Outer-membrane protein and lipopolysaccharide variation in *Pasteurella haemolytica* serotype A1 under different growth conditions

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Growth characteristics, as well as outer-membrane protein (OMP) and lipopolysaccharide (LPS) profiles in SDS-polyacrylamide gels, of two serotype A1 isolates of *Pasteurella haemolytica* were examined under different *in vitro* growth conditions. The two isolates were chosen as representatives of disease (S/C 82/1) and non-disease (W/D 83/4) isolates, respectively. The growth rates and final cell densities of both isolates increased as the degree of aeration increased. In particular, the final cell densities varied significantly according to the degree of aeration. Under anaerobic conditions, however, both the growth rate and final cell density were significantly reduced. There was reduced expression of a 40·5 kDa protein under anaerobic conditions in both isolates, whereas in S/C 82/1 expression of the 71, 77 and 100 kDa iron-regulated proteins increased as aeration decreased. There were also differences in low-molecular-mass components of LPS between cells grown anaerobically and those grown aerobically. Growth in the presence of 5% CO₂ did not significantly alter the growth rate and had little, if any, affect on OMPs or LPS. Differences in the expression of certain proteins occurred as growth progressed from the exponential to the stationary phase. Growth in the presence of the iron chelators 2,2'-dipyridyl, ethylenediamine-dihydroxyphenylacetic acid (EDDA), desferrioxamine mesylate (desferal), ovotransferrin (conalbumin) and bovine transferrin was inhibited within a very narrow concentration range. In the presence of 2,2'-dipyridyl, EDDA or desferal, 71 and 100 kDa iron-regulated OMPs increased in both isolates whereas a 77 kDa protein increased in isolate S/C 82/1 only. In the presence of ovotransferrin or bovine transferrin there was, in both isolates, increased expression of the 71 kDa protein, a slight increase in expression of the 100 kDa protein but no expression of the 77 kDa protein; there was also increased production of the 40·5 kDa protein, and synthesis of two additional proteins of 23 and 26 kDa. Other differences occurred after growth in foetal and newborn calf sera. In foetal calf serum there was enhanced expression of the 71 but not of the 100 kDa protein. In newborn calf serum there was no enhanced expression of the 71, 77 or 100 kDa proteins, but expression of novel proteins of 97 and 98 kDa as well as a high-molecular-mass protein occurred. There were also slight quantitative differences in the LPS profiles of cells grown in foetal or newborn calf sera compared to those of cells grown in other media. Addition of FeCl₃ to the various media caused repression of synthesis of the 71, 77 and 100 kDa proteins in the presence of EDDA and repression of the 71 kDa protein in the presence of ovotransferrin and in foetal calf serum, but caused no change to the expression of proteins in newborn calf serum. Thus, marked changes may occur in the OMP and LPS components of the outer membrane of *P. haemolytica* when growth conditions are altered; some of these changes may occur *in vivo*.

Introduction

Pasteurella haemolytica is a Gram-negative bacterium responsible for diseases of ruminants including bovine pneumonic pasteurellosis or shipping fever (Carter,

1967). Although two biotypes, A and T, and 16 serotypes have been identified (Biberstein, 1978; Fodor *et al.*, 1988) the majority of disease outbreaks in cattle are due to biotype A, serotype 1 (A1) strains (Frank, 1979). Experimental vaccines against bovine pneumonic pasteurellosis, including both live and killed bacteria (Wilkie *et al.*, 1980; Confer *et al.*, 1985; Purdy *et al.*, 1986) as well as various extracts (Gilmour *et al.*, 1982; Yates *et al.*, 1983), have generally been unsuccessful. Although leukotoxin-neutralizing antibodies appear to

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Abbreviations: OMP, outer-membrane protein; LPS, lipopolysaccharide; EDDA, ethylenediaminedihydroxyphenylacetic acid.

Fig. 1. OMP profiles of isolate S/C 82/1 (lanes 1-4) and W/D 83/4 (lanes 5-8) after 3.5 (lanes 1 and 5), 6.5 (lanes 2 and 6), 12 (lanes 3 and 7) and 24 (lanes 4 and 8) h of growth in BHIB with high aeration. Molecular mass markers (lane 9) are bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20.1 kDa) and α -lactalbumin (14.2 kDa) (Sigma). Numbers on the left indicate molecular masses of proteins which exhibited variation in expression during the growth cycle (see text).

be important in protection against pneumonic pasteurellosis (Shewen & Wilkie, 1983) other somatic antigens are also involved in inducing resistance (Shewen & Wilkie, 1988). As a result, recent research has attempted to identify important immunogenic components of *P. haemolytica* such as cell wall and capsular materials (Durham *et al.*, 1986), carbohydrate-protein subunits (Lesley *et al.*, 1985), lipopolysaccharide (LPS) (Confer *et al.*, 1986) and various outer-membrane proteins (OMPs) (Squire *et al.*, 1984; Nelson & Frank, 1989; Knights *et al.*, 1990; Craven *et al.*, 1991) including iron-regulated OMPs (Donachie & Gilmour, 1988; Deneer & Potter, 1989; Gilmour *et al.*, 1991).

It is now well-recognized that the expression of antigens in bacteria obtained directly from the infected host may be quite different to those expressed *in vitro* (Smith, 1990). In particular, a number of OMPs are induced in various bacteria growing *in vivo* which are not

expressed in standard laboratory media, but are expressed *in vitro* under iron-limited conditions (Griffiths *et al.*, 1983; Brown *et al.*, 1984). Such iron-regulated OMPs are immunogenic (Anwar *et al.*, 1984; Black *et al.*, 1986; Ward *et al.*, 1988), may induce bactericidal antibodies (Pettersson *et al.*, 1990), can be protective (Bolin & Jensen, 1987) and hence are likely vaccine candidates (Banerjee-Bhatnagar & Frasch, 1990). Although the *in vivo* expression of cell-surface antigens of a serotype A2 strain of *P. haemolytica* has been studied in sheep (Donachie & Gilmour, 1988; Sutherland *et al.*, 1990), little is known about the expression of such antigens in serotype A1 strains isolated from cattle. Cells of a sheep-pathogenic A2 strain of *P. haemolytica* recovered directly without subculture from the pleural fluid of an infected lamb expressed two additional envelope proteins of 70 and 100 kDa; these proteins were also induced *in vitro* under iron-restricted conditions (Donachie & Gilmour,

Fig. 2. Growth of isolate W/D 83/4 under various conditions of aeration. (a) compares growth with no aeration (○), low aeration (●), moderate aeration (□), high aeration (■) and very high aeration (△). (b) compares growth in 5% CO₂/95% air (●) with growth in air alone (○).

