Variation in outer-membrane protein and lipopolysaccharide profiles of Pasteurella haemolytica isolates of serotypes A1 and A2 obtained from pneumonic and healthy cattle

J. McCluskey,1 H. A. Gibbs2 and R. L. Davies1

Author for correspondence: R. L. Davies. Tel: +44 41 339 8855 ext. 6685. Fax: +44 41 330 4600.

The outer-membrane protein (OMP) and lipopolysaccharide (LPS) profiles of 29 isolates of Pasteurella haemolytica serotypes A1 (18 isolates) and A2 (11 isolates), obtained from pneumonic (13 isolates) or healthy (16 isolates) cattle, were compared by SDS-PAGE and Western blot analysis. Coomassie-blue-stained OMP profiles of serotype A1 and A2 isolates could be distinguished from each other by differences in both major and minor proteins. Whereas the OMP profiles of the serotype A1 isolates were extremely uniform in stained gels, there was variation in the mobilities of high-molecular-mass minor proteins and one of the major proteins of serotype A2 isolates. Differences in the OMP profiles of isolates within both the A1 and A2 serotypes were more clearly distinguished by Western blotting than by staining after SDS-PAGE. Thus, by Western blot analysis, four distinct OMP profiles were identified within the serotype A1 and A2 isolates, respectively. The profiles of the serotype A1 isolates were designated OMP types 1.1, 1.2, 1.3 and 1.4; those of the serotype A2 isolates were designated OMP types 2.1, 2.2, 2.3 and 2.4. Three distinct LPS profiles were recognized among the isolates which, by comparison with previously described LPS types, were identified as smooth LPS type 1 and rough LPS types 3 and 5. Isolates of serotype A1 consisted of LPS type 1 only, whereas isolates of serotype A2 consisted of LPS types 3 or 5. OMP and LPS analysis of P. haemolytica has applications in epidemiological and virulence studies.

Keywords: Pasteurella haemolytica, outer-membrane protein, lipopolysaccharide

INTRODUCTION

The Gram-negative bacterium Pasteurella haemolytica is the aetiological agent of bovine pneumonic pasteurellosis or 'shipping fever', a disease resulting in significant economic losses to the beef and dairy industries of both the USA and Europe (Frank, 1989). P. haemolytica comprises two biotypes, A and T, based on the fermentation of arabinose and trehalose, and 16 serotypes, based on soluble capsular antigens (Biberstein, 1978; Fodor et al., 1988). It has been proposed, however, that P. haemolytica biotype T be renamed Pasteurella trehalosi, sp. nov. (Sneath & Stevens, 1990). Whereas A1 is the predominant serotype recovered from cattle with pneumonic pasteurellosis, serotype A2 isolates are less frequently associated with pneumonic cattle although they are often isolated from the nasopharynx of healthy animals (Frank, 1989). Conversely, serotype A2 is predominantly recovered from cases of pneumonic pasteurellosis in sheep; serotype A1 isolates are recovered less frequently in this species (Gilmour & Gilmour, 1989). Very little is known, however, about strain variation within these two serotypes and whether isolates within each of the serotypes differ in other characteristics which could be used as epidemiological and/or virulence markers.

Analysis of outer-membrane protein (OMP) and lipopolysaccharide (LPS) profiles by SDS-PAGE and Western blotting has been widely used for examining strain variation within species and has applications in both epidemiological and virulence studies of bacterial patho-
Table 1. Details of *P. haemolytica* isolates used in the study

<table>
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<th>Laboratory designation</th>
<th>Date of isolation</th>
<th>Origin of isolate</th>
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<td>Healthy</td>
<td>2.2</td>
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NK, Not known.

* Numbers 1–6 indicate the different farms that isolates originated from.

† L, Lungs; NP, nasopharynx.

Although the OMP profiles of *P. haemolytica* have been examined previously (Squire et al., 1984; Deneer & Potter, 1989; Knights et al., 1990; Davies et al., 1992), these studies have been restricted to a relatively small number of isolates representative of the various serotypes; little is known about variation of OMP profiles within the species or within different serotypes. Similarly, few studies have been carried out on the variation of LPS within *P. haemolytica*, although Ali et al. (1992) described five LPS types within serotype A1 and A2 isolates, and Lacroix et al. (1993) examined the LPS of single isolates representative of each of the 16 serotypes.

