Delineation of the genus *Actinobacillus* by comparison of partial *infB* sequences

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A 426 bp fragment of *infB*, a housekeeping gene that encodes translation initiation factor 2, was sequenced from 59 clinical isolates and type strains of *Actinobacillus* species and sequences were compared. Partial sequences of 16S rRNA genes were also obtained. By comparing *infB* sequences, *Actinobacillus pleuropneumoniae*, *Actinobacillus equuli*, *Actinobacillus suis*, *Actinobacillus ureae*, *Actinobacillus arthritidis*, *Actinobacillus hominis* and two unnamed genomospecies showed more than 85 % similarity to the type strain of the type species of the genus, *Actinobacillus lignieresii*. The taxonomic position of *Actinobacillus capsulatus* was unresolved; this species is more remotely related to *A. lignieresii*. The two species *A. lignieresii* and *A. pleuropneumoniae* could not be clearly separated by *infB* sequence analysis. The phylogeny of the genus *Actinobacillus* based on *infB* analysis was essentially congruent with relationships inferred from 16S rRNA sequence comparisons and DNA hybridization studies. Discrepancies were encountered with single strains or taxa at the periphery of the genus. Greater intraspecies variation was observed with *infB* sequences than with 16S rRNA gene sequences, with notable exceptions. The apparent subdivision of some species by 16S rRNA analysis was most likely caused by RNA operon heterogeneity.

INTRODUCTION

The generic name *Actinobacillus* was introduced by Brumpt in 1910 with the binomen *Actinobacillus lignieresii*. He referred to 'l'actinobacille' associated with characteristic chronic granulomatous lesions in cattle that was described by Lignieres and Spitz in 1902. Five species were listed as members of the genus in the 1984 edition of *Bergey's Manual of Systematic Bacteriology*: *A. lignieresii*, *Actinobacillus equuli*, *Actinobacillus suis*, *Actinobacillus capsulatus* and *Actinobacillus actinomycetemcomitans* (Phillips, 1984). In the most recent edition, the number of species was 18, of which only a few are considered to belong to *Actinobacillus sensu stricto* (Olsen & Møller, 2004).

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A matrix table of *infB* sequence similarity values is available as supplementary material in IJSEM Online.

DNA hybridization results from the genus *Actinobacillus* have been presented by several groups. Based on the spectrophotometric method, Mutters *et al.* (1989) suggested that the genus *Actinobacillus* should be restricted to *A. lignieresii*, *Actinobacillus pleuropneumoniae*, *A. equuli*, *A. suis*, *A. capsulatus*, *Actinobacillus ureae*, *Actinobacillus hominis* and Bisgaard taxa 5, 9 and 11. This delineation was presented in the form of a review, and crucial information, including the number of strains and the individual DNA binding values, was not given. In a study using the S1 nuclease method on multiple strains from several species, *A. equuli*, *A. suis*, *A. ureae* and *A. capsulatus* were sufficiently related to *A. lignieresii* to justify their inclusion in the genus (Escande *et al.*, 1984). A single isolate received as *A. equuli* was unexpectedly found to differ from the other representatives of this species, but was located within the boundaries of the genus by DNA hybridization. This strain was later found to comply with Bisgaard taxon 9 and has been made the type strain of a novel species, *Actinobacillus arthritidis* (Christensen *et al.*, 2002a). Bisgaard taxon 11 is phenotypically a haemolytic *A. equuli*, closely related to the type strain of *A. equuli* by DNA hybridization, and the

taxon has been reclassified as *A. equuli* subsp. *haemolyticus* (Christensen *et al.*, 2002b).

Increasing emphasis has been directed to the division of bacteria into groups based on comparison of 16S rRNA sequences – so-called phylogenetic classification (Krieg & Garrity, 2001). Based on a large number of near-full-length 16S rRNA sequences from different members of the family *Pasteurellaceae*, single representatives of the following species or taxa constitute a monophyletic group: *A. lignieresii*, *A. pleuropneumoniae*, *A. equuli*, *A. suis*, *A. ureae*, *A. hominis*, *A. arthritidis*, Bisgaard taxon 8 and strain CCUG 28015 (a duck isolate). The other *Actinobacillus* species are pending placement in other genera of the family *Pasteurellaceae* (Olsen *et al.*, 2004).

