Genetic relationships among *Pasteurella trehalosi* isolates based on multilocus enzyme electrophoresis

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Genetic diversity among 60 British *Pasteurella trehalosi* isolates representing the four recognized capsular serotypes, T3, T4, T10 and T15, and recovered predominantly from sheep suffering from systemic pasteurellosis, was estimated by analysing electrophoretically demonstrable allelic variation at structural genes encoding 19 enzymes. Thirteen of the loci were polymorphic and 20 distinctive multilocus genotypes (electrophoretic types, ETs) were identified. The population structure of *P. trehalosi* is clonal and its genetic diversity is limited compared with most other pathogenic bacteria. ETs represent clones, and isolates of the same ET were generally associated with the same combination of serotype, LPS type and outer-membrane protein (OMP) type. The genetic diversity of isolates within each of the capsular serotypes varied. Serotype T10 was represented by 18 isolates in two related ETs and exhibited little diversity. By contrast, serotype T15 was represented by 18 isolates in nine ETs and was almost as diverse as the species as a whole. Serotype T4 was represented by 18 isolates in five ETs and was less diverse than serotype T15. Although serotype T3 was more diverse than serotype T15 it was represented by only three isolates. With the exception of the T10 isolates and those recovered from healthy sheep, 35 disease isolates belonged to 16 ETs, each of which was represented by only one to four isolates. The fact that a high proportion of disease is caused by a relatively large number of clones suggests that *P. trehalosi* is essentially an opportunistic pathogen. In addition to having the same capsular structure, isolates belonging to the two T10 clones were characterized by possession of similar, if not identical, O-antigens (LPS types 2 and 4). The occurrence of 18 serotype T10 isolates in only two ETs suggests that the T10 capsule and type 2/4 O-antigen confer enhanced virulence on members of these two clones. Multilocus enzyme electrophoresis (MLEE) had greater resolving power than did capsule/LPS/OMP analysis, being able to distinguish 20 rather than 14 sub-divisions within *P. trehalosi*. The technique demonstrated genetic identity or non-identity among strains of the same or different serotypes from different geographic localities within the UK and was a useful epidemiological tool.

Keywords: *Pasteurella trehalosi*, multilocus enzyme electrophoresis, population genetics

INTRODUCTION

Pasteurella trehalosi, a Gram-negative bacterium be-

longing to the family *Pasteurellaceae*, is a widely occurring pathogen of sheep (Biberstein & Thompson, 1966; Gilmour & Gilmour, 1989). It colonizes the tonsils of clinically normal animals (Al-Sultan & Aitken, 1985; Gilmour *et al.*, 1974) and, particularly under stressful conditions, produces an acute systemic infection affecting the upper alimentary tract and lungs of young

Abbreviations: ET, electrophoretic type; MLEE, multilocus enzyme electrophoresis; OMP, outer-membrane protein; UPGMA, unweighted-pair group method with averages; VI Centre, Veterinary Investigation Centre.

adults (Gilmour, 1980; Gilmour & Gilmour, 1989). Traditionally, *P. trehalosi* has been recognized as the T biotype of the *Pasteurella haemolytica* complex (Gilmour & Gilmour, 1989; Muters *et al.*, 1989; Smith, 1961), but significant biochemical, serological, pathogenic and epidemiological differences between the two biotypes (Biberstein & Gills, 1962; Gilmour, 1980; Smith, 1961) led to the proposal (Sneath & Stevens, 1990) that the T biotype be recognized as a distinct species, *P. trehalosi*.