1988). Sutherland *et al.* (1990) similarly identified 70 and 100 kDa proteins in A2 cells grown in chambers implanted into the peritoneal cavity of sheep. Three iron-regulated OMPs of 71, 77 and 100 kDa were demonstrated in *in-vitro*-grown strains of *P. haemolytica* belonging to 12 serotypes in response to iron-limitation by Deneer & Potter (1989) and these authors provided evidence to suggest that these proteins were expressed *in vivo* in cattle.

In addition to iron-limitation other factors which may cause changes to OMPs of Gram-negative bacteria include phase of growth (Loeb & Smith, 1980; Kelly & Parker, 1981; Blaser *et al.*, 1983; Davies, 1991) and the degree of aeration or CO₂ tension during growth (Clark *et al.*, 1987; Denyer *et al.*, 1990; Lee & Falkow, 1990; Schiemann & Shope, 1991). Although there is much less information on differences in LPS structure between *in-vivo*- and *in-vitro*-grown cells, differences have been described in *Pseudomonas aeruginosa* (Kelly *et al.*, 1989) and *Neisseria gonorrhoeae* (Mandrell *et al.*, 1990). Changes in the LPS of *Neisseria meningitidis* due to

differences in aeration and medium composition have also been described (Tsai *et al.*, 1983). However, the LPS of an *in-vitro*-grown *P. haemolytica* A2 strain did not appear to differ from that of the same strain grown in a peritoneal chamber implant in sheep (Sutherland *et al.*, 1990).

In the present study, the objectives were to examine the growth characteristics and the variation in OMP and LPS profiles of *P. haemolytica* A1 isolates under various conditions of *in vitro* growth. Two isolates were chosen for comparison, one isolate from a confirmed case of pasteurellosis and the other from a healthy animal. Previous studies on *P. haemolytica* have utilized the chelating agents 2,2'-dipyridyl, ethylenediaminedihydroxyphenylacetic acid (EDDA) or desferrioxamine mesylate (desferal) to induce iron-limited growth conditions in conventional media (Donachie & Gilmour, 1988; Deneer & Potter, 1989; Ogunnariwo & Schryvers, 1990; Lainson *et al.*, 1991). However, Chart *et al.* (1986) demonstrated that various chelating agents have differing effects on the induction of the iron-regulated OMPs of *Escherichia coli*. Thus, in addition to 2,2'-dipyridyl, EDDA and desferal, the effects of the chelators ovotransferrin (conalbumin) and bovine transferrin were also compared in the present study. As well as examining the effects of various chelating agents, bacteria were also grown in foetal and newborn calf sera. In addition, OMP and LPS profiles were examined at different stages of the growth cycle and under different conditions of aeration and CO₂ tension, both of which are additional factors which could influence *in vivo* profiles.

Methods

Bacterial strains and growth conditions. Two biotype A, serotype 1 (A1) strains (S/C 82/1 and W/D 83/4) of *P. haemolytica* were used in the study. Strain S/C 82/1 was isolated from a confirmed case of bovine pneumonic pasteurellosis whereas strain W/D 83/4 was isolated from the nasopharynx of a clinically healthy calf on a disease-free farm. The LPSs from each of these isolates have been examined previously and have been shown to be different (Davies *et al.*, 1991).

Bacteria, stored at -70 °C in brain heart infusion broth (BHIB) containing 50% (v/v) glycerol, were routinely subcultured on brain heart infusion agar (BHIA) containing 5% (v/v) sheep's blood at 37 °C. For the isolation of outer membranes and for growth studies bacteria were grown in BHIB in the absence or presence of 2,2'-dipyridyl, EDDA, ovotransferrin, bovine transferrin (Sigma) or desferal (CIBA Laboratories), as well as in decomplexed (56 °C for 20 min) foetal or newborn calf sera (Gibco) at 37 °C with orbital shaking at 120 r.p.m. For the preparation of outer membranes optimum concentrations of chelating agents were determined from growth-rate experiments where growth, in the presence of increasing concentrations of chelating agent, was followed spectrophotometrically at 660 nm. To confirm that inhibition of growth was, in fact, due to iron limitation, growth was also followed in the presence of an inhibitory concentration of EDDA or ovotransferrin and increasing concentrations of FeCl₃. Outer membranes were also obtained from cells grown under conditions where the

Fig. 3. OMP profiles of isolates S/C 82/1 (lanes 1–6) and W/D 83/4 (lanes 7–12) after growth to early stationary phase with no aeration (lanes 1 and 7), low aeration (lanes 2 and 8), high aeration (lanes 3 and 9), very high aeration (lanes 4 and 10), in 5% CO₂/95% air (lanes 5 and 11) and under anaerobic conditions (lanes 6 and 12). Molecular mass standards (lane 13) are phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α -lactalbumin (14.4 kDa) (Pharmacia).

inhibitory effects of EDDA and ovotransferrin were eliminated by FeCl₃, as well as from cells grown in foetal or newborn calf sera to which the same amount of FeCl₃ had been added.

Growth under various degrees of aeration was examined by varying the broth:flask volume ratios, by using dimpled or smooth flasks, or by covering the surface of static cultures with liquid paraffin, as follows: 50 ml of broth in 250 ml dimpled conical flask (very high aeration); 50 ml in 250 ml smooth conical flask (high aeration); 100 ml in 250 ml smooth conical flask (moderate aeration); 200 ml in 250 ml smooth conical flask (low aeration) (all shaken at 120 r.p.m.); and 200 ml in 250 ml smooth conical flask covered with liquid paraffin and grown statically (no aeration). For outer membrane preparations, 500 ml volumes were used in 2 litre dimpled flasks (very high aeration), 2 litre smooth flasks (high aeration), 500 ml smooth flasks (low aeration) (all shaken), and in 500 ml smooth flasks covered with liquid paraffin and grown statically (no aeration). Bacteria were also grown, in BHIB, in a 5% CO₂/95% air atmosphere in a controlled environment incubator shaker (New Brunswick Scientific) at 120 r.p.m. as well as anaerobically, using an anaerobe jar in conjunction with an anaerobe gas generating kit (Oxoid). In all experiments, flasks were inoculated from an overnight culture in BHIB to give a dilution of 1:500.

