Reclassification of [*Pasteurella*] *trehalosi* as *Bibersteinia trehalosi* gen. nov., comb. nov.

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[*Pasteurella*] *trehalosi* is an important pathogen of sheep, being primarily associated with serious systemic infections in lambs but also having an association with pneumonia. The aim of the present investigation was to characterize a broad collection of strains tentatively identified as [*P.*] *trehalosi* in order to reclassify and rename this taxon to support improvements in our understanding of the pathogenesis and epidemiology of this important organism. The type strain for [*P.*] *trehalosi*, strain NCTC 10370^T, was included along with 42 field isolates from sheep (21), cattle (14), goats (1), roe deer (3) and unknown sources (3). An extended phenotypic characterization was performed on all 43 strains. Amplified fragment length polymorphism (AFLP) was also performed on the isolates. Two of the field isolates were subjected to 16S rRNA gene sequencing. These sequences, along with five existing sequences for [*P.*] *trehalosi* strains and 12 sequences for other taxa in the family *Pasteurellaceae*, were subjected to a phylogenetic analysis. All the isolates and the reference strains were identified as [*P.*] *trehalosi*. A total of 17 out of 22 ovine isolates produced acid from all glycosides, while only four out of 14 bovine isolates produced acid from all glycosides. All 22 ovine isolates were haemolytic and CAMP-positive, while no other isolate was haemolytic and only two bovine isolates were CAMP-positive. Nineteen AFLP types were found within the [*P.*] *trehalosi* isolates. All [*P.*] *trehalosi* isolates shared at least 70 % similarity in AFLP patterns. The largest AFLP type included the type strain and 7 ovine field isolates. Phylogenetic analysis indicated that the seven strains studied (two field isolates and the five serovar reference strains) are closely related, with 98.6 % or higher 16S rRNA gene sequence similarity. As both genotypic and phenotypic testing support the separate and distinct nature of these organisms, we propose the transfer of [*P.*] *trehalosi* to a new genus, *Bibersteinia*, as *Bibersteinia trehalosi* comb. nov. The type strain is NCTC 10370^T (=ATCC 29703^T). *Bibersteinia trehalosi* can be distinguished from the existing genera of the family by the observation of only nine characteristics; catalase, porphyrin, urease, indole, phosphatase, acid from dulcitol, (+)-D-galactose, (+)-D-mannose and (+)-D-trehalose.

[*Pasteurella*] *trehalosi* is an important pathogen of sheep being primarily associated with serious systemic infections in lambs, but also having an association with pneumonia in sheep (Gilmour & Gilmour, 1989). The organism, first described as a separate species by Sneath & Stevens (1990), was part of the complex of species once known as the '[*Pasteurella*] *haemolytica*' complex (Angen *et al.*, 1999). The '[*Pasteurella*] *haemolytica*' complex, which consisted of biovars A and T (Smith, 1959), has been extensively reorganized. The organisms once assigned to the T biovar were named [*Pasteurella*] *trehalosi* (Sneath & Stevens, 1990). However, there is clear evidence that the species is not closely affiliated with *Pasteurella multocida*, the type species of the genus *Pasteurella* (Angen *et al.*, 1999). A new genus, *Mannheimia*, houses the A biovar organisms from the old '[*Pasteurella*] *haemolytica*' complex with five species: *Mannheimia haemolytica*, *Mannheimia glucosida*, *Mannheimia granulomatis*, *Mannheimia ruminalis* and *Mannheimia varigena* (Angen *et al.*, 1999), as well as currently un-named taxa such as Bisgaard Taxon 39 (Blackall *et al.*, 2001).

There is evidence of diversity within the species [*P.*] *trehalosi*. A serotyping scheme based on capsular polysaccharides,

Abbreviations: AFLP, amplified fragment length polymorphism; LPS, lipopolysaccharide; MLEE, multilocus enzyme electrophoresis; OMP, outer membrane protein.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains B464/94 and C1008-I are DQ361040 and DQ361041, respectively.

originally developed by Biberstein *et al.* (1960) which recognizes four serovars, termed T3, T4, T10 and T15, has been used to study strain variation (Adlam, 1989; Gilmour & Gilmour, 1989). Davies & Quirie (1996) found six lipopolysaccharide (LPS) types and four outer membrane protein (OMP) types in a collection of 60 isolates mainly from sheep in the UK and noted that this represented only a limited degree of diversity. Using multi-locus enzyme electrophoresis (MLEE), Davies *et al.* (1997) concluded that the degree of diversity within the same 60 isolates studied by LPS and OMP typing (Davies & Quirie, 1996) was lower than that of most other pathogenic species that had been studied by MLEE. In the present investigation, we have characterized a broad collection of strains tentatively identified as [*P.*] *trehalosi* to gain some insight into the diversity present in the taxon. We have then used the results of the current study and past studies to reclassify and rename this taxon. The reclassification will facilitate the study of the pathogenesis and epidemiology of this important veterinary organism.

The isolates and strains used in this study were obtained from the culture collection of the Department of Veterinary Pathobiology, The Royal Veterinary and Agricultural University, Denmark, and are shown in Table 1. The type strain of [*P.*] *trehalosi*, strain NCTC 10370^T, was included along with 45 field isolates. The isolates were obtained from sheep (22 isolates), cattle (17 isolates), goats (1 isolate) and roe deer (3 isolates), while the source of three reference serovars (FT3, T3H and T10H) remain unknown.

All of the isolates and the type strain were characterized as described previously (Bisgaard *et al.*, 1991). The CAMP reaction of all of the isolates was determined as previously described (Christie *et al.*, 1944). In addition, a specific PCR for the leukotoxin of [*P.*] *trehalosi* (Green *et al.*, 1999) was performed on a subset of the isolates.

Amplified fragment length polymorphism (AFLP) typing was carried out as reported previously (Christensen *et al.*, 2003a). Briefly, the non-selective *Bgl*II primer (FAM-5′ GAGTACACTGTCGATCT 3′) and the non-selective *Bsp*DI primer (5′ GTGTACTCTAGTCCGAT 3′) were used to amplify the fragments following restriction digestion and ligation to their corresponding adaptors. All AFLP reactions were carried out twice to determine the reproducibility of the method. Amplification products were detected on an automated DNA sequencer (ABI 377; PE Biosystems). Each lane included an internal lane size standard labelled with ROX dye (Applied Biosystems) and GeneScan 3.1 fragment analysis software (Applied Biosystems) was used for fragment size determination and pattern analysis. AFLP profiles comprising fragments in the size range 50–500 bp were considered for numerical analysis with the program GelCompar II (Applied Maths). Normalized AFLP fingerprints were compared using the Dice similarity coefficient and clustering analysis was performed by the unweighted pair group method with arithmetic means (UPGMA).