Translation initiation factor 2 is an essential protein involved in the initiation of protein synthesis in prokaryotes. So far, the encoding gene, *infB*, has only been found as a single copy in the prokaryotic genome; it encompasses 2490 bp in *Haemophilus influenzae* (Fleischmann *et al.*, 1995) and 2502 bp in *Pasteurella multocida* (May *et al.*, 2001). A partial sequence of *infB* has been used successfully in a study of the genus *Haemophilus* (Hedegaard *et al.*, 2001). In the present study, the investigation was extended to the genus *Actinobacillus*. The principal scope was species that have qualified as true members of the genus by DNA hybridization or 16S rRNA sequencing. The following were included: 11 representatives of the type species of the genus, *A. lignieresii*, four of which were equine isolates that appear to constitute a new genomospecies (genomospecies 1) of *Actinobacillus* that is distinct from *A. lignieresii* (Christensen *et al.*, 2002a); representatives of species belonging to the genus by both DNA hybridization and rRNA sequence data (*A. pleuropneumoniae*, *A. equuli*, *A. suis*, *A. ureae*, *A. hominis* and *A. arthritidis*); representatives of the sorbitol-negative subgroup of Bisgaard taxon 9 (genomospecies 2), which belong to the genus according to molecular data (Christensen *et al.*, 2002a); representatives of *A. capsulatus* and Bisgaard taxon 5, which are included in the genus by DNA hybridization data, but excluded on the basis of rRNA sequencing; and a few single isolates that are candidate species of the genus by 16S rRNA sequence comparison (CCUG 28015 and Bisgaard taxon 8) or can be used as outgroups (*Actinobacillus muris* and two biotypes of *Pasteurella pneumotropica*).

METHODS

Bacterial strains and DNA preparation. The bacterial strains sequenced in this study are listed in Table 1. DNA was released using the chelex method as described previously (Hedegaard *et al.*, 2001). PCR products were purified on silica gel columns (QIAquick PCR purification kit; Qiagen).

***infB* sequencing.** Fragments of *infB* spanning approximately 650 nt were amplified using the degenerate oligonucleotide primers 1186f and 1833r (Hedegaard *et al.*, 1999). A total of 30 cycles was run, each consisting of a denaturation step at 94 °C for 30 s, an annealing step at 51 °C for 60 s and an elongation step at 72 °C for

90 s. Purified products were cycle-sequenced using the non-degenerate internal primer 1748R (5'-GTAGCAACCGGACCACGACCTTTAT-3') and the BigDye terminator DNA sequencing kit (Applied Biosystems); analysis was on an ABI 377 DNA sequencer. Primers are named according to nucleotide positions in the *infB* gene of *Escherichia coli* (Hedegaard *et al.*, 1999). The corresponding positions in the *P. multocida* gene are nt 1018, 1580 and 1665 (May *et al.*, 2001). With *A. arthritidis* strain CCUG 24862^T and *P. pneumotropica* strain P309, a probable 3' mismatch prevented use of the internal primer, and sequencing was performed with the primers used for PCR amplification. A 426 bp fragment was obtained corresponding to amino acid positions 368–509 [with reference to *P. multocida* translation initiation factor 2 (May *et al.*, 2001)]; sequences of all bacterial strains were compared. Sequences were checked by visual examination of the electrophoretograms, by translation into amino acid sequences and finally by re-examination of polymorphic positions within individual species or taxa. Ambiguities were resolved by repeating the PCR and sequencing reactions and by sequencing in both directions with the primers used for PCR amplification.

16S rRNA gene sequencing. Fragments of the 16S rRNA genes were amplified by PCR using primers 5'-TATTACCGCGGCTGCTGGCA-3' and 5'-TCAGATTGAACGCTGGCGGC-3' derived from the *rrnB* operon of *E. coli* (Brosius *et al.*, 1978). A total of 25 cycles was run, each consisting of a denaturation step at 94 °C for 60 s, an annealing step at 60 °C for 60 s and an elongation step at 72 °C for 2 min. Purified products were cycle-sequenced from both ends using the PCR primers and a ThermoSequenase dye terminator sequencing kit (Pharmacia Biotech); analysis was on an ABI 377 DNA sequencer. Fragments of 429–437 bp (corresponding to nt 62–496 of *E. coli* 16S rRNA; GenBank/EMBL accession no. J01695) were obtained from all bacterial strains and used for analysis.

An unresolved nucleotide in one sequencing direction was ignored if the complementary position was determined unambiguously. If the position was determined ambiguously in both sequencing directions, the sequencing reactions were repeated. If the ambiguity persisted after the second round of reactions, no further resolution attempt was carried out.

Sequence analysis. Sequences were aligned using the program CLUSTAL W with a Windows interface (Thompson *et al.*, 1997). Distances were modified by the Kimura two-parameter method (Kimura, 1980). Neighbour-joining, maximum-parsimony and maximum-likelihood analysis were performed by PHYLIP (Felsenstein, 1995). Bootstrap analyses were done by 100 resamplings of the datasets. The calculation of similarity between clusters (see matrix table available as supplementary material in IJSEM Online) or sequences (Table 2) was performed with even-weighted characters. When 16S rRNA was compared, gaps and ambiguous positions were ignored.

RESULTS

Comparison of *infB* sequences

A 426 bp fragment of *infB* was unambiguously determined from the 59 bacterial strains seen in Table 1; 163 polymorphic positions were available for phylogenetic analysis. Compared with the type strain of the type species of the genus (*A. lignieresii* P151^T), 2613 nucleotide substitutions were present in the 58 other strains; when the protein sequences were compared, a total of 214 amino acid substitutions were detected.

