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

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Abstract

[*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 [*Pasteurella*] *trehalosi* in order to reclassify and rename this taxon to support improvements in our knowledge on pathogenesis and epidemiology of this important organism. The reference strain for the species [*Pasteurella*] *trehalosi* (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 characterisation was performed on all 43 isolates. As well, Amplified Fragment Length Polymorphism (AFLP) was performed on the isolates. Two of the field isolates were subjected to 16S rRNA sequencing. These sequences along with five existing sequences for [*Pasteurella*] *trehalosi* strains and 12 sequences for other taxa in the family *Pasteurellaceae* were subjected to phylogenetic analysis. All the isolates and the reference strains were identified as [*Pasteurella*] *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 [*Pasteurella*] *trehalosi* isolates. All [*Pasteurella*] *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 (2 field isolate and the five serovar reference strains) are closely related with 98.6 % or higher 16S rRNA sequence similarity. As both genotypic and phenotypic testing support the separate and distinct nature of these organisms, we propose the transfer of [*Pasteurella*] *trehalosi* to a new genus *Bibersteinia* as *Bibersteinia trehalosi* comb. nov. The type strain is NCTC 10370^T (= ATCC 29703^T). Separation of *Bibersteinia trehalosi* from the existing genera of the family is possible by application of only 9 characters (catalase, porphyrin, urease, indole, phosphatase, acid from dulcitol, D(+)-galactose, D(+)-mannose and D(+)-trehalose).

Keywords: [Pasteurella] trehalosi; Phenotype; Genetic diversity; AFLP

59 [*Pasteurella*] *trehalosi* is an important pathogen of sheep – being primarily associated with
60 serious systemic infections in lambs but also having an association with pneumonia in sheep
61 (Gilmour & Gilmour, 1989). The organism, first named as a separate species by Sneath and
62 Stevens (1990), was part of the complex of species once known as the *"*[*Pasteurella*]
63 *haemolytica"* complex (Angen *et al.*, 1999). The *"*[*Pasteurella*] *haemolytica"* complex,
64 which consisted of biovars A and T (Smith, 1959), has been extensively re-organised. The
65 organisms once assigned to the T biovar were named [*Pasteurella*] *trehalosi* (Sneath &
66 Stevens, 1990). However, there is clear evidence that the species is not closely affiliated with
67 *Pasteurella multocida,* the type species of the genus *Pasteurella* (Angen *et al.*, 1999) (the
68 reason for enclosing the genus name of [*Pasteurella*] *trehalosi* in brackets). The new genus,
69 *Mannheimia*, houses the A biovar organisms from the old *"Pasteurella haemolytica"*
70 complex with five species – *Mannheimia haemolytica, Mannheimia glucosida, Mannheimia*
71 *granulomatis, Mannheimia ruminalis* and *Mannheimia varigena* (Angen *et al.*, 1999) as well
72 as currently un-named taxa such as Bisgaard Taxon 39 (Blackall *et al.*, 2001).
73
74 There is evidence of diversity within the species [*Pasteurella*] *trehalosi*. A serotyping
75 scheme based on capsular polysaccharides, originally developed by Biberstein *et al.* (1960),
76 that recognises four serovars – termed T3, T4, T10 and T15 – has been used to study strain
77 variation (Adlam, 1989; Gilmour & Gilmour, 1989). Davies and Quirie (1996) found six
78 lipopolysaccharide (LPS) types and four outer membrane protein (OMP) types in a collection
79 of 60 isolates mainly from sheep in the UK and noted that this represented only a limited
80 degree of diversity. Using multi-locus enzyme electrophoresis, Davies *et al.* (1997)
81 concluded that the degree of diversity within the same sixty isolates studied by LPS and OMP
82 typing (Davies & Quirie, 1996) was lower than most other pathogenic species that had been
83 studied by MLEE. In the present investigation, we have characterised a broad collection of

strains tentatively identified as [*Pasteurella*] *trehalosi* to gain some insight on 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 allow progress on the pathogenesis and epidemiology of this veterinary important 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 and are shown in Table 1. The type strain for the species [*Pasteurella*] *trehalosi* (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) remained unknown.