The objectives of the present study, therefore, were to examine and compare the OMP and LPS profiles of a selection of serotype A1 and A2 *P. haemolytica* isolates, obtained from both pneumonic and healthy cattle on a small number of farms, with the aim of identifying...
Fig. 1. Coomassie-blue-stained SDS-PAGE OMP profiles of P. haemolytica isolates of serotypes A1 (isolate PH188) and A2 (isolate PH210) in lanes 1 and 2, respectively. Arrows indicate differences in the OMP profiles (see text). Molecular mass standards (kDa) are shown in lane 3.

similarities and differences within isolates which could be of epidemiological significance or prove useful as virulence markers.

METHODS

Bacterial strains and growth conditions. Twenty nine isolates of P. haemolytica were examined in the present study. The isolates comprised 18 of serotype A1, including eight from pneumonia and 10 from healthy cattle, and 11 of serotype A2, including five from pneumonia and six from healthy cattle. Isolates from healthy cattle were obtained from the nasopharynx whereas those from pneumonia were obtained from the lungs. The isolations were made over a 30 month period from six farms located in central Scotland. Details of the isolates are given in Table 1.

After primary isolation, isolates were stored in brain heart infusion broth (BHIB; Oxoid) containing 50% (v/v) glycerol at −70 °C and were routinely subcultured on brain heart infusion agar (BHIA; Oxoid) containing 5% (v/v) sheep's blood at 37 °C. For preparation of outer membranes, bacteria were grown in 500 ml BHIB at 37 °C, with shaking at 120 r.p.m., to early stationary phase (approximately 7 h).

Serotyping. Isolates were serotyped by indirect haemagglutination assay with bovine red blood cells as described by Shreeve et al. (1972).

Preparation of OMPs and LPS. OMPs were obtained by Sarkosyl extraction (Filip et al., 1973) as previously described (Davies et al., 1992). LPS was obtained by proteinase K digestion (Hitchcock & Brown, 1983) of outer membranes as previously described (Davies et al., 1992), with the exception that 2× sample buffer consisted of 0·125 M Tris/HCl (pH 6·8), 20% (v/v) glycerol, 4% (w/v) SDS, 10% (v/v) 2-mercaptoethanol and 0·0025% (w/v) bromophenol blue.

Protein assay. The protein concentration of outer membrane samples was assayed by the modified Lowry procedure described by Markwell et al. (1978).

SDS-PAGE. OMPs and LPS were separated by SDS-PAGE using the SDS discontinuous system of Laemmli (1970) as previously described (Davies et al., 1992). OMPs were separated in 12% (w/v) resolving gels whereas LPS was separated in 15% (w/v) resolving gels containing 4 M urea. The protein molecular mass standards (Pharmacia) used were 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). Proteins were visualized by staining with Coomassie Brilliant Blue and LPS by silver staining (Tsai & Frasch, 1982).

Antiserum production. Bovine antiserum was raised against live cells of isolate S/C 82/1 in a Herefordshire calf as follows. Bacteria were grown overnight in BHIB, washed and resuspended in PBS (150 mM NaCl, 7·2 mM Na₂HPO₄ 2H₂O, 28 mM NaH₂PO₄, pH 7·2) to 10⁹ c.f.u. ml⁻¹, and mixed with an equal volume of Freund’s incomplete adjuvant; 6 ml volumes of the mixture (3×10⁹ c.f.u.) were injected intramuscularly on days 0, 14, 35 and 57 and the animal was bled on day 67 after weekly test-bleeding.

Western blotting. Western blotting was performed essentially as described previously (Davies et al., 1990). OMPs were transferred to nitrocellulose (Schleicher and Schuell) in a Bio-Rad Trans-Blot cell with plate electrodes, following the manufacturer’s instructions. Transfer was carried out overnight at a constant voltage of 15 V in buffer containing 25 mM Tris/HCl and 192 mM glycine (pH 8·3). After transfer the blotting membranes were washed twice in 20 mM Tris/HCl, 500 mM NaCl buffer, pH 7·5 (TBS), for 5 min. Non-specific binding was blocked by incubation for 1 h in TBS containing 3% (w/v) gelatin. The nitrocellulose membranes were washed (two 5 min washes) in 0·05% (v/v) Tween 20 in 20 mM Tris/HCl, 500 mM NaCl buffer, pH 7·5 (TBS), for 5 min. Non-specific binding was blocked by incubation for 1 h in TBS containing 3% (w/v) gelatin. The nitrocellulose membranes were washed (two 5 min washes), followed by one wash in TBS (5 min), and incubated overnight in bovine antiserum diluted 1 in 500 in 1% (w/v) gelatin in TBS. After two 5 min washes in TBS the membranes were incubated for 4 h with horseradish peroxidase-conjugated anti-bovine IgG antibodies diluted 1 in 2000 in 1% gelatin in TBS. The membranes were washed in TBS (two 5 min washes), followed by one wash in TBS (5 min), and developed in a substrate solution containing 0·05% (w/v) 4-chloro-1-naphthol (dissolved in 20 ml ice-cold methanol) and 0·05% (v/v) H₂O₂ in 100 ml TBS. Development was stopped by immersing the nitrocellulose in distilled water for 10 min.
RESULTS