Preparation of outer membranes. Outer membranes were obtained by Sarkosyl extraction, essentially as described previously (Davies, 1991). At an appropriate stage of growth, bacteria were harvested, washed twice in 20 mM-Tris/HCl (pH 7.2) buffer, resuspended in the same buffer and sonicated, on ice, for 5 min (MSE sonicator; 4–5 μ m amplitude; 20 kHz). Unbroken cells were removed by centrifugation (10000 *g* for 30 min at 4 °C), cell envelopes pelleted by centrifugation at 60000 *g* for 1 h at 4 °C, and cytoplasmic membranes solubilized by resuspending in 0.5% sodium *N*-lauroylsarcosine (Sarkosyl) for 20 min at room temperature (Filip *et al.*, 1973). Outer membranes were pelleted by centrifugation at 60000 *g* for 1 h, washed once in 20 mM-Tris/HCl (pH 7.2), centrifuged again at 60000 *g* for 1 h, resuspended in the same buffer to a protein concentration of 2.0 mg ml⁻¹ and stored at –70 °C.

Protein assay. Protein concentrations of outer membrane samples were assayed by the modified Lowry procedure of Markwell *et al.* (1978), a method suitable for insoluble membrane proteins.

Preparation of lipopolysaccharide. Lipopolysaccharide was obtained by proteinase K digestion as described by Hitchcock & Brown (1983), except that outer membranes were the starting material rather than

Fig. 4. LPS profiles of isolates S/C 82/1 (lanes 1–6) and W/D 83/4 (lanes 7–12) after growth under the conditions described in Fig. 3. LPS of *E. coli* B4 (O:111) is shown in lane 13. Arrows indicate differences in low-molecular-mass regions (see text).

whole cells (Davies *et al.*, 1991). Outer membranes (2.0 mg protein ml^{-1}) were added to an equal volume of $\times 2$ sample buffer (0.25 M-Tris/HCl, pH 6.8, 40% v/v, glycerol, 8% w/v, SDS, 0.2% v/v, 2-mercaptoethanol, 0.005% w/v, bromophenol blue) and heated at 100 °C for 5 min. To 100 μg of protein were added 25 μg of proteinase K (type XI protease; Sigma), adjusted to 2.5 mg ml^{-1} in $\times 1$ sample buffer, and the mixture heated at 60 °C for 1 h.

SDS-PAGE. OMPs and LPS were separated by SDS-PAGE using the SDS discontinuous system of Laemmli (1970). After stacking in a 4% (w/v) gel, OMPs and LPS were separated in 12 and 15% (w/v) resolving gels, respectively, the latter also containing 4 M-urea (Davies *et al.*, 1991). A total of 25 μg protein was loaded per lane (25 μl), and 6 μl of the same proteinase-K-treated samples for LPS (approximately 5 μg LPS). Electrophoresis was performed in buffer comprising 25 mM-Tris/HCl, 192 mM-glycine and 0.1% SDS (pH 8.3), at a constant current of 20 mA per gel through the stacking gel and 30 mA per gel through the resolving gel. Proteins were visualized by staining with Coomassie blue, and LPS by silver-staining (Tsai & Frasch, 1978). Smooth lipopolysaccharide from *E. coli* B4 (O:111; Sigma) was used as a control for LPS.

Results

The OMP profiles of the two *P. haemolytica* isolates, S/C 82/1 and W/D 83/4, were very similar and comprised

four or five major proteins and at least twenty minor proteins (Fig. 1). When cells were harvested at various stages of the growth cycle, i.e. after 3.5, 6.5, 12 and 24 h of growth, under high aeration (see below), there was, in both isolates, a slight decrease in expression of a 40.5 kDa protein and an increase in expression of a 18 kDa protein, between exponential (3.5 h) and stationary (6.5 h) phases. In addition, in both isolates, a 24 kDa protein was produced during the stationary phase but not during the exponential phase and, in S/C 82/1, a 94 kDa protein was produced in greater abundance during the exponential phase (Fig. 1). In other strains examined (results not shown) there were additional differences between exponential and stationary phase cells that were not observed in the two isolates described here. Differences in banding patterns in the high-molecular-mass region of the LPS of both isolates, between exponential and stationary phase cells, also occurred and have been described previously (Davies *et al.*, 1991). To avoid variation in OMP or LPS profiles, as a result of cells being at different stages of growth, for all subsequent work bacteria were harvested during early stationary phase.

Fig. 5. Growth curves of isolate S/C 82/1 in BHIB in the absence or presence of various concentrations of the chelators desferal (a) or ovotransferrin (b). Concentrations of desferal used were 0 (○), 20 (●), 21.5 (□), 23 (■), 24.5 (△) and 26 (▲) μM; concentrations of ovotransferrin used were 0 (○), 0.5 (●), 0.75 (□), 1.0 (■), 1.25 (△) and 1.50 (▲) mg ml⁻¹.

The degree of aeration significantly affected the final cell density of the two isolates examined but, with the exception of no aeration, had a less significant effect on the growth rate (Fig. 2a). Generally, high aeration resulted in a high final cell density and as the degree of aeration decreased so did the final cell density; optimum cell density was not necessarily achieved, however, with maximum aeration, as was the case with isolate S/C 82/1. With no aeration, the growth rate and final cell density, in both isolates, were substantially lower than under the other conditions. In the presence of 5% CO₂ there was a shorter lag phase but the growth rate during the exponential phase was similar to that in air alone; the final cell density was reduced in the presence of 5% CO₂ compared to that in air alone (Fig. 2b). Similar results were obtained with both isolates. Differences were also apparent in the OMP profiles of cells grown under the various conditions of aeration and CO₂ tension (Fig. 3).

In both isolates there was reduced expression of the 40.5 kDa protein in cells grown with no aeration (lanes 1 and 7) or under anaerobic conditions (lanes 6 and 12) compared to cells grown with aeration or in the presence of 5% CO₂. In addition, in isolate S/C 82/1, there was a gradual decline in expression of proteins of 71, 77 and 100 kDa (the iron-regulated OMPs – see below) as aeration increased; these changes were not observed in isolate W/D 83/4. Maximum expression of these proteins occurred in cells grown with no aeration or under anaerobic conditions (Fig. 3, lanes 1 and 6). Less significant differences occurred in the expression of proteins in the 20–26 kDa molecular mass range. Growth in the presence of 5% CO₂ did not yield significant changes in OMP profiles (Fig. 3, lanes 5 and 11). As can be seen by comparing Figs 1 and 3 there were differences in the production of the 40.5 kDa protein in cells prepared on different occasions. The reasons for this are unknown but may have been due to slight differences in culture medium, aeration or sample preparation. The LPS profiles of cells grown with no aeration or anaerobically also differed from cells grown aerobically; these differences occurred in the low-molecular-mass region and are shown arrowed in Fig. 4. To avoid variation in OMP or LPS profiles resulting from differing degrees of aeration, for all subsequent work bacteria were grown with high aeration, i.e. a flask : culture ratio of 4:1, and at 120 r.p.m.