All the isolates and the reference strain were characterized as described previously (Bisgaard *et al.*, 1991). The CAMP reaction of all the isolates was determined as previously described (Christie *et al.*, 1944). As well, the PCR for the leukotoxin of [*Pasteurella*] *trehalosi* (Green *et al.*, 1999) was used 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 subsequent to restriction digestion and ligation to their corresponding adaptors. All AFLP reactions were carried out twice to allow evaluation of the reproducibility of the method. Amplification products were detected on an ABI 377 automated sequencer (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, Kortrijk, Belgium). Normalised AFLP fingerprints were compared using the Dice similarity coefficient and clustering analysis was performed by the unweighted pair group method with arithmetic averages (UPGMA).

16S rRNA gene sequencing of strains B464/94 and C1008-I was performed as described below. Bacteria were cultured overnight in Brain Hearth Infusion Broth (Difco) and DNA was extracted including enzymatic 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 and Dewhirst (1988). DNA sequencing was performed on the ABI 377 (Applied Biosystems) sequencer with unlabelled primers and the BigDye kit according to protocols described with the Chemistry Guide for automated DNA sequencing (Applied Biosystems). 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 [*Pasteurella*] *trehalosi*. Five sequences for the following strains were found - NCTC10370^{T}, NCTC10641, NCTC11550, NCTC10624, NCTC10626 (GenBank accession numbers AY362927, U57074, U57073, M75063, U57075 respectively). As well, the sequences for the type strains of the type species of the currently recognised genera within the family *Pasteurellaceae* were obtained. Pairwise

134 comparisons for similarity were performed by EMBOSS (Rice *et al.*, 2000). Sequences were
135 aligned by ClustalX (Thompson *et al.*, 1997).
136
137 Maximum likelihood analysis was performed by fastDNAml including bootstrap analysis
138 (Olsen *et al.*, 1994) run on a Linux compatible server. The transition/transversion ratio was
139 set to 1.5. The region *E. coli* pos. 87-1392 of the *rrnB* gene was used after removal of gaps
140 with 226 distinct data patterns analysed. The 'loop' script provided by fastDNAml was used
141 to justify that the tree with maximum lnL had been found.
142
143 By phenotypic testing, 43 isolates were identified as [*Pasteurella*] *trehalosi*. These 43
144 isolates were Gram negative, non-motile (at 22^0C and 37^0C) rods that were fermentative in
145 the Hugh and Leifson test. The isolates did not show symbiotic growth on blood agar and
146 gave positive results in the porphyrin, phosphatase and alanine aminopeptidase tests. All
147 isolates were positive in the nitrate reduction test and were negative in Simmons citrate and
148 the methyl red and Voges-Proskauer tests. No isolate produced H_2S, urease or gelatinase.
149 Indole was not produced nor were Tween 20 or Tween 80 hydrolysed. The isolates were all
150 negative in the arginine dehydrolase and lysine and ornithine decarboxylase tests.
151 Phenylalanine was not deaminated, acid was not produced from mucate and nor was alkali
152 produced in the malonate test. No isolate could grow in KCN. The isolates showed variable
153 reactions in the catalase and oxidase tests. Variable results were obtained for haemolysis (on
154 bovine blood agar), the CAMP test (performed using bovine blood cells) and ability to grow
155 on McConkey agar. Over half the isolates showed a yellowish pigment in colonies grown on
156 blood agar. Acid was produced from D(-) ribose, D(-) mannitol, D(-) sorbitol, D(-) fructose,
157 D(+) glucose, D(+) mannose, maltose, sucrose, (D)+ trehalose and dextrin. Acid was not
158 produced from meso-erythritol, adonitol, D(+) arabitol, xylitol, L(+) arabinose, D(-)