Because *P. trehalosi* has not been widely recognized as a species distinct from *P. haemolytica*, little is known about its biology, including the extent of genetic diversity, epidemiology and virulence mechanisms and their role in pathogenesis. Whereas *P. trehalosi* colonizes the tonsils and produces a systemic disease in young adult sheep, *P. haemolytica* colonizes the nasopharynx and produces pneumonia in sheep of all ages as well as in cattle (Frank, 1989; Gilmour, 1980; Gilmour & Gilmour, 1989). These important differences in host specificity and pathogenicity suggest that *P. trehalosi* and *P. haemolytica* have different virulence mechanisms. In particular, *P. trehalosi* is likely to possess mechanisms allowing it to invade the tonsils and pharyngeal mucosae, survive within the lymphatics and bloodstream, and spread to other organs (Biberstein & Thomson, 1966). Differences between *P. trehalosi* and *P. haemolytica* that may be related to differences in pathogenic mechanisms and host specificity have been described in leukotoxin structure (Gerbig *et al.*, 1992), LPS structure and profiles (Davies & Quirie, 1996; Lacroix *et al.*, 1993), outer-membrane protein (OMP) profiles (Davies & Quirie, 1996), and the presence or absence of sialoglycoprotease (Gcp) activity and the *gcp* gene (Abdullah *et al.*, 1990; Lee *et al.*, 1994).

P. trehalosi has four different capsular polysaccharide structures, T3, T4, T10 and T15, that form the basis of a serotyping scheme used for examining inter-strain variation (Adlam, 1989; Gilmour & Gilmour, 1989). However, based on a comparison of the LPS and OMP profiles of 60 isolates of *P. trehalosi*, Davies & Quirie (1996) demonstrated that capsular serotyping does not reveal the full extent of diversity within the species and concluded that it is of limited use in epidemiological studies. Although variation in cell-surface structures, including capsule, LPS and OMPs, forms the basis of important bacterial typing schemes, such variation does not necessarily reflect the underlying genetic relationships between bacteria because it is phenotypic and difficult to relate to allelic variation at specific gene loci (Selander *et al.*, 1986). In contrast, multilocus enzyme electrophoresis (MLEE) is a method that examines genetic diversity within and among populations, has been applied in numerous studies of bacterial population genetics, and is a powerful epidemiological tool (Selander *et al.*, 1986). In particular, the method has been successfully used in studies of other members of the *Pasteurellaceae*, including *Actinobacillus pleuropneumoniae* (Møller *et al.*, 1992; Musser *et al.*, 1987), *Haemophilus* (*Actinobacillus*) *actinomycescomitans*

(Haubek *et al.*, 1995; Poulsen *et al.*, 1994), and *Haemophilus influenzae* (Musser *et al.*, 1988a, b).

In the present study, MLEE was used to assess genetic diversity within a sample of 60 isolates of *P. trehalosi* which had been recovered in diverse geographic locations in the UK and had been the subject of a previous investigation of variation in cell-surface components (Davies & Quirie, 1996). The objective was to develop a population genetic framework for *P. trehalosi* that could be used to (1) identify clonal groups involved in disease, (2) correlate the presence of specific cell-surface structures with virulent clonal groups, thereby identifying those structures as putative virulence determinants, and (3) determine the geographic distribution and abundance of the various clonal groups and assess the usefulness of MLEE for epidemiological studies.

METHODS

Bacterial isolates and growth conditions. Sixty isolates of *P. trehalosi* were examined, all of which, with the exception of one from a calf, had been recovered from sheep. Except for seven strains from the National Collection of Type Cultures (NCTC) and two from the Moredun Research Institute (MRI), the isolates were obtained from nine Veterinary Investigation (VI) Centres in Scotland (five), England (two) and Wales (two). The NCTC cultures were collected 25–35 years ago. Information concerning the disease status of the host animals and tissue of origin of the isolates are provided by Davies & Quirie (1996). Properties of the isolates that are of direct relevance to the present study, including electrophoretic type (ET), capsular serotype, LPS type and OMP type, are presented in Table 1.

Growth of bacteria and electrophoresis of enzymes. Each isolate was grown overnight at 37 °C in 150 ml brain-heart infusion broth (Oxoid) on an orbital shaker (120 r.p.m.). Following incubation, samples from each culture were plated onto blood agar (brain-heart infusion agar containing 5%, v/v, defibrinated sheep's blood) to check for contamination. Bacteria were harvested by centrifugation at 5000 g for 10 min at 4 °C, resuspended in 2 ml 50 mM Tris/HCl buffer containing 1 mM EDTA and 0.05 mM NADP (pH 8.0), and disrupted by three 10 s cycles of sonication with 10 s intervals of ice-water cooling. Unbroken cells were removed by centrifugation at 15000 g for 20 min at 4 °C and the supernatant (lysate) was divided into two aliquots and stored at –70 °C.