A dendrogram constructed by the neighbour-joining

Table 1. Strains of *Actinobacillus* and *Pasteurella* included in this study

Culture designations and abbreviations: ATCC, American Type Culture Collection, Manassas, VA, USA; CCUG, Culture Collection, University of Göteborg, Göteborg, Sweden; F, from the collection of Magne Bisgaard, The Royal Veterinary and Agricultural University, Copenhagen, Denmark; HK, from the collection of M.K.; NCTC, National Collection of Type Cultures, Colindale, London, UK; P, from the collection of Wilhelm Frederiksen, Department of Clinical Microbiology, State Serum Institute, Copenhagen, Denmark.

Species	Strain	Reference(s)*	Source
<i>A. lignieresii</i>	P151 ^T (=NCTC 4189 ^T)		Cow
<i>A. lignieresii</i>	P159 (=Holm 285)		Cow
<i>A. lignieresii</i>	P160 (=Holm 339)		Cow
<i>A. lignieresii</i>	P236 (=Phillips A37)		Cow
<i>A. lignieresii</i>	P670 (=CCUG 12401)	1	Human
<i>A. lignieresii</i>	P671 (=Sakazaki 780095)	1	Human
<i>A. lignieresii</i>	P1138		Cow
<i>A. pleuropneumoniae</i> (1)†	P1595 ^T (=ATCC 27088 ^T)		Pig
<i>A. pleuropneumoniae</i> (2)	P597 (=Bertchinger2008/76)	2	Pig
<i>A. pleuropneumoniae</i> (2)	P601 (=Bertchinger204/78)	2	Pig
<i>A. pleuropneumoniae</i> (5)	HK356	3, 4	Pig
<i>A. pleuropneumoniae</i> (2)	HK361 (=NCTC 10976)	3, 4	Pig
<i>A. pleuropneumoniae</i> (3)	HK407 (=ATCC 27090)	3, 4	Pig
<i>A. pleuropneumoniae</i> (NT)	HKfemø (=NCTC 11407)	4	Pig
<i>Actinobacillus</i> genomospecies 1 (NT)‡	P680 (=6744173)	5	Human horse bite
<i>Actinobacillus</i> genomospecies 1 (3)	P1293 (=CCUG 37052)	6	Human horse bite
<i>Actinobacillus</i> genomospecies 1 (3)	CCUG 22229 (=F127)	6	Horse
<i>Actinobacillus</i> genomospecies 1 (3)	CCUG 22231 (=F264)	6	Horse
<i>Actinobacillus</i> genomospecies 2§	F141 (=CCUG 15571)	6	Horse
<i>Actinobacillus</i> genomospecies 2	H4272/4	6	Horse
<i>A. equuli</i>	P157 ^T (=NCTC 8529 ^T)		Horse
<i>A. equuli</i>	P238 (=NCTC 8644)		Horse
<i>A. equuli</i>	P240 (=NCTC 8795)		Horse
<i>A. equuli</i>	P242 (=NCTC 9435)		Horse
<i>A. equuli</i>	P861 (=F125)		Horse
<i>A. equuli</i>	I5445/4	7	Horse
<i>A. equuli</i>	CCUG 19799		Horse
<i>A. equuli</i>	F92	7	Horse
<i>A. equuli</i>	F119	7	Horse
<i>A. equuli</i>	F490	7	Horse
<i>A. equuli</i>	T355/87	7	Human horse bite
<i>A. equuli</i>	P1577 (=CCUG 15573)		Horse
<i>A. suis</i>	P635 ^T (=ATCC 33415 ^T)		Pig
<i>A. suis</i>	P954 (=CCUG 47662)		Pig
<i>A. suis</i>	P955 (=CCUG 47663)		Pig
<i>A. ureae</i>	P161 ^T (=NCTC 10219 ^T)		Human ozaena
<i>A. ureae</i>	P524 (=CCUG 47658)		Human sputum
<i>A. ureae</i>	P644 (=CCUG 47659)		Human sputum
<i>A. arthritidis</i>	CCUG 24862 ^T (=ATCC 13376 ^T)	6, 8	Horse
<i>A. arthritidis</i>	13/225/95	6	Horse
<i>A. hominis</i>	P578 ^T (=NCTC 11529 ^T)		Human sputum
<i>A. hominis</i>	P575 (=CCUG 23130)	9	Human sputum
<i>A. hominis</i>	P580 (=CCUG 11625)	9	Human sputum
<i>A. hominis</i>	P851	9	Human sputum
<i>A. hominis</i>	P880 (=CCUG 23131)	9	Human sputum
<i>A. hominis</i>	P1587	9	Human blood
<i>A. hominis</i>	AFM47	9	Human sputum
<i>A. capsulatus</i>	P243 ^T (=NCTC 11408 ^T)		Rabbit
<i>A. capsulatus</i>	P564 (=CCUG 47660)	8	Rabbit

Table 1. cont.

