Intra-specific diversity within *Pasteurella trehalosi* based on variation of capsular polysaccharide, lipopolysaccharide and outer-membrane proteins

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Intra-specific diversity within *Pasteurella trehalosi* was investigated by analysis of variation of capsular polysaccharide, and lipopolysaccharide (LPS) and outer-membrane protein (OMP) profiles. Sixty isolates of *P. trehalosi*, from diverse geographical locations within the UK, were examined. Capsular polysaccharide serotypes were determined by indirect haemagglutination assay; LPS and OMP profiles were compared by SDS-PAGE analysis. Capsular serotyping identified three isolates of serotype T3, 18 isolates each of serotypes T4, T10 and T15, and three untypable (UT) isolates. Analysis of LPS and OMP profiles identified six smooth LPS types and four OMP types among the 60 isolates. Forty-five (75%) of the isolates belonged to a single OMP type whereas 52 (87%) of the isolates possessed one of three LPS types. Each typing method, by itself, was not very discriminating but when the data from the three methods were combined, the 60 isolates could be separated into 14 distinct subgroups containing from one to 16 isolates as follows: serotype T3, two subgroups; serotype T4, four subgroups; serotype T10, two subgroups; serotype T15, five subgroups; UT isolates, one subgroup. Certain subgroups were associated with only one serotype whereas other subgroups were common to two or more serotypes. The subgroupings were capable of differentiating between isolates of the same serotype from the same and different geographical origins. Based on their LPS and OMP profiles, isolates of serotypes T4 and T15 were more closely related to each other than to isolates of serotype T10; serotype T4 and T15 isolates were also more heterogeneous than those of serotype T10. Certain isolates of serotype T10, recovered from a wide geographical area, were characterized by the possession of a unique capsule/LPS/OMP combination and represented a single clonal group which was responsible for a large proportion (31%) of recent disease outbreaks. Overall, a combination of capsular serotyping, and LPS and OMP typing, was found to be extremely useful for assessing diversity within *P. trehalosi* and should be of value for epidemiological and virulence studies.

Keywords: *Pasteurella trehalosi*, diversity, capsular polysaccharide, lipopolysaccharide, outer-membrane proteins

INTRODUCTION

Pasteurella trehalosi is an important Gram-negative bacterial pathogen of sheep, being responsible primarily for a

serious systemic infection but also being associated with pneumonia (Gilmour & Gilmour, 1989). Traditionally, *P. trehalosi* has been recognized as the T biotype of the so-called *Pasteurella haemolytica* complex, which also includes the A biotype and a number of additional groups of lesser importance and uncertain taxonomic relatedness (Gilmour & Gilmour, 1989; Mitters *et al.*, 1989). The A and T biotypes have been further subdivided into 17

Abbreviations: UT, untypable; OMP, outer-membrane protein; BHIB, brain heart infusion broth; NCTC, National Collection of Type Cultures; MRI, Moredun Research Institute; VI Centre, Veterinary Investigation Centre.

serotypes based on differences in their capsular polysaccharides (Adlam, 1989; Younan & Fodor, 1995). Biotype T isolates, or *P. trehalosi*, comprise four capsular serotypes designated T3, T4, T10 and T15. The chemical structures of the serotype T4 and T15 capsular polysaccharides have been elucidated and are very similar (Adlam *et al.*, 1985a, b). It has long been recognized that the A and T biotypes are responsible for distinct clinical syndromes in sheep. Biotype A is responsible for pneumonia in all ages of sheep and septicaemia in young lambs, whereas biotype T is responsible primarily for a well-defined systemic disease of young adult sheep (Gilmour & Gilmour, 1989). These pathogenic and epidemiological differences between the two biotypes were confirmed by Sneath & Stevens (1985) who, in a numerical taxonomic study of *Actinobacillus*, *Pasteurella* and *Yersinia*, clearly demonstrated that the A and T biotypes were very different and suggested that they should be separate species. In a later study, Sneath & Stevens (1990) proposed that biotype T of *P. haemolytica* be recognized as a new species, namely *P. trehalosi*. More recently, Dewhirst *et al.* (1992), using 16S rRNA sequence comparison, confirmed the findings of Sneath & Stevens (1985, 1990) and demonstrated that the two species belonged to different clusters within the family *Pasteurellaceae* and may, in fact, belong to different genera.