SDS-PAGE analysis of OMPs

The OMP profiles, in Coomassie-blue-stained gels, of isolates of both serotypes consisted of three or four major proteins and approximately 15–20 minor proteins (Fig. 1). The profiles of the serotype A1 isolates were similar but not identical to those of the serotype A2 isolates. There were differences in the mobilities of the upper major protein, of various high-molecular-mass proteins (>
OMP and LPS variation in *Pasteurella haemolytica*.

Fig. 3. (a) Western blot showing reaction of a 1:200 dilution of bovine anti-S/C 82/1 whole-cell antiserum against OMPs of *P. haemolytica* serotype A1 disease isolates PH162 (OMP type 1.1), PH164 and PH166 (OMP type 1.2), PH168 and PH170 (OMP type 1.3), PH172 and PH174 (OMP type 1.2), and non-disease isolates PH180 and PH184 (OMP type 1.2) and PH176, PH178 and PH182 (OMP type 1.4), in lanes 1–12, respectively. (b) Western blot showing reaction of a 1:200 dilution of bovine anti-S/C 82/1 whole cell antiserum against OMPs of *P. haemolytica* serotype A2 disease isolates PH196, PH198 and PH200 (OMP type 2.1) and PH202 and PH204 (OMP type 2.2), and non-disease isolates PH212, PH210 and PH208 (OMP type 2.3), PH214 (OMP type 2.4) and PH216 and PH218 (OMP type 2.2), in lanes 1–11, respectively. Approximate molecular masses (kDa) are indicated on the right of each part. OMP types are indicated along the bottom; arrows indicate differences between OMP types (see text).

67 kDa) and of proteins in the molecular mass ranges 20.1–30 and 43–67 kDa. These differences are shown arrowed in Fig. 1. The 18 serotype A1 isolates had very uniform OMP profiles when examined by Coomassie blue staining, although there was some quantitative variation in certain proteins, and individual isolates could not readily be distinguished from each other (Fig. 2a). The profiles of the 11 serotype A2 isolates in stained gels were more variable, however, and individual isolates could be distinguished from each other on the basis of variation in the mobilities of certain proteins (Fig. 2b). For example, differences were observed in the mobilities of high-molecular-mass proteins between isolates PH196, PH198 and PH200 (Fig. 2b, lanes 1–3; arrows), isolates PH202 and PH204 (Fig. 2b, lanes 4 and 5; arrows) and isolate PH214 (Fig. 2b, lane 9; upper arrows). In addition, the mobility of the upper major protein of isolate PH214 differed from that of the other isolates (Fig. 2b, lanes 8 and 9; lower arrows). Overall, however, differences in the OMP profiles, particularly within the serotype A1 isolates, were not easily distinguished by protein staining after SDS-PAGE. This technique was therefore supplemented with Western blotting, which identified previously unrecognized differences within the serotype A1 isolates and was able to more clearly differentiate between the various OMP profiles within the serotype A2 isolates (Fig. 3a, b). Differences in the immunological recognition of various proteins formed the basis of an OMP-subtyping scheme and enabled four distinct OMP types to be distinguished within each of the serotype A1 and A2 groups of isolates.

**Western blot profiles**

The four OMP profiles identified by Western blotting within the 18 serotype A1 isolates were designated OMP types 1.1, 1.2, 1.3 and 1.4; these are indicated in Fig. 3(a). The serotype A1 OMP types could not be differentiated in Coomassie-blue-stained gels and were based on variation in immunological reactions to minor proteins in the molecular mass range 30–40 kDa (protein bands a, b, c and d in Fig. 3a). Four different OMP profiles were also identified by Western blotting within the 11 serotype A2 isolates, which were designated OMP types 2.1, 2.2, 2.3 and 2.4; these are indicated in Fig. 3(b). The serotype A2 OMP types were distinguished by immunological reactions to minor proteins in the molecular mass range 30–50 kDa (protein bands a, b, c and d in Fig. 3b). The serotype A2 OMP types were more easily distinguished by Western blotting than were the serotype A1 OMP types. Within the serotype A2 isolates, those of OMP type 2.1 differed from the others in that a wider range of proteins was detected and high background staining occurred (Fig. 3b, lanes 1–3).
LPS analysis