16S rRNA gene sequencing of strains B464/94 and C1008-I was performed as described below. Bacteria were cultured overnight in brain heart infusion broth (Difco) and DNA was extracted, with enzymic treatments with lysozyme and proteinase K as previously reported (Leisner *et al.*, 1999). PCR amplification was performed according to the standard conditions described by Vogel *et al.* (1997). Oligonucleotides for both PCR amplification and sequencing were synthesized according to sequences and 16S rRNA positions given in Dewhirst *et al.* (1989) and Paster & Dewhirst (1988). DNA sequencing was performed on a DNA sequencer (ABI 377; Applied Biosystems) with unlabelled primers and the BigDye kit according to protocols described with the *Automated DNA Sequencing Chemistry Guide* (Applied Biosystems, 2000). Searches for DNA sequences at NCBI (Benson *et al.*, 2004; www.ncbi.nih.nlm.gov) were performed by BLAST (Altschul *et al.*, 1997).

In addition to the two sequences determined in the present study, GenBank was searched for available 16S rRNA gene sequences of other strains of [*P.*] *trehalosi*. Sequences were found for the following five strains: NCTC 10370^T, NCTC 10641, NCTC 11550, NCTC 10624 and NCTC 10626 with GenBank accession nos AY362927, U57074, U57073, M75063 and U57075, respectively. The sequences for the type strains of the type species of the currently recognized genera within the family *Pasteurellaceae* were also obtained. Pairwise comparisons for similarity were performed by EMBOSS (Rice *et al.*, 2000). Sequences were aligned by CLUSTAL_X (Thompson *et al.*, 1997).

Maximum-likelihood analysis was performed by fastDNAml including bootstrap analysis (Olsen *et al.*, 1994) run on a Linux compatible server. The transition/transversion ratio was set to 1.5. The region corresponding to *Escherichia coli* positions 87–1392 of the *rrnB* gene was used after removal of gaps with 226 distinct data patterns analysed. The 'loop' script provided by fastDNAml was used to justify that the tree with maximum lnL had been found.

By phenotypic testing, 43 isolates were identified as [*P.*] *trehalosi*. These 43 isolates were Gram-negative, non-motile (at 22 and 37 °C) rods that were fermentative in the Hugh and Leifson test. The isolates did not show symbiotic growth on blood agar and gave positive results in the porphyrin, phosphatase and alanine aminopeptidase tests. All isolates were positive in the nitrate reduction test and were negative in Simmons' citrate, methyl red and Voges–Proskauer tests. No isolate produced H_2S, urease or gelatinase. Indole was not produced and Tweens 20 and 80 were not hydrolysed. The isolates were all negative in the arginine dehydrolase and lysine- and ornithine decarboxylase tests. Phenylalanine was not deaminated, acid was not produced from mucate and alkali was not produced in the malonate test. No isolate could grow in KCN. The isolates showed variable reactions in the catalase and oxidase tests. Variable results were obtained for haemolysis (on bovine blood agar), in the CAMP test (performed using bovine blood cells) and for the ability to grow on MacConkey agar. Over half of the isolates

Table 1. Isolates used in this study

ND, No data; NT, no serotype; B, Belgium; DK, Denmark; S, Sweden; UK, United Kingdom.

Isolate	Host species	Serovar	Disease association/ Isolation site	Country	AFLP cluster/Type
34876/76	Bovine	NT	Lung	UK	1/A
34880/76	Bovine	NT	Lung	UK	1/A
NCTC 10371	Lamb	15	Septicaemia	UK	1/B
NCTC 10624	Ovine	15	Nasopharyngeal mucus	UK	1/B
4935/S945T3	Ovine	3	Disease	UK	1/B
T34682	Ovine	3	Disease	UK	1/B
S487/T3	Ovine	3	Disease	UK	1/B
T15T700B	Ovine	15	Septicaemia	UK	1/B
NCTC 10370^T	Ovine	15	Septicaemia	UK	1/B
51810/78	Ovine	NT	Septicaemia	UK	1/B
T4H	Ovine	4	Septicaemia	UK	1/C
X801	Ovine	NT	Disease	UK	1/C
T4T740C	Ovine	4	Septicaemia	UK	1/C
FT4	Ovine	4	Septicaemia	UK	1/C
FT3	ND	3	ND	UK	1/D
T3H	ND	3	Same source as FT3	UK	1/D
T3T631	Ovine	3	Septicaemia	UK	1/D
NCTC 10369	Ovine	4	Septicaemia	UK	1/E
T10H	ND	10	ND	UK	1/E
T10T676A	Ovine	10	Septicaemia	UK	1/F
5083/S261-1T	Ovine	NT	Disease	UK	1/F
35	Roe deer	NT	Brain	B	1/F
4674/S96/9-T	Ovine	NT	Disease	UK	2/G
P32	Caprine	NT	ND	B	2/H
C1019-II	Bovine	NT	Granuloma	DK	3/I
83	Bovine	NT	Intestine	B	3/J
4721	Ovine	NT	Disease	UK	4/K
5083/S261-2	Ovine	NT	Disease	UK	4/K
4954/T3	Ovine	3	Disease	UK	4/K
T10/S790	Ovine	15	Septicaemia	UK	4/K
51809/78	Ovine	10	Septicaemia	UK	4/K
B96/54	Bovine	NT	Lung	B	5/L
42	Bovine	NT	Lung	B	5/L
33	Bovine	NT	Lung	B	5/L
B96/19	Bovine	NT	Lung	B	5/L
C857-II	Bovine	NT	Pharynx	DK	5/M
B464/94	Roe deer	NT	Lung	S	5/N
B96/39	Bovine	NT	Joint	B	5/O
H12	Bovine	NT	Lung	B	5/P
1009-1	Bovine	NT	Granuloma	DK	6/Q
B817/85	Roe deer	NT	ND	S	6/Q
C1008-I	Bovine	NT	Granuloma	DK	6/R
35B	Bovine	NT	Lung	B	6/S
33B*	Bovine	NT	Intestine and lung	B	7/T
28B†	Bovine	NT	Bronchial lavage	B	8/U
42B‡	Bovine	NT	ND	B	9/V

**P. multocida* (ornithine- and indole-negative).

†Unclassified.