159 arabinose, D(+) xylose, L(-) xylose, dulcitol, D(+) fucose, L(-) fucose, D(+) galactose, L(+)
160 rhamnose, L(-) sorbose, lactose, D(+) melibose, D(+) melizitose, D(+) glycogen, inulin and
161 D(+) turanose. The isolates varied in their ability to produce acid from glycerol, meso-
162 inositol, cellobiose, raffinose, esculin, amygdalin, arbutin, gentiobiose and salicin. All
163 isolates were negative in the β-galactosidase (ONPG) test. All the isolates were negative for
164 α-fucosidase, α-galactosidase, α-mannosidase and β-xylosidase tests. Variable results were
165 obtained in the α-glucosidase, β- glucosidase and β-glucuronidase tests. For characters
166 showing variation the type strain, NCTC 10370^{T}, showed the following reactions: catalase
167 and oxidase negative, weak haemolysis on bovine blood agar, CAMP positive, weak growth
168 on McConkey agar, yellowish pigment, production of acid from cellobiose, raffinose,
169 aesculin, amygdalin, arbutin, gentiobiose, and salicin while acid was not produced from
170 glycerol and meso-inositol. The β-glucosidase and α-glucosidase tests were positive while β-
171 glucuronidase was negative. Seventeen out of 22 ovine isolates produced acid from all
172 glycosides investigated while only four out of fourteen bovine isolates had the capacity to
173 produce acid from all glycosides.
174
175 A single bovine isolate (28B), differing in ornithine decarboxylase activity and D(+) mannose
176 fermentation, remained unidentified.
177
178 Of the two remaining bovine isolates included as blind controls in the AFLP characterization,
179 one (isolate 33B) was identified as *Pasteurella multocida* (ornithine decarboxylase and indole
180 negative) and one (isolate 42B) as *Mannheimia varigena*.
181
182 The PCR for the leukotoxin of [*Pasteurella*] *trehalosi* (Green *et al.*, 1999) was used on 10
183 CAMP positive strains (NCTC 10369, NCTC 10370^{T}, NCTC 10371, NCTC 10624, FT3,

184 T3H, 4935/S945T3, T34682, 4954/T3 and S487/T3) and gave a clear positive reaction for all
185 10 isolates. When used on the six CAMP negative isolates (C1008-I, C1019-II, B96/19,
186 B96/39, B96/54 and B464/94), a suspect to weak positive result was given with all six
187 isolates.
188
189 The reproducibility of the AFLP method was evaluated by analysis of duplicates of isolates
190 with independently prepared templates, different selective PCR runs and different
191 electrophoretic runs. The reproducibility of the method was good (96.1%) with a standard
192 deviation of $\pm$2.4%. The clustering analysis is shown in Figure 1. Field isolate C857-II was
193 included as two separate cultures in the analysis. The two cultures of C857-II had an identical
194 AFLP profile and were regarded as a single field isolate. All isolates used in the study shared
195 at least 35% similarity in their AFLP profiles. Indeed, all isolates, except for three isolates
196 (numbers 33B, 28B and 42B) shared at least 70% similarity in their profiles. As noted above,
197 these three aberrant isolates represented *Pasteurella multocida,* an unclassified organism and
198 *Mannheimia varigena,* respectively. For the purpose of this study, isolates with AFLP
199 profiles of $\geq$90% similarity were defined as an AFLP type while isolates with $\geq$80%
200 similarity were defined as an AFLP cluster. As shown in Table 1, a total of 22 AFLP types (A
201 to V) were recognised and these types formed a total of 9 AFLP clusters (1 to 9). AFLP
202 types T, U and V (which corresponded to Clusters 7, 8 and 9) consisted of the three strains
203 known not to be [*Pasteurella*] *trehalosi* on the basis of phenotypic results. The exclusion of
204 these strains from [*Pasteurella*] *trehalosi* was supported by the low similarity ($\leq$50%) of
205 these strains with all other [*Pasteurella*] *trehalosi* isolates examined in the AFLP study.
206
207 Of the 22 AFLP types recognised, half were single isolate types. The largest AFLP type
208 (type B) consisted of 7 field isolates and the reference strain (NCTC 10370^T), all of ovine