Growth of both strains was examined in the presence of increasing concentrations of the chelating agents 2,2'-dipyridyl, EDDA, desferal, ovotransferrin and bovine transferrin. In the case of each of these chelating agents, growth of both strains was rapidly slowed and inhibited within a very narrow range of concentration (Fig. 5). For example, growth was slowed and completely inhibited within the concentration ranges 100–140 (S/C 82/1), 15–22.5 and 20–24.5 μM, for 2,2'-dipyridyl, EDDA and desferal, respectively. To obtain sufficient cells for outer membrane preparation, a concentration of chelating agent was chosen that gave significant growth-rate reduction, indicating iron-limited conditions, but which still resulted in relatively high cell numbers. Thus, 120 (S/C 82/1) or 150 (W/D 83/4) μM-2,2'-dipyridyl, 19 μM-EDDA, 22 μM-desferal, 0.8 mg ovotransferrin ml⁻¹ or 1.25 mg bovine transferrin ml⁻¹ were used. OMP profiles of cells grown in the presence of these chelating agents were compared with those of cells grown in BHIB alone, and of cells grown in foetal or newborn calf sera. In all cases cells were harvested during early stationary phase, i.e. after approximately 7–8 h of growth. The complete OMP profiles of isolate S/C 82/1 grown under the various conditions are shown in Fig. 6, whereas the high-molecular-mass regions of both isolates are compared in Fig. 7. There were significant differences in the

Fig. 6. OMP profiles of isolate S/C 82/1 after growth to stationary phase and with high aeration in BHIB (lane 1), BHIB + 120 μM -2,2'-dipyridyl (lane 2), BHIB + 19 μM -EDDA (lane 3), BHIB + 22 μM -desferal (lane 4), BHIB + 0.8 mg ovotransferrin ml^{-1} (lane 5), BHIB + 1.25 mg bovine transferrin ml^{-1} (lane 6), foetal calf serum (lane 7) and newborn calf serum (lane 8). The high-molecular-mass protein (see text) is shown arrowed in lane 8. Molecular mass standards (lane 9) were identical to those in Fig. 3.

expression of the three recognized iron-regulated OMPs as well as in other OMPs after growth in the various media. Growth in the presence of 2,2'-dipyridyl, EDDA or desferal gave identical results for each isolate. There were similar increases in the expression of the 71, 77 and 100 kDa proteins in the case of isolate S/C 82/1 and of the 71 and 100 kDa proteins in the case of isolate W/D 83/4; production of the 77 kDa protein did not, however, increase in isolate W/D 83/4.

In the presence of ovotransferrin and bovine transferrin different results were obtained. Although expression of the 71 kDa protein clearly increased in both isolates, expression of the 100 kDa protein was only slightly enhanced in both isolates, whereas there was no expression of the 77 kDa protein in either isolate; this was particularly noticeable in isolate S/C 82/1.

Expression of both the 71 and 100 kDa proteins was greater in the presence of bovine transferrin than in the presence of ovotransferrin, the latter chelator increasing production of the 100 kDa protein very little, if at all. In addition, in both isolates, there was increased production of the 40.5 kDa protein, as well as synthesis of two new proteins of 23 and 26 kDa after growth in the presence of these two chelators. Once again, there were differences in the expression of the 40.5 kDa protein between cells grown on different occasions but under the same conditions (compare Figs 3 and 6).

Further differences were observed after growth in foetal and newborn calf sera. In foetal calf serum there was, in both isolates, enhanced expression of the 71 kDa protein but not of the 100 kDa protein. There was no enhanced expression of the 77 kDa protein in isolate

Fig. 7. Iron-regulated OMPs of isolate S/C 82/1 (*a*) and W/D 83/4 (*b*) after growth under the conditions described in Fig. 5. Molecular mass markers (lane 9) were phosphorylase b (94 kDa) and bovine serum albumin (67 kDa). A new protein of approximate molecular mass 78 kDa expressed in isolate S/C 82/1 after growth in foetal calf serum is arrowed in (*a*), lane 7. New proteins of 97 and 98 kDa, expressed in both isolates after growth in newborn calf serum, are arrowed in lanes 8 (*a* and *b*). The new high molecular mass protein, expressed in both isolates after growth in newborn calf serum, is also indicated in lanes 8 (arrowhead, *a* and *b*).

S/C 82/1, and no expression at all in isolate W/D 83/4; an additional protein was synthesized in this region in isolate S/C 82/1 (Fig. 7*a*, lane 7, arrowed). In newborn calf serum there was, in both isolates, expression of a protein of apparent molecular mass slightly less than the 100 kDa protein (about 98 kDa). Also, in both isolates, there was no enhanced expression of the 71 kDa protein, and the 77 kDa protein was not produced. In addition, in both isolates, there was synthesis of a novel high-molecular-mass protein as well as an additional protein of apparent molecular mass 97 kDa; expression of 18 and 39.5 kDa proteins was reduced. Two diffuse protein bands of approximate molecular masses 28 and 52 kDa were also present, in both isolates, after growth in newborn calf serum; these proteins were not present in cells grown in foetal calf serum or under any of the other growth conditions. There were no significant differences in the LPS profiles of cells grown under the various conditions, although there did appear to be less LPS produced, in proportion to outer-membrane protein, in the foetal- and newborn-calf-sera-grown cells (Fig. 8).

The effects of addition of 100 μM -FeCl₃ to BHIB containing 50 μM -EDDA or 2 mg ovotransferrin ml⁻¹, or to foetal or newborn calf sera, on the expression of OMPs in isolate S/C 82/1 are shown in Fig. 9. The FeCl₃ repressed the 71, 77 and 100 kDa proteins induced by the EDDA and caused reduced expression of the 40.5 and 71 kDa proteins, repression of the 23 and 26 kDa proteins and normal synthesis of the 77 kDa protein in the presence of ovotransferrin. Addition of 100 μM -FeCl₃ to newborn calf serum did not cause repression of the 98 kDa protein and caused no change to expression of the 71 and 77 kDa proteins, which were expressed to the same extent as in BHIB alone. Furthermore, there was no change in the expression of the high-molecular-mass protein. Addition of FeCl₃ to foetal calf serum caused a marked reduction in the expression of the 71 kDa protein. Interestingly, there was slight induction of the high-molecular-mass protein after addition of FeCl₃ to foetal calf serum. Similar results were obtained for isolate W/D 83/4 after growth in the presence of EDDA or ovotransferrin and FeCl₃.

