Reclassification of equine isolates previously reported as *Actinobacillus equuli*, variants of *A. equuli*, *Actinobacillus suis* or Bisgaard taxon 11 and proposal of *A. equuli* subsp. *equuli* subsp. nov. and *A. equuli* subsp. *haemolyticus* subsp. nov.

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Members of Bisgaard taxon 11 have been isolated from horses. These bacteria are of importance in the veterinary clinic and also to the medical profession, since they may be isolated from infected wounds of humans bitten by horses. Six strains from different continents were identified as taxon 11, with 16S rRNA similarities between 98.0 and 99.7%. A single isolate that represented the so-called (+)L-arabinose-positive *Actinobacillus equuli* isolated from a diseased foal showed 99.9% 16S rRNA similarity to the type strain of *A. equuli*. DNA–DNA hybridizations showed that (+)L-arabinose-positive strains of *A. equuli* represent *A. equuli sensu stricto*. DNA–DNA hybridizations also showed that *A. equuli* and Bisgaard taxon 11 represent two genotypes. These genotypes differ with respect to disease pattern and epidemiology. For these reasons, two subspecies of *A. equuli* are proposed, *Actinobacillus equuli* subsp. *equuli* subsp. nov. (type strain NCTC 8529^T = ATCC 19392^T) and *Actinobacillus equuli* subsp. *haemolyticus* subsp. nov. (type strain F 154^T = CCUG 19799^T = NCTC 13195^T).

Keywords: *Actinobacillus equuli*, Bisgaard taxon 11, DNA hybridization, 16S rRNA, taxonomy

INTRODUCTION

Contradictory statements were given on the genus *Actinobacillus* in the first edition of *Bergey's Manual of Systematic Bacteriology*. Five species were listed as members of the genus by Phillips (1984), while Mannheim (1984) suggested *Actinobacillus sensu stricto* to include seven species, excluding *Actinobacillus actinomycetemcomitans* included by Phillips. Based upon DNA homology studies, Mutters *et al.* (1989) suggested that the genus should be limited to the following

species: *Actinobacillus lignieresii*, *Actinobacillus equuli*, *Actinobacillus capsulatus*, *Actinobacillus suis*, *Actinobacillus ureae*, *Actinobacillus hominis*, *Actinobacillus pleuropneumoniae* and Bisgaard's taxa 5, 9 and 11.

A 16S rRNA sequence comparison by Dewhirst *et al.* (1993) grouped the true actinobacilli in cluster 4A and confirmed that *Actinobacillus sensu stricto* should be confined to those taxa proposed by Mutters *et al.* (1989) with the exception of *A. capsulatus* and Bisgaard's taxa 5 and 9. In the analysis, Bisgaard's taxa 8 and 9 were contained in cluster 4B, closely related to the genus *Actinobacillus sensu stricto*. Three novel species of *Actinobacillus* from pigs were reported by Møller *et al.* (1996), of which *Actinobacillus minor* was shown to belong to Dewhirst cluster 4B together with Bisgaard's taxa 8 and 9, while *Actinobacillus porcicus* and *Actinobacillus indolicus* branched more deeply. Five additional species of *Actinobacillus*, *Actino-*

A full DNA-binding matrix is available as supplementary material in IJSEM Online (<http://ijs.sgmjournals.org/>).

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *A. equuli* and Bisgaard taxon 11 strains determined in this study are AF247713–AF247716, AF247718 and AF247720–AF247721, as shown in Fig. 1.

bacillus delphinicola (Foster *et al.*, 1996), *Actinobacillus rossii* (Sneath & Stevens, 1990), *Actinobacillus scotiae* (Foster *et al.*, 1998), *Actinobacillus seminis* (Sneath & Stevens, 1990) and *Actinobacillus succinogenes* (Guetler *et al.*, 1999), have been reported, although 16S rRNA sequence comparison showed that they should be classified outside the genus *Actinobacillus sensu stricto* as defined by DNA–DNA hybridization.

Taxon 9 has recently been split into the two taxa, *Actinobacillus arthritidis* and *Actinobacillus genomospecies 2*, while strains of *A. lignieresii* isolated from horses have been proposed as *Actinobacillus genomospecies 1* (Christensen *et al.*, 2002). In subsequent discussions in the present paper, genuine actinobacilli are defined according to Mutters *et al.* (1989) to include *A. arthritidis*, *A. equuli*, *A. lignieresii*, *A. pleuropneumoniae*, *A. suis*, *A. ureae*, Bisgaard taxa 8 and 11 and *Actinobacillus genomospecies 1* and 2, excluding *A. capsulatus*, *A. minor* and Bisgaard taxon 5.

Among the true species of *Actinobacillus*, *A. equuli*, *A. arthritidis*, Bisgaard taxon 11 and *Actinobacillus genomospecies 1* and 2 have so far been reported from horses (Bisgaard, 1993). These organisms may cause disease in horses and may be found in infected wounds of humans bitten by horses (Ashhurst-Smith *et al.*, 1998; Dibb *et al.*, 1981; Peel *et al.*, 1991). Recent studies, however, have emphasized the need for careful examination of equine isolates previously identified as *A. equuli* (Blackall *et al.*, 1997). These studies also showed that some isolates could not be assigned to any currently recognized species or taxa.

