecological niche, i.e. human mucosal surfaces and in particular the nasopharynx. In contrast to *H. parainfluenzae*, the OM of *H. influenzae* grown under iron-restricted conditions has been reported to show only minor changes in the OM protein profile (Herrington & Sparling, 1985; Williams & Brown 1986; Pidcock *et al.*, 1988). Thus, the expression of IROMPs by *H. parainfluenzae* appears to be more closely related to the enteric bacteria than to *H. influenzae*.

These high-affinity iron-uptake systems generally enable bacteria to multiply in the vertebrate host where transferrin (in serum) and lactoferrin (on mucosal surfaces) maintain the concentration of available iron at a level far below that required to support bacterial growth. Such restriction of iron constitutes an important defence against microbial infection (Griffiths & Bullen, 1987). Interestingly, Herrington & Sparling (1985) reported that neither *H. influenzae* nor *H. parainfluenzae* was capable of removing iron from lactoferrin although *H. influenzae* could
remove iron from transferrin. However, since *H. parainfluenzae* almost exclusively inhabits human mucosal surfaces, the organism’s survival may depend on its ability to obtain iron from lactoferrin. To do this, the IROMPs produced by *H. parainfluenzae* may not only function as receptors for *H. parainfluenzae* siderophores but also for those produced by other micro-organisms which are capable of removing iron from lactoferrin or transferrin. Certain *E. coli* IROMPs for example appear to function as receptors for fungal siderophores such as ferrichrome (Neilands, 1982). Further work is under way to determine whether *H. parainfluenzae* can utilize the siderophores of other micro-organisms and whether pathogenic strains are capable of removing iron from host iron-binding proteins by a siderophore-dependent or siderophore-independent mechanism.

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**REFERENCES**


