Evidence for a common gene pool and frequent recombinational exchange of the *tbpBA* operon in *Mannheimia haemolytica*, *Mannheimia glucosida* and *Bibersteinia trehalosi*

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The *tbpBA* operon was sequenced in 42 representative isolates of *Mannheimia haemolytica* (32), *Mannheimia glucosida* (6) and *Bibersteinia trehalosi* (4). A total of 27 *tbpB* and 20 *tbpA* alleles were identified whilst the *tbpBA* operon was represented by 28 unique alleles that could be assigned to seven classes. There were 1566 (34.8% variation) polymorphic nucleotide sites and 482 (32.1% variation) variable inferred amino acid positions among the 42 *tbpBA* sequences. The *tbpBA* operons of serotype A2 *M. haemolytica* isolates are, with one exception, substantially more diverse than those of the other *M. haemolytica* serotypes and most likely have a different ancestral origin. The *tbpBA* phylogeny has been severely disrupted by numerous small- and large-scale intragenic recombination events. In addition, assortative (entire gene) recombination events, involving either the entire *tbpBA* operon or the individual *tbpB* and *tbpA* genes, have played a major role in shaping *tbpBA* structure and its distribution in the three species. Our findings indicate that a common gene pool exists for *tbpBA* in *M. haemolytica*, *M. glucosida* and *B. trehalosi*. In particular, *B. trehalosi*, *M. glucosida* and ovine *M. haemolytica* isolates share a large portion of the *tbpA* gene, and this probably reflects selection for a conserved TbpA protein that provides effective iron uptake in sheep. Bovine and ovine serotype A2 lineages have very different *tbpBA* alleles. Bovine-like *tbpBA* alleles have been partially, or completely, replaced by ovine-like *tbpBA* alleles in ovine serotype A2 isolates, suggesting that different transferrin receptors are required by serotype A2 isolates for optimum iron uptake in cattle and sheep. Conversely, the *tbpBA* alleles of bovine-pathogenic serotype A1 and A6 isolates are very similar to those of closely related ovine isolates, suggesting a recent and common evolutionary origin.

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INTRODUCTION

Mannheimia haemolytica is the aetiological agent of bovine and ovine pneumonic pasteurellosis. These are serious respiratory tract infections that cause considerable economic losses to the cattle and sheep industries (Frank, 1989; Gilmour & Gilmour, 1989). Infections of cattle are usually associated with isolates of capsular serotypes A1 and A6 whereas infections of sheep are most frequently caused by isolates of serotypes A2 and A7 (Frank, 1989; Gilmour & Gilmour, 1989). Evidence from previous studies (Davies &

Donachie, 1996; Davies & Lee, 2004; Davies *et al.*, 1997a, 2001, 2002) suggests that natural populations of *M. haemolytica* consist of genetically distinct subpopulations that are differentially adapted to, and elicit disease in, either cattle or sheep. However, the molecular basis of host adaptation in bovine and ovine lineages of *M. haemolytica* remains unclear. *Mannheimia glucosida* and *Bibersteinia trehalosi* were previously recognized as distinct serotypes of *M. haemolytica* before being reclassified as separate species and genera (Angen *et al.*, 1999; Blackall *et al.*, 2007; Sneath & Stevens, 1990). *M. glucosida* represents a heterogeneous group of bacteria that are mainly opportunistic pathogens of sheep (Angen *et al.*, 1999; Davies *et al.*, 1997a). *B. trehalosi* is associated predominantly with sheep, in which it causes a systemic infection that is pathologically distinct from pneumonic pasteurellosis (Davies *et al.*, 1997b; Gilmour & Gilmour, 1989).

Abbreviation: ET, electrophoretic type.

The GenBank/EMBL/DDBJ accession numbers for the *tbpBA* sequences (including the non-coding intergenic regions) obtained during this study are AY850230–AY850258.

Two supplementary tables and eight supplementary figures are available with the online version of this paper.

The ability to sequester iron is recognized as a key virulence factor of pathogenic bacteria. Bacterial species representing the families *Neisseriaceae*, *Pasteurellaceae* and *Moraxellaceae* have evolved highly specific uptake systems to obtain iron direct from host transferrin (Cornelissen, 2003). These transferrin iron uptake systems consist of (i) two receptor proteins, TbpA and TbpB, responsible for binding transferrin and transport of iron across the outer membrane, (ii) a periplasmic-protein-dependent transport system, consisting of three proteins, FbpA, FbpB and FbpC, that is responsible for transport of iron across the periplasm and cytoplasmic membrane, and (iii) the TonB–ExbB–ExbD energy transduction system (Cornelissen, 2003). TbpA is a TonB-dependent integral membrane protein that is essential for iron uptake. Based on the crystal structure of the TonB-dependent FepA (Buchanan *et al.*, 1999), TbpA is thought to consist of 22 putative amphipathic β -strands, 11 putative surface-exposed loops and a plug domain. The protein forms a pore in the outer membrane through which iron passes after its release from transferrin. TbpB is a putatively surface-exposed lipoprotein that is thought to increase the efficiency of iron uptake from transferrin. The protein has a bilobed structure where each lobe consists of an eight-stranded β barrel flanked by a ‘handle’ domain (Moraes *et al.*, 2009).