The diversity of *A. equuli* was reported by Bisgaard (1993), who classified trehalose-negative strains as taxon 9. Since production of acid from (+)L-arabinose was shown to be of taxonomic significance in the reclassification of [*Haemophilus*] *avium* into three novel species (Mutters *et al.*, 1985), Bisgaard (1993) also drew attention to the existence of (+)L-arabinose-positive strains of *A. equuli*, the taxonomy of which remains to be investigated. The diversity of *A. equuli* was subsequently confirmed by ribotyping (Blackall *et al.*, 1998; Sternberg & Brändström, 1999). (+)L-Arabinose-positive strains clustered separately, joining the *A. equuli* cluster at approximately 50% similarity, while taxon 9 branched more deeply (Blackall *et al.*, 1998).

Equine strains of bacteria described as *A. suis* or haemolytic variants of *A. equuli* were classified as taxon 11 with four biovars, of which only three biovars have been kept (Bisgaard *et al.*, 1984). Further investigations have confirmed the existence of taxon 11, since haemolytic *A. lignieresii*-like strains isolated from horses had a PAGE profile different from that of the bovine reference strain (Jang *et al.*, 1987; Samitz & Biberstein, 1991). Lower airway inflammation of horses was significantly associated with taxon 11-like bacteria and *A. lignieresii* (Ward *et al.*, 1998).

The uncertain taxonomy of the above-mentioned equine taxa represents a diagnostic problem and prevents progress on studies of the virulence and epidemiology of these organisms. In the present investigation, we decided to undertake a study involving 16S rRNA sequencing of selected strains representing equine taxa of uncertain taxonomy and to compare the sequences obtained with those published previously, in order to clarify the phylogeny of these organisms further. In addition, DNA–DNA hybridizations were carried out to classify organisms previously reported as equine *A. suis*, haemolytic variants of *A. equuli* or Bisgaard taxon 11.

METHODS

Bacterial strains and phenotypic characterization. Selection of strains (Table 1) was based on previous investigations (Bisgaard *et al.*, 1984; Blackall *et al.*, 1997, 1998; Carman & Hodges, 1982; Mráz *et al.*, 1968; Peel *et al.*, 1991) and strains retained in our laboratory. Phenotypic characterization was performed as reported previously (Bisgaard *et al.*, 1991).

Sequencing of 16S rRNA genes. Bacteria were cultured overnight in brain/heart infusion broth (Difco) at 37 °C. Cells from 1 ml culture were harvested by centrifugation and DNA extraction was performed according to Leisner *et al.* (1999). PCR amplification was performed as described by Vogel *et al.* (1997). Oligonucleotides for both PCR amplification and sequencing were synthesized according to sequences and 16S rRNA positions given by Dewhirst *et al.* (1989) and Paster & Dewhirst (1988). PCR-amplified fragments were purified and cycle-sequenced as reported by Christensen *et al.* (2002).

Analysis of sequence data. Searches for 16S rRNA sequences were performed by FASTA and BLAST in GenBank by use of the Wisconsin Sequence Analysis package (Genetics Computer Group, Madison, WI, USA). Sequences were aligned manually to the *Escherichia coli rrsB* sequence and to the consensus sequence (Lane, 1991). The region corresponding to *E. coli* positions 28–1480 could be aligned and 1378 nucleotides (87 distinct data patterns analysed) were left after removal of ambiguous positions. Maximum-likelihood analysis was performed by fastDNAmL including bootstrap analysis (Felsenstein, 1981; Olsen *et al.*, 1994) run on an HP9000/819 computer (UNI-C, Lyngby, Denmark). Parsimony and neighbour-joining analysis were performed by PHYLIP (Felsenstein, 1995).

DNA–DNA hybridization. DNA similarities were determined according to the micro-well method (Christensen *et al.*, 2000) with the modifications that only DNA of high concentration (> 150 µg ml⁻¹) was used and coated plates were hybridized within a few days. To eliminate 'edge' effects during binding of DNA to wells and hybridization, these steps were carried out in a small, closed, humidified chamber in addition to sealing wells with tape, as reported previously (Christensen *et al.*, 2000). Hybridizations were repeated if the difference between DNA binding values obtained by reversal of strains for binding and hybridization was more than 20%. A dendrogram was generated by Fitch–Margoliash analysis with the 'contemporary tips' option (KITSCH) as provided in the PHYLIP package.

Table 1. Strains of equine taxa investigated

Strain	Taxon	Source	Country of isolation	Reference(s)
NCTC 4189 ^T	<i>A. lignieresii</i>	Cow	UK	
T 355/87	Bisgaard 11 bv. 1	Horse bite	Australia	Peel <i>et al.</i> (1991)
F 92	Bisgaard 11 bv. 1	Horse	Denmark	Bisgaard <i>et al.</i> (1984)
Ac 7	Bisgaard 11 bv. 1	Horse	Zimbabwe	Mohan <i>et al.</i> (1997)
F 154 ^T (= Carman 8-11272 ^T = CCUG 19799 ^T = NCTC 13195 ^T)	Bisgaard 11 bv. 2	Horse	New Zealand	Carman & Hodges (1982), Bisgaard <i>et al.</i> (1984)
F 490 (= CCM 5587)	Bisgaard 11 bv. 2	Horse	Czech Republic	Mráz <i>et al.</i> (1968), Bisgaard <i>et al.</i> (1984)
F 119	Bisgaard 11 bv. 3	Horse	Denmark	Bisgaard <i>et al.</i> (1984)
NCTC 8529 ^T (= F 102 ^T = ATCC 19392 ^T)	<i>A. equuli</i>	Horse	UK	
I 5445/4	<i>A. equuli</i>	Horse	Australia	Blackall <i>et al.</i> (1997, 1998)

bv., Biovar.