Unfortunately, the A and T biotypes of *P. haemolytica* have not been widely recognized as distinct and separate species and have been treated as a single species in a number of studies since 1990 (Abdullah *et al.*, 1990; Gerbig *et al.*, 1992; Lacroix *et al.*, 1993; Lee *et al.*, 1994). The taxonomic status of the A and T biotypes as separate species accounts for the various differences between the two biotypes described by these authors. For example, Gerbig *et al.* (1992) examined the neutralizing activity of a monoclonal antibody against the leukotoxin of isolates belonging to the major A and T serotypes and found that the monoclonal antibody neutralized the leukotoxin produced by biotype A isolates but did not neutralize the leukotoxin of biotype T isolates. Similarly, Abdullah *et al.* (1990) and Lee *et al.* (1994) examined sialoglycoprotease (Gcp) activity and the presence of the *gcp* gene among isolates belonging to each of the A and T biotypes and found that the *gcp* gene and Gcp activity were present in biotype A isolates but not in biotype T isolates. In addition, Lacroix *et al.* (1993) described major differences in the LPS structure of biotype A and T isolates. All of these results are not surprising in view of the taxonomic status of the A and T biotypes as separate species and they illustrate the importance of taxonomy to other areas of bacteriological research.

Due to the fact that *P. trehalosi* has yet to gain wide acceptance as a species distinct from *P. haemolytica*, the organism has not been studied in any great detail. With the exception of a single study on DNA variation in a small sample of isolates from a restricted source, using ribotyping and restriction enzyme analysis (Jaworski *et al.*, 1993), very little is known about genetic diversity and cell-surface variation within the species, nor about the epidemiology and virulence of, or host immunity to, the

pathogen. In previous comparative and epidemiological studies, the focus has been placed primarily on capsular serotyping alone, although lipopolysaccharide (LPS) and outer-membrane protein (OMP) profiles of single, representative isolates of each serotype have occasionally been examined during the course of studies on *P. haemolytica* (Knights *et al.*, 1990; Lacroix *et al.*, 1993). The present study was initiated, therefore, as part of a wider investigation, using a variety of techniques, into the diversity and taxonomy of the *P. haemolytica* complex. Analysis of intra-specific variation in the capsular polysaccharide, LPS and OMPs was carried out for three reasons. (1) Basic information about these important cell-surface structures of *P. trehalosi* is lacking. (2) An analysis of the natural variation occurring in the capsular polysaccharide, LPS and OMPs of *P. trehalosi* will lead to an improvement in typing methods, which are currently based only on capsular variation, and thereby to a better understanding of the epidemiology of systemic pasteurellosis. (3) The identification of specific surface characteristics associated with disease isolates will provide a stimulus to further studies aimed at understanding the virulence mechanisms of this pathogen and host immunity against it.

METHODS

Bacterial isolates and growth conditions. Sixty isolates of *P. trehalosi* were examined in the present study, including the type strain NCTC 10370. For comparative purposes, two *P. haemolytica* isolates of serotypes A1 and A2 were also included in the study. All of the *P. trehalosi* isolates were obtained from sheep, with the exception of isolate PH604, which was obtained from a calf. Except for seven isolates obtained from the National Collection of Type Cultures (NCTC) and two isolates obtained from the Moredun Research Institute (MRI), all of the isolates were obtained from one of nine Veterinary Investigation (VI) Centres in Scotland (5), England (2) and Wales (2). The sample of isolates therefore represented widespread geographical areas within the UK. With the following exceptions, all of the isolates were derived from the lungs of different infected sheep. Five groups of three isolates were obtained from the lungs, liver and spleen, respectively, of the same infected sheep; a single isolate was obtained from the nasopharynx of a healthy sheep; and eight isolates were of unknown tissue origin. Thirty-eight isolates were obtained from sheep with systemic infection (septicaemia), eleven isolates from sheep with pneumonia, two isolates from sheep with both systemic infection and pneumonia, two isolates from apparently healthy sheep, and six isolates from sheep and one isolate from a calf of unknown disease status. Details of the isolates, including their serotype, the disease status of the host, the tissue of origin and the geographical origin, are given in Table 1.