Fig. 8. LPS profiles of isolate S/C 82/1 after growth under the conditions described in Fig. 6. Samples were standardized with respect to the protein content of the outer membrane (see text). LPS of *E. coli* B4 (O:111) is shown in lane 9.

Discussion

The OMP profiles of the two *P. haemolytica* isolates, SC 82/1 and WD 83/4, were very similar and comprised four or five major proteins and at least twenty minor proteins (Fig. 1). Proteins of apparent molecular masses 18, 29, 39.5, 40.5 and 42 kDa could be classed as major proteins, the last three being in the same molecular mass range as the porin proteins of *E. coli* and other members of the *Enterobacteriaceae* (Lugtenberg *et al.*, 1977). Proteins in the molecular mass range 70–100 kDa included the 71, 77 and 100 kDa iron-regulated proteins of *P. haemolytica* previously described by others (Donachie & Gilmour, 1988; Deneer & Potter, 1989; Ogunnariwo & Schryvers, 1990).

Variation in OMP profiles at different stages of the growth cycle probably reflects changes in nutrient

availability at the different growth stages, although changes due to differences in growth rate *per se* cannot be discounted (Brown & Williams, 1985). It is important to note, furthermore, that changes due to nutrient depletion can actually occur three cell divisions before the onset of stationary phase (Brown & Williams, 1985). In the present study, clear differences in the expression of certain minor OMPs occurred between exponential and stationary phase cells and were, most likely, due to specific nutrient depletion. However, minor proteins, as differentiated by Coomassie blue staining of SDS-polyacrylamide gels, may be more immunogenic than major proteins and appear as dominant bands when analysed by Western blotting (Davies *et al.*, 1990). Such differences in expression of minor proteins may also occur *in vivo* and may be very important in terms of the success or failure of the organism to survive and produce

Fig. 9. OMP profiles of isolate S/C 82/1 after growth in BHIB (lane 1), in BHIB containing 50 μM -EDDA (lanes 2 and 3) or 2 mg ovotransferrin ml^{-1} (lanes 4 and 5), in newborn calf serum (lanes 6 and 7) or in foetal calf serum (lanes 8 and 9). Cells were grown in the absence of added iron (lanes 2, 4, 6 and 8) or in the presence of 100 μM - FeCl_3 (lanes 3, 5, 7 and 9). Molecular mass standards (lane 10) were identical to those shown in Fig. 3.

disease. There were no obvious changes in the OMPs of cells as growth progressed from early stationary phase (6.5 h) to late stationary phase (24 h) thus indicating that the critical period for changes in the expression of certain OMPs was between the exponential phase and early stationary phase.

Growth of *Salmonella* spp. under oxygen-limited conditions has been demonstrated to cause repression of an outer-membrane protein (Schiemann & Shope, 1991) or increased synthesis of certain proteins (Lee & Falkow, 1990), these latter changes in turn leading to an increase in invasiveness. At least three novel OMPs are induced in *N. gonorrhoeae* when grown anaerobically (Clark *et al.*, 1987), one of these being a major antigen recognized by patients' sera (Clark *et al.*, 1988). In a comparison of coagulase-negative staphylococci grown in air or in air containing 5% CO_2 , Denyer *et al.* (1990) demonstrated significant differences in surface-exposed carbohydrates and proteins, as well as changes in cell-wall protein profiles and adherence, and concluded that the *in vivo* gaseous tension is an important factor to be considered in

in vitro models. In the present study, growth, OMPs and LPS were examined under varying degrees of aeration and in an atmosphere of 5% CO_2 /95% air. In general, with the exception of anaerobic growth, the growth rate was not greatly affected by the degree of aeration, whereas the final cell density was significantly affected. In the presence of CO_2 , initial growth was more rapid although the final cell density was less than the corresponding growth in air. Although the presence of CO_2 did not significantly affect the OMP profiles, the degree of aeration affected a number of minor proteins as well as the 40.5 kDa major protein. Synthesis of this latter protein was significantly reduced after growth under anaerobic conditions. The enhanced expression of the 71, 77 and 100 kDa proteins under anaerobic conditions, and their reduced expression as aeration increased, in isolate S/C 82/1, was also significant and suggested that these proteins may be regulated by oxygen as well as by iron. This phenomenon, which has not been described before, did not occur in isolate W/D 83/4, and deserves further attention.

The growth patterns of the two isolates in the presence of the various chelators were unexpected in that growth was rapidly slowed and completely inhibited within a very narrow range of chelator concentration. In additional experiments (results not shown), the inhibitory effects of 50 μM -EDDA and 2 mg ovotransferrin ml^{-1} were overcome by similar small incremental increases in FeCl_3 concentration. For example, 25 μM - FeCl_3 had no effect on inhibition whereas 50 μM - FeCl_3 completely restored growth of both isolates in the presence of 50 μM -EDDA. These results suggest that growth of *P. haemolytica* is finely regulated by micromolar concentrations of iron.

The variation in expression of different OMPs in the presence of the various chelators and in foetal and newborn calf sera was highly significant. In terms of their effects on the induction of various OMPs, the chelators could be divided into two groups, Group 1 consisting of 2,2'-dipyridyl, EDDA and desferal, and Group 2 consisting of ovotransferrin and bovine transferrin. Whereas the Group 1 chelators caused significant increases in the 71, 77 and 100 kDa proteins, as has been described previously (Deneer & Potter, 1989), the Group 2 chelators induced increases in the 71 and 100 (less marked) kDa proteins but not of the 77 kDa protein. In addition, there was increased production of the 40.5 kDa protein and synthesis of two additional proteins, of 23 and 26 kDa, after growth in the presence of Group 2 chelators. Increased synthesis of the 71 and 100 kDa proteins was slightly greater in SC 82/1 than in WD 83/4. Furthermore, the increase in synthesis of the 100 kDa protein in particular was not as great for the Group 2 chelators as it was for the Group 1 chelators. Since previous investigators have used only 2,2'-dipyridyl, EDDA or desferal in iron-limitation experiments with *P. haemolytica* (Donachie & Gilmour, 1988; Deneer & Potter, 1989; Ogunnariwo & Schryvers, 1990; Lainson *et al.*, 1991) these observations are highly significant because they suggest that results obtained with Group 1 chelators may not be strictly representative of the *in vivo* situation. In contradiction to the observation of Deneer & Potter (1989) that the 77 kDa protein was completely absent from iron-replete cells, whereas the 71 and 100 kDa proteins were detectable in iron-replete cells, this study demonstrated that all three proteins were present in iron-replete cells and that their synthesis was increased individually by specific and different conditions. The study also demonstrated that the increased synthesis of the 77 kDa protein in the presence of the Group 1 chelators may be strain dependent. This would explain the failure to detect this protein in an A2 strain of *P. haemolytica* grown under iron-limitation by Donachie & Gilmour (1988).