RESULTS AND DISCUSSION

Phenotypic characterization

The phenotypic characterization of strains of Bisgaard taxon 11 and (+)L-arabinose-positive *A. equuli* summarizes results published previously (Bisgaard *et al.*, 1984; Blackall *et al.*, 1997; Christensen *et al.*, 2002). All equine strains of *A. equuli*, (+)L-arabinose-positive variants of *A. equuli*, Bisgaard taxon 11, *A. arthritidis*, *Actinobacillus* genomospecies 2 and the type strain of *A. lignieresii* were positive in Hugh–Leifson medium with (+)D-glucose (fermentative), porphyrin test, nitrate reduction, urease, alanine aminopeptidase, phosphatase and ONPG and produced acid from (–)D-ribose, (–)D-fructose, (+)D-glucose, (+)D-mannose, (+)D-xylose, maltose, dextrin and sucrose. The bacteria were Gram-negative. Negative results were obtained in the following tests: motility at 22 and 37 °C, symbiotic growth, Simmons citrate, mucate-acid, malonate-base, H₂S/tri-sugar iron (TSI), growth in the presence of KCN, methyl red and Voges–Proskauer tests at 37 °C, production of gas from nitrate, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, phenylalanine deaminase, indole production, gelatinase, hydrolysis of Tweens 20 and 80, pigment formation, α-fucosidase (*o*-nitrophenyl α-L-fucopyranoside; ONPF), α-glucuronidase (*p*-nitrophenyl β-D-glucopyranosiduronic acid; PGUA), α-mannosidase and formation of acid from *m*-erythritol, adonitol, (+)D-arabitol, xylitol, (–)L-xylose, dulcitol, *m*-inositol, (+)D-fucose, (+)L-rhamnose, (–)L-sorbose, (+)D-melezitose, (+)D-glycogen, inulin, (+)D-turanose and β-*N*-CH₃-glucosamid. Formation of gas from (+)D-glucose was not observed.

Results for the 25 characters for which the strains varied are given in Table 2. Only differences in oxidase and production of acid from (+)L-arabinose separate strain I 5445/4 from the type strain of *A. equuli*. This group can be separated from the type strain of *A. lignieresii* by differences in production of acid from (–)L-fucose, (+)D-melibiose, trehalose and raffinose,

α-galactosidase, *o*-nitrophenyl β-D-xylanopyranoside (ONPX) and *p*-nitrophenyl α-D-glucopyranoside (PNPG). The presence of β-haemolysis separates taxon 11 from the other equine groups. With the exception of F 119 (biovar 3), this group produces acid from glycosides. From a phenotypic point of view, biovar 3 of taxon 11 appears as a (–)D-mannitol-negative strain of *A. equuli*. Within biovars 1 and 2 of taxon 11, variations are observed in formation of acid from (+)L-arabinose, (–)D-mannitol, (–)D-sorbitol and aesculin and in the PNPG test. Differences in (–)D-mannitol and PNPG separate biovars 1 and 2 of taxon 11.

16S rRNA sequence comparison

16S rRNA sequences were obtained for seven strains of equine actinobacilli. The sequences were identical for strains CCUG 19799^T (GenBank accession no. M75067) and F 154^T (GenBank accession no. AF247716; this investigation). These strains represent identical depositions under different strain designations. It should be noted that strain CCUG 19799^T belongs to taxon 11. However, this strain was misnamed *A. capsulatus* when the sequence was deposited under accession number M75067. The type strain of [*Haemophilus*] *parahaemolyticus* showed only 0.1% difference in 16S rRNA sequence from *A. equuli*, but was excluded from the analysis, since it has recently been shown not to be the sequence of the strain reported (Hedegaard *et al.*, 2001).

The phylogenetic tree shown in Fig. 1 is based on the seven sequences determined in the present study and published sequences of genuine actinobacilli, with *A. minor* included as an outgroup. A phylogenetic analysis performed by inclusion of representative taxa of the remaining part of the family *Pasteurellaceae* (Pohl 1981) showed that the actinobacilli included in Fig. 1 formed a monophyletic unit (data not shown). The phylogeny was poorly resolved and only the group with *A. lignieresii*, the type species of the genus, *A.*

Table 2. Phenotypic characters for which the selected equine actinobacilli investigated were variable

Strains are identified as: 1, NCTC 8529^T; 2, I 5445/4; 3, F 92; 4, T 355/87; 5, Ac 7; 6, F 154^T; 7, F 490; 8, F 119; 9, *Actinobacillus* genomospecies 2 strain F 141; 10, *A. arthritidis* CCUG 24862^T; 11, *A. lignieresii* NCTC 4189^T. Characters are scored as: +, positive within 1–2 days; (+), delayed positive (≥ 3 days); –, negative after 14 days; w, weakly positive. NPG, *p*-Nitrophenyl β -D-glucopyranoside; ONPX, *o*-nitrophenyl β -D-xylanopyranoside; PNPG, *p*-nitrophenyl α -D-glucopyranoside; TMPD, tetramethyl *p*-phenylenediamine.

