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.

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The present paper presents the conclusions of a polyphasic investigation of the taxonomy of the trehalose-negative [*Pasteurella*] *haemolytica* complex. Clusters previously identified by ribotyping and multilocus enzyme electrophoresis (MEE) have been evaluated by 16S rRNA sequencing and DNA–DNA hybridizations. Results obtained by the different techniques were highly related and indicated that the [*P.*] *haemolytica* complex contains distinct genetic and phenotypic groups. At least seven species were outlined, five of which were named. We refrained in formal naming of more groups until additional strains are characterized. Five 16S rRNA clusters were identified corresponding to distinct lineages previously outlined by MEE. Within 16S rRNA cluster I two distinct genotypic groups have been outlined in addition to [*P.*] *haemolytica sensu stricto* (biogroup 1). Each of the clusters II, III, IV and V represent at least one new species. The investigations underline that [*P.*] *haemolytica sensu stricto* only contains strains that do not ferment L-arabinose even though they are referred to as 'biotype A' of [*P.*] *haemolytica*. The five 16S rRNA clusters identified had a common root relative to the other species within the family *Pasteurellaceae*, and the overall sequence similarity among these five clusters was higher than what is observed within the existing genera of the family. The allocation of the trehalose-negative [*P.*] *haemolytica* complex to a new genus seems to be indicated. Based on the polyphasic investigation performed a new genus *Mannheimia* is proposed for the trehalose-negative [*P.*] *haemolytica* complex. At the present stage two previously named species are transferred to this new genus and three new species are described. [*P.*] *haemolytica* is reclassified as *Mannheimia haemolytica* comb. nov., whereas *Pasteurella granulomatis*, Bisgaard taxon 20 and [*P.*] *haemolytica* biovar 3J are reclassified and combined in the species *Mannheimia granulomatis* comb. nov. *Mannheimia glucosida* sp. nov.

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Abbreviations: MEE, multilocus enzyme electrophoresis; UG, untypable group; VP, Voges–Proskauer.

The GenBank accession numbers for the sequences reported in this paper are listed in Table 1.

corresponds to [*P.*] *haemolytica* biogroups 3A–3H and the β -glucosidase and meso-inositol-positive strains of [*P.*] *haemolytica* biogroup 9. All typable strains within *M. glucosida* belong to serotype 11. *Mannheimia ruminalis* sp. nov. consists of strains previously classified as Bisgaard taxon 18 and [*P.*] *haemolytica* biogroup 8D. Finally, *Mannheimia varigena* sp. nov. includes [*P.*] *haemolytica* biogroup 6 as well as Bisgaard taxon 15 and Bisgaard taxon 36. The type strains are NCTC 9380^T (*M. haemolytica*), ATCC 49244^T (*M. granulomatis*), CCUG 38457^T = P925^T (*M. glucosida*), CCUG 38470^T = HPA92^T (*M. ruminalis*) and CCUG 38462^T = 177^T (*M. varigena*).

Keywords: *Pasteurella haemolytica*, *Mannheimia* gen. nov., DNA–DNA hybridization, 16S rRNA sequencing, polyphasic taxonomy

INTRODUCTION

In 1921, Jones reported on three groups of bovine pasteurellae and placed atypical strains in group I of '*Bacillus bovissepticus*' (Jones, 1921). These strains were further characterized by Newsom & Cross (1932), who proposed the name [*Pasteurella*] *haemolytica* for group I of *Bacillus bovissepticus*. Two biotypes of [*P.*] *haemolytica* were described by Smith (1959, 1961) based upon a number of phenotypic characters as well as epidemiological and pathological differences. The biotypes were designated A and T referring to their ability to ferment either L-arabinose or trehalose. A third taxon of [*P.*] *haemolytica* was proposed by Frederiksen (1973) for strains which did not fit into the A/T biotyping pattern.