Lysates were electrophoresed on starch gels and selectively stained for 19 metabolic enzymes by the methods described by Selander *et al.* (1986). The enzymes studied were as follows: acid phosphatase (ACP), adenylate kinase (ADK), carbamate kinase (CAK), esterase-1 and -2 (ES1 and ES2), glyceraldehyde-3-phosphate dehydrogenase (G3P), glutamate dehydrogenase (GLD), glucose-6-phosphate dehydrogenase (G6P), glutamic-oxalacetic transaminase (GOT), malate dehydrogenase (MDH), mannitol-1-phosphate dehydrogenase (M1P), nucleoside phosphorylase (NSP), 6-phosphogluconate dehydrogenase (6PG), phenylalanyl-leucine peptidase-1 and -2 (PP1 and PP2), leucine-glycine-glycine peptidase (LGG), phosphoglucose isomerase (PGI), phosphoglucosmutase (PGM), and shikimate dehydrogenase (SKD). GLD, G6P, PGM and SKD were electrophoresed in buffer system A; GOT was electrophoresed in buffer system B; ADK, MDH and M1P were electrophoresed in buffer system C; ACP, 6PG, PP1, PP2 and

Table 1. Characteristics of *P. trehalosi* isolates

ET	Reference isolate	No. of isolates	Capsular serotype	LPS type	OMP type	Origin of isolates (no.)
Cluster A1						
1	PH728	2	T4	3	1	Dumfries (2)
2	PH246	5	T4	3	1	NCTC (2), Penrith (3)
2	PH636	3	T4	6	1	Edinburgh (3)
3	PH356	7	T10	2	1	Aberystwyth (1), Auchincruvie (1), Carmarthen (2), Penrith (2), St Boswells (1)
3	PH662	2	T10	4	1	Carmarthen (2)
4	PH492	8	T10	2	1	Aberystwyth (1), Carmarthen (1), Dumfries (1), Edinburgh (1), Penrith (3), St Boswells (1)
4	PH252	1	T10	2	2	NCTC (1)
5	PH328	4	T4	4	1	Aberystwyth (1), Carmarthen (1), Edinburgh (1), Penrith (1)
6	PH750	3	T15	4	1	Penrith (3)
7	PH520	2	T15	4	1	St Boswells (1), MRI (1)
Cluster A2						
8	PH604	1	T3	R	4	Edinburgh (1)
9	PH780	1	T15	3	2	Carmarthen (1)
10	PH722	1	T15	3	1	Carmarthen (1)
11	PH718	1	T15	3	1	Carmarthen (1)
12	PH664	2	T15	3	1	Carmarthen (2)
Cluster B						
13	PH732	3	T4	3	2	Penrith (3)
14	PH688	1	T4	3	1	Carmarthen (1)
15	PH70	4	T15	3	3	NCTC (3), Penrith (1)
Cluster C						
16	PH694	2	T15	3	2	Aberdeen (2)
17	PH648	2	T15	5	1	Carmarthen (1), Shrewsbury (1)
Cluster D						
18	PH792	3	UT	3	3	Penrith (3)
Cluster E						
19	PH68	1	T3	1	1	NCTC (1)
20	PH674	1	T3	1	1	MRI (1)

LGG were electrophoresed in buffer system E; CAK, ES1, ES2, G3P, NSP and PGI were electrophoresed in buffer system I.

For each enzyme, distinctive mobility variants were designated as electromorphs and numbered in order of decreasing rate of anodal migration. Electromorphs of an enzyme were equated with alleles at the corresponding structural gene locus, and an absence of enzyme activity was attributed to a null allele, designated as 0. Because most isolates showed activity for all 19 enzymes, it was assumed that the corresponding structural gene loci are located on the chromosome rather than on plasmids. Isolates with identical combinations of alleles at the 19 enzyme loci corresponded to a unique multilocus genotype and were designated as an ET.