The bacterial isolates from the MRI and from the VI Centres were submitted to Glasgow on blood agar slopes. At the VI Centres the isolates were identified as *P. trehalosi* based on clinical diagnosis, colony morphology, Gram-staining and cell morphology, and preliminary biochemical characterization. Confirmation of identity was obtained by capsular serotyping, at the MRI, as described below. Cultures from the NCTC were obtained in a freeze-dried state and reconstituted on sheep's blood agar (brain heart infusion agar containing 5%, v/v, defibrinated sheep's blood). All bacterial isolates were stored at -70°C in 50% (v/v) glycerol in brain heart infusion broth

Table 1. Details of isolates of *P. trehalosi* examined in the present study

Isolate	Capsular serotype	LPS type	OMP type	Disease status*	Tissue of origin	VI Centre
PH604	T3	R	4	P	Lung	Edinburgh
NCTC 11550	T3	1	1	—	—	—
PH674	T3	1	1	—	—	Moredun
NCTC 10626	T4	3	1	H	Nasopharynx	—
NCTC 10627	T4	3	1	P	Lung	—
PH688	T4	3	1	S	Lung	Carmarthen
PH728	T4	3	1	S	Lung	Dumfries
PH730	T4	3	1	P	Lung	Dumfries
PH738†	T4	3	1	S	Liver	Penrith
PH740†	T4	3	1	S	Lung	Penrith
PH742†	T4	3	1	S	Spleen	Penrith
PH732†	T4	3	2	S	Liver	Penrith
PH734†	T4	3	2	S	Lung	Penrith
PH736†	T4	3	2	S	Spleen	Penrith
PH328	T4	4	1	—	—	Edinburgh
PH716	T4	4	1	S	Lung	Carmarthen
PH768	T4	4	1	H	Lung	Aberystwyth
PH804	T4	4	1	S	Lung	Penrith
PH636	T4	6	1	S	Lung	Edinburgh
PH638	T4	6	1	S	Lung	Edinburgh
PH640	T4	6	1	S	Liver	Edinburgh
PH356	T10	2	1	—	—	Auchincruvie
PH492	T10	2	1	S	Lung	St Boswells
PH504	T10	2	1	S	Lung	St Boswells
PH650	T10	2	1	P	Lung	Carmarthen
PH658	T10	2	1	P	Lung	Carmarthen
PH668	T10	2	1	S	Lung	Carmarthen
PH724	T10	2	1	P	Lung	Edinburgh
PH726	T10	2	1	P	Lung	Dumfries
PH746	T10	2	1	S	Liver	Penrith
PH748	T10	2	1	S	Lung	Penrith
PH764	T10	2	1	P	Lung	Aberystwyth
PH766	T10	2	1	P	Lung	Aberystwyth
PH798†	T10	2	1	S	Lung	Penrith
PH800†	T10	2	1	S	Liver	Penrith
PH802†	T10	2	1	S	Spleen	Penrith
NCTC 10641	T10	2	2	—	Liver	—
PH662	T10	4	1	S	Lung	Carmarthen
PH712	T10	4	1	S	Lung	Carmarthen
PH664	T15	3	1	S	Lung	Carmarthen
PH710	T15	3	1	S	Lung	Carmarthen
PH718	T15	3	1	S	Lung	Carmarthen
PH722	T15	3	1	S	Lung	Carmarthen
PH694	T15	3	2	P/S	Lung	Aberdeen
PH696	T15	3	2	P/S	Lung	Aberdeen
PH780	T15	3	2	S	Lung	Carmarthen
NCTC 10624	T15	3	3	—	—	—
NCTC 10370 ^T	T15	3	3	S	—	—
NCTC 10371	T15	3	3	S	—	—
PH744	T15	3	3	S	Lung	Penrith
PH520	T15	4	1	P	Lung	St Boswells
PH676	T15	4	1	—	—	Moredun
PH750†	T15	4	1	S	Lung	Penrith
PH752†	T15	4	1	S	Liver	Penrith
PH754†	T15	4	1	S	Spleen	Penrith
PH648	T15	5	1	S	Lung	Carmarthen
PH684	T15	5	1	P	Lung	Shrewsbury
PH792†	UT	3	3	S	Lung	Penrith
PH794†	UT	3	3	S	Liver	Penrith
PH796†	UT	3	3	S	Spleen	Penrith

* P, pneumonia; S, systemic infection; H, healthy.

† Indicates isolates from the lung, liver and spleen of the same animal.

(BHIB). Subsequently, bacteria were streaked onto sheep's blood agar and grown overnight at 37 °C. For preparation of outer membranes, bacteria were grown for approximately 6 h in 500 ml volumes of BHIB in 2 litre Erlenmeyer flasks at 37 °C with shaking at 120 r.p.m. To assess the effect of the stage of growth on the expression of LPS and OMPs, cultures of isolate PH328 (serotype T4) were grown for 4, 8, 12 and 24 h. To assess the effect of repeated subculture on the expression of LPS and OMPs, isolate PH328 was subcultured 20 times on sheep's blood agar and outer membranes prepared after 5, 10, 15 and 20 subcultures.