M. haemolytica possesses a transferrin receptor complex that is involved in iron uptake from host transferrin (Ogunnariwo *et al.*, 1997). The transferrin receptor proteins TbpA and TbpB have molecular masses of approximately 107 and 63 kDa, respectively, and the *tbpA* and *tbpB* genes are arranged in an operon arrangement of *tbpB–tbpA*. The transferrin receptor of *M. haemolytica* is highly specific for ruminant transferrin (Ogunnariwo & Schryvers, 1990; Yu *et al.*, 1992) and is likely to contribute to host specificity. The protective capacity of antibodies to TbpA and TbpB has been studied in serotype A1 and A2 *M. haemolytica* isolates in cattle (Potter *et al.*, 1999) and sheep (Gilmour *et al.*, 1991), respectively. Very little is known about TbpA and TbpB diversity in *M. haemolytica* and, in particular, how such diversity might relate to transferrin specificity and host adaptation. Previously, we have shown that horizontal DNA transfer and recombination have made significant contributions, complementing mutation, to the evolution of the leukotoxin operon (*lktCABD*) and *ompA* gene of *M. haemolytica* (Davies & Lee, 2004; Davies *et al.*, 2001, 2002). Interestingly, *M. glucosida* and *B. trehalosi* have played important roles in the diversification of these genes in *M. haemolytica*. The aim of the present study was to investigate the genetic diversity and evolutionary relationships of the *tbpA* and *tbpB* genes in these three species. In particular, we wished to determine the role of horizontal recombinational exchange in *tbpA* and *tbpB* evolution, examine the influence of *M. glucosida* and *B. trehalosi* on *tbpA* and *tbpB* diversity in *M. haemolytica*, and assess how TbpA and TbpB diversity might relate to known differences in virulence and host specialization of *M. haemolytica*.

METHODS

Bacterial strains and growth conditions. Partial sequences of the *tbpB* and *tbpA* genes were determined in 32 *M. haemolytica*, six *M. glucosida* and four *B. trehalosi* isolates. The 42 isolates have been well characterized in previous studies (Davies & Donachie, 1996; Davies & Quirie, 1996; Davies & Lee, 2004; Davies *et al.*, 1996, 1997a, b, 2001, 2002) and were selected to represent specific evolutionary lineages, capsular serotypes and hosts of origin. Properties of these isolates are presented in Table 1. Isolates were stored at -85°C in 50% (v/v) glycerol in brain–heart infusion (BHI) broth (Oxoid) and subcultured on blood agar [BHI agar (Oxoid) containing 5% (v/v) sheep’s blood] by overnight aerobic incubation at 37°C . For preparation of DNA, a few colonies were inoculated into 15 ml volumes of BHI broth and grown aerobically overnight at 37°C at 120 r.p.m.

Preparation of chromosomal DNA. Cells from 1.0 ml overnight cultures were harvested by centrifugation for 1 min at 13 000 g and washed once in sterile distilled H_2O . DNA was prepared with InstaGene Matrix (Bio-Rad) according to the manufacturer’s instructions and stored at -20°C .

PCR amplification and DNA sequence analysis. Four forward (192, 193, 223 and 426) and three reverse (191, 198 and 224) primers have previously been shown to amplify transferrin binding protein genes in several members of the *Pasteurellaceae* (Ogunnariwo & Schryvers, 1996). These primers were tested against the panel of 42 isolates in various combinations with mixed results. For those isolates that gave negative results, new primers were designed using the *tbpB* and *tbpA* gene sequences from *M. haemolytica* and the related species *Aggregatibacter actinomycetemcomitans*, *Haemophilus ducreyi*, *Haemophilus influenzae* and *Pasteurella multocida*. These sequences were downloaded from the GenBank database and aligned using the Lasergene MEGALIGN (DNA Star) software application. Conserved regions were identified and these were used to design new PCR amplification primers. The extensive sequence variation that exists within the *tbpBA* operon necessitated the use of a complex PCR amplification and sequencing strategy that is summarized in Supplementary Fig. S1 (available with the online version of this paper). Based on the initial PCR amplification scheme required to amplify overlapping segments of the *tbpB* and *tbpA* genes, the *M. haemolytica* isolates could be subdivided into seven groups (Mh1–Mh7) and the *B. trehalosi* isolates into two groups (Bt1 and Bt2); the *M. glucosida* isolates were represented by a single group (Mg) (Supplementary Fig. S1). In most cases the amplification primers were also used for the first stage of sequencing although separate sequencing primers were designed in some cases. Both strands of the PCR amplicons were sequenced and internal sequencing primers were designed as sequence data became available. The primers were designed with the computer program Primer Designer Version 2.0 (Scientific and Educational Software) and synthesized by Sigma-Genosys. Full details of the amplification and sequencing primers are provided in Supplementary Table S1 (available with the online version of this paper).

PCR fragments containing partial segments of the *tbpB* or *tbpA* genes were amplified from chromosomal DNA by using a *Taq* DNA polymerase kit (Boehringer Mannheim) according to the manufacturer’s instructions. PCRs were carried out in a Perkin-Elmer 480 DNA thermal cycler using, in most cases, the following amplification parameters: denaturation at 94°C for 45 s, annealing at 55°C for 45 s, and extension at 72°C for 2 min. However, different annealing temperatures were used for some primer pairs as follows: 50°C for 223a/287 (Bt1 and Bt2); 52°C for 399/380 and 379/339 (Mh5); 56°C for 345/198a (Mh7); 57°C for 335/224a (Bt2); and 58°C for 223a/287 (Mh5). Thirty cycles were performed and a final extension step of 72°C for 10 min was used. The production of PCR amplicons of the

expected size was confirmed by agarose gel electrophoresis and the DNA was purified with a Qiaquick PCR purification kit (Qiagen). DNA was finally eluted in 30 µl sterile distilled H₂O and stored at -20 °C. Sequencing reactions were performed with an ABI Prism Big Dye Terminator cycle sequencing kit (Applied Biosystems) in a GeneAmp PCR System 9700 (Applied Biosystems) thermal cycler. Sequence analysis was performed with an Applied Biosystems 377 DNA sequencer (University of Glasgow Sequencing Service).

Analysis of nucleotide and protein sequence data. Nucleotide sequence data were analysed and edited with Seqed (Applied Biosystems) and Lasergene (DNASTAR) sequence analysis software. Phylogenetic and molecular evolutionary analyses were conducted with MEGA, version 4.0 (Kumar *et al.*, 2001), in conjunction with alignment programs written by T.S. Whittam (Michigan State University). Split decomposition trees were constructed with SplitsTree 4.0 (<http://www.splitsree.org>) to test for recombination (Huson & Bryant, 2006). ClonalFrame version 1.1 was used for further phylogenetic analysis and to identify regions likely to have undergone recombination (Didelot & Falush, 2007). We used 50 000 iterations, 50 000 burn-in iterations and every hundredth tree was sampled. Phylogenetic trees generated by ClonalFrame were imported into MEGA for further annotation.