A serotyping system using an indirect haemagglutination test (IHA) was developed by Biberstein *et al.* (1960). Biberstein & Gills (1962) subsequently reported a consistent association between serotypes and biotypes. The number of recognized serotypes has subsequently increased to 17 (Pegram *et al.*, 1979; Fraser *et al.*, 1982; Fodor *et al.*, 1987; Younan & Wallmann, 1989). Serotypes 3, 4, 10 and 15 are associated with biotype T, and the remaining serotypes with biotype A. Untypable strains by IHA have subsequently been characterized by counter-current immunoelectrophoresis (Donachie *et al.*, 1984), demonstrating nine additional serogroups.

A number of investigators have reported difficulties in obtaining L-arabinose fermentation with some trehalose-negative strains even though these strains by other criteria represent strains of biotype A (Mraz, 1969; Bisgaard, 1984). Additionally, conflicting results have been obtained in different laboratories (Mraz, 1969). Extended phenotypic investigations in the mid-1980s showed that strains from ruminants classified as [*P.*] *haemolytica* could be divided into 12 biogroups based upon ornithine decarboxylase and fermentation of L-arabinose, D-sorbitol, glucosides (aesculin, amygdalin, arbutin, cellobiose, gentibiose and salicin) and trehalose (Mutters *et al.*, 1989). DNA–DNA hybridizations (Mutters *et al.*, 1986) have shown that some of the biogroups represent separate species and

that [*P.*] *haemolytica sensu stricto* ([*P.*] *haemolytica* biogroup 1) should only consist of strains negative for fermentation of L-arabinose. However, biotyping has only been used in a few laboratories, so at present there is little knowledge about the prevalence of the different biogroups and their veterinary importance. This situation is undesirable as it makes interpretation of studies on strains classified as [*P.*] *haemolytica* difficult, and it may hinder progress in studies on epidemiology and pathogenesis.

[*P.*] *haemolytica* was excluded from the genus *Pasteurella sensu stricto* in 1985 on the basis of DNA–DNA hybridizations (Mutters *et al.*, 1985), and its separation from the three recognized genera within the family *Pasteurellaceae* was later supported by DNA–rRNA hybridizations (De Ley *et al.*, 1990) and 16S rRNA sequencing (Dewhirst *et al.*, 1992, 1993). Genotypic investigations have also shown that the trehalose-positive strains represent a separate species (Biberstein & Francis, 1968; Pohl, 1979; Mannheim, 1983). Although these strains show no close affiliation to the genus *Pasteurella*, they were recently named *Pasteurella trehaliosi* (Sneath & Stevens, 1990).

Studies on the 16S rRNA sequence variation among the different serotypes of [*P.*] *haemolytica* have recently been published showing that most of the typable 'biotype A' isolates had identical 16S rRNA sequences. Serotypes 2 and 11, however, showed deviations in two and four sequence positions, respectively (Davies *et al.*, 1996). Untypable groups (UGs) identified by outer-membrane proteins and LPS variation were also described. UG4 and UG5 only showed 95.3–96.0% sequence similarity with the type strain, indicating that they might represent new species. However, no attempt was made to classify these groups with the previously described biogroups.

The phenotypic and genotypic diversity of the trehalose-negative [*P.*] *haemolytica* complex has recently been investigated using quantitative evaluation of phenotypic data, ribotyping and multilocus enzyme electrophoresis (MEE) (Angen *et al.*, 1997a, b, c). These studies also included taxa related to the [*P.*] *haemolytica* complex including: *Pasteurella granuloma-*

matris, Bisgaard taxon 18 and Bisgaard taxon 36, all of which have been isolated from ruminants, Bisgaard taxon 15 isolated from pigs and Bisgaard taxon 20 isolated from hares and rabbits. It was shown that the clusters found by numerical analysis of quantitative phenotypic data generally corresponded to genotypic clusters identified by ribotyping and MEE, and that sub-groups within the previously described biogroups also could be outlined.