Character	<i>A. equuli</i> subsp. <i>equuli</i>		<i>A. equuli</i> subsp. <i>haemolyticus</i>								
			Biovar 1			Biovar 2		Biovar 3			
	1	2	3	4	5	6	7	8	9	10	11
Catalase	+	w	+	w	+	+	w	w	+	+	+
Oxidase, TMPD	+	–	+	+	+	+	+	+	+	+	+
β -Haemolysis	–	–	w	w	w	w	+	w	–	–	–
Growth on MacConkey agar	–	–	–	–	–	–	–	w	w	–	–
Production of acid from:											
Glycerol	(+)	(+)	(+)	+	+	(+)	(+)	(+)	(+)	(+)	w
(+)-L-Arabinose	–	+	–	–	–	–	+	–	–	–	–
(–)-D-Arabinose	–	–	–	–	–	–	–	–	(+)	–	–
(–)-D-Mannitol	+	+	–	–	–	+	+	–	+	+	+
(–)-D-Sorbitol	–	–	–	–	–	+	–	–	–	+	–
(–)-L-Fucose	–	–	–	–	–	–	–	–	+	–	+
(+)-D-Galactose	+	+	+	+	+	+	+	+	+	(+)	+
Cellobiose	–	–	+	+	+	+	+	–	–	–	–
β -Glucosidase (NPG)	–	–	+	+	+	+	+	–	–	–	–
Production of acid from:											
Lactose	+	+	+	+	+	+	+	+	+	+	w
(+)-D-Melibiose	+	+	+	+	+	+	+	+	(+)	(+)	–
Trehalose	+	+	+	+	+	+	+	+	–	–	–
Raffinose	+	+	+	+	+	+	+	+	(+)	+	–
Aesculin	–	–	+	+	–	–	–	–	–	–	–
Amygdalin	–	–	(+)	(+)	(+)	(+)	(+)	–	–	–	–
Arbutin	–	–	+	+	+	+	+	–	–	–	–
Gentiobiose	–	–	+	+	+	+	+	–	–	–	–
Salicin	–	–	+	+	+	+	+	–	–	–	–
α -Galactosidase	+	+	+	+	+	+	+	+	+	+	–
α -Glucosidase (PNPG)	+	+	+	+	+	–	–	+	–	–	–
β -Xylosidase (ONPX)	+	+	–	w	+	+	+	+	–	–	–

pleuropneumoniae and, distantly related to these species, taxon 8 was consistent with all three phylogenetic methods. The lowest similarity within this group was 97%, between the strain of Bisgaard taxon 8 and the type strain of *A. pleuropneumoniae*. A second group was formed by the type strain of *A. ureae*, the type strain of *A. arthritidis*, *Actinobacillus* genomospecies 2 and one strain of taxon 11, but was only supported by maximum-likelihood analysis. The lowest similarity within this group was 98%, observed between *A. ureae* and *Actinobacillus* genomospecies 2. The remaining strains, representing Bisgaard taxon 11, *A. equuli*, *A. suis* and *A. hominis*, did not form monophyletic groups but instead gave a comb-like topology without consistency compared with results obtained by neighbour-joining and parsimony analysis. A similar topology was recently reported for some taxa of *Mannheimia*

(Blackall *et al.*, 2001). The reason for the poorly resolved phylogeny is probably that there is too little sequence variation between the taxa, with the result that random variation gives noise in relation to the phylogenetic signal, since the highest variation was only 1.3%, observed between strains F 92 and F 119 and the type strain of *A. hominis*. A high similarity of 99.9% was found between strain I 5445/4, reported as (+)-L-arabinose-positive *A. equuli*, and the type strain of *A. equuli*. The lowest similarity between the strains of Bisgaard taxon 11 was between strain Ac 7 and the five other strains, with 98–99% similarity, compared with 98.7–99.7% similarity between these five strains.

The lowest similarity between *A. lignieresii*, *A. hominis*, *A. arthritidis*, *A. pleuropneumoniae*, *A. equuli*, *A. hominis*, *A. suis*, Bisgaard taxa 8 and 11 and *Actino-*

Fig. 1. Phylogenetic relationships between selected taxa associated with horses and published sequences of genuine actinobacilli based on maximum-likelihood analysis of 16S rRNA sequences. The significance for specific nodes obtained in bootstrap analysis ($n = 100$) is indicated for values greater than 50%. Nodes supported in phylogenetic trees obtained by neighbour-joining and parsimony methods are respectively indicated by + and *. Strains sequenced in the present study are in bold. Strain and GenBank accession numbers are given in brackets.