Statistical analysis. Genetic diversity at an enzyme locus (h) among either ETs or isolates was calculated from the allele frequencies by $h = (1 - \sum x_i^2)/(n-1)$ where x_i is the frequency of the i th allele and n is the number of ETs or isolates (Selander *et al.*, 1986). Mean genetic diversity per locus (H) is the arithmetic average of h values for all loci. Genetic distance between pairs of ETs was expressed as the proportion of enzyme loci at which different alleles were represented (mismatches). A dendrogram showing relationships among

ETs was constructed from the matrix of distance coefficients by the unweighted-pair group method with averages (UPGMA) clustering strategy (Sneath & Sokal, 1973) with the program MEGA (Molecular Evolutionary Genetics Analysis). Various statistical programs, together with a conversion program for MEGA, were provided by T. S. Whittam (Institute of Molecular and Evolutionary Genetics, Pennsylvania State University).

Capsular serotyping. The method described by Fraser & Donachie (1983) was used for capsular serotyping.

LPS and OMP profiles. Methods for the analysis of LPS and OMP profiles have been described by Davies & Quirie (1996).

RESULTS

Overall genetic diversity

In the collection of 60 isolates of *P. trehalosi* examined by MLEE, 13 of the 19 enzyme loci were polymorphic for two to seven alleles encoding electrophoretically distinguishable variant proteins, and six loci were monomorphic (Table 2). The mean number of alleles

Table 2. Allele profiles at 19 enzyme loci in 20 ETs of *P. trehalosi*

Abbreviations: ADK, adenylate kinase; MDH, malate dehydrogenase; M1P, mannitol-1-phosphate dehydrogenase; CAK, carbamate kinase; ES1 and ES2, esterase; G3P, glyceraldehyde-3-phosphate dehydrogenase; NSP, nucleoside phosphorylase; PGI, phosphoglucose isomerase; GOT, glutamic-oxalacetic transaminase; GLD, glutamate dehydrogenase; G6P, glucose-6-phosphate dehydrogenase; PGM, phosphoglucomutase; SKD, shikimate dehydrogenase; ACP, acid phosphatase; 6PG, 6-phosphogluconate dehydrogenase; PP1 and PP2, phenylalanyl-leucine peptidase; LGG, leucine-glycine-glycine peptidase.

ET	Reference isolate	No. of isolates	Allele at the indicated enzyme locus																		
			ADK	MDH	M1P	CAK	ES1	ES2	G3P	NSP	PGI	GOT	GLD	G6P	PGM	SKD	ACP	6PG	PP1	PP2	LGG
1	PH728	2	2	1	1	2	2	6	1	1	1	3	2	2	1	2	1	3	4	1	4
2	PH246	8	2	1	1	2	2	5	1	1	1	3	2	2	1	2	1	3	4	1	4
3	PH356	9	2	1	1	2	2	0	1	1	1	3	1	2	1	2	1	3	4	1	4
4	PH492	9	2	1	1	2	2	0	1	1	1	1	1	2	1	2	1	3	4	1	4
5	PH328	5	2	1	1	2	2	0	1	1	1	1	2	2	1	1	1	3	4	1	4
6	PH750	3	2	1	1	2	2	0	1	1	1	1	1	2	1	1	1	3	4	1	4
7	PH520	2	2	1	1	2	2	0	1	1	1	1	1	1	1	1	1	3	4	1	4
8	PH604	1	2	1	1	2	1	5	1	1	1	3	2	2	1	3	1	1	4	1	4
9	PH780	1	2	1	1	2	1	3	1	1	1	3	1	2	1	3	1	3	4	1	4
10	PH722	1	2	1	1	2	1	3	1	1	1	3	2	2	1	3	1	2	4	1	4
11	PH718	1	2	1	1	2	1	3	1	1	1	1	2	2	1	3	1	3	4	1	4
12	PH664	2	2	1	1	2	1	3	1	1	1	3	2	2	1	3	1	3	4	1	4
13	PH732	3	2	1	1	2	1	4	1	1	1	1	2	2	1	3	1	3	3	1	3
14	PH688	1	2	1	1	2	2	2	1	1	1	1	2	2	1	3	1	3	1	1	1
15	PH70	4	2	1	1	2	2	6	1	1	1	1	2	2	1	3	1	3	1	1	1
16	PH694	2	2	1	1	2	2	1	1	1	1	3	2	2	1	3	1	1	1	2	1
17	PH648	2	2	1	1	2	2	0	1	1	1	4	2	2	1	3	1	3	5	2	5
18	PH792	3	2	1	2	2	2	6	1	1	1	2	2	2	1	3	1	4	2	1	2
19	PH68	1	1	1	1	1	2	0	1	1	1	5	1	2	1	2	1	4	3	1	3
20	PH674	1	1	1	1	1	2	0	1	1	1	5	1	2	1	2	1	3	3	1	3