The taxonomic consequences of these observations are only indicative. However, DNA–DNA hybridizations performed between a few selected strains representing biogroups 1, 3A, 3G, 3H, 9, taxon 15 and [*P.*] *trehalosi* (Mutters *et al.*, 1986) in addition to selected serotypes of [*P.*] *haemolytica* and [*P.*] *trehalosi* (Bingham *et al.*, 1990) indicate that the trehalose-negative group might represent a new genus within the family *Pasteurellaceae* Pohl 1981 (Mutters *et al.*, 1986). 16S rRNA sequences subsequently available for selected strains representing biogroup 1, taxon 15, taxon 18, taxon 20 and [*P.*] *trehalosi* also show that with the exception of [*P.*] *trehalosi* these organisms are phylogenetically related (Davies *et al.*, 1996). In the present investigation, strains representing the genotypic clusters previously outlined have been examined by 16S rRNA sequencing and DNA–DNA hybridizations to achieve information about the phylogenetic structure and taxonomic distances within the [*P.*] *haemolytica* complex.

METHODS

Bacterial strains and phenotypic characterization. The selection of strains was based upon previous investigations including quantitative evaluation of phenotypic test results from 339 isolates representing different taxa within the trehalose-negative [*P.*] *haemolytica* complex (Angen *et al.*, 1997a, b), ribotyping of 201 isolates (Angen *et al.*, 1997c) and characterization of 59 isolates by MEE (Angen *et al.*, 1997c). The polyphasic approach was used to ensure that strains chosen for 16S rRNA sequencing and DNA–DNA hybridization covered the genotypic diversity known so far. Strains selected for 16S rRNA sequencing and sequences from GenBank used for comparison are listed in Table 1. The bacterial strains used in the DNA homology studies are listed in Table 2. Materials and methods used to determine the phenotypic and genotypic (ribotyping and MEE) characteristics of the strains have been described previously (Angen *et al.*, 1997a, c).

16S rRNA sequencing. One millilitre of an overnight culture grown with shaking in Tryptic Soy Broth (Difco) was centrifuged at 12000 r.p.m. for 2 min, washed in 0.9% NaCl, resuspended in 1 ml dH₂O, and heated at 94 °C for 20 min. After centrifugation at 12000 r.p.m. for 2 min, 5 µl lysate was added to 95 µl PCR mixture consisting of 10 × MIX (USB), 2.5 mM MgCl₂, 5 pmol biotinylated primer, 10 pmol non-biotinylated primer, 0.2 nmol each of the four NTPs and 2.5 U *Taq* DNA polymerase (USB). Amplification was carried out in a thermocycler (GeneAmp RCP System 2400; Perkin Elmer) and initiated by 2 min denaturation at 94 °C and then followed by 35 cycles, each cycle consisting of 30 s denaturation at 94 °C, 1 min

annealing at 55 °C and 2 min extension at 72 °C. The solution was left at 72 °C for 7 min after the last cycle.

Oligonucleotides were synthesized according to sequences and positions reported by Paster & Dewhirst (1988) and Dewhirst *et al.* (1992) (Table 3). For the PCR amplification, one of the oligonucleotide primers was labelled with biotin (HPLC-purified; Pharmacia Biotech). This allowed DNA strand separation and purification of amplified DNA using streptavidin-coated magnetic beads (Dynabeads M-280) to trap the biotin-labelled PCR fragments. DNA strand separation was performed as recommended by the manufacturer. Briefly, the double-stranded DNA bound to the magnetic beads was incubated in 0.1 M NaOH for 5 min, allowing the non-biotinylated strand to be removed. After neutralization of the solution containing the non-biotinylated strand, and additional washings of the bead fraction, both strands could be used as templates for DNA sequencing.