Capsular serotyping. In order to identify their capsular antigen types, the isolates were serotyped by the rapid indirect haemagglutination assay, with bovine red blood cells, as described by Fraser & Donachie (1983).

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 $\times 2$ 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 modified Lowry procedure of Markwell *et al.* (1978), specific for membrane proteins, was used to standardize the protein concentrations of the outer membrane samples to 2.0 mg ml⁻¹.

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 (20 µg per lane) were separated in 12% (w/v) resolving gels; LPS (5 µg per lane) 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). OMPs were visualized by staining with Coomassie Brilliant Blue and LPS by silver staining (Tsai & Frasch, 1982).

RESULTS

Serotyping

All but three of the isolates could be assigned to one of the four recognized serotypes of *P. trehalosi*. Three isolates were of serotype T3 and there were 18 isolates each of serotypes T4, T10 and T15 (Table 1). The three isolates which could not be serotyped (PH792, PH794 and PH796) were classified as untypable (UT).

SDS-PAGE analysis of LPS

To ascertain whether any variation in LPS profiles occurred at different stages of the growth cycle or after repeated subculture, LPS was prepared from cells of the serotype T4 isolate PH328 after 4, 8, 12 and 24 h of growth in BHIB and after 5, 10, 15 and 20 subcultures on sheep's blood agar. The times at which bacteria were harvested corresponded to late exponential and early, middle and late stationary phases of growth, respectively. As can be seen from Fig. 1, there were no changes in the LPS profiles either at different stages of growth (lanes

Fig. 1. LPS profiles of *P. trehalosi* isolate PH328 (serotype T4) after 4, 8, 12 and 24 h of growth (lanes 1–4, respectively) and after 5, 10, 15 and 20 subcultures (lanes 5–8, respectively).

1–4) or after repeated subculture (lanes 5–8). The LPS profile of this isolate had the characteristic ladder pattern typical of so-called 'smooth' LPS, the ladder pattern representing the additional repeat units of the O-antigen component of the molecule.

The LPS profiles of the 60 isolates of *P. trehalosi* were compared to each other and to the profiles of serotype A1 and A2 isolates of *P. haemolytica*. As can be seen from Fig. 2(a), the LPS profile of the *P. haemolytica* serotype A1 strain (PH468) lacked the well-defined ladder pattern seen in all but one of the *P. trehalosi* isolates and the same was true of other *P. haemolytica* isolates (profiles not shown). In addition, the core-oligosaccharide region of the A1 LPS of *P. haemolytica* possessed four bands whereas that of *P. trehalosi* possessed only one or two bands, although the resolution of the four bands was poor due to overstaining in order to demonstrate the O-chains of the A1 LPS (see also Davies *et al.*, 1991, for further details). The LPS profiles of the *P. trehalosi* isolates could be differentiated into six 'smooth' LPS types, designated LPS types 1–6 and, as already mentioned, a single isolate, PH604, of serotype T3, did not possess a ladder pattern and was classified as 'rough' (Fig. 2a, lane 2).

Differentiation of the LPS into distinct types was based (1) on variation in the fastest-migrating low-molecular-mass bands representing the core-oligosaccharide region and

Fig. 2. (a) LPS profiles of *P. haemolytica* isolate of serotype A1 (lane 1) and *P. trehalosi* isolates with rough LPS (lane 2) and smooth LPS type 1 (lanes 3 and 4), LPS type 2 (lanes 5-7) and LPS type 4 (lanes 8-13). The isolates and their serotypes in lanes 1-13 are as follows: 1, PH468 (T3); 2, PH604 (T3); 3, PH68 (T3); 4, PH674 (T3); 5, PH726 (T10); 6, PH746 (T10); 7, PH252 (T10); 8, PH328 (T4); 9, PH804 (T4); 10, PH662 (T10); 11, PH712 (T10); 12, PH676 (T15); 13, PH750 (T15). (b) LPS profiles of *P. trehalosi* isolates of LPS type 4 (lane 1), LPS type 1 (lane 2), LPS type 3 (lanes 3-9), LPS type 5 (lanes 10 and 11) and LPS type 6 (lanes 12 and 13). The isolates and their serotypes in lanes 1-13 are as follows: 1, PH676 (T15); 2, PH674 (T3); 3, PH734 (T4); 4, PH740 (T4); 5, PH664 (T15); 6, PH688 (T4); 7, PH694 (T15); 8, PH744 (T15); 9, PH792 (UT); 10, PH648 (T15); 11, PH684 (T15); 12, PH636 (T4); 13, PH638 (T4).