209 origin. AFLP types C and K only included ovine isolates too. Only AFLP clusters B and C
210 included strains producing acid from all glycosides investigated. The other large AFLP type
211 was type K which contained 5 ovine field isolates of intermediate capacity as to produce acid
212 from glycosides. AFLP type B included serovars 3 and 15 while serovar 4 was associated
213 with types C and E and serovar 10 with types E, F and K. However, serovar 3 was also
214 associated with types D and K.
215
216 Of the nine AFLP clusters recognised, six contained more than one isolate. AFLP Cluster 1
217 was the largest cluster recognised and consisted of AFLP types A, B, C, D, E and F. AFLP
218 Cluster 1 included a total of 21 field isolates and the reference strain (NCTC 10370^{T}). The
219 next largest cluster was Cluster 5 – which included eight isolates. Of the six multi-member
220 clusters, five were associated with a single host species. The only AFLP cluster that involved
221 more than one host was Cluster F where two isolates were of ovine origin and one isolate was
222 of cervine origin.
223
224 In the 16S rRNA analysis, the major AFLP clusters were represented. To supplement the 5
225 strains already sequenced and representing all four serovars, two additional strains B464/94
226 and C1008-I were sequenced in the present study and deposited in GenBank with accession
227 numbers DQ361040 and DQ361041, respectively. The lowest 16S rRNA similarity within
228 [*Pasteurella*] *trehalosi* was 98.3 % between strains NCTC 10624 and B464/94 isolated from
229 sheep and roe deer, respectively. The highest similarity outside of [*Pasteurella*] *trehalosi* was
230 96.4 % found between the type strain of [*Pasteurella*] *trehalosi* to the type strain of
231 *Mannheimia ruminalis*.
232

233 The phylogenetic analysis showed that the strains of [*Pasteurella*] *trehalosi* formed a
234 monophyletic group that was unrelated to other members of the *Pasteurellaceae* including
235 *Mannheimia* (Figure 2).
236
237 This study has confirmed earlier findings that [*Pasteurella*] *trehalosi* is a monophyletic group
238 unrelated to other taxa within the family *Pasteurellaceae*. The full neighbor joining tree of
239 *Pasteurellaceae* by Olsen *et al.* (2005) showed a deep branching of the type strain of
240 [*Pasteurella*] *trehalosi*, unrelated to other members of the family. The deep branching was
241 also found by Christensen *et al.* (2004c) with application of maximum likelihood analysis.
242 Phylogenetic analysis based on 16S rRNA sequences has previously indicated that the four
243 isolates of [*Pasteurella*] *trehalosi*, representing the four recognised serovars within the taxon
244 (T3, T4, T10 and T15), are closely related (showing at least 98.7% similarity) and form a
245 monophyletic group (Davies *et al.*, 1996). These 16S rRNA based results have been
246 supported by phylogenies based on two house-keeping genes (Christensen *et al.*, 2004c). As
247 well, only 62% or less DNA relatedness has been found between [*Pasteurella*] *trehalosi* and
248 other members of the *Pasteurellaceae* (Biberstein & Francis, 1968; Mutters *et al.*, 1986;
249 Mutters *et al.*, 1985; Pohl, 1981). The uniqueness of [*Pasteurella*] *trehalosi* has also been
250 demonstrated in terms of polyamine profiles (Busse *et al.*, 1997). As well, [*Pasteurella*]
251 *trehalosi* strain NCTC 10624 (serovar T3) was not located on any of the seven rRNA
252 branches outlined by De Ley *et al.* (1990). Indeed, this strain was located at the root of the
253 large *Haemophilus-Pasteurella-Actinobacillus* rRNA branch (De Ley *et al.*, 1990).
254
255 Hence, our work using 16S rRNA gene sequence analysis, AFLP typing and phenotypic
256 characterisation is in full accord with all existing knowledge – [*Pasteurella*] *trehalosi* is a
257 distinct and separate genus within the family *Pasteurellaceae*. On the basis of our results,

plus this existing knowledge, we propose that [*Pasteurella*] *trehalosi* should be housed within a separate genus for which we propose the name *Bibersteinia* gen. nov.