bacillus genomospecies 1 and 2, considered as *Actinobacillus sensu stricto* in the present study, was 95.6%, found between the strain of Bisgaard taxon 8 and strain T355/87 of taxon 11. However, this range covered a continuum of closely related taxa. Comparison of 16S rRNA sequences showed that *A. actinomycetemcomitans*, *A. capsulatus*, *A. delphinicola*, *A. indolicus*, *A. minor*, *Actinobacillus muris*, *A. porcicus*, *A. scotiae*, *A. seminis* and *A. succinogenes* were distantly related to *A. lignieresii*, *A. pleuropneumoniae*, *A. equuli*, *A. hominis*, *A. suis*, *A. arthritis*, Bisgaard taxa 8 and 11 and *Actinobacillus* genomospecies 1 and 2. The highest similarities observed between these two groups of taxa were 95.4 (*A. suis*) to 96.6% (T355/87) for *A. minor*, followed by 94.0 (Ac 7, F 119, F 141) to 94.8% (*A. hominis*) for *A. delphinicola*.

In conclusion, equine actinobacilli tentatively classified as taxon 11 and within the species *A. equuli* all belong to the genuine actinobacilli according to the definition

Fig. 2. Phylogenetic relationships between selected taxa associated with horses and genuine actinobacilli based on pairwise DNA-DNA hybridization experiments. The phylogram was constructed by the Fitch-Margoliash program with the 'contemporary tips' option on the basis of the complete DNA-binding matrix (available as supplementary material in IJSEM Online; <http://ijs.sgmjournals.org/>).

given above. It has to be emphasized that further investigation is necessary to obtain a strict definition of *Actinobacillus*, especially with respect to the classification of *A. minor*.

DNA-DNA hybridization

DNA binding of 98% was observed between (+)L-arabinose-positive and -negative strains of *A. equuli*, underlining that differences in production of acid from (+)L-arabinose are taxonomically insignificant within this group. DNA-binding values between 78 and 100% were observed for strains belonging to taxon 11. The lowest DNA-binding value was observed between isolates from the Czech Republic and the Zimbabwe isolates, respectively representing biovars 2 and 1. 16S rRNA analysis showed one strain of taxon 11 to be closely related to the type strain of *A. ureae*. This relationship was investigated further by DNA-DNA hybridization. However, only 45% DNA binding was found between these strains. A full DNA-binding matrix is available as supplementary material in IJSEM Online (<http://ijs.sgmjournals.org/>).

The result of the phylogenetic analysis of DNA binding is shown in Fig. 2. Selected strains of all biovars of taxon 11 formed a monophyletic group at or above 78% DNA binding. The type strain of *A. equuli* and strain I 5445/4 were separated from strains of taxon 11 at 88% DNA binding. The type strain of *A. suis* branched more deeply (43–77% DNA binding), with less than 77% DNA binding to the equine strains

investigated, and so did *Actinobacillus* genomospecies 2 (Bisgaard taxon 9).

16S rRNA sequence comparison showed that the equine strains formed only weakly defined groups. One strain was found closely related to *A. ureae*, *A. arthritidis* and *Actinobacillus* genomospecies 2, while other strains formed a loose, comb-like structure (Fig. 1). The groups recognized by phylogenetic analysis of 16S rRNA sequences were not supported by DNA–DNA hybridization results, as this analysis grouped all strains of taxon 11 together (Fig. 2). This discrepancy is related to inappropriate resolution of 16S rRNA sequence comparison at the species level for genuine actinobacilli, as demonstrated by the high 16S rRNA similarities observed between these strains. Except for *A. pleuropneumoniae*, 16S rRNA sequences have only been available for type strains of species of genuine actinobacilli. Published sequences of *A. pleuropneumoniae* show more than 99.7% 16S rRNA sequence similarity. Similarity of 98% between strains of *A. equuli* and taxon 11 resulted in polyphyletic groupings of this taxon and showed that phylogenetic analysis based on 16S rRNA sequence analysis is of little value for resolving species within the genuine actinobacilli. Since the aim of the present study was the resolution of equine actinobacilli, hybridizations were not performed to all actinobacilli; however, because 16S rRNA analysis showed that a single strain of taxon 11 was closely related to the type strain of *A. ureae*, this relationship was investigated further by DNA–DNA hybridization and found to be only 45%. Previous investigations have shown 75% DNA binding between *A. ureae* and *A. suis* and, at most, 66% to *A. equuli* (Escande *et al.*, 1984; Mutters *et al.*, 1986). A solution to the problem of the failure of 16S rRNA phylogenetic analysis to resolve individual species might include sequences of other genes and the use of this information for phylogenetic analysis.