‡*M. varigena.*

showed a yellowish pigment in colonies grown on blood agar. Acid was produced from (−)-D-ribose, (−)-D-mannitol, (−)-D-sorbitol, (−)-D-fructose, (+)-D-glucose, (+)-D-mannose, maltose, sucrose, (+)-D-trehalose and dextrin. Acid was not produced from *myo*-erythritol, adonitol, (+)-D-arabitol, xylitol, (+)-L-arabinose, (−)-D-arabinose, (+)-D-xylose, (−)-L-xylose, dulcitol, (+)-D-fucose, (−)-L-fucose, (+)-D-galactose, (+)-L-rhamnose, (−)-L-sorbose, lactose, (+)-D-melibiose, (+)-D-melezitose, (+)-D-glycogen, inulin or (+)-D-turanose. The isolates varied in their ability to produce acid from glycerol, *myo*-inositol, cellobiose, raffinose, aesculin, amygdalin, arbutin, gentiobiose and salicin. All isolates were negative in the β-galactosidase (ONPG) test. All the isolates were negative in tests for α-fucosidase, α-galactosidase, α-mannosidase and β-xylosidase. Variable results were obtained in the α-glucosidase, β-glucosidase and β-glucuronidase tests. For characteristics showing variation, strain NCTC 10370^T showed the following reactions: catalase- and oxidase-negative, weak haemolysis on bovine blood agar, CAMP-positive, weak growth on MacConkey agar, yellowish pigment, production of acid from cellobiose, raffinose, aesculin, amygdalin, arbutin, gentiobiose and salicin, but no acid produced from glycerol and *myo*-inositol. The β-glucosidase and α-glucosidase tests were positive, while a β-glucuronidase test was negative. Seventeen out of 22 ovine isolates produced acid from all glycosides investigated, while only four out of fourteen bovine isolates had the capacity to produce acid from all glycosides.

A single bovine isolate (28 bronchial lavage), differing in ornithine decarboxylase activity and (+)-D-mannose fermentation, remained unidentified.

Of the two remaining bovine isolates included as blind controls in the AFLP characterization, one (isolate 33B) was identified as *P. multocida* (ornithine decarboxylase- and indole negative) and one (isolate 42B) was identified as *M. varigena*.

The PCR for the leukotoxin of [*P.*] *trehalosi* (Green *et al.*, 1999) was used on ten CAMP-positive strains (NCTC 10369, NCTC 10370^T, NCTC 10371, NCTC 10624, FT3, T3H, 4935/S945T3, T34682, 4954/T3 and S487/T3) and gave a clear positive reaction for all ten isolates. When used on the six CAMP-negative isolates (C1008-I, C1019-II, B96/19, B96/39, B96/54 and B464/94), a suspect to weak-positive result was given with all six isolates.

The reproducibility of the AFLP method was evaluated by the analysis of duplicates of isolates with independently prepared templates, different selective PCR runs and different electrophoretic runs. The reproducibility of the method was good (96.1 %) with a standard deviation of $\pm$2.4 %. The clustering analysis is shown in Fig. 1. Field isolate C857-II was included as two separate cultures in the analysis. The two cultures of C857-II had an identical AFLP profile and were regarded as a single field isolate. All isolates used in the study shared at least 35 % similarity in their AFLP profiles. Indeed, all isolates, except for three isolates (numbers 33B, 28B and 42B) shared at least 70 % similarity in their profiles. As noted above, these three aberrant isolates represented *P. multocida*, an unclassified organism and *M. varigena*, respectively. For the purpose of this study, isolates with AFLP profiles of $\geqslant$90 % similarity were defined as an AFLP type, while isolates with $\geqslant$80 % similarity were defined as an AFLP cluster. As shown in Table 1, a total of 22 AFLP types (A to V) were recognized and these types formed a total of nine AFLP clusters (1 to 9). AFLP types T, U and V (which corresponded to clusters 7, 8 and 9) consisted of the three strains known not to be representatives of [*P.*] *trehalosi* on the basis of phenotypic results. The exclusion of these strains from [*P.*] *trehalosi* was supported by the low similarity ($\leqslant$50 %) of these strains with all other [*P.*] *trehalosi* isolates examined in the AFLP study.

Of the 22 AFLP types recognized, half were single isolate types. The largest AFLP type (type B) consisted of seven field isolates and the type strain (NCTC 10370^T), all of ovine origin. AFLP types C and K also only included ovine isolates. Only AFLP clusters B and C included strains producing acid from all of the glycosides investigated. The other large AFLP type was type K which contained five ovine field isolates that had an intermediate capacity to produce acid from glycosides. AFLP type B included serovars 3 and 15, while serovar 4 was associated with types C and E and serovar 10 with types E, F and K. However, serovar 3 was also associated with types D and K.

Of the nine AFLP clusters recognized, six contained more than one isolate. AFLP cluster 1 was the largest cluster recognized and consisted of AFLP types A, B, C, D, E and F. AFLP cluster 1 included a total of 21 field isolates and the type strain NCTC 10370^T. The next largest cluster was cluster 5 which included eight isolates. Of the six multi-member clusters, five were associated with a single host species. The only AFLP cluster that involved more than one host was cluster F, where two isolates were of ovine origin and one isolate was of cervine origin.

In the 16S rRNA gene sequence analysis, the major AFLP clusters were represented. To supplement the five strains already sequenced and representing all four serovars, two additional strains B464/94 and C1008-I were sequenced in the present study and deposited in GenBank with accession numbers DQ361040 and DQ361041, respectively. The lowest 16S rRNA gene sequence similarity within [*P.*] *trehalosi* was 98.3 % between strains NCTC 10624 and B464/94, isolated from sheep and roe deer, respectively. The highest similarity outside of [*P.*] *trehalosi* was 96.4 % which was found between the type strain of [*P.*] *trehalosi* and the type strain of *M. ruminalis*.

The phylogenetic analysis showed that the strains of [*P.*] *trehalosi* formed a monophyletic group that was unrelated to other members of the *Pasteurellaceae*, including *Mannheimia* (Fig. 2).