Table 3. Allele frequencies and genetic diversity (h) at 19 enzyme loci in 20 ETs of *P. trehalosi*

Enzyme abbreviations are defined in the legend to Table 2.

Enzyme locus	Frequency of the indicated allele:							h^*
	1	2	3	4	5	6	0	
ADK	0.100	0.900						0.189
MDH	1.000							0.000
M1P	0.950	0.050						0.100
CAK	0.100	0.900						0.189
ES1	0.300	0.700						0.442
ES2	0.050	0.050	0.200	0.050	0.100	0.150	0.400	0.800
G3P	1.000							0.000
NSP	1.000							0.000
PGI	1.000							0.000
GOT	0.400	0.050	0.400	0.050	0.100			0.700
GLD	0.350	0.650						0.479
G6P	0.050	0.950						0.100
PGM	1.000							0.000
SKD	0.150	0.300	0.550					0.616
ACP	1.000							0.000
6PG	0.100	0.050	0.750	0.100				0.437
PP1	0.150	0.050	0.150	0.600	0.050			0.621
PP2	0.900	0.100						0.189
LGG	0.150	0.050	0.150	0.600	0.050			0.621

* Mean genetic diversity per locus, $H = 0.289$.

Fig. 1. Genetic relationships of ETs of *P. trehalosi* isolates. The dendrogram was generated by UPGMA clustering from a matrix of coefficients of pairwise genetic distances based on 19 enzyme loci. ETs are numbered sequentially from top to bottom in the order of listing in Table 2. *N* is the number of isolates in each ET represented by multiple isolates; all other ETs were represented by single isolates. Five lineages, identified at a genetic distance of 0.14, are indicated by the letters A–E; lineages A1 and A2 diverged at a genetic distance of 0.1.

Table 4. Mean genetic diversity (*H*) at 19 enzyme loci among ETs of *P. trehalosi* classified by serotype

Serotype	No. of:		Diversity among ETs	
	Isolates	ETs	<i>H</i>	Variance
T4	18	5	0.221	0.080
T10	18	2	0.053	0.053
T15	18	9	0.263	0.064
T3	3	3	0.368	0.084
Untypable	3	1	0.000	0.000

per locus was 2.6. Twenty ETs were identified (Table 2), among which the mean genetic diversity per locus (*H*) was 0.289 (Table 3). There was less diversity among the isolates (*H* = 0.225), because 13 of the ETs were represented by two or more isolates (mean 4.1 isolates; range 2–9).

Genetic relationships among multilocus genotypes

Estimates of the genetic relationships among the 20 ETs are summarized in the dendrogram in Fig. 1. The smallest observed genetic distance (0.03) between ETs corresponds to a single locus difference. At a genetic distance of 0.14 there were five lineages or clusters of lineages designated A–E (Fig. 1). Cluster A consisted of 12 ETs distributed in two sub-clusters, A1 and A2,

which separated at a genetic distance of 0.1. Lineage A1 consisted of 37 (62%) isolates in seven ETs, whereas lineage A2 consisted of only six (10%) isolates in five ETs. Clusters B and C diverged from cluster A at a genetic distance of 0.16 and consisted of 12 (20%) isolates in five ETs. Clusters D and E diverged from clusters A–C at genetic distances of 0.22 and 0.23, respectively, and consisted of five isolates in three ETs.