Low variation has also been reported for some species of *Vibrio* and lactobacilli (Wiik *et al.*, 1995; Vandamme *et al.*, 1996), and no definitive limits for the separation of species on the basis of 16S rRNA sequence variation can be drawn (Stackebrandt & Goebel, 1994; Vandamme *et al.*, 1996). A threshold of 70% DNA binding has been used to separate species of bacteria; however, thresholds may vary for different bacterial groups (Vandamme *et al.*, 1996) and for different DNA hybridization methods (Grimont *et al.*, 1980). For *Mannheimia*, a limit of 85% DNA binding was found (Angen *et al.*, 1999; Mutters *et al.*, 1989); however, it seems likely that a threshold for actinobacilli is slightly lower. Using 80% DNA binding as a threshold, *A. equuli* (including strains of taxon 11) can be separated from *A. hominis* (73%) and *A. suis* (58–77% DNA binding). The binding between the two strains classified as *A. equuli* was 98%; however, strains classified as taxon 11 showed values between 78 and 100%, while DNA-binding values between strains classified as *A. equuli* and taxon 11 varied between 78 and 88%. The limit for separation of the two groups of

A. equuli will then be 78% DNA binding, corresponding to the level used for separation between species.

Previously published data on selected strains showed 83% DNA binding between *A. equuli* strain NCTC 8529^T and strain F 154^T of taxon 11 biovar 2 (Bisgaard *et al.*, 1984). Using the recently developed micro-well method for DNA–DNA hybridization (Christensen *et al.*, 2000), 84% DNA binding was found between the same strains. DNA binding of 62% was observed between biovar 2 of taxon 11 (F 154^T) and *A. suis* (CCM 5586^T) by the spectrophotometric method (Bisgaard *et al.*, 1984). The same strains showed 66% DNA binding by the micro-well method in the present study. Using the S1 nuclease–trichloroacetic acid method, Escande *et al.* (1984) found 80% DNA binding between strains NCTC 8529^T of *A. equuli* and CCM 5587 (= F 490) of Bisgaard taxon 11. According to the micro-well method, 78% DNA binding was observed between the same pair of strains. In conclusion, the results obtained by the micro-well DNA–DNA hybridization method were in agreement with results published previously and based on other methods.

Although Bisgaard taxon 11 did not form a monophyletic unit by rRNA sequence comparison, DNA–DNA hybridizations showed that these organisms form a subgroup of *A. equuli*. Equine strains previously classified as taxon 11, *A. suis* or haemolytic variants of *A. equuli* (Bisgaard *et al.*, 1984) seem to represent a group of opportunistic pathogens that have been associated with different disease conditions in foals and horses, including stillborn foetuses, metritis, mastitis, septicaemia, arthritis, endocarditis, meningitis, respiratory infections and inflamed wounds (Bisgaard, 1993). *A. equuli*, however, is an opportunistic pathogen of both horses and pigs, found in association with various clinical conditions, especially in young animals. The most prominent and usual syndrome associated with this organism in foals is an acute, highly fatal septicaemia of newborn foals (sleepy foal disease) that may become chronic (joint ill) with lesions of purulent nephritis and arthritis (Bisgaard, 1993).

Although DNA-binding data allowed the classification of *Pasteurella multocida* as three distinct species (Mutters *et al.*, 1985), the authors resisted doing so from a clinical viewpoint and instead proposed the division of *P. multocida* into three subspecies, which was supposed to be more useful for epidemiological purposes. For the same reasons, it is suggested that *A. equuli* (Van Straten) Haupt 1934 be divided into two subspecies, *Actinobacillus equuli* subsp. *equuli* subsp. nov., representing non-haemolytic (CAMP-negative) strains and associated with sleepy foal disease/joint ill and having a reservoir in horses and pigs, and *Actinobacillus equuli* subsp. *haemolyticus* subsp. nov., representing haemolytic or weakly haemolytic but CAMP-positive strains and having a reservoir so far involving horses only. The CAMP reaction is a co-haemolytic reaction on a blood-agar plate previously

affected by staphylococcal β -haemolysin (Christie *et al.*, 1944).

Description of *Actinobacillus equuli* subsp. *equuli* subsp. nov.

Actinobacillus equuli subsp. *equuli* (e.qu.u'li. L. gen. n. *equuli* of a foal).

Under Rule 46 of the Bacteriological Code, the creation of *Actinobacillus equuli* subsp. *haemolyticus* automatically leads to the creation of a second subspecies, *Actinobacillus equuli* subsp. *equuli* subsp. nov. In addition to the phenotypic characters consistent for all members of the genus, reactions common to both subspecies of *A. equuli* are positive reactions for catalase, glycerol, (+)D-xylose, (+)D-galactose, lactose, maltose, (+)D-melibiose, trehalose, raffinose, dextrin and α -galactosidase. The G+C content of both subspecies ranges from 40.0 to 43.4 mol %, with genome sizes of 1.7–1.8 GDa (Bisgaard *et al.*, 1984; Boháček & Mráz, 1967; Piechulla *et al.*, 1985). The main characters used for differentiation of the two subspecies are haemolysis and the CAMP reaction; *A. equuli* subsp. *equuli* subsp. nov. is non-haemolytic and CAMP-negative. Considerable amounts of extracellular slime are often associated with this subspecies. In this study, strain I 5445/4 was allocated to *A. equuli* subsp. *equuli*. Isolated from horses and pigs. Strain NCTC 8529^T (= ATCC 19392^T) is the type strain.

Description of *Actinobacillus equuli* subsp. *haemolyticus* subsp. nov.

Actinobacillus equuli subsp. *haemolyticus* (hae.mo.ly'ti.cus. Gr. n. *haima* blood; Gr. adj. *lytikos* loosening, dissolving; N.L. adj. *haemolyticus* blood-dissolving).