Species	Strain	Reference(s)*	Source
<i>A. capsulatus</i>	P662		Rabbit
<i>A. capsulatus</i>	P796 (= CCUG 47661)	10	Rabbit
<i>A. capsulatus</i>	P1364		Rabbit
Bisgaard taxon 8	P1572 (= CCUG 16494)		Guinea pig
<i>P. pneumotropica</i> Jawetz	P421 ^T (= NCTC 8141 ^T)		Mouse
<i>P. pneumotropica</i> Heyl	P309 (= ATCC 12555)	9	Mouse
<i>Actinobacillus</i> sp.	CCUG 28015 (= NCTC 11410)	10	Duck
Bisgaard taxon 5	CCUG 16493		Guinea pig
Bisgaard taxon 5	CCUG 24854		Guinea pig
<i>A. muris</i>	P1573 ^T (= CCUG 16938 ^T)		Mouse

*1, Fussing *et al.* (1998); 2, Pohl *et al.* (1983); 3, Kilian (1976); 4, Kilian *et al.* (1978); 5, Dibb *et al.* (1981); 6, Christensen *et al.* (2002a); 7, Christensen *et al.* (2002b); 8, Escande *et al.* (1984); 9, Friis-Møller *et al.* (2001); 10, Olsen *et al.* (2004).

†Numbers in parentheses refer to serotypes of *H. pleuropneumoniae*. Typing was also performed with strains of *Actinobacillus* genomospecies 1. NT, Not typable.

‡Equine isolates phenotypically identified as *A. lignieresii*.

§Sorbitol-negative subgroup of Bisgaard taxon 9.

||Haemolytic variant; *A. equuli* subsp. *haemolyticus* according to Christensen *et al.* (2002b).

method is shown in Fig. 1, with 52 strains clustering in nine sequence groups. Phylogenetic trees were also generated by the maximum-parsimony and maximum-likelihood methods (dendrograms not shown). These analyses generally supported the major topology of Fig. 1; some contradictory findings are mentioned in the text.

A. lignieresii branch

Twenty strains, encompassing clusters 1, 2 and 3 from Fig. 1, were placed on a common branch by all three phylogenetic methods. The branch was supported by a moderate bootstrap value of 69 (of 100 resamplings) (Fig. 1) and relatively large differences were observed between individual clusters in the branch. The branch contained *A. lignieresii*, *A. pleuropneumoniae* and two unnamed taxa.

Fourteen strains of *A. lignieresii* and *A. pleuropneumoniae*, including the two type strains, were found in a common cluster with a sequence similarity in excess of 96 % (cluster 1). The cluster was further divided into four subgroups, with some distinction between *A. lignieresii* and *A. pleuropneumoniae*: one subgroup included only strains of *A. lignieresii* and two subgroups contained only *A. pleuropneumoniae*. In a fourth subgroup, a strain of *A. pleuropneumoniae* (HK356) carried an *infB* sequence that was almost identical to that of the type strain of *A. lignieresii* (Fig. 1).

Four equine isolates phenotypically identified as *A. lignieresii* constituted cluster 2. These were clearly distinct from the type strain and the other bovine isolates of *A. lignieresii* located in cluster 1. However, these isolates were related to strains in cluster 1 (similarity of 89–92 %). Equine isolates of *A. lignieresii* have recently been described as a unique genomospecies (genomospecies 1) based on DNA hybridization and 16S rRNA gene sequencing (Christensen *et al.*,

2002a). Two other equine isolates, F141 and H4272/4, constituted a separate cluster on the *A. lignieresii* branch (cluster 3). These isolates comply phenotypically with the sorbitol-negative subgroup of Bisgaard taxon 9 (genomospecies 2) (Christensen *et al.*, 2002a).

A. equuli branch

Six clusters (4–9) representing *A. equuli*, *A. suis*, *A. ureae*, *A. arthritis*, *A. hominis* and *A. capsulatus* were placed on a common branch by the neighbour-joining method (Fig. 1). The branch was supported by a bootstrap value of 64. The *A. capsulatus* cluster (cluster 9) branched rather deeply and its location on the *A. equuli* branch was not confirmed by maximum-parsimony or maximum-likelihood analyses (dendrograms not shown).

Twelve bacterial strains representing *A. equuli* and its haemolytic variants constituted cluster 4. The latter phenotype has been designated Bisgaard taxon 11 or *A. equuli* subsp. *haemolyticus* (Christensen *et al.*, 2002b). Although some division of the cluster into subgroups was apparent, subgrouping according to *infB* genotype did not correlate with haemolytic activity. A bootstrap value of only 20 was found for cluster 4, indicating difficulties in the distinction between *A. equuli* and *A. suis* (cluster 5). The three representatives of *A. suis* carried almost identical *infB* sequences, with only a single polymorphic position found in the studied fragment. A similar situation was found in the three representatives of *A. ureae* (cluster 6); however, these strains were more clearly separated from sequences in the *A. equuli* cluster. A similarity of 95–97 % was found between clusters 4 and 6, whereas the differences between clusters 4 and 5 were of the same magnitude as the differences found within cluster 4 (see supplementary material in IJSEM Online).