Sequencing was performed by the dideoxy chain-termination method (Sanger *et al.*, 1977) using Autoread Sequencing Kit (Pharmacia Biotech) set up with 10 ng fluorescein labelled primers using 3 U *T7* DNA polymerase (Pharmacia Biotech) and 1–2 µg DNA template. Sequence reactions were analysed on the ALF Automatic DNA Sequencer (Pharmacia Biotech). For all strains 1467 bases were sequenced in both directions between positions 26 and 1492 according to the type strain of [*P.*] *haemolytica* (GenBank no. M75080).

Analysis of sequence data. Sequences were aligned manually to the 16S sequence of *Escherichia coli* (GenBank J01695) published by Brosius *et al.* (1978) and the consensus sequence given by Lane (1991). For alignment of the 16S rRNA sequences of strains R19.2, HPA121 and the GenBank sequence U57072 to other sequences, one base (guanine) at position 1126 (*E. coli* position 1135) was removed. Maximum-likelihood analysis and bootstrap analysis (100 repeats) were performed by fastDNAm1 (Olsen *et al.*, 1994) run on a HP9000/819 computer. Similarities within and between the clusters obtained were calculated as the mean similarity between all pairs of sequences transformed to evolutionary distances by accounting for superimposed nucleotide substitutions by the method of Jukes & Cantor (1969).

GenBank accession numbers. The GenBank accession numbers for the 16S rRNA sequences of the strains examined are listed in Table 1.

Preparation of DNA for DNA–DNA hybridization. Cells were grown under shaking at 37 °C in Tryptic Soy Broth (Difco) supplemented with 0.4% (w/v) filter-sterilized yeast extract (Oxoid) and 0.01 M NaHCO₃. The cells were harvested in the early-stationary phase of growth, examined microscopically for purity, washed in 1 × SSC (0.15 M NaCl plus 0.015 M trisodium citrate) and resuspended in 1 × SSC containing 0.01 M EDTA. High-molecular-mass DNA was isolated by combining the methods of Marmur (1961) and Kirby *et al.* (1967) as proposed by De Ley *et al.* (1970). Briefly, harvested cells were lysated at 60 °C with SDS and treated with Pronase (Serva). DNA was isolated by repeated chloroform extractions and ethanol precipitations. After RNAase treatment, extraction with phenol/m-kresol was performed. DNA was then precipitated by m-kresol in 20% sodium-benzoate to remove carbohydrates. After dialysis in 0.1 × SSC, the DNA solution was treated with potassium-phosphate and 2-methoxyethanol to remove lipids. DNA

Table 1. Strains investigated by 16S rRNA sequencing

ATCC, American Type Culture Collection, Manassas, VA, USA; CCM, Czech Collection of Microorganisms, Brno, Czech Republic; CCUG, Culture Collection, University of Gothenburg, Sweden; NCTC, National Collection of Type Cultures, Colindale, UK.