(2) on variation in the appearance of the ladder pattern representing the O-antigen region. LPS types 1 and 2 were characterized by possessing two bands in the core-oligosaccharide region (Fig. 2a, lanes 3–7 and Fig. 2b, lane 2); by contrast, all other LPS types had a single band in this region. Unfortunately, optimum resolution of the individual bands has been partially lost during the photographic process. LPS types 1 and 2 could be differentiated on the basis that the individual bands within the ladders of LPS types 1 (Fig. 2a, lanes 3 and 4) and 2 (Fig. 2a, lanes 5–7), respectively, were in alignment with each other but were not in alignment with the bands of the other LPS type. This is best observed by placing a ruler across Fig. 2(a) and comparing the relative positions of bands in the LPS in lanes 3–7. The differences in molecular masses of individual bands within the ladders of LPS types 1 and 2 indicated different structures of the O-antigen repeat units. In addition, the ladder pattern of LPS type 1 consistently stained less intensely in the upper region of the gel than that of LPS type 2. This is best seen by comparing lanes 4 and 5 in Fig. 2(a). Of the 60 isolates of *P. trehalosi* examined, only two possessed LPS type 1 and both of these were of serotype T3 (Table 1). Sixteen of the 60 isolates possessed LPS type 2 and all of these were of serotype T10 (Table 1).

LPS types 3 and 4 could be differentiated from LPS types 1 and 2 in having a single band in the low-molecular-mass core-oligosaccharide region (Fig. 2a, lanes 8–13; Fig. 2b, lanes 3–9). LPS type 3 could be differentiated from LPS type 4 in the same way that LPS types 1 and 2 could be differentiated. That is, the spacing of the individual bands in LPS types 3 and 4 differed and the ladder pattern of LPS type 3 stained less intensely in the upper region of the gel than that of LPS type 4. The ladder pattern of LPS type 4 had the same appearance, in terms of both spacing of the bands and intensity of staining, as that of LPS type 2 (Fig. 2a, compare lanes 5–7 with lanes 8–13). The ladder pattern of LPS type 3 was characterized by some of the bands having the appearance of doublets; there was also some micro-heterogeneity of the patterns assigned to this group. The doublet effect was more evident in the LPS of some isolates than others and is quite clearly seen in the LPS of isolates PH688 and PH694 in Fig. 2(b) (lanes 6 and 7). The ladder patterns of LPS types 1 and 3 were also very similar, even to the extent of clearly visible doublet bands in isolate PH68 (Fig. 2a, lane 3), although the absence of short chain LPS in the type 1 ladder patterns meant that the two profiles were not identical. Of the 60 isolates of *P. trehalosi*, 25 possessed LPS type 3 and 11 possessed LPS type 4 (Table 1). LPS type 3 was associated with isolates of serotypes T4, T15 and the UT isolates; LPS type 4 was associated with isolates of serotypes T4, T10 and T15.

The remaining LPS types, 5 and 6, were not common and were associated with two isolates of serotype T15 and three isolates of serotype T4, respectively (Table 1). Both LPS types had ladder patterns which were dissimilar to any of the others (Fig. 2b, lanes 10–13), although LPS type 5 also had a low-molecular-mass band which was expressed more abundantly than in any of the other LPS types (Fig. 2b, lanes 10 and 11).

Fig. 3. OMP profiles of *P. trehalosi* isolate PH328 (serotype T4) after 4, 8, 12 and 24 h of growth in BHIB (lanes 1–4, respectively) and after 5, 10, 15 and 20 subcultures on sheep's blood agar (lanes 5–8, respectively).

SDS-PAGE analysis of OMPs

To determine whether any variation in OMP profiles occurred at different stages of the growth cycle or after repeated subculture, the OMP profiles of isolate PH328 were compared after 4, 8, 12 and 24 h of growth and after 5, 10, 15 and 20 subcultures. As can be seen from Fig. 3 (lanes 1–3) the OMP profiles of cells grown for 4, 8 and 12 h were identical. However, after 24 h of growth an additional protein of approximate molecular mass 19 kDa was expressed (Fig. 3, lane 4, arrow). There was no change in the OMP profiles of isolate PH328 after repeated subculture (Fig. 3, lanes 5–8).