Fig. 1. Neighbour-joining nucleotide distance tree based on partial sequences of *infB* with evolutionary distances given in terms of base substitutions weighted by the Kimura two-parameter model. Type strains in bold and nucleotide sequence accession numbers are given. Values to the left of nodes are percentages of bootstrap replications supporting the node. The *infB* sequence from *E. coli* was used as the outgroup. Superscript letters are defined as follows: a, equine isolates phenotypically identified as *A. lignieresii*; b, sorbitol-negative subgroup of Bisgaard taxon 9; c, haemolytic variant (*A. equuli* subsp. *haemolyticus* according to Christensen *et al.*, 2002b).

Two representatives of the newly described species *A. arthritidis* were positioned on the *A. equuli* branch by all three phylogenetic methods (cluster 7). This species was formed from the sorbitol-positive subgroup of Bisgaard taxon 9 (Christensen *et al.*, 2002a).

Identical *infB* sequences were observed in seven strains of *A. hominis* (cluster 8) and in five strains of *A. capsulatus* (cluster 9). The relationship of *A. hominis* to clusters 4–7 was supported by a bootstrap value of 77 and the topology was also seen in dendrograms constructed by maximum-parsimony and maximum-likelihood analyses (not shown). The location of the *A. capsulatus* cluster was more questionable, as the two other phylogenetic methods placed this cluster on another branch together with the group consisting of *P. pneumotropica* (P421^T), P309, CCUG 28015 and P1572; indeed, by maximum-likelihood analyses, the *A. capsulatus* cluster was even less related to the ‘true’

actinobacilli (clusters 1–8) than was the *P. pneumotropica* group. The simple index of similarity also reveals the uncertain position of the *A. capsulatus* cluster, as this cluster (cluster 9) differed more from the *A. lignieresii* cluster (cluster 1) than from the other groups of actinobacilli (see supplementary material in IJSEM Online).

Strains more remotely related to the genus *Actinobacillus*

Compared to the other strains studied, the type strain of *P. pneumotropica* and an additional strain (biotype Heyl, P309), a duck isolate (CCUG 28015), one representative of Bisgaard taxon 8 (P1572), two representatives of Bisgaard taxon 5 (CCUG 16493 and CCUG 24854) and the type strain of *A. muris* were more remotely related to the type species of the genus *Actinobacillus* using *infB* sequence analysis (Fig. 1).

Comparison of partial 16S rRNA gene sequences

A fragment from the 5' end of the 16S rRNA gene was sequenced from all 59 bacterial strains included in the present study. The fragment encompassed 437 nt in strains of *A. lignieresii* and *A. pleuropneumoniae* (cluster 1); 435 nt in clusters 2–9, CCUG 28015, P1572 and the two representatives of Bisgaard taxon 5; 431 nt in *P. pneumotropica* biotype Heyl; and 429 nt in the type strains of *P. pneumotropica* and *A. muris* (GenBank/EMBL accession nos AJ438630–AJ438688). Phylogenetic analysis based on near-full-length 16S rRNA sequences from 114 representatives of the family *Pasteurellaceae* has recently been published (Olsen *et al.*, 2004), so the 16S rRNA sequencing in the present study was carried out to ascertain the identity of the isolates and to study intraspecies variation. In most cases, minor differences were found between the sequence of the rRNA gene fragment determined by us and the sequences available in the databases for the same strains. The majority of discrepancies concerned single bases that were either resolved in our sequence, but undetermined in GenBank/EMBL, or vice versa.

The subgrouping of cluster 1 observed by *infB* analysis was not supported by 16S rRNA gene sequencing. Near-full-length 16S rRNA sequences obtained with the type strains of *A. lignieresii* and *A. pleuropneumoniae* were almost identical and differed in only 4 nt positions (Fussing *et al.*, 1998). Two of the polymorphic positions occurred in the fragment used in the present study and these two signature bases were repeatedly confirmed in the rRNA sequence of the other 12 strains of *infB* cluster 1, allowing a distinction of *A. lignieresii* from *A. pleuropneumoniae*. The variation within the two species was restricted to a single polymorphic position in each species (0.2%) in the fragment of the 16S rRNA gene studied (GenBank/EMBL accession nos AJ438630–AJ438642 and AJ438688; alignment not shown).

With the five representatives of *A. capsulatus*, three distinct sequences were obtained (GenBank/EMBL accession nos AJ438675–AJ438679). The previously published sequences of the type strain and P796 were essentially confirmed, whereas the three remaining isolates carried mutually identical 16S rRNA gene fragment sequences with 98.6% similarity to the type strain of the species.

Relatively large differences between the multiple *rrn* operons were indicated in some species of the genus *Actinobacillus*. With the type strain of *A. hominis* and an additional strain (P575, GenBank/EMBL accession nos AJ438666–AJ438667), the nucleotides GCAA were found in positions where the *rrnB* operon of *E. coli* carries the sequence TTCG (nt 208–211, numbering with reference to *E. coli* 16S rRNA). With the five other isolates of *A. hominis*, the sequencing in this area was ambiguous, as two distinct nucleotides at each position were discernible in both sequencing directions. The 16S rRNA sequence of the type strain, available as L06076, recognizes the ambiguity at position 208, but not at

209–211. With all seven representatives of *A. hominis* (GenBank/EMBL accession nos AJ438666–AJ438672), as well as the deposited sequence (L06076), positions 199 and 218 were ambiguously determined.