Nucleotide sequence accession numbers. The GenBank accession numbers for the *tbpBA* sequences (including the non-coding intergenic regions) obtained in this study are provided in Table 1.

RESULTS

Nucleotide and amino acid variation within *tbpBA*

Approximately 97 % of the *tbpBA* operon was sequenced in 32 *M. haemolytica* isolates representing 11 capsular serotypes and the 22 electrophoretic types (ETs) previously defined by multilocus enzyme electrophoresis (Davies *et al.*, 1997a). The *tbpBA* operon was also sequenced in six isolates representing different ETs of *M. glucosida* and in four isolates representing each of the capsular serotypes T3, T4, T10 and T15 of *B. trehalosi* (Davies *et al.*, 1997a, b). PCR errors have been previously shown to be insignificant by duplicate amplification and sequencing of the *lktA* and *ompA* genes in selected isolates (Davies & Lee, 2004; Davies *et al.*, 2001). The total number of nucleotides sequenced varied from 4496 for the *B. trehalosi* isolates to 4531 for the *M. haemolytica* isolates PH550, PH202 and PH470. The *tbpB* and *tbpA* sequences varied from 1668 to 1695 nt (96 % of *tbpB*) and 2736 to 2766 nt (98 % of *tbpA*), respectively (Table 1). The predicted amino acid sequences varied from 556 to 565 aa and 912 to 922 aa for *tbpB* and *tbpA*, respectively (Table 1). The non-coding intergenic regions varied from 71 to 85 nt in length and were deleted for the sequence analyses. The total aligned length (including gaps) of the 42 modified *tbpBA* sequences was 4503 nt.

Twenty-seven unique *tbpB* sequences and 20 unique *tbpA* sequences, each representing a distinct allele, were identified. The 27 *tbpB* alleles were assigned to six subclasses, *tbpB1* to *tbpB6*, based on their clustering in a neighbour-joining tree (see below), and individual alleles within each subclass were

designated *tbpB1.1*, *tbpB1.2*, etc. (Table 1). Similarly, the 20 *tbpA* alleles could also be assigned to six subclasses, *tbpA1* – *tbpA6*, based on their clustering in a neighbour-joining tree (see below), and individual alleles within each subclass were designated *tbpA1.1*, *tbpA1.2*, etc. (Table 1). The *tbpB* and *tbpA* genes were also considered as a single operon, *tbpBA*, and 28 unique sequences or alleles were identified. These alleles were assigned to seven subclasses, *tbpBA1*–*tbpBA7*, based on their clustering in a neighbour-joining tree (see below), and individual alleles within each subclass were designated *tbpBA1.1*, *tbpBA1.2*, etc. (Table 1). There were 1566 (34.8 %) polymorphic nucleotide sites and 482 (32.1 %) variable inferred amino acid positions among the 28 *tbpBA* sequences. Pairwise differences in nucleotide and inferred amino acid sequences between representative pairs of the seven *tbpBA* allele types ranged from 25 to 1143 (0.6 to 25.4 %) nucleotide sites and 16 to 365 (1.1 to 24.3 %) amino acid positions (see Supplementary Table S2, available with the online version of this paper).

Phylogenetic relationships of the *tbpB*, *tbpA* and *tbpBA* alleles

The phylogenetic relationships of the 42 *tbpB* and *tbpA* sequences are shown in Supplementary Figs S2 and S3 (available with the online version of this paper), respectively. The *tbpB1*-, *tbpB3*-, *tbpB4*- and *tbpB6*-type alleles were associated exclusively with *M. haemolytica*, whereas the *tbpB2*-type alleles were present in *M. haemolytica* and *M. glucosida* and the *tbpB5*-type alleles in *B. trehalosi*. The *tbpA1*-, *tbpA4*-, *tbpA5*- and *tbpA6*-type alleles were associated exclusively with *M. haemolytica*, whereas the *tbpA2*-type alleles were present in *M. glucosida* and the *tbpA3*-type alleles in *B. trehalosi*. Visual inspection of the *tbpB* and *tbpA* phylogenetic trees revealed them to be non-congruent. In the case of *tbpB*, the *tbpB6*-type ovine serotype A2 alleles were the most divergent, followed by the *tbpB5*-type *B. trehalosi* alleles and the *tbpB4*- and *tbpB3*-type bovine serotype A2 alleles. However, in the case of *tbpA*, the *tbpA5*- and *tbpA6*-type bovine serotype A2 alleles were the most divergent, followed by the *tbpA4*-type ovine serotype A2 alleles and the *tbpA3*-type *B. trehalosi* alleles. We also compared the phylogenetic relationships of *tbpB* and *tbpA* sequences representative of the major lineages/clusters of *M. haemolytica*, *M. glucosida* and *B. trehalosi* with those of other members of the *Pasteurellaceae* and *Neisseria meningitidis* (see Supplementary Figs S4 and S5 available with the online version of this paper). The *tbpB* and *tbpA* sequences of the other species were highly divergent from those of *M. haemolytica*, *M. glucosida* and *B. trehalosi*.

A neighbour-joining phylogenetic tree of the 42 *tbpBA* sequences is shown in Fig. 1. The 42 sequences form five distinct lineages, I–V, and lineage I consists of three closely related clusters, A–C. The majority (28) of the sequences are represented in clusters A–C of lineage I. Cluster A comprises *tbpBA1*-type alleles that are present in bovine

Table 1. Properties of 32 *M. haemolytica*, six *M. glucosida* and four *B. trehalosi* isolates