Haemolytic and CAMP-positive. Reactions for the separation of *A. equuli* subsp. *haemolyticus* into biovars are shown in Table 2. Strains Ac 7, F 92, F 119, F 490 and T 355/87, tentatively classified as Bisgaard taxon 11, are allocated to *A. equuli* subsp. *haemolyticus*. Strains have so far been isolated from horses only. Strain F 154^T (= CCUG 19799^T = NCTC 13195^T) is the type strain.

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REFERENCES

- Angen, Ø., Muters, 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 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.
- Ashhurst-Smith, C., Norton, R., Thoreau, W. & Peel, M. M. (1998). *Actinobacillus equuli* septicemia: an unusual zoonotic infection. *J Clin Microbiol* **36**, 2789–2790.
- Bisgaard, M. (1993). Ecology and significance of *Pasteurellaceae* in animals. *Zentbl Bakteriol* **279**, 7–26.
- Bisgaard, M., Piechulla, K., Ying, Y.-T., Frederiksen, W. & Mannheim, W. (1984). Prevalence of organisms described as *Actinobacillus suis* or haemolytic *Actinobacillus equuli* in the oral cavity of horses. Comparative investigations of strains obtained and porcine strains of *A. suis sensu stricto*. *Acta Pathol Microbiol Immunol Scand Sect B* **92**, 291–298.
- Bisgaard, M., Houghton, S. B., Muters, 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., Bisgaard, M. & McKenzie, R. A. (1997). Characterisation of Australian isolates of *Actinobacillus capsulatus*, *Actinobacillus equuli*, *Pasteurella caballi* and Bisgaard taxa 9 and 11. *Aust Vet J* **75**, 52–55.
- Blackall, P. J., Christensen, J. P. & Bisgaard, M. (1998). Diversity among isolates of *Actinobacillus equuli* and related organisms as revealed by ribotyping. *Aust Vet J* **76**, 423–425.
- Blackall, P. J., Angen, Ø., Fegan, N., Blackall, L., Muters, R. & Bisgaard, M. (2001). Characterisation of a novel *Mannheimia* sp. from Australian feedlot cattle. *Aust Vet J* **79**, 634–639.
- Boháček, J. & Mráz, O. (1967). Basengehalt der Desoxyribonukleinsäure bei den Arten *Pasteurella haemolytica*, *Actinobacillus lignieresii* und *Actinobacillus equuli*. *Zentbl Bakteriol Parasitenkd Infektionskr Hyg Abt 1 Orig* **202**, 468–478.
- Carman, M. G. & Hodges, R. T. (1982). *Actinobacillus suis* infection of horses. *N Z Vet J* **30**, 82–84.
- Christensen, H., Angen, Ø., Muters, R., Olsen, J. E. & Bisgaard, M. (2000). DNA–DNA hybridization determined in micro-wells using covalent attachment of DNA. *Int J Syst Evol Microbiol* **50**, 1095–1102.
- Christensen, H., Bisgaard, M., Angen, Ø. & Olsen, J. E. (2002). Final classification of Bisgaard taxon 9 as *Actinobacillus arthritidis* sp. nov. and recognition of a novel genomospecies for equine strains of *Actinobacillus lignieresii*. *Int J Syst Evol Microbiol* **52**, 1239–1246.
- 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* **2**, 197.
- 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.
- Dewhirst, F. E., Paster, B. J., Olsen, I. & Fraser, G. J. (1993). Phylogeny of the *Pasteurellaceae* as determined by comparison of 16S ribosomal ribonucleic acid sequences. *Zentbl Bakteriol* **279**, 35–44.
- Dibb, W. L., Digranes, A. & Tønjum, S. (1981). *Actinobacillus lignieresii* infection after a horse bite. *Br Med J* **283**, 583–584.
- Escande, F., Grimont, F., Grimont, P. A. D. & Bercovier, H. (1984). Deoxyribonucleic acid relatedness among strains of *Actinobacillus* spp. and *Pasteurella ureae*. *Int J Syst Bacteriol* **34**, 309–315.
- Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *J Mol Evol* **17**, 368–376.
- Felsenstein, J. (1995). PHYLIP (Phylogeny Inference Package) version 3.5c. Seattle: Department of Genetics, University of Washington.
- Foster, G., Ross, H. M., Malnick, H., Willems, A., Garcia, P., Reid, R. J. & Collins, M. D. (1996). *Actinobacillus delphinicola* sp. nov., a new member of the family *Pasteurellaceae* Pohl (1979) 1981 isolated from sea mammals. *Int J Syst Bacteriol* **46**, 648–652.
- Foster, G., Ross, H. M., Patterson, I. A. P., Hutson, R. A. & Collins, M. D. (1998). *Actinobacillus scotiae* sp. nov., a new member of the