The OMP profiles of the 60 isolates of *P. trehalosi* were compared to each other and to the profiles of serotype A1 and A2 isolates of *P. haemolytica* (Fig. 4). Based on the above results, outer membranes of all the isolates were prepared from cells grown for approximately 6 h, i.e. to late exponential phase. As can be seen from Fig. 4(a), the OMP profiles of *P. haemolytica* (lanes 1 and 2) and *P. trehalosi* (lanes 3–12) were very different. Important differences between *P. haemolytica* and *P. trehalosi* were apparent in the mobilities of major proteins in the molecular mass range 29–43 kDa (Fig. 4a, lane 3, arrows). In addition, a 17 kDa protein was present in both serotypes of *P. haemolytica* but not in *P. trehalosi* (Fig. 4a, lane 2, arrow). Finally, differences between *P. haemolytica* and *P. trehalosi* were also apparent in the high-molecular-

Fig. 4. (a) OMP profiles of *P. haemolytica* isolates of serotypes A1 and A2 (lanes 1 and 2) and *P. trehalosi* isolates of serotypes T3, T4, T10 and T15 and OMP type 1 (lanes 3–12). The isolates and their serotypes in lanes 1–12 are as follows: 1, PH468 (A1); 2, PH776 (A2); 3, PH68 (T3); 4, PH674 (T3); 5, PH716 (T4); 6, PH728 (T4); 7, PH738 (T4); 8, PH724 (T10); 9, PH650 (T10); 10, PH764 (T10); 11, PH710 (T15); 12, PH752 (T15). Arrows (lane 3) indicate differences in mobilities of major proteins between *P. haemolytica* serotype A1 and A2 isolates and *P. trehalosi* isolates (see text for details). (b) OMP profiles of *P. trehalosi* isolates of OMP type 1 (lanes 1 and 12), OMP type 2 (lanes 2–6), OMP type 3 (lanes 8–11) and OMP type 4 (lane 7). The isolates and their serotypes in lanes 1–12 are as follows: 1, PH728 (T4); 2, PH734 (T4); 3, PH252 (T10); 4, PH780 (T15); 5, PH694 (T15); 6, PH696 (T15); 7, PH604 (T3); 8, PH70 (T15); 9, PH744 (T15); 10, PH792 (UT); 11, PH794 (UT); PH728 (T4). Arrows indicate differences in mobilities of major proteins between OMP types 1, 2, 3 and 4 (see text for details).

mass region 67–100 kDa (Fig. 4a). However, the resolution of proteins in the high-molecular-mass region of all the *P. trehalosi* isolates was poor compared to that in the same region of the *P. haemolytica* isolates.

Within *P. trehalosi* itself, four distinct OMP profiles were evident and these were designated OMP types 1–4. OMP

type 1 was the most common OMP profile and was present in isolates belonging to each of the four recognized serotypes as can be seen from Fig. 4(a) (lanes 3–12), which shows the OMP profiles of representative isolates of each of the four serotypes. In total, 45 (75%) of the 60 isolates of *P. trehalosi* possessed OMP profiles of type 1 (Table 1). OMP type 2 was present in seven isolates, including isolates of serotypes T4, T10 and T15, and is shown in Fig. 4(b) (lanes 2–6). OMP type 2 differed from OMP type 1 in that the 35.5 kDa protein of OMP type 1 (Fig. 4b, lane 1, arrow) appeared to have shifted in mobility to an apparent molecular mass of approximately 37 kDa (Fig. 4b, lane 6, arrow), with the result that there now appeared to be three proteins in the range 37–39 kDa. This resulted in poor resolution of the proteins in this range (Fig. 4b, lanes 2–6). OMP type 3 was present in seven isolates and is shown in Fig. 4(b) (lanes 8–11). Isolates possessing OMP type 3 were restricted to serotype T15 and to the UT isolates, although three of the T15 isolates were cultures obtained from the NCTC and were at least 25 years old. In the OMP type 3 profile the 29 kDa protein was expressed more abundantly than in OMP types 1 and 2 (Fig. 4b, lane 7, arrow) and the 30 kDa protein appeared not to be expressed. Also, the 35.5 kDa protein of OMP type 1 appeared to have shifted in mobility to an apparent molecular mass of 37 kDa, as in OMP type 2 (Fig. 4b, lane 8, arrow), but there appeared to be only a single protein, of 39 kDa, above the 37 kDa protein (compare Fig. 4b, lanes 11 and 12). OMP type 4 was present in only a single isolate, PH604 (Fig. 4b, lane 7). OMP type 4 was characterized by having two major proteins in the molecular mass range 37.5–38.5 kDa, but in the 29–31 kDa range was identical to OMP type 3.