Representatives of *A. equuli* were divided into two groups, one group being almost identical to *A. suis* and the other showing more resemblance to *A. hominis*. These separate sequence groups have been previously represented as GenBank/EMBL accession numbers M75072 (the type strain of *A. equuli*) and M75067 (CCUG 19799). The latter strain has erroneously been referred to as *A. capsulatus* (Dewhirst *et al.*, 1992), but it is a haemolytic variant of *A. equuli*, formerly designated Bisgaard taxon 11 (Berthoud *et al.*, 2002; Christensen *et al.*, 2002b). Haemolytic activity was not correlated with 16S rRNA sequence, as both groups contained haemolytic as well as non-haemolytic strains (dendrogram not shown). In the fragment of 16S rRNA studied, the difference between the two groups of *A. equuli* was determined by 12 polymorphic positions (nt 456–461 and 471–476, *E. coli* numbering). However, with the type strain of *A. equuli* (P157^T, GenBank/EMBL accession no. AJ438649), electrophoretograms were obtained from both sequencing directions that were clearly an average of the two sequences at the 12 polymorphic positions. The sequencing was repeated with bacteria subcultured twice from a single colony to ascertain the purity of the strain and the same results were obtained.

Delineation of the genus *Actinobacillus* by different molecular methods

In Table 2, the results of partial *infB* sequencing are shown along with results obtained by 16S rRNA gene sequencing and DNA hybridization data. The type strains of different species (or reference strains from unnamed taxa) are compared with the type strain of the type species of the genus, *A. lignieresii*. The results from DNA hybridization experiments were compiled from two separate sources and are incomplete. Gaps and ambiguous positions are ignored in the calculation of rRNA similarity.

The results from DNA hybridization are not precise. Rather different results were obtained using the two methods with the type strain of *A. suis* or using the same method on highly related biovars of *A. equuli* (Table 2). The DNA relationship is high between the type strain of *A. lignieresii* and the reference strain of equine *A. lignieresii* (genomospecies 1, CCUG 22229), but decreases to a level of around 40% in relation to members of the *A. equuli* branch. With 16S rRNA gene comparison, 97.0–98.3% similarity was seen between the type strains of *A. equuli*, *A. suis*, *A. ureae*, *A. arthritidis* and *A. hominis* (all of the *A. equuli* branch) and *A. lignieresii*. A similar level was observed with Bisgaard taxon 8, whereas the duck isolate CCUG 28015 was as different from *A. lignieresii* as Bisgaard taxon 5 (95.1–95.7%), which is unclustered by phylogenetic analysis (Olsen *et al.*, 2004). A conspicuous result is seen with the type strain of *A. capsulatus*, which is about as different from

Table 2. Delineation of the genus *Actinobacillus* as determined by DNA hybridization plus 16S rRNA and partial *infB* sequencesColumns headed Escande and Christensen respectively give data from Escande *et al.* (1984) and Christensen *et al.* (2002a). —, Not reported.

Strain	16S rRNA gene accession no.	Similarity to the type strain of <i>A. lignieresii</i> (%)			
		DNA hybridization		Nucleotide similarity	
		Escande	Christensen	16S rRNA*	<i>infB</i>
<i>A. pleuropneumoniae</i> (type strain)	M75074	—	—	99.7	96.2
CCUG 22229 (genomospecies 1)	AF247722	—	70	96.3	89.9
CCUG 15571 (genomospecies 2)	AF247717	—	48	96.8	88.3
<i>A. equuli</i> (type strain)	M75072	44	40	98.3	86.9
CCUG 19799 ('haemolytic equuli')	AF247716	—	48	97.5	86.9
<i>A. suis</i> (type strain)	M75071	36	46	98.0	87.3
<i>A. ureae</i> (type strain)	M75075	37	—	98.1	87.1
<i>A. arthritidis</i> (type strain)	AF247712	43	—	97.0	86.6
<i>A. hominis</i> (type strain)	L06076	—	38	97.3	88.8
<i>A. capsulatus</i> (type strain)	M75062	41	—	93.4	84.5
CCUG 16494 (Bisgaard taxon 8)	L06083	—	—	97.1	84.3
<i>P. pneumotropica</i> (type strain)	M75083	4	—	93.5	81.7
CCUG 28015 (duck isolate)	AF224284	—	—	95.7	81.0
CCUG 16493 (Bisgaard taxon 5)	L06080	—	—	95.1	81.2
<i>A. muris</i> (type strain)	AF024526	—	—	93.7	78.9

*Full-length (>1400 nt) 16S rRNA gene sequences were compared with that of the type strain of *A. lignieresii* (accession no. M75068).

A. lignieresii as *A. muris* or *P. pneumotropica*. By *infB* comparison, the similarity values of members on the *A. equuli* branch (omitting *A. capsulatus*) with the type species of the genus *Actinobacillus* are in the range 86.9–88.8%, the similarity level of *A. capsulatus* and Bisgaard taxon 8 (which is of unresolved taxonomic position) is 84.3–84.5%, whereas strains clearly located outside the genus *Actinobacillus* showed similarity to *A. lignieresii* of 81.7% or less (Table 2).

