Outer membrane proteins of bovine strains of *Pasteurella multocida* type A and their doubtful role as protective antigens

M. Z. ABDULLAHI,* N. J. L. GILMOUR and I. R. POXTONT

Moredun Research Institute, 408 Gilmerton Road, Edinburgh EH17 7JH, and tDepartment of Bacteriology, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG

Summary. Outer membranes were prepared by the Sarkosyl method from 30 strains of *Pasteurella multocida* and the closely related Taxon 13, which had been isolated from cattle. The patterns of the outer membrane proteins (OMPs) on SDS-PAGE were generally similar to one another, though the four major proteins (a–d) varied somewhat in molecular mass; these patterns allowed the strains to be arranged into 12 groups. Taxon 13 strains and typical *P. multocida* strains were indistinguishable, both types being found within the same group. Mice were vaccinated with heat-killed bacteria of three strains and challenged with 10 LD50 of homologous and heterologous live bacteria, representing groups based on OMP patterns; the best protection was afforded by strain W674, which protected against nine of the 17 challenge strains; but there was no correlation between protection and PAGE pattern. Pre-vaccination and pre-challenge sera were used in immunoblotting to probe OMPs from protective and non-protective strains. All three vaccines produced antibody to proteins a and d; these proteins appeared to be common to all strains, varying in molecular mass but not in overall antigenic expression. The antibody response to the other two major OMPs appeared to be PAGE-group specific. There was no correlation between protection and the antigen pattern seen by immunoblotting.

Introduction

*Pasteurella multocida* is a small gram-negative, saccharolytic, indole-positive, facultatively anaerobic cocco-bacillus. Carter’s capsular serotype A is associated with several animal diseases of worldwide importance such as bovine and porcine pneumonia, fowl cholera and rabbit septicaemia.1–3 Type A can be distinguished from other types4–5 by indirect haemagglutination (IHA) and hyaluronidase decapsulation (HYD). Similar organisms6 that do not produce indole and are unable to ferment mannitol and sorbitol have been designated Taxon 13.

Many studies of the pathogenic mechanisms and immunogenicity of surface extracts of *P. multocida* serotypes have been made with the aim of developing vaccines.7–11 However, effective vaccines have been developed only for types B and E, the haemorrhagic septicaemia serotypes.12 Lugtenburg et al.13,14 have shown a correlation between cell-envelope-protein type and pathogenicity of strains, presumably type D,15 isolated from swine atrophic rhinitis. For type A strains, an experimental vaccine for rabbits has been reported recently. It is based on a potassium thiocyanate extract of whole bacteria16 and the protective immunogen has been identified as a 37.5-Kda outer membrane protein (OMP).17

The aim of this study was to analyse the OMP profiles of *P. multocida* type A and the related Taxon 13 strains isolated from bovine pneumonia, to determine the heterogeneity of this group, and to attempt to define a protective immunogen in a mouse septicaemia model, by investigation of the immunogenicity of the OMPs.

Materials and methods

Bacteria

With the exception of the reference strain NCTC 10322 which was obtained from the National Collection of Type Cultures, London NW9 5HT, all the strains were isolated at Veterinary Investigation Centres from the lungs of calves with pneumonia, and were identified...
provisionally as *P. multocida*. They were sent to the Moredun Research Institute (MRI) on blood-agar plates, and subcultured there once on plates of Dextrose Starch Agar (DSA; Gibco). After overnight incubation, pure cultures were suspended in skimmed milk and stored by freeze drying. Strains were typed by IHA and HYD, and identified further by biochemical reactions in a combination of conventional tube tests and API 20E test strips. The IHA technique was similar to that of Carter, except that glutaraldehyde-fixed sheep erythrocytes were used instead of human group 0 cells. Tests were performed in 50-μl volumes in U-shaped wells of microtitration plates (Sterilin) with rabbit antiserum kindly supplied by Dr V. Norring (Statens Veterinaire Serum Laboratorium, Denmark). From the 93 strains submitted, 30 were selected for this study: 24 of *P. multocida* type A, and six of Taxon 13. More details of the bacterial identification have been published elsewhere.

**Vaccine strains and preparation of vaccine**

In a preliminary study, vaccine preparations from nine strains, containing capsule, lipopolysaccharide, formalin-killed bacteria or heat-killed bacteria, were tested for protective ability against homologous and heterologous challenge with 10 LD50 of live bacteria. The heat-killed cell vaccines of *P. multocida* strains W674 and W829 afforded the best heterologous protection. These two strains, and a Taxon 13 strain (X120) which gave no protection, were selected for the present study.

**Preparation of vaccine.** Freeze-dried bacteria were plated on DSA and incubated overnight at 37°C in a moist sealed chamber to enhance hyaluronic acid production.