The LPS of serotype A1 and A2 isolates of *P. haemolytica* exists either in the smooth or rough form, depending on the presence or absence, respectively, of O-antigen side-chains (Davies *et al.*, 1991; Ali *et al.*, 1992). The O-antigen side-chains are discernible as a series of closely spaced high-molecular-mass bands forming a distinctive ladder-like pattern (Davies *et al.*, 1991). Two smooth (types 1 and 2) and three rough (types 3, 4 and 5) LPS types have previously been described in serotype A1 and A2 isolates of *P. haemolytica* (Ali *et al.*, 1992). In the present study three LPS types were demonstrated, one smooth and two rough, which were compared with the LPS types described previously and demonstrated to be equivalent to LPS types 1, 3 and 5, respectively (Fig. 4).

The 18 serotype A1 isolates all possessed smooth LPS of type 1 (Fig. 4, lanes 2 and 3). However, the 11 serotype A2 isolates possessed one of two different rough LPS types, this variation being based on differences in the mobilities of low-molecular-mass bands representing the core-oligosaccharide region. Three isolates possessed rough LPS of type 3 (Fig. 4, lanes 6 and 7), whereas the remaining eight isolates possessed rough LPS of type 5 (Fig. 4, lanes 10 and 11).

The relationship between serotype, OMP type, LPS type, origin of the isolates and disease status of the host is shown in Table 1. Serotype A1 disease isolates, all of which possessed LPS of type 1, were obtained from farms 1, 2, 3 and 4 and could be differentiated on the basis of their OMP types, i.e. types 1.1, 1.2 or 1.3. Serotype A1 non-disease isolates, all of which also possessed LPS of type 1, were obtained from farm 5 and the majority were of OMP type 1.4; two isolates were of OMP type 1.2. The situation was more complex within the serotype A2 isolates because two different LPS types were present, i.e. types 3 and 5. Isolates of OMP type 2.1 were associated only with LPS type 3; the remaining isolates possessed LPS type 5 but could be distinguished by having one of three different OMP types, i.e. types 2.2, 2.3 or 2.4. Serotype A2 disease isolates were obtained from farm 6 and formed two groups which could clearly be distinguished by both their OMP and LPS types, i.e. OMP/LPS types 2.1/3 and 2.2/5. Serotype A2 non-disease isolates were obtained from farm 5, were all of LPS type 5 and could be differentiated by their OMP types, i.e. types 2.2, 2.3 or 2.4. When serotype A1 and A2 isolates from farm 5 were considered together, at least 5 groups of isolates were present, i.e. OMP/LPS types 1.2/1, 1.4/1, 2.2/5, 2.3/5 and 2.4/5. However, these isolates were obtained on three separate occasions (1.85, 2.85 and 3.85) and, as can be seen from Table 1, the various OMP/LPS types were isolated on only one of the three occasions.

**DISCUSSION**

The objective of the present study was to examine and compare the OMP and LPS profiles of a selection of *P. haemolytica* isolates of serotypes A1 and A2, obtained from both pneumonic and healthy cattle, with the aim of detecting differences and similarities which could prove useful in epidemiological and virulence studies. The OMP and LPS profiles of the 29 isolates examined in SDS-polyacrylamide gels were similar to those described in previous studies (Ali *et al.*, 1992; Davies *et al.*, 1992). The OMP profiles consisted of three to four major proteins and approximately 15–20 minor proteins, and the LPS profiles were either of the smooth or rough chemotypes. Based on variation in the OMP and LPS profiles the study revealed significant heterogeneity both between and within the serotype A1 and A2 isolates. Thus, the serotype A1 isolates differed from the serotype A2 isolates in the mobilities of a major protein and various minor proteins, as well as in the possession of smooth as opposed to rough LPS. Variation of OMP profiles within the serotype A1 isolates could only be detected by Western blotting, but variation within the serotype A2 isolates was detected both in stained gels and by Western blotting. Four distinct OMP types were identified within both the serotype A1 and A2 groups of isolates by Western blotting. The serotype A2 isolates being more clearly differentiated than the serotype A1 isolates. The LPS profiles were compared to those described previously (Ali *et al.*, 1992) and demonstrated to be of smooth type 1 or rough types 3 or 5. The 18 serotype A1 isolates comprised OMP types 1.1, 1.2, 1.3 and 1.4 and LPS type 1; the 11
serotype A2 isolates comprised OMP types 2.1, 2.2, 2.3 and 2.4 and LPS types 3 and 5.