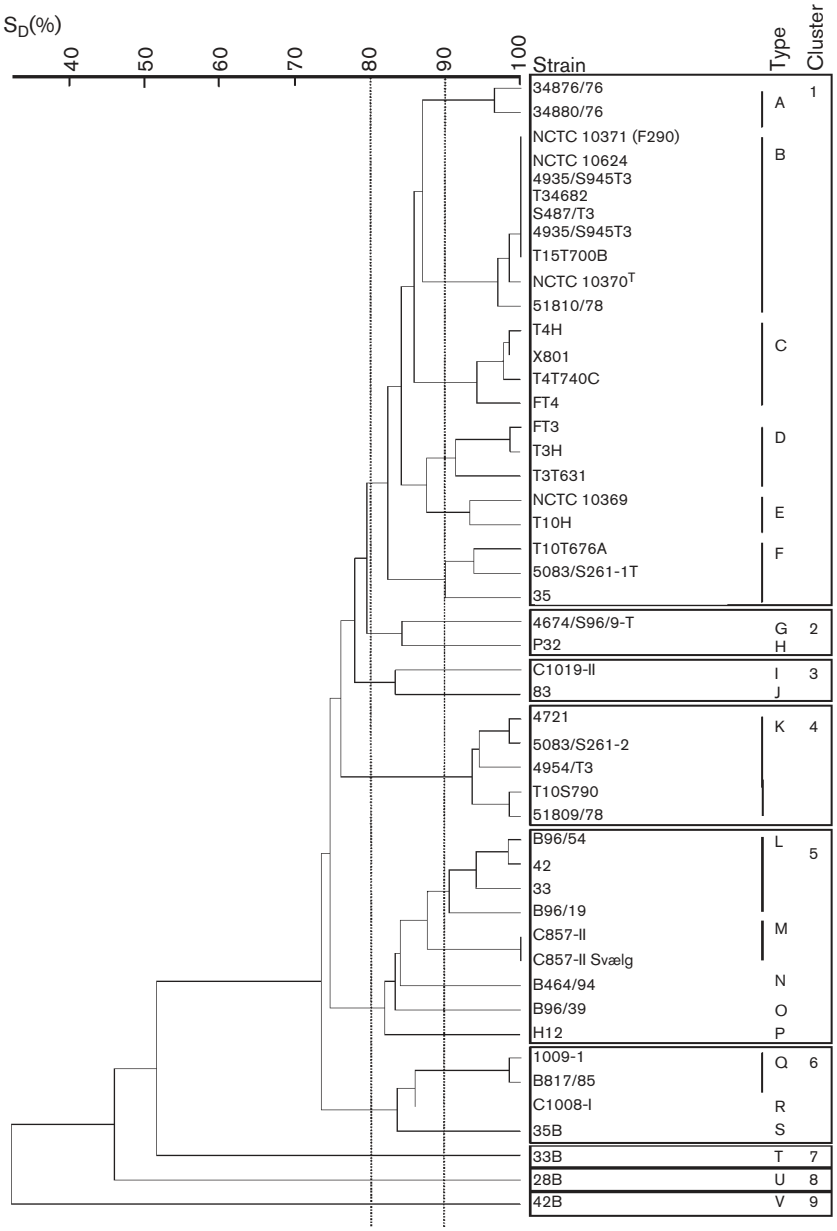

Fig. 1. Dendrogram (UPGMA) of AFLP similarities (Dice coefficient) between the strains of [*P.*] *trehalosi*. S_D, Dice similarity.

This study has confirmed earlier findings that [*P.*] *trehalosi* is a monophyletic group unrelated to other taxa within the family *Pasteurellaceae*. The full neighbour-joining tree of the family *Pasteurellaceae* constructed by Olsen *et al.* (2005) showed a deep branching of the type strain of [*P.*] *trehalosi*, unrelated to other members of the family. The deep branching was also found by Christensen *et al.* (2004c) with the application of maximum-likelihood analysis. Phylogenetic analysis based on 16S rRNA gene sequences has previously indicated that the four isolates of [*P.*] *trehalosi*, representing the four recognized serovars within the taxon (T3, T4, T10 and T15), are closely related (showing at least 98.7 % similarity) and form a monophyletic group (Davies *et al.*, 1996). These 16S rRNA gene sequence-based results have been supported by phylogenies based on two housekeeping genes (Christensen *et al.*, 2004c). In addition, only 62 % or less DNA–DNA relatedness has been found between [*P.*] *trehalosi* and other members of the family *Pasteurellaceae* (Biberstein & Francis, 1968; Mutters *et al.*, 1985, 1986; Pohl, 1981). The uniqueness of [*P.*] *trehalosi* has also been demonstrated in terms of polyamine profiles (Busse *et al.*, 1997). [*P.*] *trehalosi* strain NCTC 10624 (serovar T3) was not located on any of the seven rRNA branches outlined by De Ley *et al.* (1990). Indeed, this strain was located at the root of the large *Haemophilus–Pasteurella–Actinobacillus* rRNA branch (De Ley *et al.*, 1990).

Hence, our study using 16S rRNA gene sequence analysis, AFLP typing and phenotypic characterization is in full accord with all existing evidence – [*P.*] *trehalosi* represents a distinct and separate genus within the family *Pasteurellaceae*. On the basis of our results, plus this existing evidence, we