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

AFLP was used in this study to assess diversity within *Bibersteinia 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 trehalosi* strains were all members of other taxa (one unidentified strain, one *Pasteurella multocida* strain and one *Mannheimia varigena* strain). The clusters recognised 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 if there is an association between clonal lines of *Bibersteinia trehalosi* and host species.

Most standard texts refer to the fact that *Bibersteinia ([Pasteurella]) trehalosi* is a pathogen of sheep and make no mention of any role in bovine disease e.g. Gilmour and Gilmour (1989). In our study, 14 of 43 strains of *Bibersteinia 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 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 trehalosi* (nine strains) were isolated from the respiratory tract and it is difficult to assess their role as primary pathogens. While *Bibersteinia trehalosi* does not appear to have been commonly reported from cattle, the organism was found to be the most common *Pasteurellaceae* 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 trehalosi* in bovine disease. Our study, plus the comprehensive description of the genus *Mannheimia* with five species, *Mannheimia haemolytica, Mannheimia glucosida, Mannheimia granulomatis, Mannheimia ruminalis* and *Mannheimia 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 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 CAMP test – all 22 ovine strains were positive while only the 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 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 frankly 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 frankly 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 bighorn sheep isolates 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 both isolates from domestic and wild ruminants – are necessary before a full understanding of the means of detecting the leukotoxin gene is available. 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 characterisation work on this organism including the creation of the serotyping scheme and some of the earliest DNA:DNA homology 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, grayish or yellowish, semi-transparent at the periphery and are about 2 mm in diameter after 24 h at 37^0C. 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 Tween 20 and 80 hydrolysis. 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, *meso*-erythritol, dulcitol, D(+) fucose, L(-) fucose, D(+) galactose, D-glycogen, inulin, lactose, D(+) melibiose, D(+) melizitose, L(+) rhamnose, L(-) sorbose, D(+) turanose, xylitol, D(+) Xylose or L(-) xylose.

Reactions for α-fucosidase, α-galactosidase β-galactosidase, α-mannosidase and β-xylosidase are negative. Variable reactions occur for the catalase, oxidase tests and the production of acid from glycerol, meso-inositol, cellobiose, raffinose, esculin, amygdalin, arbutin, gentibiose and salicin. Variable reactions were also obtained in the α-glucosidase, β-glucosidase and β-glucuronidase tests.

The DNA G+C content has been reported as 42.6 mol% (Mutters *et al.*, 1986; Mutters *et al.*, 1985). The type species of the genus is *Bibersteinia trehalosi* NCTC 10370^T originally described as [*Pasteurella*] *trehalosi* in 1990 by Sneath and Stevens (1990).

Description of *Bibersteinia trehalosi* (Sneath & Stevens, 1990) comb. nov.

Basonym: *Pasteurella trehalosi* Sneath and Stevens 1990

The key tests that allow the separation of *Bibersteinia trehalosi* from the other genera of the *Pasteurellaceae* are shown in Table 2. The type strain is NCTC 10370^T (= ATCC 29703^T). For characters showing variation in Table 2, the type strain, NCTC 10370^T, showed the following reactions: catalase and oxidase negative, weak haemolysis on bovine blood agar, CAMP positive, weak growth on McConkey agar, yellowish pigment, production of acid from cellobiose, raffinose, aesculin, amygdalin, arbutin, gentiobiose, and salicin while acid was not produced from glycerol and meso-inositol.