GenBank accession no.	Strain	Taxon*	Source
AF060699	NCTC 9380 ^T	[<i>P.</i>] <i>haemolytica</i> biogroup 1	Ovine isolate
AF053887	UT26	[<i>P.</i>] <i>haemolytica</i> biogroup 1	Ovine isolate
AF053888	P731	[<i>P.</i>] <i>haemolytica</i> biogroup 3A	Ovine nose
AF053889	P925 ^T	[<i>P.</i>] <i>haemolytica</i> biogroup 3B	Ovine lung
AF053890	UT18	[<i>P.</i>] <i>haemolytica</i> biogroup 3C	Ovine isolate
AF053891	P737	[<i>P.</i>] <i>haemolytica</i> biogroup 3G	Ovine nose
AF053892	P733	[<i>P.</i>] <i>haemolytica</i> biogroup 3H	Ovine nose
AF053893	177 ^T	[<i>P.</i>] <i>haemolytica</i> biogroup 6	Bovine pneumonia
AF053894	R19.2	[<i>P.</i>] <i>haemolytica</i> biogroup 7	Bovine rumen
AF053895	HPA102	[<i>P.</i>] <i>haemolytica</i> biogroup 8A	Ovine rumen
AF053896	HPA98	[<i>P.</i>] <i>haemolytica</i> biogroup 8D	Ovine rumen
AF053897	P730	[<i>P.</i>] <i>haemolytica</i> biogroup 9	Ovine nose
AF053898	HPA121	[<i>P.</i>] <i>haemolytica</i> biogroup 10	Bovine rumen
AF053899	CCM 5786 (= F155)	Bisgaard taxon 15 biovar 1	Pig sepsis
AF053900	HPA92 ^T	Bisgaard taxon 18 biovar 1	Ovine rumen
AF053901	Ph13	Bisgaard taxon 20 biovar 1	Hare lung
AF053902	ATCC 49244 ^T	<i>Pasteurella granulomatis</i>	Bovine panniculitis
Sequences from GenBank included in maximum-likelihood analysis			
M75080	NCTC 9380 ^T , PH208, PH380	[<i>P.</i>] <i>haemolytica</i> serotype 2	Ovine isolates
U57066	PH2, PH8, PH66, PH236, PH284, PH296, PH344, PH374, PH376, PH388, PH568, PH570, PH706, UT3	[<i>P.</i>] <i>haemolytica</i> serotypes 1, 5–9, 12–14, 16 and UG1†	Bovine and ovine isolates
U57067	PH344	[<i>P.</i>] <i>haemolytica</i> serotype 11	Ovine isolate
U57068	PH152	[<i>P.</i>] <i>haemolytica</i> UG3†	Bovine pneumonia
U57069	PH290	[<i>P.</i>] <i>haemolytica</i> UG3†	Ovine abortion
U57070	PH40 (= UT35)	[<i>P.</i>] <i>haemolytica</i> UG4†	Bovine pneumonia
U57071	PH360	[<i>P.</i>] <i>haemolytica</i> UG4†	Bovine liver
U57072	PH704	[<i>P.</i>] <i>haemolytica</i> UG5†	Bovine abortion
U57075	NCTC 10626	[<i>P.</i>] <i>trehalosi</i> serotype T4	Ovine isolate
U57076	CCUG 16500 (= F454)	Bisgaard taxon 15 biovar 1	Pig pneumonia
U57077	CCUG 20954 (= HPA81)	Bisgaard taxon 18 biovar 1	Ovine rumen
U57078	CCUG 16505‡	Bisgaard taxon 20	Rabbit isolate
M75062	NCTC 11408 ^T	<i>Actinobacillus capsulatus</i>	Rabbit arthritis
M59769	NCTC 10322 ^T	<i>Pasteurella multocida</i>	Bovine isolate
M75068	NCTC 4189 ^T	<i>Actinobacillus lignieresii</i>	Bovine granuloma
M59433	NCTC 8143 ^T	<i>Haemophilus influenzae</i>	Human

*Taxa according to Angen *et al.* (1997a, b).

† Untypable groups according to Davies *et al.* (1996).

‡ According to Davies *et al.* (1996), U57078 refers to CCUG 16504. However, this is not a taxon 20 strain. According to a personal communication between Curator E. Falsen, CCUG, and M. Bisgaard of 16 September 1996, CCUG 16505 was sent to Dr I. Olsen. This means that CCUG 16504 should be replaced by CCUG 16505.

was then repeatedly precipitated with ethanol and dialysed against 0.1 × SSC. Only high-molecular-mass DNA that could be spun up on a glass rod was used for hybridizations.

DNA–DNA hybridization. Initial renaturation rates of homologous or heterologous solutions of DNA were determined by the method of De Ley *et al.* (1970), using a model 250 spectrophotometer (Gilford Instruments). A DNA concentration of 80 µg ml⁻¹, a salt milieu containing 2 × SSC,

and a reassociation temperature of 68 °C were used. The reassociation temperature was calculated as proposed by Gillis *et al.* (1970). The DNA binding values (D%) were calculated according to the formula given by De Ley *et al.* (1970). Each experiment was repeated at least three times.