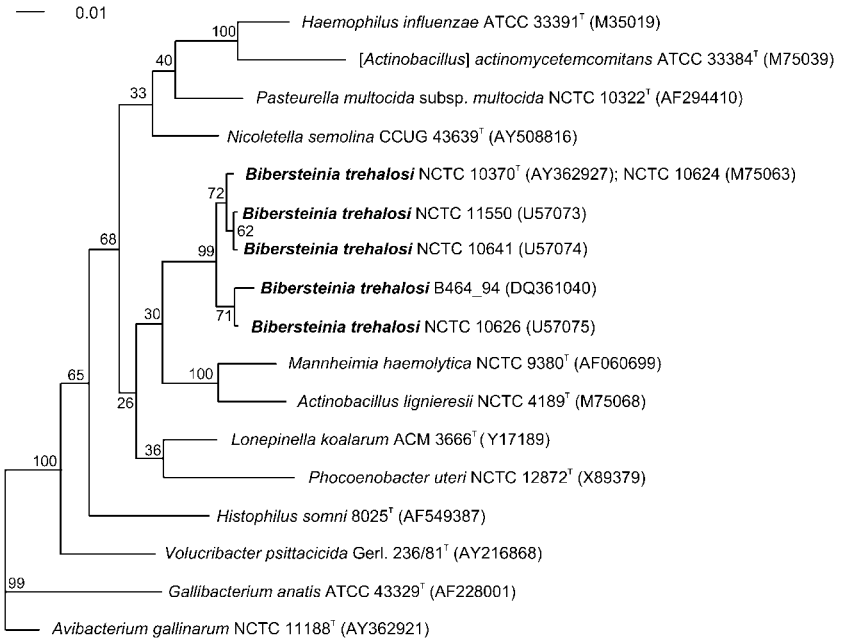

Fig. 2. Phylogenetic relationships based upon maximum-likelihood analysis of 16S rRNA gene sequences of members of the genus *Bibersteinia* gen. nov. and members of representative genera in the family *Pasteurellaceae*. Support for specified nodes obtained in bootstrap analysis is indicated. Strains sequenced in the present study are shown in bold. Bar, 0.01 evolutionary distance.

propose that [*P.*] *trehalosi* should be housed within a separate genus, for which we propose the name *Bibersteinia* gen. nov.

At the genus level, the genus *Bibersteinia* is clearly phenotypically distinguishable from all other genera in the family *Pasteurellaceae* (Table 2). The genus *Bibersteinia* can be separated from the existing genera of *Pasteurellaceae* by the following characteristics: catalase, porphyrin test, urease, indole production, phosphatase, α-glucosidase and production of acid without gas from dulcitol, (+)-D-galactose, (+)-D-mannose, (+)-D-trehalose and glycosides.

AFLP was used in this study to assess diversity within *Bibersteinia* ([*Pasteurella*]) *trehalosi*. The collection of strains used in our study was as diverse as we could assemble, representing four host species (bovine, caprine, cervine and ovine) and four countries (Belgium, Denmark, Sweden and the UK). Despite this diversity, the AFLP patterns all showed a similarity of at least 70 %. The only strains in this study that shared less than 55 % similarity in AFLP profiles with the 43 *Bibersteinia* ([*Pasteurella*]) *trehalosi* strains were all members of other taxa (one unidentified strain, one *P. multocida* strain and one *M. varigena* strain). The clusters recognized by AFLP did show some association with host species; only one of the five multi-member clusters included isolates from more than one host species. The examination of more isolates, including from geographical regions not covered in this study, is necessary to determine whether there is an association between clonal lines of *Bibersteinia* ([*Pasteurella*]) *trehalosi* and host species.

Most standard texts refer to the fact that *Bibersteinia* ([*Pasteurella*]) *trehalosi* is a pathogen of sheep and do not mention any role in bovine disease, e.g. Gilmour & Gilmour (1989). In our study, 14 of 43 strains of *Bibersteinia* ([*Pasteurella*]) *trehalosi* were isolated from cattle. As this was a retrospective study, detailed case histories were not available for these bovine isolates. However, the fact that three *Bibersteinia* ([*Pasteurella*]) *trehalosi* strains were associated with granulomas (strains C1019-II, 1009-1 and C1008-I) and one (B96/39) was obtained from a joint suggests at least the possibility that these bovine strains may have a role in pathogenic processes. Most of the bovine isolates of *Bibersteinia* ([*Pasteurella*]) *trehalosi* (nine strains) were isolated from the respiratory tract and it is difficult to assess their role as primary pathogens. While *Bibersteinia* ([*Pasteurella*]) *trehalosi* does not appear to have been commonly reported from cattle, the organism was found to be the most common member of the family *Pasteurellaceae* to be cultured from the tonsils of commercially reared American bison (*Bison bison*) (Ward *et al.*, 1999). The past tendency to lump field isolates within the '[*Pasteurella*] *haemolytica*' complex may have obscured the role of *Bibersteinia* ([*Pasteurella*]) *trehalosi* in bovine disease. Our study, plus the comprehensive description of the genus *Mannheimia* with five species, *M. haemolytica*, *M. glucosida*, *M. granulomatis*, *M. ruminalis* and *M. varigena* (Angen *et al.*, 1999), now allows diagnostic laboratories to effectively and accurately identify quite distinct taxa that would have once been simply called '[*Pasteurella*] *haemolytica*'. Thorough identification of all *Pasteurellaceae*-like organisms should be undertaken where possible.

Of the 43 *Bibersteinia* ([*Pasteurella*]) *trehalosi* strains examined in this study, 22 strains, all of ovine origin, showed haemolytic activity (albeit weak haemolysis) against bovine red blood cells. All 14 bovine isolates, the sole caprine isolate and the three cervine isolates failed to show haemolytic activity. This pattern was essentially repeated with the

Table 2. Key characteristics for the differentiation of genera within the family *Pasteurellaceae*