DISCUSSION

Since phenotypic characters such as LPS and OMPs are influenced by growth conditions and can change after subculture, the stability of the LPS and OMP profiles of *P. trehalosi* under different growth conditions was assessed at the outset. In particular, phase of growth, degree of aeration and iron-limitation have previously been shown to influence the OMP profiles of *P. haemolytica* (Davies *et al.*, 1992). In the present study bacteria were grown under iron-sufficient conditions and the degree of aeration was maintained constant. The effects of phase of growth and subculture on the LPS and OMP profiles of a serotype T4 isolate were assessed. The LPS profiles were found to be the same both after repeated subculture and at different stages of the growth cycle. Although the OMP profiles of the same isolate did not change after repeated subculture, there was variation at different stages of growth. The OMP profiles of bacteria grown for 4–12 h were identical but an additional protein of 19 kDa was expressed after 24 h. In subsequent experiments bacteria were grown for approximately 6 h to minimize any effects due to cells entering stationary phase. Since there were no changes in either the LPS or OMP profiles after 20 subcultures it was concluded that the number of times an isolate had been subcultured before arriving in the laboratory was not important.

Table 2. Summary of subdivision of *P. haemolytica* based on capsular serotypes, LPS types and OMP types

Serotype	LPS type	OMP type	No. of isolates
T3	R	4	1
T3	1	1	2
T4	3	1	8*
T4	3	2	3*
T4	4	1	4
T4	6	1	3
T10	2	1	16*
T10	4	1	2
T15	3	1	4
T15	3	2	3
T15	3	3	4*
T15	4	1	5
T15	5	1	2
UT	3	3	3*

* Three isolates within each group derived from same host animal.

Significant differences between the OMP profiles of *P. haemolytica* and *P. trehalosi* confirmed the findings of other investigators which suggest that biotypes A and T of *P. haemolytica* are, in fact, different species (Sneath & Stevens, 1985, 1990; Dewhirst *et al.*, 1992). The high degree of variation in the molecular masses of the major proteins, in particular, indicated significant taxonomic differences between *P. haemolytica* and *P. trehalosi*, supporting the suggestion of Dewhirst *et al.* (1992) that the two species belong in different genera. By contrast, there was a low degree of intra-specific variation between the profiles of serotype A1 and A2 isolates of *P. haemolytica* (see also McCluskey *et al.*, 1994) and between those of the *P. trehalosi* serotypes. Although there were differences in the nature of the O-antigen side-chains of *P. haemolytica* (Davies *et al.*, 1991) and *P. trehalosi*, further immunological characterization of the LPS of the two species, particularly of the core-oligosaccharide region, is required to demonstrate species-specific variation. Nevertheless, the well-defined ladder pattern present in all but one of the *P. trehalosi* isolates contrasted with the absence of a ladder pattern in serotype A2 isolates and the faint high-molecular-mass ladder pattern in serotype A1 isolates of *P. haemolytica* (Davies *et al.*, 1991).

None of the three methods employed to assess diversity within *P. trehalosi* – variation in the capsular polysaccharides, and LPS and OMP profiles – was particularly discriminating when used alone because the degree of heterogeneity in each group of surface structures was limited. Only four capsular types, six smooth LPS types and four OMP types were identified within the population of 60 isolates examined. OMP typing was less useful than the other methods because 45 (75%) of the isolates possessed the same OMP type, i.e. type 1, seven isolates possessed OMP types 2 or 3, respectively, and a single

isolate possessed OMP type 4. Overall, LPS typing was perhaps more useful than capsular serotyping, since six groups rather than four could be distinguished. However, there were differences in the association of the various LPS types with each of the capsular serotypes T4, T10 and T15 (Table 2). For example, LPS type 2 was associated only with serotype T10, LPS type 3 was associated with serotypes T4 and T15, and LPS type 4 was associated with each of the serotypes T4, T10 and T15. It should be noted that within serotype T10, LPS types 2 and 4 appear to have identical O-antigen types and differ only in the number of bands in the core-oligosaccharide region. Previous studies have shown that the O-antigen structures of 'T4' and 'T10' LPS (Perry & Babiuk, 1984; Richards & Leitch, 1989) and 'T3' and 'T15' LPS (Leitch & Richards, 1988; Lacroix *et al.*, 1993) are identical. However, these authors assumed that the LPS structures of the isolates chosen were representative of the T3, T4, T10 and T15 capsular groups, respectively. From the results presented here, this is clearly not the case, since LPS structure varies within capsular serotypes. Based on the observations described here, it seems likely that Perry & Babiuk (1984) and Richards & Leitch (1989) analysed the O-antigens of LPS types 2 (T10) and/or 4 (T4/T10), respectively, and Leitch & Richards (1988) and Lacroix *et al.* (1993) analysed the O-antigens of LPS types 1 (T3) and 3 (T15), respectively. If this is the case, these structural studies support the observations of the present study regarding the relationship of LPS types 2 and 4, and 1 and 3 although, as mentioned above, immunological analyses are required to confirm this.