Isolate	ET*	Serotype	Host species	Country of origin	<i>tbpB</i>			<i>tbpA</i>			<i>tbpBA</i> allele	GenBank accession no.
					Allele	No. of nt	No. of aa	Allele	No. of nt	No. of aa		
<i>M. haemolytica</i>												
PH2	1	A1	Bovine	UK	<i>tbpB1.1</i>	1677	559	<i>tbpA1.1</i>	2748	916	<i>tbpBA1.1</i>	AY850230
PH30	1	A1	Bovine	UK	<i>tbpB1.2</i>	1671	557	<i>tbpA1.1</i>	2748	916	<i>tbpBA1.2</i>	AY850231
PH376	1	A6	Bovine	UK	<i>tbpB1.1</i>	1677	559	<i>tbpA1.1</i>	2748	916	<i>tbpBA1.1</i>	
PH346	1	A12	Ovine	UK	<i>tbpB2.1</i>	1680	560	<i>tbpA1.5</i>	2748	916	<i>tbpBA2.1</i>	AY850232
PH540	2	A1	Bovine	Germany	<i>tbpB1.3</i>	1671	557	<i>tbpA1.1</i>	2748	916	<i>tbpBA1.3</i>	AY850233
PH338	3	A9	Ovine	UK	<i>tbpB2.1</i>	1680	560	<i>tbpA1.5</i>	2748	916	<i>tbpBA2.1</i>	
PH388	4	A7	Ovine	UK	<i>tbpB2.2</i>	1680	560	<i>tbpA1.5</i>	2748	916	<i>tbpBA2.2</i>	AY850234
PH50†	5	A5	Ovine	UK	<i>tbpB2.2</i>	1680	560	<i>tbpA1.5</i>	2748	916	<i>tbpBA2.2</i>	
PH56†	5	A8	Ovine	UK	<i>tbpB1.5</i>	1671	557	<i>tbpA1.3</i>	2748	916	<i>tbpBA1.4</i>	AY850235
PH238†	5	A9	Ovine	UK	<i>tbpB1.4</i>	1671	557	<i>tbpA1.2</i>	2748	916	<i>tbpBA1.5</i>	AY850236
PH8	6	A1	Ovine	UK	<i>tbpB2.2</i>	1680	560	<i>tbpA1.4</i>	2748	916	<i>tbpBA2.3</i>	AY850237
PH398	7	A1	Ovine	UK	<i>tbpB2.2</i>	1680	560	<i>tbpA1.4</i>	2748	916	<i>tbpBA2.3</i>	
PH284	8	A6	Ovine	UK	<i>tbpB2.2</i>	1680	560	<i>tbpA1.4</i>	2748	916	<i>tbpBA2.3</i>	
PH232†	9	A6	Ovine	UK	<i>tbpB2.2</i>	1680	560	<i>tbpA1.4</i>	2748	916	<i>tbpBA2.3</i>	
PH66†	10	A14	Ovine	Ethiopia	<i>tbpB1.6</i>	1677	559	<i>tbpA1.5</i>	2748	916	<i>tbpBA1.6</i>	AY850238
PH706	11	A16	Ovine	Unknown	<i>tbpB1.7</i>	1674	559	<i>tbpA1.7</i>	2748	916	<i>tbpBA1.7</i>	AY850239
PH296	12	A7	Ovine	UK	<i>tbpB2.3</i>	1680	560	<i>tbpA1.6</i>	2748	916	<i>tbpBA2.4</i>	AY850240
PH396	13	A7	Ovine	UK	<i>tbpB2.3</i>	1680	560	<i>tbpA1.6</i>	2748	916	<i>tbpBA2.4</i>	
PH484	14	A7	Ovine	UK	<i>tbpB2.3</i>	1680	560	<i>tbpA1.6</i>	2748	916	<i>tbpBA2.4</i>	
PH588	15	A13	Ovine	UK	<i>tbpB2.2</i>	1680	560	<i>tbpA1.4</i>	2748	916	<i>tbpBA2.3</i>	AY850241
PH494	16	A2	Ovine	UK	<i>tbpB6.1</i>	1696	565	<i>tbpA4.1</i>	2736	912	<i>tbpBA5.1</i>	AY850242
PH550	17	A2	Bovine	Germany	<i>tbpB4.1</i>	1680	560	<i>tbpA6.1</i>	2766	922	<i>tbpBA7.1</i>	AY850243
PH196	18	A2	Bovine	UK	<i>tbpB3.1</i>	1677	559	<i>tbpA5.1</i>	2766	922	<i>tbpBA6.1</i>	AY850244
PH786	18	A2	Bovine	UK	<i>tbpB3.2</i>	1677	559	<i>tbpA5.1</i>	2766	922	<i>tbpBA6.2</i>	AY850245
PH526	19	A2	Ovine	UK	<i>tbpB6.2</i>	1686	562	<i>tbpA4.2</i>	2754	918	<i>tbpBA5.2</i>	AY850246
PH598	20	A2	Ovine	UK	<i>tbpB6.3</i>	1686	562	<i>tbpA4.2</i>	2754	918	<i>tbpBA5.3</i>	AY850247
PH202	21	A2	Bovine	UK	<i>tbpB4.2</i>	1680	560	<i>tbpA6.1</i>	2766	922	<i>tbpBA7.2</i>	AY850248
PH470	21	A2	Bovine	UK	<i>tbpB4.3</i>	1680	560	<i>tbpA6.1</i>	2766	922	<i>tbpBA7.3</i>	AY850249
PH278	21	A2	Ovine	UK	<i>tbpB1.8</i>	1674	558	<i>tbpA1.8</i>	2748	916	<i>tbpBA1.8</i>	AY850250
PH372	21	A2	Ovine	UK	<i>tbpB1.8</i>	1674	558	<i>tbpA1.8</i>	2748	916	<i>tbpBA1.8</i>	
PH292	22	A2	Ovine	UK	<i>tbpB6.2</i>	1686	562	<i>tbpA4.2</i>	2754	918	<i>tbpBA5.2</i>	
PH392	22	A2	Ovine	UK	<i>tbpB6.2</i>	1686	562	<i>tbpA4.2</i>	2754	918	<i>tbpBA5.2</i>	
<i>M. glucosida</i>												
PH344	1	A11	Ovine	UK	<i>tbpB2.8</i>	1674	558	<i>tbpA2.4</i>	2766	922	<i>tbpBA3.1</i>	AY850251
PH498	3	A11	Ovine	UK	<i>tbpB2.8</i>	1674	558	<i>tbpA2.4</i>	2766	922	<i>tbpBA3.1</i>	
PH240†	5	A11	Ovine	Norway	<i>tbpB2.5</i>	1674	558	<i>tbpA2.1</i>	2766	922	<i>tbpBA3.2</i>	AY850252
PH496	7	UG3	Ovine	UK	<i>tbpB2.4</i>	1674	558	<i>tbpA2.3</i>	2766	922	<i>tbpBA3.3</i>	AY850253

Table 1. cont.