DISCUSSION

At present, bacterial systematics relies heavily on comparisons of 16S rRNA gene sequences. The method is relatively simple and rapid and can be performed with any bacterial isolate. A number of phylogenetic computer programs is available to do routine allocation to a sequence group of a novel isolate. However, potential problems of both a technical and fundamental nature do exist.

Comparison of 16S rRNA gene sequences must often be carried out with sequences of unequal lengths. While substitutions can be handled easily, either as evenly weighted characters or by assignment of a value according to the type of substitution, it is not easy to score differences in length of sequences. Between the type strains of *A. muris* and *A. lignieresii*, a difference in length of nearly 2% was found in the studied fragment of 16S rRNA. This difference is likely to be of phylogenetic significance, but, due to the inability

of currently used algorithms to take this into account, the information is ignored. The allowance for gaps in the comparison of sequences most likely underestimates the phylogenetic distance between bacterial strains.

Another problem arises when differences are encountered between the multiple *rrn* operons in the same bacterial strain. PCR amplification results in an average product, which may be biased towards any of the individual sequences. The method used in the present study does not permit the certain determination of different sequences in separate operons; however, a clear indication of this phenomenon was observed with *A. hominis* and *A. equuli*. With the type strain of the latter species, 12 polymorphic positions were found within a stretch of 21 nt, which were also seen in the sequence deposited by Berthoud *et al.* (2002) (GenBank/EMBL accession no. AF381186). The equivocally determined nucleotides were not seen with all representatives of the various species. It is possible that repeated sequencing would have revealed ambiguities in a larger number of strains, but this was not attempted, as the sequences were accepted on defined criteria and because another sequencing method would be needed in order to identify the individual operons with certainty. The accepted strategy when comparing 16S rRNA sequences is to ignore positions with ambiguously determined nucleotides. However, even when accurate information is available, e.g. from whole-genome sequencing projects, it is difficult to make use of the information, as different phylogenies could be inferred

from different operons. The taxonomic problems associated with *rrn* operon heterogeneity in the genus *Veillonella* have recently been addressed (Marchandin *et al.*, 2003).

In general, greater intraspecies variation was observed with *infB* sequences than with 16S rRNA gene sequences, with *A. capsulatus* as the notable exception. Five representatives of this species all carried an identical *infB* sequence, whereas 21 polymorphic positions were encountered in the studied fragment of the 16S rRNA gene. Low similarity (97.9%) has previously been noted between the near-full-length sequences deposited in databases for the type strain of *A. capsulatus* and *A. capsulatus* strain P796 (Olsen *et al.*, 2004). Identical gene sequences in different bacterial strains could indicate intraspecies horizontal spread of a preferred sequence; however, in four positions in the 16S rRNA gene where the type strain differed from three other representatives of *A. capsulatus*, nucleotides in the fifth strain (P796) were ambiguously determined. It is therefore possible that the apparent differences between strains of *A. capsulatus* reflect, to some extent, differences in the multiple rRNA operons, and further studies on the homogeneity of this species are needed.

DNA hybridization determinations are reported in the literature with decreasing frequency, which underscores the necessity for other molecular methods to be used for validating conclusions based on 16S rRNA sequence comparisons. Phylogenetic analysis of DNA sequences from translated genes is an approach with theoretical advantages (Palys *et al.*, 1997). Ideally, the method should be based on a number of universally distributed genes and multiple strains of each taxon should be included to avoid unrepresentative sequences, which may be the result of recombination events. One candidate gene is *infB*, encoding the translation initiation factor 2, which has worked well with the genus *Haemophilus* (Hedegaard *et al.*, 2001).

Using *infB* sequence comparison, the genus *Actinobacillus sensu stricto* should include *A. lignieresii*, *A. pleuropneumoniae*, *A. equuli*, *A. suis*, *A. ureae*, *A. arthritidis* and *A. hominis*, plus the unnamed taxa of 'equine *lignieresii*' (genomospecies 1) and the sorbitol-negative subgroup of Bisgaard taxon 9 (genomospecies 2). These taxa were all related to the type species of the genus at a level above 85% and constituted a monophyletic cluster by the neighbour-joining, maximum-likelihood and maximum-parsimony methods. With five representatives of *A. capsulatus* and a single strain of Bisgaard taxon 8, the *infB* sequences obtained were related to the type species of the genus at a level below 85%, and these taxa were positioned with the *P. pneumotropica* cluster by the majority of the phylogenetic methods employed. These taxa may not belong to the genus *Actinobacillus sensu stricto*.

A. pleuropneumoniae is the cause of porcine necrotizing pleuropneumonia and was originally allocated to the genus *Haemophilus* as a consequence of its V factor (NAD) requirement. The description of V factor-independent

strains led to an investigation of its generic position. It was found to be so closely related to *A. lignieresii* that individual speciation was not justified on the basis of DNA relatedness (Pohl *et al.*, 1983). Because of the differences in host range and pathological lesions, *A. lignieresii* and *A. pleuropneumoniae* were upheld as separate species. The close relationship of these species is reflected in their *infB* sequences (Fig. 1). A single strain of *A. pleuropneumoniae* (HK356) may have acquired a fragment of the *infB* gene by recombination with a strain of *A. lignieresii*.