Genetic variation in relation to capsular serotypes, LPS types and OMP types

Estimates of the extent of genetic variation among ETs of the various capsular serotypes are presented in Table 4. Diversity (*H*) among ETs of a given serotype was, on average, 0.226, which is 78% of that in the total sample of 20 ETs. It ranged from 0.053 for serotype T10, represented by 18 isolates in two closely related ETs in cluster A, to 0.368 for serotype T3, represented by three isolates in three ETs. There were no cases of isolates of different capsular serotypes occurring within the same ET. The three untypable (UT) isolates, which originated from the same animal and probably represent a new serotype, were genetically identical.

Specific combinations of capsular serotype, LPS type and OMP type were associated with each ET or closely related groups of ETs (Table 1). Within cluster A1, isolates of ETs 2–4 could be sub-divided on the basis of variation in either LPS (ETs 2 and 3) or OMP (ET 4) type. ETs 1 and 2, 3 and 4, and 5–7, formed three distinct groups based on the associations of serotypes, LPS types and OMP types. By contrast, ETs within cluster A2 were more homogeneous, with the exception of ET 8, in terms of their capsular serotypes, LPS types and OMP types. Clusters B and C consisted of serotype T4 and T15 isolates in five ETs. These isolates generally possessed the same LPS types as those in cluster A2 (i.e. LPS type 3), but had more variable OMP types. Clusters D and E included the group of previously unrecognized untypable isolates (ET 18), together with two serotype T3 isolates in ETs 19 and 20.

In most of the cases described above, isolates having the same capsule/LPS/OMP types belonged to the same or to closely related multilocus genotypes. However, a small number of isolates had identical capsule/LPS/OMP types but belonged to unrelated ETs. For example, isolates with capsular serotype T4/LPS type 3/OMP type 1 occurred in ETs 1, 2 and 14; similarly, isolates having capsular serotype T15/LPS type 3/OMP type 2 occurred in ETs 9 and 16.

Genetic diversity within geographic region

The geographical distribution of the isolates is summarized in Table 1. Those isolates of ETs in lineage A1 were the most numerous and geographically widespread. In particular, ETs 3 and 4, which consisted of 18 serotype T10 isolates and accounted for 30% of the total number of isolates, originated from seven of the nine VI Centres (Aberystwyth, Auchincruvie, Carmarthen,

Dumfries, Edinburgh, Penrith and St Boswells) and represented two geographically widespread and important clonal groups. ET 5 consisted of only four isolates, but each was recovered by a different VI Centre in Scotland, England or Wales (Aberystwyth, Carmarthen, Edinburgh and Penrith) and was therefore also geographically widely distributed. Other ETs in this lineage consisted of only two to three isolates which originated from one or two generally different VI Centres; for example ET 1 isolates originated from Dumfries, ET 2 isolates originated from Edinburgh and Penrith, ET 6 isolates originated from Penrith and ET 7 isolates originated from St Boswells (Table 1). By contrast, lineage A2 included four ETs (ETs 9–12) that were represented by only one or two isolates recovered from the same VI Centre, Carmarthen. Clusters B and C consisted of ETs 13–17 and included isolates from Aberdeen, Carmarthen, Penrith and Shrewsbury. However, with the exception of ET 17, each ET was represented by isolates from a single VI Centre only. The previously unrecognized untypable isolates belonged to ET 18 and were recovered from a single VI Centre, Penrith.

DISCUSSION

Clonal nature of *P. trehalosi*

The clone concept of bacterial population structure, first demonstrated in *Escherichia coli* (Ochman & Selander, 1984; Ørskov *et al.*, 1976), is now well accepted, although it is also recognized that not all bacterial species have a clonal population structure (Go *et al.*, 1996; Maynard-Smith *et al.*, 1993; Selander & Musser, 1990). The recovery of the same multilocus genotypes at different localities and times within the UK suggests that chromosomal recombination occurs relatively infrequently in natural populations of *P. trehalosi* and that, consequently, the genetic structure of the species is basically clonal. For example, isolates of ETs 3, 4 and 5 were recovered from VI Centres in Scotland, England and Wales; and isolates of ETs 2 and 15, which included NCTC strains and recent field isolates, were recovered at least 25–35 years apart. The fact that isolates of the same capsule/LPS/OMP types frequently belong to the same ET, or to closely related ETs, is also consistent with a basically clonal population structure. ETs 3 and 4, which included 30% of the total number of isolates, were recovered from seven of the nine VI Centres in Scotland, England and Wales and represent two important geographically widespread clones.