**Isolation of outer membrane (OM)**

A modification of the method of Filip et al., for the preparation of OM from *E. coli*, was used. Confluent growth from each of five DSA plates was washed off with 5 ml of 0.01 M HEPES buffer, pH 7.4. The bacteria were deposited by centrifugation (20 000 g, 15 min), resuspended in 20 ml of buffer, and disrupted in a French pressure cell (Aminco, Silver Springs, MD, USA) at 6000 psi. After removal of unbroken cells by centrifugation (10 000 g, 15 min), c. 400 μl of Sarkosyl (sodium N-lauroyl sarcosinate; Sigma; 30% w/v) was added to give a final concentration of 0.7%. The OM was deposited by centrifugation at 50 000 g for 1 h, washed in distilled water, resuspended in 1 ml of distilled water, and stored at −20°C.

**Results**

**SDS-PAGE of outer membrane protein (OMP)**

Fig. 1 shows the Sarkosyl OM preparations from 30 strains of *P. multocida* type A and Taxon 13, analysed by SDS-PAGE, and stained with Coomassie Blue. Overall, the patterns are very similar, revealing two, three or four major proteins (labelled a, b, c and d) with molecular masses (M₉) in the range 36.5–19 Kda, though each protein varied in M₉ over a limited range. By visual examination, the strains could be arranged in 12 groups based on the mobility of these major OMPs (table I). Protein a was absent or incompletely determined previously by the method of Reed and Muench; it ranged from 7.5 × 10⁶ to 1.2 × 10⁹ organisms.

A 50-μl sample of blood was collected from the tail vein of each mouse pre-vaccination and pre-challenge. The blood from each group was pooled, and the serum was stored at −20°C.
Fig. 1. SDS-PAGE gels of Sarkosyl OM preparations from strains of *P. multocida* and Taxon 13,* stained with Coomassie Blue; a, b, c, d represent major OMPs. (a) Track 1, strain X200; 2, W493; 3, X113; 4, W311; 5, X195; 6, W499; 7, W674; 8, X1442; 9, X995; 10, X1437; 11, NCTC 1022; 12, W419; 13, X869; 14, W1500; 15, W570; 16, X109. (b) Track 16, strain X109; 17, X12; 18, X9; 19, W828; 20, X699; 21, X1122; 22, X918; 23, X1053; 24, X110; 25, X120; 26, W571; 27, X834; 28, W820; 29, X139; 30, W839; 31, X134; 32, X109; 33, X145; 34, X103; 35, X101; 36, X1049.
Table I. Grouping of 24 strains of *P. multocida* and six strains of Taxon 13 with patterns produced by major OMPs on SDS-PAGE as shown in fig. 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Tracks (fig. 1)</th>
<th>Strains of <em>P. multocida</em> or Taxon 13</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 2 3 ∗</td>
<td>X200 W829 X1113</td>
</tr>
<tr>
<td>2</td>
<td>4 6 7</td>
<td>W571 X1016 X195</td>
</tr>
<tr>
<td>3</td>
<td>8 9</td>
<td>W599 W674</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>X1043*</td>
</tr>
<tr>
<td>5</td>
<td>5 11</td>
<td>X198 NCTC10322</td>
</tr>
<tr>
<td>6</td>
<td>12 13</td>
<td>W819* X969</td>
</tr>
<tr>
<td>7</td>
<td>14</td>
<td>W590*</td>
</tr>
<tr>
<td>8</td>
<td>15 16 17 18 19</td>
<td>W833 X109 X12 X9 W828</td>
</tr>
<tr>
<td>9</td>
<td>20 21</td>
<td>W669 X1122</td>
</tr>
<tr>
<td>10</td>
<td>22 23 24 25</td>
<td>X918 X1053 X110 X120*</td>
</tr>
<tr>
<td>11</td>
<td>26</td>
<td>W577*</td>
</tr>
<tr>
<td>12</td>
<td>27 28 29 30</td>
<td>X483 X971 W820* W1049</td>
</tr>
</tbody>
</table>

* Six strains of Taxon 13.

resolved in strains X200, W829, X1113, W571, X198, X1016, X195 and NCTC 10322; whereas present, its M, ranged from 36.5 to 33.5 Kda. The M, of protein d ranged from 21 to 19 Kda.

There were no features in the OMP patterns which allowed differentiation between the typical *P. multocida* and the Taxon 13 strains.