Determination of G + C content of the DNA. G + C contents of the DNA preparations were determined from thermal

Table 2. Origin and G + C content of strains investigated by DNA–DNA hybridization

Strain	CCUG no.	Taxon*	New name	Source	G + C mol % (SD)
NCTC 9380 ^T	12392	[<i>P.</i>] <i>haemolytica</i> biogroup 1	<i>M. haemolytica</i>	Ovine isolate	43.6 (0.1)
265	38454	[<i>P.</i>] <i>haemolytica</i> biogroup 1	<i>M. haemolytica</i>	Bovine pneumonia	–
UT26	38455	[<i>P.</i>] <i>haemolytica</i> biogroup 1	†	Ovine isolate	42.6 (0.1)
P731	38456	[<i>P.</i>] <i>haemolytica</i> biogroup 3A	<i>M. glucosida</i>	Ovine nose	43.2 (0.5)
P925 ^T	38457	[<i>P.</i>] <i>haemolytica</i> biogroup 3B	<i>M. glucosida</i>	Ovine lung	41.6 (0.1)
UT18	38458	[<i>P.</i>] <i>haemolytica</i> biogroup 3C	<i>M. glucosida</i>	Ovine isolate	41.8 (0.1)
P737	38459	[<i>P.</i>] <i>haemolytica</i> biogroup 3G	<i>M. glucosida</i>	Ovine nose	43.5 (0.2)
P733	38460	[<i>P.</i>] <i>haemolytica</i> biogroup 3H	<i>M. glucosida</i>	Ovine nose	42.7 (0.2)
W4672/1	38461	[<i>P.</i>] <i>haemolytica</i> biogroup 3J	<i>M. granulomatis</i>	Bovine tongue	–
177 ^T	38462	[<i>P.</i>] <i>haemolytica</i> biogroup 6	<i>M. varigena</i>	Bovine pneumonia	41.7 (0.1)
R19.2	38463	[<i>P.</i>] <i>haemolytica</i> biogroup 7	†	Bovine rumen	–
HPA102	38464	[<i>P.</i>] <i>haemolytica</i> biogroup 8A	†	Ovine rumen	40.0 (0.4)
M14.4	38465	[<i>P.</i>] <i>haemolytica</i> biogroup 8C	†	Bovine mouth	–
HPA98	38466	[<i>P.</i>] <i>haemolytica</i> biogroup 8D	<i>M. ruminalis</i>	Ovine rumen	–
P730	38467	[<i>P.</i>] <i>haemolytica</i> biogroup 9	<i>M. glucosida</i>	Ovine nose	43.9 (0.3)
HPA121	38468	[<i>P.</i>] <i>haemolytica</i> biogroup 10	†	Bovine rumen	40.2 (0.4)
UT27	38469	[<i>P.</i>] <i>haemolytica</i> biogroup 10	†	Ovine isolate	43.7 (0.2)
HPA92 ^T	38470	Bisgaard taxon 18 biovar 1	<i>M. ruminalis</i>	Ovine rumen	–
HPA109	38471	Bisgaard taxon 18 biovar 3, <i>xylose</i> [–]	<i>M. ruminalis</i>	Ovine rumen	–
P655	38472	Bisgaard taxon 15 biovar 1	<i>M. varigena</i>	Porcine sepsis	41.4 (0.1)
P411	38473	Bisgaard taxon 20 biovar 1	<i>M. granulomatis</i>	Rabbit isolate	40.8 (0.5)
Ph13	26828	Bisgaard taxon 20 biovar 1	<i>M. granulomatis</i>	Hare lung	–
BJ1680.3	38474	Bisgaard taxon 20 biovar 2	<i>M. granulomatis</i>	Hare isolate	–
H39	38475	Bisgaard taxon 36	<i>M. varigena</i>	Bovine mastitis	–
ATCC 49244 ^T	–	<i>P. granulomatis</i>	<i>M. granulomatis</i>	Bovine panniculitis	39.2 (2.1)
NCTC 11408 ^T	12396	<i>A. capsulatus</i>		Hare arthritis	–
NCTC 10624	–	[<i>P.</i>] <i>trehalosi</i> serotype T15		Ovine isolate	42.6