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547

548 **Table 1.** Isolates used in this study.
549

Isolate	Host Species	Sero-var	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[a]	Bovine	NT	Intestine and lung	B	7/T
28B[b]	Bovine	NT	Bronchial Lavage	B	8/U
42B[c]	Bovine	NT	ND	B	9/V

550
551 ND: No data
552 NT: No serotype
553 a : *P. multocida* (ornithine and indole negative)
554 b : Unclassified
555 c : *M. varigena*

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

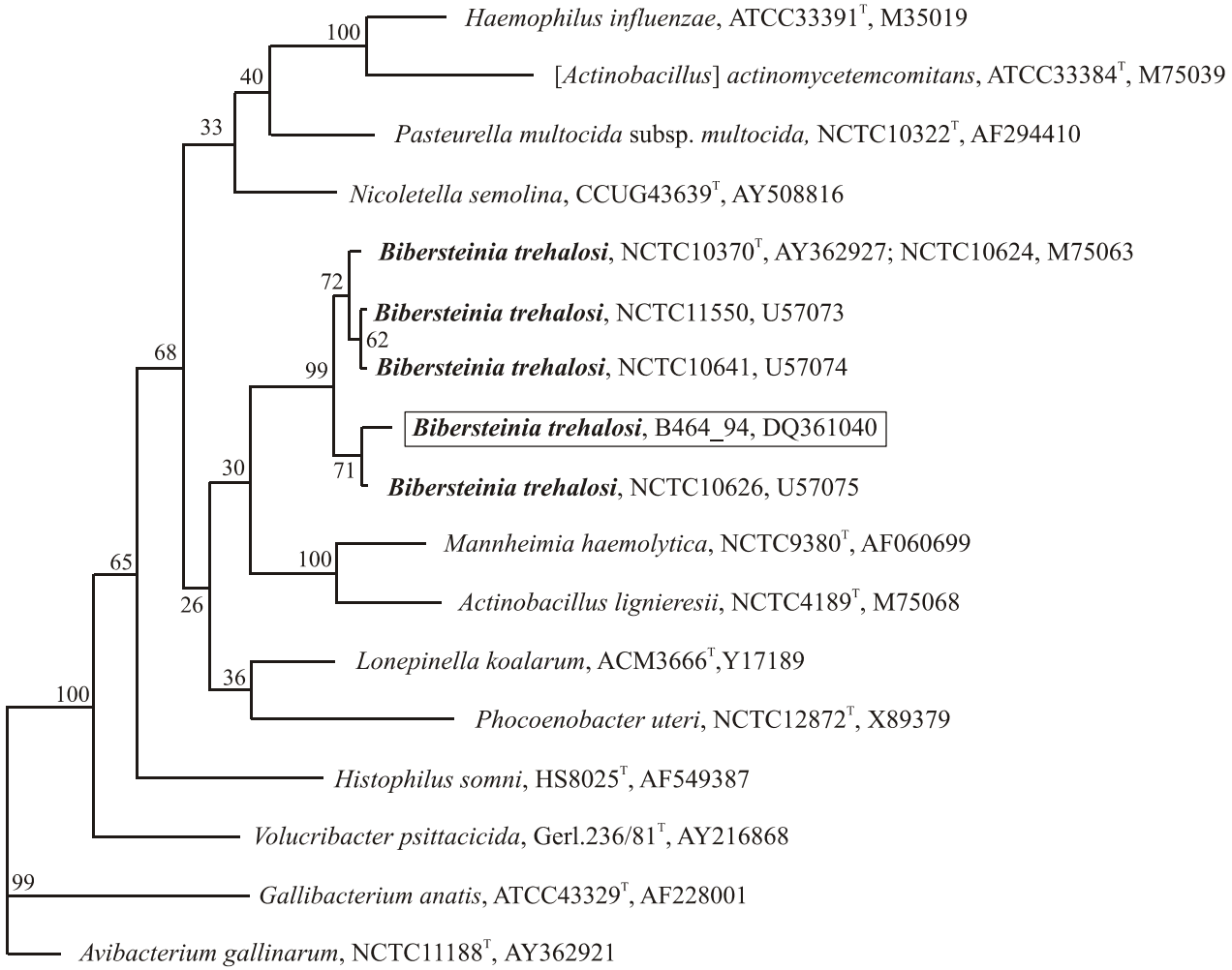
Genera: 1, *Actinobacillus sensu stricto*; 2, *Pasteurella sensu stricto*; 3, *Haemophilus sensu stricto* (includes *H. influenzae*, *H. haemolyticus* and *H. aegypticus* – results for *H. parainfluenzae* and *H. pittmania* are given in []); 4, *Mannheimia*; 5, *Lonepinella*; 6, *Phocoenobacter*; 7, *Gallibacterium*; 8, *Volucribacter*; 9, *Histophilus*; 10, *Avibacterium*, 11, *Nicoletella*, 12, *Bibersteinia* gen. nov. Data based on Angen *et al.* (1999; 2003), Bisgaard and Mutters (1986), Blackall *et al.* (2005), Christensen and Bisgaard (2003; 2004), Christensen *et al.* (2003a; 2003b), Christensen *et al.* (2004a; 2004b), Kuhnert *et al.* (2004) Mutters *et al.* (1985) and this study. Characters are scored as: +, 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, weak positive.