Mouse protection

Groups of mice were vaccinated with heat-killed cells of strains W674 and W829 (*P. multocida*) and strain X120 (Taxon 13). Vaccinated mice and non-vaccinated controls were challenged with 10 LD50 of the homologous strain and 16 heterologous strains representing most of the groups defined by SDS-PAGE of the OMPs (table II). Strain W674 appeared to afford the best protection—at least 50% protection against nine of the 17 challenge strains. Strain W829 gave this degree of protection against four strains, but the Taxon 13 strain X120 protected only against strain X9. Strain X120 was also the only strain that did not afford homologous protection at this level. There was no apparent correlation between the SDS-PAGE group pattern and protection.

Discussion

The patterns produced on SDS-PAGE by Sarkosyl extracts of *P. multocida* are similar to the patterns produced by the OMPs of other gram-negative bacteria, in which there are a small number of major proteins and several minor ones. The four major proteins which we have labelled a–d have allowed us to arrange strains of *P. multocida* into groups (table I). It is apparent from this grouping that strains of Taxon 13 share profiles of major OMPs with strains of *P. multocida*, and we suggest that these taxa cannot be distinguished on this basis.
### Table II. Percentage protection of mice in groups of 10 vaccinated with heat-killed organisms and challenged with strains of *P. multocida* or Taxon 13

<table>
<thead>
<tr>
<th>Vaccine strain</th>
<th>W674 (3)</th>
<th>W829 (1)</th>
<th>W669 (9)</th>
<th>W571 (2)</th>
<th>X9 (8)</th>
<th>X12 (8)</th>
<th>X110 (10)</th>
<th>X198 (5)</th>
<th>X971 (12)</th>
<th>X1016 (2)</th>
<th>X1053 (10)</th>
<th>X1113 (1)</th>
<th>X1122 (9)</th>
<th>X120 (10)</th>
<th>W577 (11)</th>
<th>W590 (7)</th>
<th>W819 (6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W674</td>
<td>70</td>
<td>50</td>
<td>50</td>
<td>0</td>
<td>20</td>
<td>60</td>
<td>60</td>
<td>20</td>
<td>20</td>
<td>0</td>
<td>50</td>
<td>60</td>
<td>80</td>
<td>70</td>
<td>0</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>W829</td>
<td>50</td>
<td>90</td>
<td>0</td>
<td>0</td>
<td>60</td>
<td>0</td>
<td>0</td>
<td>...</td>
<td>10</td>
<td>0</td>
<td>30</td>
<td>50</td>
<td>40</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>X120</td>
<td>40</td>
<td>30</td>
<td>0</td>
<td>0</td>
<td>60</td>
<td>20</td>
<td>20</td>
<td>...</td>
<td>10</td>
<td>0</td>
<td>20</td>
<td>40</td>
<td>0</td>
<td>20</td>
<td>0</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>

*Percentage protection = \( \frac{\text{number of vaccinated survivors} - \text{number of non-vaccinated survivors}}{10} \times 100\)

† In parenthesis: SDS-PAGE group no.—see table I.
When mice were vaccinated with heat-killed cells they produced an antibody response to two or more of the major OMPs (fig. 2b). This response was a combination of species-specific and PAGE-group-specific antibodies, but the latter did not correlate with protection. The proteins were antigenic in mice but not protective. Moreover, although proteins may differ from one another in $M_r$, they did not necessarily differ antigenically. This finding applies also to other groups of closely related species, e.g., some of the major OMPs of Enterobacteriaceae share epitopes but differ in $M_r$.29

Our conclusion is that, in this mouse model of pasteurellosis, the major OMPs are not protective antigens, and therefore may be unlikely candidates for vaccines. However, this appears to contradict the findings of Lu et al.17 who showed that the 37-5 Kda OMP (presumably our protein $a$) was protective against homologous challenge in a rabbit model; but in a mouse model, protection was afforded against heterologous challenge only if the challenge strain was shown, by probing with a monoclonal antibody, to express the 37-5-Kda antigen.30 Protection was not provided against strains lacking this antigen. Thus, they showed that this OMP can be a protective immunogen in the strains that possess it; but they concede that other antigens may be more important as vaccine candidates, because only 24% of their strains of P. multocida expressed this antigen.

We acknowledge the award of a scholarship to MZA by the Sokoto State Government and the University of Sokoto, Nigeria; and we thank Thula Wijewardana for useful discussion.

Fig. 2. (a) SDS-PAGE gels of Sarkosyl OM preparations from strains of P. multocida and Taxon 13* against which mice were or were not protected by vaccination with strain W829 (see table II). Track 1, strain W829 (protected); 2, W674 (protected); 3, X1113 (protected); 4, X12 (not protected); 5, W577* (not protected); 6, X9 (protected); 7, X120* (not protected). (b) Corresponding immunoblot probed with pre-challenge serum from mice vaccinated with heat-killed cells of strain W829. (a, b, c, d represent major OMPs).
REFERENCES