Genetic diversity compared with that of other bacteria

P. trehalosi is genetically less diverse ($H = 0.289$) than most other species of pathogenic bacteria that have been examined, with the exceptions of *Bordetella* spp. ($H = 0.284$) (Musser *et al.*, 1986) and *Staphylococcus aureus* ($H = 0.289$) (Musser & Selander, 1990). The restricted diversity of *P. trehalosi* could, in theory, be explained by

a low mutation rate, recent evolutionary origin, niche specialization or small effective population size. The role that a low mutation rate may have is impossible to assess in the absence of data on the spontaneous mutation rate of this organism. That *P. trehalosi* colonizes the tonsils of, and causes disease only in, sheep indicates a high level of tissue tropism and host specificity, which, in turn, might suggest that niche specialization is a factor involved in the low genetic diversity of this pathogen. But studies of a number of other bacteria, including *E. coli*, *Neisseria meningitidis*, *Legionella pneumophila* and *Bordetella bronchiseptica*, have failed to demonstrate a relationship between niche width and genetic diversity (Musser *et al.*, 1987). A possible explanation for the low genetic diversity of *P. trehalosi* is a recent evolutionary origin and consequent low effective population size. However, the fact that the sample was derived from a relatively small geographic area and was represented by isolates recovered only from diseased animals might also account for the low diversity. Since *P. trehalosi* is recovered at high rates from the tonsils of healthy sheep (Al-Sultan & Aitken, 1985; Gilmour *et al.*, 1974), studies of isolates from healthy animals and from other countries might reveal a higher diversity. In support of this, Møller *et al.* (1992) obtained a mean genetic diversity of only 0.247 for *A. pleuropneumoniae* isolates obtained from Denmark, whereas Musser *et al.* (1987) reported a value of 0.370 for *A. pleuropneumoniae* isolates recovered from 14 different countries.

Genetic variation in relation to capsular serotypes, LPS types and OMP types

In a previous study of LPS and OMP variation within *P. trehalosi*, Davies & Quirie (1996) suggested that serotype T10 isolates are less diverse than those of serotypes T4 and T15. This conclusion was confirmed in the present investigation. Serotype T15 ($H = 0.263$) isolates belonged to nine ETs occurring in each of the clusters A, B and C, and were more diverse than isolates of serotypes T4 or T10. Serotype T4 ($H = 0.221$) isolates belonged to five ETs occurring in lineages A1 and B, whereas serotype T10 ($H = 0.053$) isolates belonged to only two ETs, both in lineage A1. The T4 and T15 capsules are structurally very similar, differing only in the linkage of a phosphate group to galactose at C4 in the T4 capsule and at C6 in the T15 capsule (Adlam *et al.*, 1985a, b). The structure of the T10 capsule is not known, but, based on the genetic relationships of *P. trehalosi* isolates, it seems likely that it may more closely resemble the T4 capsule than that of T15. Although, in some cases, closely related clusters of ETs possessed different capsules (e.g. ETs 4–7), each ET was represented by only a single capsular serotype. This situation is similar to that in *H. influenzae* (Musser *et al.*, 1988a, b) and *A. pleuropneumoniae* (Musser *et al.*, 1987) and differs markedly from that in *E. coli* (Caugant *et al.*, 1985) and *N. meningitidis* (Caugant *et al.*, 1987) in which many ETs are represented by isolates of two or more polysaccharide capsule serotypes.