Isolate	ET*	Serotype	Host species	Country of origin	tbpB		tbpA		tbpBA allele	GenBank accession no.
					Allele	No. of nt	Allele	No. of nt		
PH574	10	UG3	Ovine	UK	tbpB2.6	1674	tbpA2.5	2766	tbpBA3.4	AY850254
PH290	16	UG3	Ovine	UK	tbpB2.7	1674	tbpA2.2	2766	tbpBA3.5	AY850255
<i>B. trehalosi</i>										
PH246†	2	T4	Ovine	UK	tbpB5.1	1668	tbpA3.1	2757	tbpBA4.1	AY850256
PH252†	4	T10	Ovine	UK	tbpB5.2	1668	tbpA3.2	2757	tbpBA4.2	AY850257
PH254†	15	T15	Ovine	UK	tbpB5.3	1668	tbpA3.3	2757	tbpBA4.3	AY850258
PH68†	19	T3	Ovine	UK	tbpB5.1	1668	tbpA3.1	2757	tbpBA4.1	

*ET, Electrophoretic type (see Davies *et al.*, 1997a, b).

†PH50, NCTC10630; PH56, NCTC10636; PH238, NCTC10638; PH232, NCTC10633; PH66, NCTC11303; PH240, NCTC10208; PH246, NCTC10626; PH252, NCTC10641; PH254, NCTC10370[†]; PH68, NCTC11550.

and ovine *M. haemolytica* isolates, cluster B includes *tbpBA2*-type alleles that occur in ovine *M. haemolytica* isolates, and cluster C consists of *tbpBA3*-type alleles that are present in *M. glucosida* isolates. Lineage II contains *tbpBA4*-type alleles that are present in *B. trehalosi*. The most divergent *tbpBA* sequences are those from bovine and ovine serotype A2 *M. haemolytica* isolates. Lineage III includes *tbpBA5*-type alleles present in ovine serotype A2 isolates and lineages IV and V consist of *tbpBA6*- and *tbpBA7*-type alleles, respectively, that occur in bovine serotype A2 isolates. Genealogical analysis of the 42 *tbpBA* sequences using ClonalFrame resolved the sequences into six distinct lineages (Fig. 2). The three closely related clusters of lineage I in Fig. 1 (IA, IB and IC) were resolved into three distinct lineages; the *tbpBA1*- and *tbpBA2*-type alleles of *M. haemolytica* were resolved into two lineages (IA and IB) and the *tbpBA3*-type alleles of *M. glucosida* represented a third lineage (IC). The *tbpBA6*- and *tbpBA7*-type alleles (IV and V) and the *tbpBA5*-type alleles (III) of bovine and ovine serotype A2 *M. haemolytica* isolates, respectively, and the *tbpBA4*-type alleles (II) of *B. trehalosi* were resolved into three further lineages.

Recombination within *tbpBA*

It was clear from the non-congruence of the *tbpB* and *tbpA* gene trees (Supplementary Figs S2 and S3) that the *tbpBA* operon of *M. haemolytica*, *M. glucosida* and *B. trehalosi* has been disrupted by recombinational exchange. Splits decomposition analysis of the 28 unique *tbpBA* sequences produced a network consistent with recombination (see Supplementary Fig. S6, available with the online version of this paper). ClonalFrame analysis provided further evidence of recombinational exchange among *tbpBA* alleles from all three species (see Supplementary Fig. S7). However, there was no evidence from *tbpB* and *tbpA* trees incorporating the available sequences from a wider range of species that these recombinational exchanges have involved other members of the *Pasteurellaceae* or *N. meningitidis* (Supplementary Figs S4 and S5). None of the other sequences branched within the *M. haemolytica*, *M. glucosida* and *B. trehalosi* cluster and the pattern of branching of the other species was more or less identical for both *tbpB* and *tbpA*. Also, comparison of these *tbpB* and *tbpA* sequences (and those of *Histophilus somni*, in particular) with those of *M. haemolytica*, *M. glucosida* and *B. trehalosi* revealed no regions of identity or similarity indicative of recombination.

Visual inspection of the distribution of polymorphic nucleotide sites among the *tbpB* sequences revealed clear evidence for intragenic recombinational exchanges among the *tbpB1*- to *tbpB3*-type alleles of *M. haemolytica* and *M. glucosida* which exhibited varying degrees of mosaicism (Fig. 3). There was no evidence of intragenic recombination within the *M. haemolytica* alleles *tbpB1.1* to *tbpB1.6*, but short segments in alleles *tbpB1.7* (nt 1510–629) and *tbpB1.8* (nt 942–994 and 1510–1629) were identical, or

Fig. 1. An unrooted bootstrapped phylogenetic tree of 42 *tbpBA* sequences from *M. haemolytica*, *M. glucosida* and *B. trehalosi* constructed with the neighbour-joining algorithm based on the Jukes–Cantor model of nucleotide substitution. Lineages are marked on the tree. B-Mh, Bovine *M. haemolytica* isolates; O-Mh, ovine *M. haemolytica* isolates; Mg, *M. glucosida* isolates; Btr, *B. trehalosi* isolates.

almost identical, to the same regions in *M. haemolytica* alleles *tbpB2.1*–*tbpB2.3* or in *M. glucosida* alleles *tbpB2.4*, *tbpB2.5* and *tbpB2.6*. The *M. haemolytica* *tbpB2.1*–*tbpB2.3* alleles and the *M. glucosida* *tbpB2.4*–*tbpB2.8* alleles had complex mosaic structures because these alleles possessed regions of complete identity (segment B), regions that were unique to each species (segments A and C) and also regions of mixed association (segments D and E). Finally, the *M. glucosida* *tbpB2.8*-type allele contained a recombinant segment (nt 1419–1629) that shows complete identity between nucleotides 1449 and 1552 with the *tbpB3*-type alleles of *M. haemolytica*.