Further differences occurred in the expression of

OMPs during growth in foetal and newborn calf sera. Expression of the 77 and 100 kDa proteins was not increased, compared to that in BHIB, in either foetal calf serum or newborn calf serum. Although expression of the 71 kDa protein was increased in foetal calf serum, compared to its expression in BHIB, and completely inhibited by the addition of FeCl_3 , it was expressed to the same extent as in BHIB in both neat and iron-supplemented newborn calf serum. These results suggest that foetal calf serum provides an iron-limited environment, and that the 71 kDa protein is an iron-regulated OMP, but that the 77 and 100 kDa proteins may not be important iron-regulated proteins, as the results with Group 1 chelators suggest (Deneer & Potter, 1989). In particular, results with the Group 2 chelators and the animal sera suggest that the 77 kDa protein may not be an important protein *in vivo*. These results support the findings of Donachie & Gilmour (1988), in fact, who were not able to demonstrate synthesis of this protein in an A2 strain of *P. haemolytica* isolated directly from the lungs of an infected sheep, although Deneer & Potter (1989) described synthesis of this protein in iron-restricted A2 cells grown in the presence of 2,2'-dipyridyl. However, as explained above, these discrepancies may have been due to strain variation. It is also notable that the former workers were not able to demonstrate, by Western-blotting with serum from infected sheep, recognition of the 100 kDa protein in the same *in-vivo*-obtained cells, although a faint band was present in a Coomassie-blue-stained SDS-polyacrylamide gel. The non-expression of the 100 kDa protein, the receptor for bovine transferrin (Ogunnariwo & Schryvers, 1990), within the detection limits of Coomassie blue staining at least, in either foetal calf serum or newborn calf serum was unexpected and suggests that the protein may not be produced in significant quantity *in vivo* and may not, therefore, be a good vaccine candidate. This may also help to explain the inability of Donachie & Gilmour (1988) to detect this protein by Western blotting in *in-vivo*-obtained cells. Although the 71 kDa protein was expressed in abundance in the presence of Group 2 chelators as well as in foetal calf serum, indicating its potential as a vaccine candidate, the non-expression of this protein in newborn calf serum raises questions against this possibility which need to be resolved. The results in newborn calf serum suggest that the medium is not iron-limiting, since none of the three previously recognized iron-regulated proteins were produced and addition of FeCl_3 made no difference to the OMP profiles. However, in this medium, at least three additional OMPs were produced, one of which, the 98 kDa protein is highly immunogenic (results not shown). The 98 kDa protein, it should be pointed out, could easily be mistaken for the 100 kDa

protein. Confirmation of their being different proteins was achieved with monospecific polyclonal rabbit antisera raised against each of the proteins (results not shown). The apparent non-regulation of these three additional proteins by iron was unexpected and raises the question as to exactly what induces their synthesis. Reduction in expression of the 18 kDa protein in cells grown in newborn calf serum was probably analogous to the reduced expression of a 17 kDa protein observed in A2 cells obtained from pleural fluid of a sheep, compared to those grown in artificial media, described by Donachie & Gilmour (1988). This observation is significant because it indicates that the *in vitro* growth conditions for the A1 isolates in bovine sera, as described in the present study, are similar to the *in vivo* growth conditions for the A2 strain described by these authors. Identical results, it should be noted, were obtained with two different batches of foetal and newborn calf sera. The two diffuse protein bands of approximate molecular masses 28 and 52 kDa, which occurred in cells grown in newborn calf serum only, probably represented cell-surface-bound IgG light and heavy chains, respectively. Surface-bound IgG light and heavy chains have also been detected in *in-vivo*-grown *E. coli* (Finn *et al.*, 1982) and *P. haemolytica* A2 (Sutherland *et al.*, 1990). Bound serum albumin was not observed, however, as it has been for *in-vivo*-grown *P. haemolytica* A2 (Sutherland *et al.*, 1990).

Although there was no variation in LPS profiles in the presence of various chelators, there was quantitative variation in LPS, relative to the amount of protein in the outer membrane, in cells grown in foetal and newborn calf sera compared to cells grown under other conditions. In addition, there were differences in the LPS of cells grown aerobically compared to cells grown anaerobically, as well as differences between exponential and stationary phase cells (Davies *et al.*, 1991). Changes in LPS, due to differences in aeration, have also been described in *N. meningitidis* (Tsai *et al.*, 1983). It might be expected from these observations, therefore, that there would be some quantitative differences in the LPS of cells that had been grown *in vivo* compared to cells grown in standard laboratory media. Although Sutherland *et al.* (1990) observed no differences in the LPS profiles of *P. haemolytica* cells grown *in vivo* compared to cells grown *in vitro*, their study was carried out with an A2 strain, possessing a rough-type LPS. Differences in the high-molecular-mass region of the LPS molecule, i.e. the O-antigen, may well occur between *in-vivo*- and *in-vitro*-grown A1 cells.

In conclusion, this study has demonstrated significant differences in the synthesis of certain *P. haemolytica* OMPs under various growth conditions. In particular, the OMP profiles of cells grown in foetal or newborn calf

sera are different to each other and to those of cells grown in the presence of artificial chelating agents. The study raises questions as to the use of these chelators and the conclusions derived from previous studies. Subtle differences were also observed in the LPS profiles of cells grown under various conditions. Finally, the study has highlighted the importance of examining the OMP and LPS profiles of cells grown *in vivo* and this work is currently underway.