Genera: 1, *Actinobacillus sensu stricto*; 2, *Pasteurella sensu stricto*; 3, *Haemophilus sensu stricto* (includes *H. influenzae*, *H. haemolyticus* and *H. aegypticus*; results for *H. parainfluenzae* and *H. pittmania* are given in [ ]); 4, *Mannheimia*; 5, *Lonepinella*; 6, *Phocoenobacter*; 7, *Gallibacterium*; 8, *Volucribacter*; 9, *Histophilus*; 10, *Avibacterium*; 11, *Nicoletella*; 12, *Bibersteinia* gen. nov. Data are based on Angen *et al.* (1999, 2003), Bisgaard & Mutters (1986), Blackall *et al.* (2005), Christensen & Bisgaard (2003, 2004), Christensen *et al.* (2003a, b, 2004a, b), Kuhnert *et al.* (2004), Mutters *et al.* (1985) and this study. +, 90 % or more of the strains positive within 1–2 days; (+), 90 % or more of the strains positive within 3–14 days; −, less than 10 % of the strains are positive within 14 days; D, 11–89 % of the strains are positive; W, weakly positive.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12
Catalase	D	D	D	D	−	−	+	D	−	D	+	D
NAD requirement	−*	−†	+[D]	−	−	−	−	−	−	+‡	−	−
Porphyrin	+	+	−[+]	+	+	+	+	+	−	+	ND	+
Methyl red	−	−	ND	−	−	ND	W	+	ND	−	ND	−
Voges–Proskauer	−	−	ND	−	D	+	−	−	−	−	ND	−
Urease	+	−§	+[D]	−	−	−	−	−	−	−	+	−
Indole	−	+	D	−	−	−	−	−	+	−	−	−
Phosphatase	+	+	+	+	−	+	+	+/W	+	+‖	D	+
Pigment	−	−	ND	−	−	ND	−	−	+	D	ND	D
Acid from:												
(−)-D-Arabinose	−	D	ND	D	(+)	ND	(+)	−	ND	D	ND	−
Dulcitol	−	D	−	−	+	−	−	−	ND	−	−	−
(−)-D-Mannitol	+¶	D	−	+	−	−	+	−	ND	D	−	+
(−)-D-Fructose	+	+	−[D]	+	+	ND	+	+	−	+	−	+
(+)-D-Galactose	D	+	+	+	ND	ND	+	+	ND	D	−	−
(+)-D-Mannose	D	+	−[+]	−	+	−	+	+	ND	+	−	+
Maltose	+	−	+	D	+	−	D	D	−	D	−	+
Sucrose	+	+	−[D]	+	D	−	+	+	−	+	−	+
(+)-D-Trehalose	D	D	−	−	−	−	D	−	−	D	−	+
Glycosides	D	−	−	D	+	D	−	−	ND	−	ND	D
ONPG	D	D	−[D]	D	+	+	+	D	ND	D	D	−
α-Glucosidase	D	+	−	−	−	ND	+	−	ND	+	ND	D
β-Glucuronidase	−	−	−	−	+	ND	−	−	ND	−	ND	−

Discrepant results are indicated by: **Actinobacillus pleuropneumoniae* biovar 1 is positive; †*P. multocida* might be positive; ‡*Avibacterium gallinarum* is negative; some isolates of *Avibacterium paragallinarum* are also negative (biovar 2); §*Pasteurella dagmatis* is positive; ‖*Avibacterium paragallinarum* biovar 1 might be negative; ¶*Actinobacillus suis* is negative.

CAMP test; all 22 ovine strains were positive while the only other strains to be positive in the CAMP test were two bovine isolates. We explored the use of the PCR developed by Green *et al.* (1999) for the identification of the leukotoxin gene in *Bibersteinia* ([*Pasteurella*]) *trehalosi*. While we did not examine the full set of isolates, we found that the ten haemolytic and CAMP-positive strains (all ovine in origin) were clearly positive in this PCR, while five non-haemolytic and CAMP-negative isolates (four bovine and one cervine) gave a weak positive reaction. As the haemolytic activity of *Bibersteinia* ([*Pasteurella*]) *trehalosi* has been reported to correlate with the production of leukotoxin (Ward *et al.*, 1999), our finding that the haemolytic and CAMP-positive strains were all clearly positive in the leukotoxin PCR was an expected finding. The finding that the non-haemolytic and CAMP-negative isolates gave an unclear result (weak positive) was not expected. The initial development and validation of the leukotoxin PCR by Green *et al.* (1999) noted that the test was highly reproducible. Green *et al.* (1999) used isolates from bighorn sheep and reported clear cut results with a correlation between a positive PCR result and the ability to produce cytotoxic effects *in vitro*. The difficulties we have encountered suggest that further detailed studies using isolates from both domestic and wild ruminants are necessary before a full understanding of the means of detecting the leukotoxin gene is reached. This requirement for further study on isolates from domestic ruminants was noted by Green *et al.* (1999) and is still a relevant cautionary note.

Description of *Bibersteinia* gen. nov.

Bibersteinia (Bi.ber.stei′ni.a. N.L. fem. n. *Bibersteinia* bacterial genus named after Ernst L. Biberstein, who did much of the early characterization work on this organism, including the creation of the serotyping scheme and some of the

earliest DNA–DNA relatedness studies that indicated the unique nature of this taxon).

A member of the family *Pasteurellaceae* as defined by Olsen *et al.* (2005). Gram-negative, non-motile, rod-shaped or pleomorphic with cells occurring singly and in pairs or short chains depending upon the growth stage. Colonies on bovine blood agar are round, regular, greyish or yellowish, semi-transparent at the periphery and are about 2 mm in diameter after 24 h at 37 °C. Some isolates are haemolytic and are CAMP-positive. Endospores are not formed. Growth is mesophilic and facultatively anaerobic or microaerophilic. Nitrate is reduced without gas production. The reaction in Hugh–Leifson medium with (+)-D-glucose is fermentative without gas production. Porphyrin, phosphatase and alanine aminopeptidase tests are positive. Negative reactions occur for Simmons' citrate, malonate-base, growth in the presence of KCN, Voges–Proskauer, methyl red and urease tests. Negative tests are further observed with ONPG, arginine dehydrolase, lysine decarboxylase, ornithine decarboxylase, phenylalanine deaminase, indole, gelatinase and hydrolysis of Tweens 20 and 80. Acid is formed from (−)-D-ribose, (−)-D-mannitol, (−)-D-sorbitol, (−)-D-fructose, (+)-D-glucose, (+)-D-mannose, maltose, sucrose, (+)-D-trehalose and dextrin. Acid is not produced from adonitol, (+)-D-arabitol, (−)-D-arabinose, (+)-L-arabinose, *myo*-erythritol, dulcitol, (+)-D-fucose, (−)-L-fucose, (+)-D-galactose, D-glycogen, inulin, lactose, (+)-D-melibiose, (+)-D-melezitose, (+)-L-rhamnose, (−)-L-sorbose, (+)-D-turanose, xylitol, (+)-D-xylose or (−)-L-xylose. Reactions for α-fucosidase, α-galactosidase β-galactosidase, β-glucuronidase (PGUA), α-mannosidase and β-xylosidase are negative. Variable reactions occur for the catalase and oxidase tests and the production of acid from glycerol, *myo*-inositol, cellobiose, raffinose, aesculin, amygdalin, arbutin, gentiobiose and salicin. Variable reactions are also obtained in the α-glucosidase and β-glucosidase tests.

The DNA G+C content is reported as 42.6 mol% (Mutters *et al.*, 1985, 1986). The type species of the genus is *Bibersteinia trehalosi*, originally described as [*Pasteurella*] *trehalosi* by Sneath & Stevens (1990).