* Taxa according to Angen *et al.* (1997a, b).† Unnamed species in *Mannheimia*.**Table 3.** Oligonucleotide primers used for PCR amplification and sequencing of 16S rRNA

Primer no.	Type	Sequence (5' → 3')*	Position†	Orientation
1	PCR biotinylated	B-AGAGTTTGATYMTGGCT	8–24	Forward
2	PCR	TACGGYTACCTTGTTACGACT	1493–1513	Reverse
3	Sequencing	F-AGAGTTTGATYMTGGCT	8–24	Forward
4	Sequencing	F-ACTGCTGCCTCCCGT	344–358	Reverse
5	Sequencing	F-ACGGGAGGCAGCAGT	344–358	Forward
6	Sequencing	F-CTACCAGGGTATCTAATC	786–803	Reverse
7	Sequencing	F-GATTAGATACCTGGTAG	786–803	Forward
8	Sequencing	F-GGTTGCGCTCGTTGCGGG	1096–1113	Reverse
9	Sequencing	F-CCCGCAACGAGCGCAACC	1096–1113	Forward
10	Sequencing	F-TACGGYTACCTTGTTACGACT	1493–1513	Reverse

* Y, C or T; M, A or C; B, biotin; F, fluorescein.

† Numbers according to *E. coli* positions (Brosius *et al.*, 1978).

denaturation midpoints (T_m) (Marmur & Doty, 1962) and were calculated according to De Ley *et al.* (1970).

Analysis of hybridization data. Single linkage clustering (Sneath & Sokal, 1973) was performed on the matrix of D-values using the taxonomy program NTSYS (Rohlf, 1993).

Comparison of 16S rRNA sequence similarities and DNA binding levels. For 78 pairs of strains both DNA reassociation values and 16S rRNA sequences were available. A plot of the sequence similarities [Jukes & Cantor (1969) corrected] against the DNA binding levels and

standard deviations was performed using Fig.P version 2.7 (BIOSOFT).

RESULTS

Phylogenetic analysis by 16S rRNA comparison

The result of the maximum-likelihood analysis of the 16S rRNA sequences is shown in Fig. 1. The trehalose-

negative [*P.*] *haemolytica* complex formed five distinct, but affiliated clusters, indicated by numerals I–V. The mean sequence differences within each cluster ranged from 0.9 to 2.5% (Table 4). The mean sequence differences between the five clusters ranged from 2.8 to 4.4%, clusters I + II (2.9%) and IV + V (2.8%) being the most affiliated ones.

The significance of the phylogenetic analysis was

Fig. 1. Phylogenetic relationships within the [*P.*] *haemolytica* complex as revealed by maximum-likelihood analysis of 16S rRNA sequences. Five major clusters are indicated as well as the five proposed species within *Mannheimia*. The boundaries of the new species are only indicative as some of the strains (PH152, PH290, PH344, PH360) have only been investigated by 16S rRNA sequencing. Bg, Biogroup of [*P.*] *haemolytica*; Tax, Bisgaard taxon; St, serotype; UG, untypable group; A, sequence representing serotypes 1, 5–9, 12–14, 16 and UG1. Numbers adjacent to branches indicate how often (out of 100 repeats) the clusters and strains branched together in bootstrap analyses.

Table 4. Mean 16S rRNA sequence differences within and between 16S rRNA clusters of the trehalose-negative [*P.*] *haemolytica* complex

16S rRNA clusters are according to Fig. 1. Values are Jukes–Cantor corrected distances as % ($\pm$ SD).