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References

- ANWAR, H., BROWN, M. R. W., DAY, A. & WELLER, P. H. (1984). Outer membrane antigens of mucoid *Pseudomonas aeruginosa* isolated directly from the sputum of a cystic fibrosis patient. *FEMS Microbiology Letters* **24**, 235–239.
- BANERJEE-BHATNAGAR, N. & FRASCH, C. E. (1990). Expression of *Neisseria meningitidis* iron-regulated outer membrane proteins, including a 70-kilodalton transferrin receptor, and their potential for use as vaccines. *Infection and Immunity* **58**, 2875–2881.
- BIBERSTEIN, E. L. (1978). Biotyping and serotyping of *Pasteurella haemolytica*. *Methods in Microbiology* **10**, 253–268.
- BLACK, J. R., DYER, D. W., THOMPSON, M. K. & SPARLING, P. F. (1986). Human immune response to iron-repressible outer membrane proteins of *Neisseria meningitidis*. *Infection and Immunity* **54**, 710–713.
- BLASER, M. J., HOPKINS, J. A., BERKA, R. M., VASIL, M. L. & WANG, W.-L. L. (1983). Identification and characterization of *Campylobacter jejuni* outer membrane proteins. *Infection and Immunity* **42**, 276–284.
- BOLIN, C. A. & JENSEN, A. E. (1987). Passive immunization with antibodies against iron-regulated outer membrane proteins protects turkeys from *Escherichia coli* septicaemia. *Infection and Immunity* **55**, 1239–1242.
- BROWN, M. R. W. & WILLIAMS, P. (1985). The influence of environment on envelope properties of bacteria in infections. *Annual Review of Microbiology* **39**, 527–556.
- BROWN, M. R. W., ANWAR, H. & LAMBERT, P. A. (1984). Evidence that mucoid *Pseudomonas aeruginosa* in the cystic fibrosis lung grows under iron-restricted conditions. *FEMS Microbiology Letters* **21**, 113–117.
- CARTER, G. R. (1967). Pasteurellosis: *Pasteurella multocida* and *Pasteurella haemolytica*. *Advances in Veterinary Science* **11**, 321–379.
- CHART, H., BUCK, M., STEVENSON, P. & GRIFFITHS, E. (1986). Iron regulated outer membrane proteins of *Escherichia coli*: variations in expression due to the chelator used to restrict the availability of iron. *Journal of General Microbiology* **132**, 1373–1378.
- CLARK, V. L., CAMPBELL, L. A., PALERMO, D. A., EVANS, T. E. & KLIMPEL, K. W. (1987). Induction and repression of outer membrane proteins by anaerobic growth of *Neisseria gonorrhoeae*. *Infection and Immunity* **55**, 1359–1364.
- CLARK, V. L., KNAPP, J. S., THOMPSON, S. & KLIMPEL, K. W. (1988). Presence of antibodies to the major anaerobically induced gonococcal outer membrane protein in sera from patients with gonococcal infections. *Microbial Pathogenesis* **5**, 381–390.
- CONFER, A. W., LESSLEY, B. A., PANCIERA, R. J., FULTON, R. W. & RUMMAGE, J. A. (1985). Effect of vaccination with live or killed *Pasteurella haemolytica* on resistance to experimental bovine pneumonic pasteurellosis. *American Journal of Veterinary Research* **46**, 342–347.
- CONFER, A. W., PANCIERA, R. J. & MOSIER, D. A. (1986). Serum antibodies to *Pasteurella haemolytica* lipopolysaccharide: relation-