Description of *Bibersteinia trehalosi* comb. nov.

Basonym: *Pasteurella trehalosi* Sneath & Stevens 1990.

Key tests that allow the separation of *Bibersteinia trehalosi* from the other genera of the *Pasteurellaceae* are shown in Table 2. For characteristics showing variation in Table 2, the type strain shows the following reactions: catalase and oxidase-negative, weak haemolysis on bovine blood agar, CAMP-positive, weak growth on MacConkey agar, yellowish pigment, production of acid from cellobiose, raffinose, aesculin, amygdalin, arbutin, gentiobiose and salicin, no acid production from glycerol and *myo*-inositol. The type strain is NCTC 10370^T (=ATCC 29703^T).

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References

Adlam, C. (1989). The structure, function and properties of cellular and extracellular components of *Pasteurella haemolytica*. In *Pasteurella and Pasteurellosis*, pp. 75–92. Edited by C. F. Adlam & J. M. Rutter. London: Academic Press.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.

Angen, O., Mutters, R., Caugant, D. A., Olsen, J. E. & Bisgaard, M. (1999). Taxonomic relationships of the [*Pasteurella*] *haemolytica* complex as evaluated by DNA-DNA hybridizations and 16S rRNA sequencing with the proposal of *Mannheimia haemolytica* gen. nov., comb. nov., *Mannheimia granulomatis* comb. nov., *Mannheimia glucosida* sp. nov., *Mannheimia ruminalis* sp. nov. and *Mannheimia varigena* sp. nov. *Int J Syst Bacteriol* **49**, 67–86.

Angen, O., Ahrens, P., Kuhnert, P., Christensen, H. & Mutters, R. (2003). Proposal of *Histophilus somni* gen. nov., sp. nov. for the three species incertae sedis '*Haemophilus somnus*', '*Haemophilus agni*' and '*Histophilus ovis*'. *Int J Syst Evol Microbiol* **53**, 1449–1456.

Applied Biosystems (2000). *Automated DNA Sequencing Chemistry Guide*, 264 pp. Foster City, CA: Applied Biosystems.

Benson, D. A., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J. & Wheeler, D. L. (2004). GenBank: update. *Nucleic Acids Res* **32** (Database issue), D23–26.

Biberstein, E. L. & Francis, C. K. (1968). Nucleic acid homologies between the A and T types of *Pasteurella haemolytica*. *J Med Microbiol* **1**, 105–108.

Biberstein, E. L., Gills, M. & Knight, H. (1960). Serological types of *Pasteurella haemolytica*. *Cornell Vet* **50**, 283–300.

Bisgaard, M. & Mutters, R. (1986). Characterization of some previously unclassified "*Pasteurella*" spp. obtained from the oral cavity of dogs and cats and description of a new species tentatively classified with the family *Pasteurellaceae* Pohl 1981 and provisionally called taxon 16. *Acta Pathol Microbiol Immunol Scand [B]* **94**, 177–184.

Bisgaard, M., Houghton, S. B., Mutters, R. & Stenzel, A. (1991). Reclassification of German, British and Dutch isolates of so-called *Pasteurella multocida* obtained from pneumonic calf lungs. *Vet Microbiol* **26**, 115–124.

Blackall, P. J., Angen, O., Fegan, N., Blackall, L. L., Mutters, R. & Bisgaard, M. (2001). Characterisation of a novel *Mannheimia* sp from Australian feedlot cattle. *Aust Vet J* **79**, 634–639.

Blackall, P. J., Christensen, H., Beckenham, T., Blackall, L. L. & Bisgaard, M. (2005). Reclassification of *Pasteurella gallinarum*, [*Haemophilus*] *paragallinarum, Pasteurella avium* and *Pasteurella volantium* as *Avibacterium gallinarum* gen. nov., comb. nov., *Avibacterium paragallinarum* comb. nov., *Avibacterium avium* comb. nov. and *Avibacterium volantium* comb. nov. *Int J Syst Evol Microbiol* **55**, 353–362.

Busse, H. J., Bunka, S., Hensel, A. & Lubitz, W. (1997). Discrimination of members of the family *Pasteurellaceae* based on polyamine patterns. *Int J Syst Bacteriol* **47**, 698–708.

Christensen, H. & Bisgaard, M. (2003). The genus *Pasteurella*. In *The Prokaryotes*. Edited by M. Dworkin. New York: Springer.

Christensen, H. & Bisgaard, M. (2004). Revised definition of *Actinobacillus sensu stricto* isolated from animals: A review with special emphasis on diagnosis. *Vet Microbiol* **99**, 13–30.

Christensen, H., Bisgaard, M., Bojesen, A. M., Mutters, R. & Olsen, J. E. (2003a). Genetic relationships among avian isolates classified as *Pasteurella haemolytica*, '*Actinobacillus salpingitidis*' or *Pasteurella anatis* with proposal of *Gallibacterium anatis* gen. nov., comb. nov. and description of additional genomospecies within *Gallibacterium* gen. nov. *Int J Syst Evol Microbiol* **53**, 275–287.

Christensen, H., Foster, G., Christensen, J. P., Pennycott, T., Olsen, J. E. & Bisgaard, M. (2003b). Phylogenetic analysis by 16S rDNA gene sequence comparison of avian taxa of Bisgaard and characterization and description of two new taxa of *Pasteurellaceae*. *J Appl Microbiol* **95**, 354–363.

Christensen, H., Bisgaard, M., Aalbaek, B. & Olsen, J. E. (2004a). Reclassification of Bisgaard taxon 33, with proposal of *Volucribacter psittacicida* gen. nov., sp. nov. and *Volucribacter amazonae* sp. nov. as new members of the *Pasteurellaceae*. *Int J Syst Evol Microbiol* **54**, 813–818.

Christensen, H., Angen, O., Olsen, J. E. & Bisgaard, M. (2004b). Revised description and classification of atypical isolates of *Pasteurella multocida* from bovine lungs based on genotypic characterization to include variants previously classified as biovar 2 of *Pasteurella canis* and *Pasteurella avium*. *Microbiology* **150**, 1757–1767.