- family *Pasteurellaceae* Pohl (1979) 1981 isolated from porpoises (*Phocoena phocoena*). *Int J Syst Bacteriol* **48**, 929–933.
- Grimont, P. A. D., Popoff, M. Y., Grimont, F., Coynault, C. & Lemelin, M. (1980). Reproducibility and correlation study of three deoxyribonucleic acid hybridization procedures. *Curr Microbiol* **4**, 325–330.
- Guettler, M. V., Rumler, D. & Jain, M. K. (1999). *Actinobacillus succinogenes* sp. nov., a novel succinic-acid-producing strain from the bovine rumen. *Int J Syst Bacteriol* **49**, 207–216.
- Hedegaard, J., Okkels, H., Bruun, B., Kilian, M., Mortensen, K. K. & Nørskov-Lauritsen, N. (2001). Phylogeny of the genus *Haemophilus* as determined by comparison of partial *infB* sequences. *Microbiology* **147**, 2599–2609.
- Jang, S. S., Biberstein, E. L. & Hirsch, D. C. (1987). *Actinobacillus suis*-like organisms in horses. *Am J Vet Res* **48**, 1036–1038.
- Lane, D. (1991). 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–147. Edited by E. Stackebrandt & M. Goodfellow. New York: Wiley.
- 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.
- Mannheim, W. (1984). Family III. *Pasteurellaceae* Pohl 1981a, 382^{VP}. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 550–552. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.
- Mohan, K., Muvavarirwa, P. & Pawandiwa, A. (1997). Strains of *Actinobacillus* spp. from diseases of animals and ostriches in Zimbabwe. *Onderstepoort J Vet Res* **64**, 195–199.
- Møller, K., Fussing, V., Grimont, P. A. D., Paster, B. J., Dewhirst, F. E. & Kilian, M. (1996). *Actinobacillus minor* sp. nov., *Actinobacillus porcinus* sp. nov., and *Actinobacillus indolicus* sp. nov., three new V factor-dependent species from the respiratory tract of pigs. *Int J Syst Bacteriol* **46**, 951–956.
- Mráz, O., Zakopal, J. & Matoušek, Z. (1968). The finding of a haemolytic variant of *Actinobacillus equuli* as the cause of septicaemia in a herd of breeding mares. *Acta Univ Agric Brno* **37**, 263–275.
- Mutters, R., Piechulla, K., Hinz, K.-H. & Mannheim, W. (1985). *Pasteurella avium* (Hinz and Kunjara 1977) comb. nov. and *Pasteurella volantium* sp. nov. *Int J Syst Bacteriol* **35**, 5–9.
- Mutters, R., Pohl, S. & Mannheim, W. (1986). Transfer of *Pasteurella ureae* Jones 1962 to the genus *Actinobacillus* Brumpt 1910: *Actinobacillus ureae* comb. nov. *Int J Syst Bacteriol* **36**, 343–344.
- Mutters, R., Mannheim, W. & Bisgaard, M. (1989). Taxonomy of the group. In *Pasteurella and Pasteurellosis*, pp. 3–34. Edited by C. Adlam & J. M. Rutter. London: Academic Press.
- Olsen, G. J., Matsuda, H., Hagström, R. & Overbeek, R. (1994). fastDNAmL: a tool for construction of phylogenetic trees of DNA sequences using maximum likelihood. *Comput Appl Biosci* **10**, 41–48.
- Paster, B. J. & Dewhirst, F. E. (1988). Phylogeny of campylobacters, wolliellas, *Bacteroides gracilis*, and *Bacteroides ureolyticus* by 16S ribosomal ribonucleic acid sequencing. *Int J Syst Bacteriol* **38**, 56–62.
- Peel, M. M., Hornidge, K. A., Luppino, M., Stacpoole, A. M. & Weaver, R. E. (1991). *Actinobacillus* spp. and related bacteria in infected wounds of humans bitten by horses and sheep. *J Clin Microbiol* **29**, 2535–2538.
- Phillips, J. E. (1984). Genus III. *Actinobacillus* Brumpt 1910, 849^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 570–575. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.
- Piechulla, K., Bisgaard, M., Gerlach, H. & Mannheim, W. (1985). Taxonomy of some recently described avian *Pasteurella/Actinobacillus*-like organisms as indicated by deoxyribonucleic acid relatedness. *Avian Pathol* **14**, 281–311.
- Samitz, E. M. & Biberstein, E. L. (1991). *Actinobacillus suis*-like organisms and evidence of hemolytic strains of *Actinobacillus lignieresii* in horses. *Am J Vet Res* **52**, 1245–1251.
- Sneath, P. H. A. & 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.
- Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA–DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.
- Sternberg, S. & Brändström, B. (1999). Biochemical fingerprinting and ribotyping of isolates of *Actinobacillus equuli* from healthy and diseased horses. *Vet Microbiol* **66**, 53–65.
- Vandamme, P., Pot, B., Gillis, M., De Vos, P., Kersters, K. & Swings, J. (1996). Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiol Rev* **60**, 407–438.
- Vogel, B. F., Jørgensen, 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, C. L., Wood, J. L. N., Houghton, S. B., Mumford, J. A. & Chanter, N. (1998). *Actinobacillus* and *Pasteurella* species isolated from horses with lower airway disease. *Vet Rec* **143**, 277–279.
- Wiik, R., Stackebrandt, E., Valle, O., Daae, F. L., Rødseth, O. M. & Andersen, K. (1995). Classification of fish-pathogenic vibrios based on comparative 16S rRNA analysis. *Int J Syst Bacteriol* **45**, 421–428.