When the three methods were used in combination, 14 distinct subgroups were delineated within the four serotypes as follows: T3 isolates, 2 subgroups; T4 isolates, 4 subgroups; T10 isolates, 2 subgroups; T15 isolates, 5 subgroups; UT isolates, 1 subgroup (summarized in Table 2). These subgroupings were clearly able to differentiate isolates of the same serotype and from the same VI Centre. For example, isolates of serotype T4 from Penrith VI Centre could be separated into three subgroups: LPS type 3/OMP type 1; LPS type 3/OMP type 2; and LPS type 4/OMP type 1. Some subgroups were restricted to one serotype only, namely LPS/OMP types R/4, 1/1, 6/1, 2/1 and 5/1. Other subgroups were found in up to three serotypes, e.g. types 3/1 and 3/2 among both serotype T4 and T15 isolates, type 3/3 within serotype T15 and the UT group, and type 4/1 within the three serotypes T4, T10 and T15. The relative homogeneity of the OMP profiles was a possible indication of limited genetic diversity within the species. The T10 group of isolates, which comprised isolates possessing LPS types 2 and 4, and OMP profiles only of type 1, was clearly more homogeneous than the T4 and T15 groups, each of which possessed a wider range of LPS and OMP profiles. The frequent occurrence of serotype T10 isolates of LPS/OMP type 2/1, originating from diverse geographical areas (samples within this group were obtained from seven different VI Centres in England, Scotland and Wales), marked the existence of an important clonal group within *P. trehalosi* which was responsible for a high

proportion (31 %) of the recent disease outbreaks. LPS and OMP types, used in combination, are useful markers for the identification of clonal groups (Achtman & Pluschke, 1986; Kapur *et al.*, 1992). In addition, the occurrence of LPS/OMP type 3/3 in both recent field isolates and NCTC cultures isolated 25–35 years ago marked the existence of a clonal group which has remained relatively unchanged for over 35 years. However, the fact that three of the field isolates belonging to this clone were untypable suggests either that the capsular structure of these isolates has changed or that this capsular type has remained unrecognized over this period of time.

The fact that the population was not dominated by a single capsular serotype suggests that, with the exception of the T3 capsule (see below), there were no significant differences in the virulence potential of the various capsular polysaccharides. That the T4 and T15 capsules do not vary significantly in their potential as virulence determinants is supported by the fact that the chemical structures of these capsular polysaccharides are almost identical (Adlam *et al.*, 1985a, b). The similarity of the T4 and T15 capsules, together with the existence of isolates of each serotype having the same LPS/OMP profiles, namely 3/1, 3/2 and 4/1, suggests that a high proportion of T4 and T15 isolates are closely related. Conversely, T10 isolates of LPS/OMP type 2/1 are probably less closely related to the T4 and T15 isolates. The high incidence of the T10 clone possessing LPS/OMP type 2/1, and its recovery from diverse geographical locations within the UK, suggests that the T10/LPS type 2/OMP type 1 combination might be an important virulence determinant. Of these surface structures it is likely that the type 2 LPS is the most important since OMP profiles of type 1 are commonly associated with other serotypes and LPS types, and the T10 capsule is no more common than the T4 or T15 capsules, as mentioned above. Similarly, the low incidence of serotype T3 isolates from disease cases, which agrees with previous observations (Gilmour & Gilmour, 1989), suggests that this serotype is possibly less virulent than the other serotypes. Although these conclusions are speculative they are, nevertheless, concordant with observations in other clonal populations, where a small number of virulent clones are responsible for a majority of the disease outbreaks (Selander & Musser, 1990). Based on the incidence of these surface components among the disease isolates examined, the study has highlighted important areas for future research regarding the role of specific surface components in disease. In particular, the immunological relationships of the different LPS types to each other, and the role of the different capsular and LPS structures in virulence, need to be assessed experimentally.

In summary, our analysis of variation of the capsular polysaccharide serotypes, and LPS and OMP profiles, has shown that by comparison to other species, *P. trehalosi* is relatively homogeneous: only four capsular serotypes, six LPS types and four OMP types were identified in a geographically diverse sample of isolates. However, when the three typing methods were employed together, they were demonstrated to be very effective as an epidemio-

logical tool, and capable of identifying important clonal groups and specific surface components likely to be involved in virulence. The underlying genetic diversity of *P. trehalosi* is currently being investigated by multilocus enzyme electrophoresis.

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