Previous studies on the OMP profiles of *P. haemolytica* have not demonstrated significant differences either between serotype A1 and A2 isolates or within serotypes. In a study of envelope proteins of *P. haemolytica* isolates representing each of the 16 serotypes, Knights *et al.* (1990) were unable to distinguish individual serotypes from one another. Deneer & Potter (1989) described variation between serotypes in the expression of certain iron-regulated OMPs when cells were grown under iron-restricted growth conditions. However, these authors did not observe significant differences between serotype A1 and A2 isolates. The present study has confirmed previous findings that serotype A1 and A2 isolates of *P. haemolytica* differ in their LPS profiles (Ali *et al.*, 1992); it has also demonstrated that serotype A1 and A2 isolates differ in the expression of various OMPs. However, it should be noted that certain similarities also exist between some isolates of serotypes A1 and A2. For example, the core-oligosaccharide regions of LPS types 1 and 3, which are present in certain serotype A1 and A2 isolates, respectively, are identical (Ali *et al.*, 1992). It is, in fact, the presence of type 3 LPS which accounts for the enhanced immunological staining of the OMP type 2.1 profiles (Fig. 3b, lanes 1–3), since the primary antiserum was raised against an isolate containing type 1 LPS. Type 3 LPS associated with the OMPs of serotype A2 isolates of OMP type 2.1/LPS type 3 would account for this cross-reactivity. Such an association between OMPs and LPS in Western blots has been described in *Pseudomonas aeruginosa* by Poxton *et al.* (1985). The differences observed in both the OMP and LPS profiles of isolates of serotypes A1 and A2, as well as differences in capsule structure (Adlam *et al.*, 1984, 1987) and host specificity, suggest that isolates of these two serotypes may not be as closely related as previously considered. By contrast, similar work within this laboratory suggests that isolates of other biotype A serotypes are more closely related to isolates of serotype A1 than to those of serotype A2.

In the present study we have examined and compared the OMP and LPS profiles of *P. haemolytica* isolates of serotypes A1 and A2 in stained SDS-polyacrylamide gels; we have further investigated the OMP profiles by Western blotting. Although SDS-PAGE alone was able to differentiate between some isolates of serotype A2, the method was unable to discriminate between isolates of serotype A1. Western blotting, however, was able to differentiate more clearly between isolates of serotype A2 and was also able to distinguish between isolates of serotype A1. Similar findings were reported by Mulligan *et al.* (1988) and Hansman & Lawrence (1993), who found Western blotting to have greater resolving power than SDS-PAGE alone in studies on *Clostridium difficile* and *H. influenzae*, respectively. It was concluded, therefore, that Western blotting has greater powers of discrimination than SDS-PAGE alone and is a more useful tool for investigating strain variation within *P. haemolytica*. Although only a single antiserum was used in the present study, it was still able to differentiate between isolates of both serotypes. It is likely that the LPS type, rather than the serotype, of the isolate used to generate the antiserum will have a greater influence on the pattern of protein recognition in Western blots for reasons discussed above. LPS analysis was found to be less discriminating than OMP analysis. However, since additional LPS types occur within serotype A1 and A2 isolates of *P. haemolytica* (Ali *et al.*, 1992) it is important that LPS analysis should also be included in such studies.

Based on differences in OMP and LPS profiles the present study has demonstrated that serotype A1 and A2 populations of *P. haemolytica* are more diverse than previously thought. In addition, the study has provided evidence to suggest that these differences may be useful in epidemiological studies. For example, the serotype A1 isolates from farms 1, 2, 3 and 4 could be distinguished in terms of their OMP types even though the differences were slight. In addition, isolates obtained from farm 5 on three separate occasions could be distinguished in terms of their serotypes, OMP types and LPS types. Although the number of isolates in the present study was relatively small the examples described above nevertheless demonstrate that variation in the OMP and LPS profiles of serotype A1 and A2 isolates of *P. haemolytica* does occur, and that this variation can be detected by the methods described. Although there was no clear correlation of a particular OMP or LPS type with virulence in the isolates examined, with the possible exception of serotype A2 isolates of OMP type 2.1/LPS type 3, further work on a larger numbers of isolates may be useful in demonstrating such a relationship.

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**REFERENCES**