The O-antigen structures of LPS from T4 and T10 isolates (Perry & Babiuk, 1984; Richards & Leitch, 1989) and from T3 and T15 isolates (Lacroix *et al.*, 1993; Leitch & Richards, 1988), respectively, have been shown to be identical, although Davies & Quirie (1996) demonstrated that isolates of the same capsular serotype may have different O-antigen profiles. It was previously suggested that the O-antigen side-chains of type 2 and type 4 LPS are identical, and that these LPS types differ only in the nature of the core-oligosaccharide region (Davies & Quirie, 1996). The present study demonstrated that isolates possessing LPS types 2 and 4 are genetically related ($H = 0.095$; results not shown); isolates possessing these LPS types belonged to the related ETs 3–7 in sub-cluster A1 (Table 1 and Fig. 1). The type 2/4 O-antigen is therefore an important marker of this genetically related group of ETs which was represented by 27 (45%) isolates. By comparison, isolates possessing LPS type 3 were genetically more diverse ($H = 0.248$; results not shown), being distributed in 11 ETs occurring in clusters A–D. Based on the MLEE data, it appears that LPS types 5 and 6 might be related to LPS type 3, since isolates of LPS types 5 and 6 were genetically related to those of LPS type 3.

Only three major OMP profiles were present in the *P. trehalosi* population, and isolates of the same OMP type were not necessarily genetically related. For example, OMP type 1, which occurred in 75% of isolates, was found in all clusters except D; OMP type 2 occurred in four ETs (4, 9, 13 and 16) in four lineages; and OMP type 3 occurred in two ETs (15 and 18) in two lineages.

The findings of the present study indicate that capsular serotyping is an ineffective method for assessing the genetic relationships of isolates within *P. trehalosi* and is of limited use in epidemiological studies. MLEE identified distinctive multilocus genotypes among isolates of the same serotype obtained from the same VI Centre (e.g. T15 isolates of ETs 9–12 from Carmarthen), differentiated between isolates of the same serotype from different VI Centres (e.g. T4 isolates of ETs 1, 13 and 14 from Dumfries, Penrith and Carmarthen, respectively) and demonstrated clonal identity of isolates of the same serotype from different VI Centres (e.g. T4 isolates of ET 5 from Aberystwyth, Carmarthen, Edinburgh and Penrith). MLEE had greater resolving power than capsule/LPS/OMP typing, being able to distinguish 20 groups rather than 14 and to differentiate between isolates of the same capsule/LPS/OMP type. However, in three cases (ETs 2, 3 and 4) LPS and OMP analysis differentiated between isolates of the same multilocus genotypes. The present study demonstrated that the three untypable isolates (ET 18) were genetically distinct, confirming that these isolates probably represent a new serotype.

Genetic variation in relation to disease

In those pathogenic bacteria that have a clonal population structure, the majority of cases of serious disease

is caused by a small proportion of the total number of extant clones (Selander & Musser, 1990). With the exception of nine isolates that were either recovered from healthy sheep (two isolates) or were of unknown origin (seven isolates), 51 of the total sample of 60 *P. trehalosi* isolates were recovered from sheep diagnosed as suffering from systemic infection (40 isolates) or pneumonia (11 isolates) (for further information see Davies & Quirie, 1996). Furthermore, with the exception of ETs 19 and 20, all of the ETs were represented by one or more isolates that had originated from diseased sheep. Discounting isolates representing the two T10 clones (ETs 3 and 4), 35 isolates (58%) representing 16 ETs, each of which included just one to four isolates, were found to be associated with disease. With the exception of the T10 clones, therefore, it appears that there is no significant variation in pathogenic potential among the genetic divisions of *P. trehalosi*. The observed lack of association of specific multilocus genotypes with disease suggests that the role of *P. trehalosi* in systemic and pneumonic pasteurellosis in sheep is largely opportunistic. It has been postulated (Gilmour, 1980) that disease outbreaks caused by *P. trehalosi* may be related to stress associated with husbandry changes, such as the movement of sheep and change of diet in the autumn. In addition, experimental reproduction of infection by *P. trehalosi* is difficult to induce in sheep (Gilmour, 1978). These observations are consistent with the conclusion of the present study that *P. trehalosi* is primarily an opportunistic pathogen. However, the occurrence of 18 serotype T10 isolates in only two ETs suggests a higher degree of virulence for these isolates. In addition to having the same capsular structure, the two T10 clones were characterized by possession of similar, if not identical, O-antigens (LPS types 2 and 4). These data suggest, therefore, that the T10 capsule and type 2/4 O-antigen confer enhanced virulence on members of these two clones. Clearly, further work is required to elucidate the precise role in the disease process of the different cell-surface structures of this pathogen.

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