- ship to experimental bovine pneumonic pasteurellosis. *American Journal of Veterinary Research* **47**, 1134-1138.
- CRAVEN, R. C., CONFER, A. W. & GENTRY, M. J. (1991). Cloning and expression of a 30 kDa surface antigen of *Pasteurella haemolytica*. *Veterinary Microbiology* **27**, 63-78.
- DAVIES, R. L. (1991). Outer membrane protein profiles of *Yersinia ruckeri*. *Veterinary Microbiology* **26**, 125-140.
- DAVIES, R. L., WALL, R. A. & BORRIELLO, S. P. (1990). Comparison of methods for the analysis of outer membrane proteins of *Neisseria meningitidis* by Western blotting. *Journal of Immunological Methods* **134**, 215-225.
- DAVIES, R. L., ALI, Q., PARTON, R., COOTE, J. G., GIBBS, A. H. & FREER, J. H. (1991). Optimal conditions for the analysis of *Pasteurella haemolytica* lipopolysaccharide by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. *FEMS Microbiology Letters* **90**, 23-28.
- DENEER, H. G. & POTTER, A. A. (1989). Iron-repressible outer membrane proteins of *Pasteurella haemolytica*. *Journal of General Microbiology* **135**, 435-443.
- DENYER, S. P., DAVIES, M. C., EVANS, J. A., FINCH, R. G., SMITH, D. G. E., WILCOX, M. H. & WILLIAMS, P. (1990). Influence of carbon dioxide on the surface characteristics and adherence potential of coagulase-negative *Staphylococci*. *Journal of Clinical Microbiology* **28**, 1813-1817.
- DONACHIE, W. & GILMOUR, N. J. L. (1988). Sheep antibody response to cell wall antigens expressed *in vivo* by *Pasteurella haemolytica* serotype A2. *FEMS Microbiology Letters* **56**, 271-276.
- DURHAM, J. A., CONFER, A. W., MOSIER, D. A. & LESLEY, B. A. (1986). Comparison of the antigens associated with saline, potassium thiocyanate, and sodium salicylate extracts of *Pasteurella haemolytica* serotype 1. *American Journal of Veterinary Research* **47**, 1946-1951.
- FILIP, C., FLETCHER, G., WULF, J. L. & EARHART, C. F. (1973). Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium lauryl sarcosinate. *Journal of Bacteriology* **115**, 717-722.
- FINN, T. M., ARBUTHNOTT, J. P. & DOUGAN, G. (1982). Properties of *Escherichia coli* *in vivo* using a chamber implant system. *Journal of General Microbiology* **128**, 3083-3091.
- FRANK, G. H. (1979). *Pasteurella haemolytica* and respiratory disease in cattle. *Proceedings of the United States Animal Health Association* **83**, 153-160.
- FODOR, L., VARJA, J., HAJTOS, J., DONACHIE, W. & GILMOUR, N. J. L. (1988). Characterization of a new serotype of *P. haemolytica* isolated in Hungary. *Research in Veterinary Science* **44**, 399.
- GILMOUR, N. J. L., ANGUS, K. W., DONACHIE, W. & FRASER, J. (1982). Vaccination against experimental pneumonic pasteurellosis. *Veterinary Record* **110**, 450.
- GILMOUR, N. J. L., DONACHIE, W., SUTHERLAND, A. D., GILMOUR, J. S., JONES, G. E. & QUIRIE, M. (1991). Vaccination containing iron-regulated proteins of *Pasteurella haemolytica* A2 enhances protection against experimental pasteurellosis in lambs. *Vaccine* **9**, 137-140.
- 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.
- HITCHCOCK, P. J. & BROWN, T. M. (1983). Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. *Journal of Bacteriology* **154**, 269-277.
- KELLY, J. T. & PARKER, C. D. (1981). Identification and preliminary characterization of *Vibrio cholerae* outer membrane proteins. *Journal of Bacteriology* **145**, 1018-1024.
- KELLY, N. M., BELL, A. & HANCOCK, R. E. W. (1989). Surface characteristics of *Pseudomonas aeruginosa* grown in a chamber implant model in mice and rats. *Infection and Immunity* **57**, 344-350.
- KNIGHTS, J. M., ADLAM, C. & OWEN, P. (1990). Characterization of envelope proteins from *Pasteurella haemolytica* and *Pasteurella multocida*. *Journal of General Microbiology* **136**, 495-505.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680-685.
- LAINSON, F. A., HARKINS, D. C., WILSON, C. F., SUTHERLAND, A. D., MURRAY, J. E., DONACHIE, W. & BAIRD, G. D. (1991). Identification and localization of an iron-regulated 35 kDa protein of *Pasteurella haemolytica* serotype A2. *Journal of General Microbiology* **137**, 219-226.
- LEE, C. A. & FALKOW, S. (1990). The ability of *Salmonella* to enter mammalian cells is affected by bacterial growth state. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 4304-4308.
- LESLEY, B. A., CONFER, A. W., MOSIER, D. A., GENTRY, M. J., DURHAM, J. A. & RUMMAGE, J. A. (1985). Saline-extracted antigen of *Pasteurella haemolytica*: separation by chromatofocusing. Preliminary characterization and evaluation of immunogenicity. *Veterinary Immunology and Immunopathology* **10**, 279-296.
- LOEB, M. R. & SMITH, D. H. (1980). Outer membrane protein composition in disease isolates of *Haemophilus influenzae*: pathogenic and epidemiological implications. *Infection and Immunity* **30**, 709-717.
- LUGTENBERG, B., BRONSTEIN, H., VAN SELM, N. & PETERS, R. (1977). Peptidoglycan-associated outer membrane proteins in Gram-negative bacteria. *Biochimica et Biophysica Acta* **465**, 571-578.
- MANDRELL, R. E., LESSE, A. J., SUGAL, J. V., SHERO, M., GRIFFISS, J. M., COLE, J. A., PARSONS, N. J., SMITH, H., MORSE, S. & APICELLA, M. A. (1990). *In vitro* and *in vivo* modifications of *Neisseria gonorrhoeae* lipopolysaccharide epitope structure by sialylation. *Journal of Experimental Medicine* **171**, 1649-1664.
- MARKWELL, M. A. K., HAAS, S. M., BIEBER, L. L. & TOLBERT, N. E. (1978). A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Analytical Biochemistry* **87**, 206-210.
- NELSON, S. L. & FRANK, G. H. (1989). Purification and characterization of a 94 kDa *Pasteurella haemolytica* antigen. *Veterinary Microbiology* **21**, 57-66.
- OGUNNARIWO, J. A. & SCHRYVERS, A. B. (1990). Iron acquisition in *Pasteurella haemolytica*: expression and identification of a bovine-specific transferrin receptor. *Infection and Immunity* **58**, 2091-2097.
- PETTERSSON, A., KUIPERS, B., PELZER, M., VERHAGEN, E., TIESJEMA, R. H., TOMMASSEN, J. & POOLMAN, J. T. (1990). Monoclonal antibodies against the 70-kilodalton iron-regulated protein of *Neisseria meningitidis* are bactericidal and strain specific. *Infection and Immunity* **58**, 3036-3041.
- PURDY, C. W., LIVINGSTON, C. W., FRANK, G. H., CUMMINS, J. H., COLE, N. A. & LOAN, R. W. (1986). A live *Pasteurella haemolytica* vaccine efficacy trial. *Journal of American Veterinary Medical Association* **188**, 589-591.
- SCHIEHMANN, D. A. & SHOPE, S. R. (1991). Anaerobic growth of *Salmonella typhimurium* results in increased uptake by Henle 407 epithelial and mouse peritoneal cells *in vitro* and repression of a major outer membrane protein. *Infection and Immunity* **59**, 437-440.
- SHEWEN, P. E. & WILKIE, B. N. (1983). *Pasteurella haemolytica* cytotoxin neutralizing activity in sera from Ontario beef cattle. *Canadian Journal of Comparative Medicine* **47**, 497-498.
- SHEWEN, P. E. & WILKIE, B. N. (1988). Vaccination of calves with leukotoxic culture supernatant from *Pasteurella haemolytica*. *Canadian Journal of Veterinary Research* **52**, 30-36.
- SMITH, H. (1990). Pathogenicity and the microbe *in vivo*. *Journal of General Microbiology* **136**, 377-383.
- SQUIRE, P. G., SMILEY, D. W. & CROSKELL, R. B. (1984). Identification and extraction of *Pasteurella haemolytica* membrane proteins. *Infection and Immunity* **45**, 667-673.
- SUTHERLAND, A. D., JONES, G. E. & POXTON, I. R. (1990). The susceptibility of *in vivo*-grown *Pasteurella haemolytica* to ovine defence mechanisms *in vitro*. *FEMS Microbiology Immunology* **64**, 269-278.
- TSAI, C. M., BOYKINS, R. & FRASCH, C. E. (1983). Heterogeneity and variation among *Neisseria meningitidis* lipopolysaccharides. *Journal of Bacteriology* **155**, 498-504.
- TSAI, C. M. & FRASCH, C. E. (1982). A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Analytical Biochemistry* **119**, 115-119.
- WARD, K. H., ANWAR, H., BROWN, M. R. W., WALE, J. & GOWAR, J. (1988). Antibody response to outer membrane antigens of *Pseudomonas aeruginosa* in human burn wound infection. *Journal of Medical Microbiology* **27**, 179-190.

WILKIE, B. N., MARKHAM, R. J. F. & SHEWEN, P. E. (1980). Response of calves to lung challenge exposure with *Pasteurella haemolytica* after parental or pulmonary immunization. *American Journal of Veterinary Research* **41**, 1773–1778.

YATES, W. D. G., STOCKDALE, P. H. G., BABIUK, L. A. & SMITH, R. J. (1983). Prevention of experimental bovine pneumonic pasteurellosis with an extract of *Pasteurella haemolytica*. *Canadian Journal of Comparative Medicine* **47**, 250–256.