Visual inspection of the distribution of polymorphic nucleotide sites among the *tbpA* sequences revealed clear evidence of intragenic recombinational exchanges among the *tbpA1*- to *tbpA4*-type alleles of *M. haemolytica*, *M. glucosida* and *B. trehalosi* because these alleles exhibited varying degrees of mosaicism (Fig. 4). Interestingly, intragenic recombination affected the *tbpA1*- to *tbpA2*-type alleles only after nucleotide position 631. With the

exception of allele *tbpA2.1*, all of the *tbpA1*- and *tbpA2*-type alleles have identical nucleotide sequences from positions 1 to 630 (segment A); these alleles represent both *M. haemolytica* and *M. glucosida* isolates. In contrast, the *B. trehalosi* *tbpA3*-type alleles and the *M. haemolytica* *tbpA4*-type alleles are strikingly divergent in this region (Fig. 4). Nt 631–2393 of the *M. glucosida* *tbpA2*- and *B. trehalosi* *tbpA3*-type alleles are highly conserved (segment B) and this region represents a recombination event(s) between these two species (compare segment A). The 3'-end of the gene (nt 2394–2759) is, overall, more variable in these two species but three *M. glucosida* alleles and a single *B. trehalosi* allele have identical nucleotide sequences (Fig. 4, shaded regions) from positions 2394 to 2745 (segment C); these segments are also identical to the corresponding regions of the *tbpA1*-type alleles. A recombinant segment (nt 1254–1635) is present in *M. haemolytica* alleles *tbpA1.4* to *tbpA1.8* and *tbpA4.1*, in *M. glucosida* alleles *tbpA2.1* to *tbpA2.5* (differs at a single nucleotide site in all five alleles), and in *B. trehalosi* alleles *tbpA3.1* to *tbpA3.3*. The *M. haemolytica* alleles *tbpA1.7* and *tbpA4.1* contain an identical

Fig. 2. A 50 % majority rule consensus ClonalFrame radial tree of 42 *tbpBA* sequences from *M. haemolytica*, *M. glucosida* and *B. trehalosi*. Groups of isolates are circled: B-Mh, bovine *M. haemolytica* isolates; O-Mh, ovine *M. haemolytica* isolates; Mg, *M. glucosida* isolates; Btr, *B. trehalosi* isolates. The lineages IA, IB, IC, II, III, IV and V correspond to those shown in Fig. 1.

recombinant segment [nt 820–1839 (shaded region)] but otherwise differ enormously in the remainder of the gene; allele *tbpA1.8* also contains a large part of this segment [nt 820–1635 (shaded region)]. Finally, a small recombinant segment (nt 1664–1839) is present in alleles *tbpA1.8* and *tbpA4.2* which occur in different ovine serotype A2 *M. haemolytica* isolates.

The distribution of polymorphic nucleotide sites among 17 representative *tbpBA* alleles is shown schematically in Fig. 5. The mosaic structures of the *B. trehalosi* *tbpBA4*-type alleles and *M. haemolytica* *tbpBA5*-type alleles, in particular, are dramatic and provide clear evidence that intragenic recombination has had a significant effect on the composition of the *tbpBA* operon in the three species. The presence of very similar *tbpBA* alleles in phylogenetically divergent *M. haemolytica* and *M. glucosida* isolates also indicates that horizontal DNA transfer and assortative (entire gene) recombination have played important roles in the transmission of the entire *tbpBA* operon between different strains and species. Overall, the *tbpBA* operon is remarkably conserved among non-serotype A2 *M. haemolytica* and *M. glucosida* isolates. The most striking feature of the *tbpBA* sequences is that a large segment (nt 1864–4503 in Fig. 5) of *tbpA* is, to a large extent, highly conserved in all of the ovine *M. haemolytica* *tbpBA1*-, *tbpBA2*- and *tbpBA5*-type alleles, as well as in the *M. glucosida*

tbpBA3-type alleles and *B. trehalosi* *tbpBA4*-type alleles (see Supplementary Fig. S8b, available with the online version of this paper). Conversely, this region is divergent in the bovine serotype A2 *M. haemolytica* *tbpBA6*- and *tbpBA7*-type alleles. The *tbpB* gene and a small 5' segment of *tbpA* (nt 1–1863 in Fig. 5) are highly conserved in the *M. haemolytica* *tbpBA1*- and *tbpBA2*-type alleles and in the *M. glucosida* *tbpBA3*-type alleles. However, this region exhibits varying degrees of divergence in the bovine serotype A2 *M. haemolytica* *tbpBA6*- and *tbpBA7*-, in the *B. trehalosi* *tbpBA4*-, and in the ovine serotype A2 *M. haemolytica* *tbpBA5*-type alleles (see Supplementary Fig. S8a). Significantly, the ovine serotype A2 *M. haemolytica* *tbpBA5* segment is more divergent than the *B. trehalosi* *tbpBA4* segment.

Distribution of *tbpBA* alleles among *M. haemolytica* isolates

The distribution of the *tbpBA* alleles among *M. haemolytica* isolates representing all of the previously described ETs (Davies *et al.*, 1997a) is shown in Fig. 6. The *tbpBA1*- and *tbpBA2*-type alleles were associated with a diverse range of isolates representing ETs 1 to 15 and 21 and serotypes A1, A2, A5–A9, A13, A14 and A16. Notably, the *tbpBA1.1*- to *tbpBA1.3*-type alleles were associated exclusively with bovine serotype A1 and A6 isolates of ETs 1 and 2 whereas the *tbpBA1.4*- to *tbpBA1.8*-type alleles occurred in ovine serotype A2, A8, A9, A14 and A16 isolates of diverse ETs (Figs 1 and 6). The *tbpBA5*-type alleles were associated exclusively with ovine serotype A2 isolates of ETs 16, 19, 20 and 22. The *tbpBA6*-type alleles were restricted to bovine serotype A2 isolates of ET18 whereas the *tbpBA7*-type alleles were present in bovine serotype A2 isolates of ETs 17 and 21. Notably, ET21 was represented by ovine serotype A2 isolates associated with the *tbpBA1.8* allele and by bovine serotype A2 isolates that possessed the very different *tbpBA7.2* allele.