	Genus											
Characteristic	1	2	3	4	5	6	7	8	9	10	11	12
Catalase	d	d	d	d	-	-	+	d	-	d	+	d
NAD requirement	-[a*]	-[b]	+ [d]	-	-	-	-	-	-	+[c]	-	-
Porphyrin	+	+	- [+]	+	+	+	+	+	-	+	nd	+
Methyl red	-	-	nd	-	-	nd	w	+	nd	-	nd	-
Voges Proskauer	-	-	nd	-	d	+	-	-	-	-	nd	-
Urease	+	-[d]	+ [d]	-	-	-	-	-	-	-	+	-
Indole	-	+	d	-	-	-	-	-	+	-	-	-
Phosphatase	+	+	+	+	-	+	+	+/w	+	+[e]	d	+
Pigment	-	-	nd	-	-	nd	-	-	+	d	nd	d
Acid from												
D(-) arabinose	-	d	nd	d	(+)	nd	(+)	-	nd	d	nd	-
Dulcitol	-	d	-	-	+	-	-	-	nd	-	-	-
D(-)-mannitol	+[f]	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: a, *Actinobacillus pleuropneumoniae* biovar 1 positive; b *Pasteurella multocida* might be positive; c, *Avibacterium gallinarum* negative; some isolates of *Avibacterium paragallinarum* also negative (biovar 2); d, *Pasteurella dagmatis* positive; e, *Avibacterium paragallinarum* biovar 1 might be negative; f, *Actinobacillus suis* negative

Fig 1. Dendrogram (UPGMA) of AFLP similarities (Dice coefficient) between the strains of [*Pasteurella*] *trehalosi*.