A. lignieresii is associated with characteristic granulomatous lesions in cattle and sheep (Christiansen, 1917; Olsen & Møller, 2004) and bacteria of indistinguishable phenotype have been isolated from horses and human horse bites (Dibb *et al.*, 1981). It was recently shown that equine isolates of *A. lignieresii* represent a unique genomospecies (genomospecies 1; Christensen *et al.*, 2002a). This finding was substantiated by *infB* sequence comparison (cluster 2 in Fig. 1). Two representatives of the sorbitol-negative subgroup of Bisgaard taxon 9 (genomospecies 2) were also located on the *A. lignieresii* branch (cluster 3). Clusters 1–3 were placed on a common branch by all three phylogenetic methods, yet it is apparent that relatively large differences separate clusters 2 and 3 from cluster 1. It is therefore possible that the topology of the *A. lignieresii* branch is not robust and that inclusion of other species or taxa in the comparison will lead to a shift in the position of clusters 2 and 3. Their affiliation to the genus *Actinobacillus* is, however, supported by both DNA relatedness and nucleotide sequence similarities (Table 2).

A relatively large number of strains of *A. equuli* were included in the study. Haemolytic variants of *A. equuli* have been designated 'equine isolates of *A. suis*' or Bisgaard taxon 11; recently, a reclassification as *A. equuli* subsp. *haemolyticus* was proposed (Christensen *et al.*, 2002b). The subspeciation has not been substantiated by 16S rRNA (Berthoud *et al.*, 2002) or *infB* sequences (Fig. 1). The differences in *infB* sequence between the 12 haemolytic and non-haemolytic strains of *A. equuli* (cluster 4) were comparable with the differences encountered between the 14 representatives of cluster 1. However, in contrast to the latter cluster, *A. equuli* was not well separated from the other species of its branch, particularly *A. suis* (Fig. 1). A similar result was seen in a study of near-full-length 16S rRNA sequences of eight strains of *A. equuli* together with representative taxa of the remaining part of the genus *Actinobacillus*. The phylogeny was poorly resolved and representatives of *A. equuli* were scattered in the dendrogram among other *Actinobacillus* species (Christensen *et al.*, 2002b).

Four other species on the *A. equuli* branch, *A. suis*, *A. ureae*, *A. arthritidis* and *A. hominis*, were very homogeneous with respect to their *infB* sequences. These species were each represented by two to seven isolates. Because of the restricted sample size, it is not clear whether the apparent similarity of these species is real.

A. capsulatus presents a particular taxonomic problem. A thorough investigation of DNA relatedness on multiple strains has shown this species to be a true member of the genus *Actinobacillus* (Escande *et al.*, 1984). In contrast, analysis based on 16 rRNA sequences of five strains showed that the species constituted a separate cluster only remotely related to *Actinobacillus sensu stricto* (Olsen *et al.*, 2004). Some additional strains were included in this study and the peculiar sequence of the 16S rRNA gene was confirmed. In the studied fragment, the type strains of *A. capsulatus*, *A. muris* and *P. pneumotropica* were equally different from that of *A. lignieresii* (Table 2); however, the type strains of *A. muris* and *P. pneumotropica* carried a 16S rRNA sequence that was considerably shorter (429 nt) than the 435 nt observed with all species on the *A. equuli* branch, including *A. capsulatus*. With respect to *infB* sequence, *A. capsulatus* took up an intermediate position (Table 2) and, although the cluster was placed on the *A. equuli* branch by the neighbour-joining method (Fig. 1), this was not the case with the maximum-likelihood and maximum-parsimony methods (dendrograms not shown). Further investigations are needed in order to clarify the taxonomic position of *A. capsulatus*.

Analysis based on comparison of 16S rRNA sequences has clustered the duck isolate CCUG 28015 and the reference strain of Bisgaard taxon 8 with *Actinobacillus sensu stricto* (Olsen *et al.*, 2004). This inclusion into the genus was not apparent from investigations of *infB* sequences (Fig. 1; Table 2). The taxonomic position of these candidate species awaits analysis of a greater number of representative strains. Bisgaard taxon 5, included in the genus by anecdotal evidence, was clearly located outside the genus *Actinobacillus sensu stricto* by *infB* sequence comparison (Fig. 1; Table 2).

A large number of provisional taxa and species of the family *Pasteurellaceae* have been described in recent years, and the family is likely to undergo rearrangement and reclassification in the future. If relatively high levels of similarity are insisted upon within individual genera, a large number of genera will be needed in order to encompass the diversity of the family. Using *infB* sequence comparison, an 85 % similarity demarcation line appeared appropriate for delineation of the genus *Actinobacillus sensu stricto*. Further data on DNA sequence similarity in the genus and in the family are needed before taxonomic conclusions are drawn.

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