Christensen, H., Kuhnert, P., Olsen, J. E. & Bisgaard, M. (2004c). Comparative phylogenies of the housekeeping genes *atpD*, *infB* and *rpoB* and the 16S rRNA gene within the *Pasteurellaceae*. *Int J Syst Evol Microbiol* **54**, 1601–1609.

Christie, R., Atkins, N. E. & Munch-Petersen, E. (1944). A note on a lytic phenomenon shown by group B streptococci. *Aust J Exp Biol Med Sci* **22**, 197–200.

Davies, R. L. & Quirie, M. (1996). Intra-specific diversity within *Pasteurella trehalosi* based on variation of capsular polysaccharide, lipopolysaccharide and outer-membrane proteins. *Microbiology* **142**, 551–560.

Davies, R. L., Paster, B. J. & Dewhirst, F. E. (1996). Phylogenetic relationships and diversity within the *Pasteurella haemolytica* complex based on 16S rRNA sequence comparison and outer membrane protein and lipopolysaccharide analysis. *Int J Syst Bacteriol* **46**, 736–744.

Davies, R. L., Arkinsaw, S. & Selander, R. K. (1997). Genetic relationships among *Pasteurella trehalosi* isolates based on multilocus enzyme electrophoresis. *Microbiology* **143**, 2841–2849.

De Ley, J., Mannheim, W., Mutters, R., Piechulla, K., Tytgat, R., Segers, P., Bisgaard, M., Frederiksen, W., Hinz, K. H. & Vanhoucke, M. (1990). Inter- and intrafamilial similarities of rRNA cistrons of the Pasteurellaceae. *Int J Syst Bacteriol* **40**, 126–137.

Dewhirst, F. E., Paster, B. J. & Bright, P. L. (1989). *Chromobacterium, Eikenella, Kingella, Neisseria, Simonsiella*, and *Vitreoscilla* species comprise a major branch of the beta group *Proteobacteria* by 16S ribosomal ribonucleic acid sequence comparison: transfer of *Eikenella* and *Simonsiella* to the family *Neisseriaceae* (emend.). *Int J Syst Bacteriol* **39**, 258–266.

Gilmour, N. J. L. & Gilmour, J. S. (1989). Pasteurellosis of sheep. In *Pasteurella and Pasteurellosis*, pp. 223–261. Edited by C. F. Adlam & J. M. Rutter. London: Academic Press.

Green, A. L., DuTeau, N. M., Miller, M. W., Triantis, J. M. & Salman, M. D. (1999). Polymerase chain reaction techniques for differentiating cytotoxic and noncytotoxic *Pasteurella trehalosi* from Rocky Mountain bighorn sheep. *Am J Vet Res* **60**, 583–588.

Kuhnert, P., Korczak, B., Falsen, E., Straub, R., Hoops, A., Boerlin, P., Frey, J. & Mutters, R. (2004). *Nicoletella semolina* gen. nov., sp. nov., a new member of *Pasteurellaceae* isolated from horses with airway disease. *J Clin Microbiol* **42**, 5542–5548.

Leisner, J. J., Pot, B., Christensen, H., Rusul, G., Olsen, J. E., Wee, B. W., Muhamad, K. & Ghazali, H. M. (1999). Identification of lactic acid bacteria from chili bo, a Malaysian food ingredient. *Appl Environ Microbiol* **65**, 599–605.

Mutters, R., Ihm, P., Pohl, S., Frederiksen, W. & Mannheim, W. (1985). Reclassification of the genus *Pasteurella* Trevisan 1887 on the basis of deoxyribonucleic acid homology, with proposals for the new species *Pasteurella dagmatis, Pasteurella canis, Pasteurella stomatis, Pasteurella anatis*, and *Pasteurella langaa*. *Int J Syst Bacteriol* **35**, 309–322.

Mutters, R., Bisgaard, M. & Pohl, S. (1986). Taxonomic relationships of selected biogroups of *Pasteurella haemolytica* as revealed by DNA:DNA hybridizations. *Acta Pathol Microbiol Immunol Scand [B]* **94**, 195–202.

Olsen, G. J., Matsuda, H., Hagstrom, R. & Overbeek, R. (1994). FastDNAml: a tool for construction of phylogenetic trees of DNA sequences using maximum likelihood. *Comput Appl Biosci* **10**, 41–48.

Olsen, I., Dewhirst, F. E., Paster, B. J. & Busse, H.-J. (2005). Family *Pasteurellaceae*. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol 2, part 2, pp. 851–856. Edited by D. J. Brenner, N. R. Kreig & J. T. Staley. New York: Springer.

Paster, B. J. & Dewhirst, F. E. (1988). Phylogeny of campylobacters, wolinellas, *Bacteroides gracilis*, and *Bacteroides ureolyticus* by 16S ribosomal ribonucleic acid sequencing. *Int J Syst Bacteriol* **38**, 56–62.

Pohl, S. (1981). DNA relatedness among members of *Haemophilus, Pasteurella* and *Actinobacillus*. In *Haemophilus, Pasteurella and Actinobacillus*, pp. 245–253. Edited by M. Kilian, W. Frederiksen & E. L. Biberstein. London: Academic Press.

Rice, P., Longden, I. & Bleasby, A. (2000). EMBOSS: the European Molecular Biology Open Software Suite. *Trends Genet* **16**, 276–277.

Smith, G. R. (1959). Isolation of two types of *Pasteurella haemolytica* from sheep. *Nature* **183**, 1132–1133.

Sneath, P. H. & Stevens, M. (1990). *Actinobacillus rossii* sp. nov., *Actinobacillus seminis* sp. nov., nom. rev., *Pasteurella bettii* sp. nov., *Pasteurella lymphangitidis* sp. nov., *Pasteurella mairi* sp. nov., and *Pasteurella trehalosi* sp. nov. *Int J Syst Bacteriol* **40**, 148–153.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.

Vogel, B. F., Jorgensen, K., Christensen, H., Olsen, J. E. & Gram, L. (1997). Differentiation of *Shewanella putrefaciens* and *Shewanella alga* on the basis of whole-cell protein profiles, ribotyping, phenotypic characterization, and 16S rRNA gene sequence analysis. *Appl Environ Microbiol* **63**, 2189–2199.

Ward, A. C., Dyer, N. W. & Fenwick, B. W. (1999). *Pasteurellaceae* isolated from tonsillar samples of commercially-reared American bison (*Bison bison*). *Can J Vet Res* **63**, 161–165.