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.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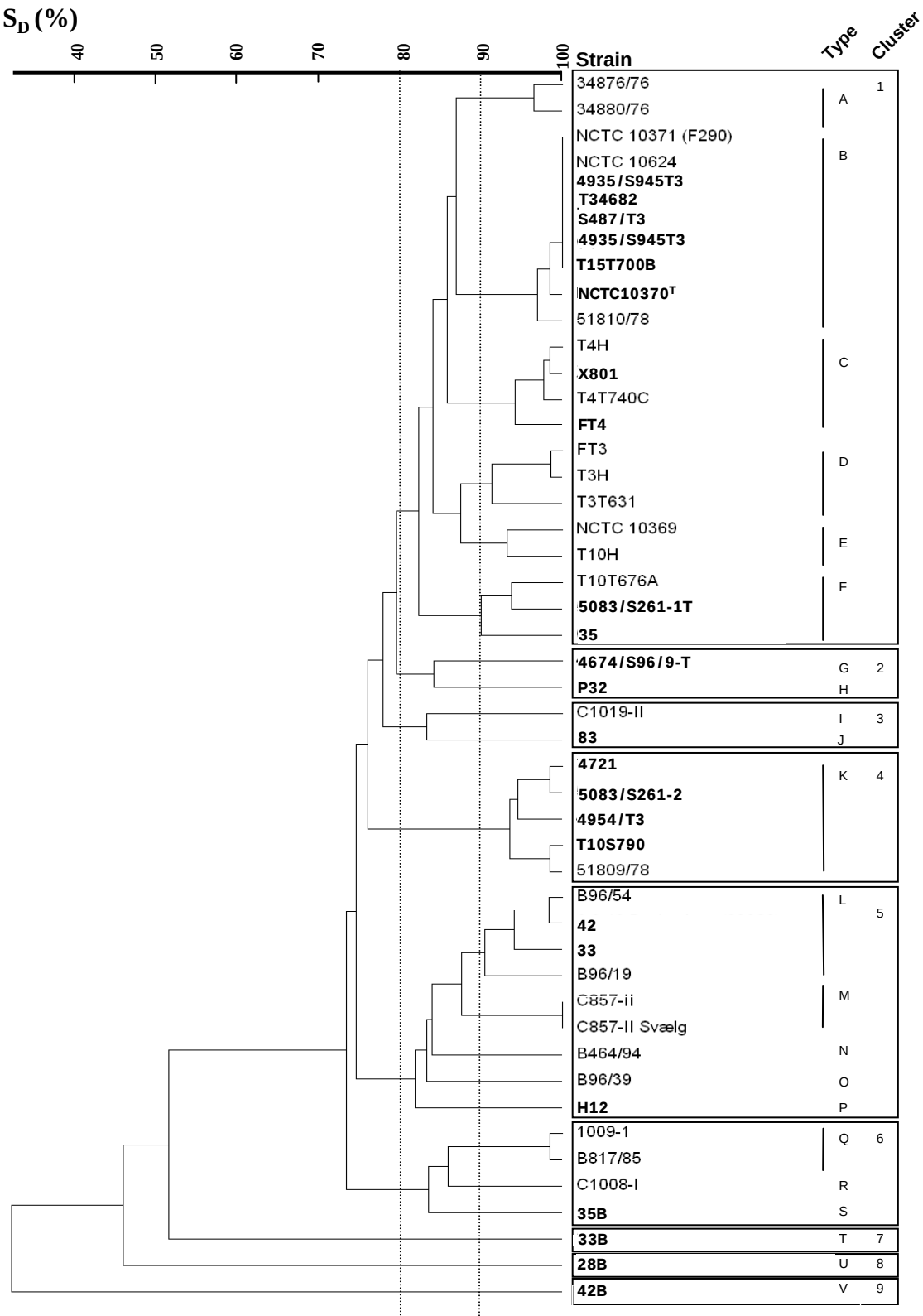
Haemophilus influenzae, ATCC33391T, M35019
[Actinobacillus] actinomycetemcomitans, ATCC33384T, M75039
Pasteurella multocida subsp. multocida, NCTC10322T, AF294410
Nicoletella semolina, CCUG43639T, AY508816
Bibersteinia trehalosi, NCTC10370T, AY362927; NCTC10624, M75063
Bibersteinia trehalosi, NCTC11550, U57073
Bibersteinia trehalosi, NCTC10641, U57074
Bibersteinia trehalosi, B464_94, DQ361040
Bibersteinia trehalosi, NCTC10626, U57075
Mannheimia haemolytica, NCTC9380T, AF060699
Actinobacillus lignieresii, NCTC4189T, M75068
Lonepinella koalarum, ACM3666T,Y17189
Phocoenobacter uteri, NCTC12872T, X89379
Histophilus somni, HS8025T, AF549387
Volucribacter psittacicida, Gerl.236/81T, AY216868
Gallibacterium anatis, ATCC43329T, AF228001
Avibacterium gallinarum, NCTC11188T, AY362921
0.01 Evolutionary distance


S_D (%)
40
50
60
70
80
90
100
Strain
Type
Cluster
34876/76
34880/76
NCTC 10371 (F290)
NCTC 10624
4935/S945T3
T34682
S487/T3
4935/S945T3
T15T700B
NCTC10370T
51810/78
T4H
X801
T4T740C
FT4
FT3
T3H
T3T631
NCTC 10369
T10H
T10T676A
5083/S261-1T
35
4674/S96/9-T
P32
C1019-II
83
4721
5083/S261-2
4954/T3
T10S790
51809/78
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B96/19
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C857-II Svælg
B464/94
B96/39
H12
1009-1
B817/85
C1008-I
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33B
28B
42B
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