DISCUSSION

The phylogenetic relationships of the *tbpBA* sequences of *M. haemolytica*, *M. glucosida* and *B. trehalosi* (Fig. 1) do not correspond to the species phylogenies based on comparative nucleotide sequence analysis of the 16S rRNA gene (Angen *et al.*, 1999; Davies *et al.*, 1996) and various housekeeping enzyme genes (Kuhnert & Korczak, 2006; Lee, 2004). Although *M. haemolytica* and *M. glucosida* are closely related (Angen *et al.*, 1999; Kuhnert & Korczak, 2006), comparative sequence analysis of various housekeeping enzyme genes (Kuhnert & Korczak, 2006; Lee, 2004) indicates that the two species have diverged from a common ancestor and represent distinct lineages; *B. trehalosi* is more distantly related to both species. The *tbpBA* sequences of non-serotype A2 *M. haemolytica* isolates (including the *tbpBA1.8* allele of ovine serotype A2 isolates) and *M. glucosida* isolates are very similar and

represent a single lineage (Fig. 1). In contrast, the *tbpBA* sequences of bovine and ovine serotype A2 *M. haemolytica* (with the exception of the *tbpBA*1.8 allele), together with those of *B. trehalosi* isolates, represent distinct and divergent lineages. Furthermore, the former sequences are more divergent than those of *B. trehalosi*. These differences in the *tbpBA* and species phylogenies provided initial evidence that the *tbpBA* operon has been subjected to significant recombinational exchanges within the three species. Further evidence for the disruption of the *tbpBA* operon in these three species was provided by the non-congruence of the individual *tbpB* and *tbpA* gene trees, the identification of recombination events by Splits decomposition and ClonalFrame analyses, and the visual identification of complex mosaic structures within the *tbpB* and *tbpA* genes. The presence of partial or complete *tbpB* and *tbpA* sequences within bovine and ovine serotype A2 isolates of *M. haemolytica* that were more divergent than those of *B. trehalosi* suggested that these regions of the operon have been imported from species more divergent from *B. trehalosi*. However, the analysis of additional *tbpB* and *tbpA* sequences from other members of the

Two types of recombinational exchanges can be distinguished within the *tbpBA* operon. First, numerous small-scale intragenic recombination events involving all three species have occurred, resulting in the exchange of small segments of DNA (tens or hundreds of nucleotides in length). Second, larger-scale assortative (entire gene) recombination events have occurred involving either the entire *tbpBA* operon or the individual *tbpB* and *tbpA* genes. The overall similarity of the *tbpBA1*- and *tbpBA2*-type alleles of non-serotype A2 *M. haemolytica* isolates (including *tbpBA1.8*) and the *tbpBA3*-type alleles of *M. glucosida* isolates can be most easily explained by one or more early assortative recombination events involving the entire operon followed by the subsequent accumulation of point mutations and further small-scale intragenic recombination events. A second major event has been the exchange of a large segment of the *tbpA* gene (nt 1864–4503) between

Fig. 4. Distribution of polymorphic nucleotides sites among *tbpA* alleles. The numbers written vertically above the sequences represent the positions of polymorphic nucleotide sites from the 5' end of the gene. The dots represent sites where the nucleotides match those of the first sequence (*tbpA1.1*). The shaded and unshaded boxes highlight regions of sequence identity that represent proposed recombinant segments (see text). *Mh*, *M. haemolytica*; *Mg*, *M. glucosida*; *Bt*, *B. trehalosi*.

ovine serotype A2 *M. haemolytica* and *B. trehalosi* isolates, and non-serotype A2 *M. haemolytica* and *M. glucosida* isolates (Fig. 5). This recombination event has occurred on at least two separate occasions (in *tbpBA4*- and *tbpBA5*-type alleles). The more-or-less identical position of the recombination break-point within the *tbpA* gene in the *tbpBA4*- and *tbpBA5*-type alleles represents a likely 'hot-spot' of recombination and probably marks the presence of chi sequences within this region of the *tbpA* gene.

Further evidence to suggest that the *tbpBA* operon has undergone extensive assortative recombination within *M. haemolytica* is provided by the association of very similar, or identical, *tbpBA* alleles with divergent phylogenetic lineages (ETs) and by the association of different *tbpBA* alleles with isolates of the same lineage (Fig. 6). For example, the *tbpBA1*-, *tbpBA2*-, *tbpBA5*- and *tbpBA7*-type alleles are all widely distributed among isolates of divergent phylogenetic lineages, and ETs 1, 5 and 21 comprise isolates that have different *tbpBA* alleles. Of particular interest is the presence of the very different *tbpBA1.8* and *tbpBA7.2* alleles within ovine and bovine serotype A2 *M. haemolytica* isolates of ET21, respectively (Fig. 6). Ovine

isolates of ET21 are significant because they represent a major pathogenic lineage of sheep (Davies *et al.*, 1997a). These ovine and bovine isolates have the same underlying genetic background and therefore the same ancestral origin, but possess very different *tbpBA* alleles. For the same reasons as argued previously (Davies *et al.*, 2001), we propose that the ancestral bovine *tbpBA*7.2-type allele has been replaced by the ovine *tbpBA*1.8 allele after transmission of a bovine serotype A2 isolate from cattle to sheep. It is interesting to speculate that the *tbpBA*1.8 allele has been selected because the encoded TbpB and TbpA proteins provide more efficient uptake of iron from ovine transferrin than the same proteins encoded by the bovine *tbpBA*7.2 allele. Similarly, the *tbpBA*5-type allele present in ovine serotype A2 isolates of ETs 16, 19, 20 and 22 may have evolved as a consequence of the transmission of a bovine serotype A2 isolate from cattle to sheep and a subsequent recombination event. The highly divergent 1–1863 bp segment most likely represents part of the ancestral bovine allele whereas the 1864–4503 fragment has probably been derived from an ovine donor allele (Fig. 5). The *tbpBA*5-type allele has subsequently become disseminated among different serotype A2 lineages by

Fig. 5. Distribution of polymorphic nucleotide sites among 17 representative *tbpBA* sequences. Vertical lines represent polymorphic nucleotide sites with respect to the uppermost sequence, *tbpBA1.1*. The positions of *tbpB* and *tbpA* are indicated by the vertical line.

further recombinational exchanges and accumulated point mutations. Notably, the *tbpBA5.1* allele is present in isolates of ET22 which has emerged as a second major pathogenic lineage of sheep (Davies *et al.*, 1997a).

The wide distribution of very similar *tbpBA1*-, *tbpBA2*- and *tbpBA3*-type operons among ovine *M. haemolytica* and *M. glucosida* isolates suggests that there has been selection for these operon types due to the effective interaction of the

Fig. 6. Distribution of *tbpBA* alleles among *M. haemolytica* isolates representing 22 ETs previously identified from variation of 18 housekeeping enzymes (Davies *et al.*, 1997a). The boxes highlight the major pathogenic lineages of cattle and sheep.

encoded proteins with ovine transferrin and their successful involvement in iron uptake. It is also significant that the *tbpBA4*- and *tbpBA5*-type operons present in *B. trehalosi* and ovine serotype A2 isolates, respectively, contain *tbpA* alleles that, with the exception of a short 5' segment, are very similar to the *tbpA* alleles found in the *tbpBA1*- to *tbpBA3*-type operons (Fig. 5). Thus, the TbpA protein is, overall, highly conserved in *B. trehalosi*, *M. glucosida* and in ovine isolates of *M. haemolytica*. These *tbpA* alleles have presumably been maintained within the bacterial populations by selection because the encoded TbpA proteins play effective roles in iron uptake within the ovine host and provide the organisms with enhanced fitness. In contrast, there is significantly more variation of the *tbpB* gene within the same isolates. These observations reflect differences in TbpB and TbpA function and are in agreement with those for other bacteria in which *tbpB* diversity is much greater than *tbpA* diversity (Cornelissen, 2003; Cornelissen *et al.*, 1997, 2000; Rokbi *et al.*, 1993, 1995, 1997, 2000).

Bovine serotype A1 and A6 isolates of ETs 1 and 2 are responsible for the majority of bovine disease (Davies *et al.*, 1997a) and yet possess *tbpBA1.1*, *tbpBA1.2* and *tbpBA1.3* alleles that are very closely related to the *tbpBA1.4* and *tbpBA1.5* alleles present in ovine isolates of serotypes A8 and A9 (Fig. 1). The TbpB proteins of these isolates have 16 polymorphic amino acid sites whereas the TbpA proteins have only two. The highly conserved nature of the TbpA protein, in particular, among these bovine and ovine isolates suggests that the transferrin receptors may function equally well in both cattle and sheep. This conclusion is supported by the finding that the transferrin receptors from bovine *M. haemolytica* isolates bind to both bovine and ovine transferrin (Yu & Schryvers, 1994; Yu *et al.*, 1992) although these authors did not investigate iron uptake. On the other hand, if the ancestral host of bovine serotype A1 and A6 isolates is sheep, and these isolates have only recently (in evolutionary terms) transferred to cattle, we might expect the transferrin receptor to be less efficient in iron uptake in cattle. Under these circumstances, these bovine isolates might utilize other iron-uptake mechanisms. *M. haemolytica* possesses mechanisms to acquire iron from haemoglobin and haemopexin via the haemoglobin receptors, HmbR1 and HmbR2, and the haemophore, HxuA, respectively (Gioia *et al.*, 2006). Clearly, further studies are required to investigate differences in the interactions and specificities of the various TbpB/TbpA types with bovine and ovine transferrins and to elucidate the roles of the different receptor types in iron uptake in cattle and sheep.

In the present study, we have shown that the *tbpBA* operon has a complex mosaic structure and that various regions of the operon are shared by *M. haemolytica*, *M. glucosida* and *B. trehalosi*. Furthermore, the 1–1863 bp segment of the *tbpBA5*-type alleles is substantially more divergent than the corresponding region of the *tbpBA4*-type alleles from *B. trehalosi*, suggesting that this segment originates from a species more distantly related than *B. trehalosi*. Thus, it is

clear that a common gene pool exists for *tbpBA* in *M. haemolytica*, *M. glucosida* and *B. trehalosi* and that this gene pool has made a significant contribution to the diversification of *tbpBA* in these species. We have also described similar findings for the *lktCABD* operon (Davies *et al.*, 2001, 2002) and *ompA* gene (Davies & Lee, 2004). Common gene pools involving different species have also been documented for certain *Neisseria* antigens including fHbp (Brehony *et al.*, 2009), PorB2 (Derrick *et al.*, 1999), FetA (Bennett *et al.*, 2009) and TbpB (Harrison *et al.*, 2008; Linz *et al.*, 2000). In these cases, horizontal DNA transfer events involving various non-pathogenic *Neisseria* species have contributed to the diversification of these genes in the pathogenic species *N. meningitidis*. It has been proposed that diversity at the *tbpAB* locus in *N. meningitidis* is due to the close proximity of neisserial intergenic mosaic elements (NIMes) which may act as binding sites for site-specific recombinases (Bentley *et al.*, 2007). However, NIME arrays have not been observed in non-neisserial genomes and may not explain the horizontal transfer of segments of the *tbpBA* operon in *M. haemolytica*, *M. glucosida* and *B. trehalosi*.

In conclusion, the *tbpBA* operon of *M. haemolytica*, *M. glucosida* and *B. trehalosi* has a complex mosaic structure and evolutionary history. The data suggest a history of frequent recombinational exchange involving all three species and the existence of a common gene pool that also involves other species. Our findings have implications not only for understanding the evolution and diversification of these three species but also for the use of the transferrin receptor as a potential vaccine component against *M. haemolytica* (Cornelissen, 2003; Gilmour *et al.*, 1991; Potter *et al.*, 1999).

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