Cluster	I	II	I + II	III	IV	V
I	1.4 ($\pm$ 0.5)					
II	2.9 ($\pm$ 1.2)	2.2 ($\pm$ 1.3)				
I + II	–	–	2.0 ($\pm$ 1.2)			
III	3.6 ($\pm$ 0.4)	4.4 ($\pm$ 1.1)	3.7 ($\pm$ 0.6)	2.5 ($\pm$ 0.3)		
IV	4.3 ($\pm$ 0.4)	4.2 ($\pm$ 1.0)	4.2 ($\pm$ 0.6)	3.7 ($\pm$ 0.4)	1.6 ($\pm$ 0.9)	
V	3.7 ($\pm$ 0.3)	3.8 ($\pm$ 1.0)	3.8 ($\pm$ 0.6)	3.6 ($\pm$ 0.2)	2.8 ($\pm$ 0.4)	0.9 ($\pm$ 0.4)

Fig. 2. Phylogenetic relationships between the five clusters representing the [*P.*] *haemolytica* complex and selected reference strains of the family *Pasteurellaceae* as revealed by maximum-likelihood analysis of 16S rRNA sequences.

assessed by bootstrap analysis. The numbers above the branches in Fig. 1 indicate how often (in %) the clusters were found as monophyletic groups. Clusters II–V were found as monophyletic in more than 80 % of the repeats, whereas the sequences in cluster I only clustered together in 39 % of the repeats. Three pairs of sequences within cluster I clustered together in more than 50 % of the repeats (indicated in Fig. 1).

Cluster I contained the type strain of [*P.*] *haemolytica* (biogroup 1). The sequence found was identical to the sequence found by Davies *et al.* (1996), but differed at 18 positions from a GenBank sequence having the same accession number and downloaded in 1995 (14 of these differences due to Ns). The closest affiliated sequence to that of the type strain was sequence U57066 (designated A in Fig. 1) which was common for serotypes 1, 5–9, 12–14, 16 and untypable group 1 (UG1), differing in two nucleotide positions. These two sequences clustered together in 71 % of the bootstrap trees. The other strains found within cluster I represent biogroups 3A, 3B, 3C, 3G, 3H, 8A and 9 of

[*P.*] *haemolytica* (Angen *et al.*, 1997a) and Davies' sequences representing UG3 and serotype 11 (Davies *et al.*, 1996). From Fig. 1 it can be seen that although the different sub-groups of biogroup 3 are found within the same 16S rRNA cluster, their sequences differ considerably, and they do not constitute a separate phylogenetic branch in the tree. Furthermore, they do not form a separate branch in any of the 100 bootstrap analyses performed. Cluster II consisted of four strains representing biogroup 8D of [*P.*] *haemolytica*, Bisgaard taxon 18 biovar 1 and a strain of biogroup 1 (UT26). Cluster III contained representatives of *P. granulomatis* and Bisgaard taxon 20. Cluster IV consisted of strains representing Bisgaard taxon 15 and biogroup 6 of [*P.*] *haemolytica*, in addition to representatives of Davies' untypable group 4 (UG4). One UG4 strain had a 16S rRNA sequence identical to strain 177 of biogroup 6. Cluster V contained representatives of biogroups 7 and 10 of [*P.*] *haemolytica* as well as a strain representing Davies' untypable group 5 (UG5).

A maximum-likelihood analysis including selected reference strains within the family *Pasteurellaceae* is shown in Fig. 2. The five clusters of [*P.*] *haemolytica* had a common root relative to all these strains. In the bootstrap analysis the five clusters were found as monophyletic in 33 % of the repeats. The five clusters including *Actinobacillus lignieresii* had a common branching relative to the other taxa investigated in 45 % of the repeats. [*P.*] *trehalosi* joined this group in 28 % of the repeats, and *Pasteurella multocida* in 13 % of the repeats. The mean distances between strains of the trehalose-negative [*P.*] *haemolytica* complex and the selected reference strains are shown in Table 5. The trehalose-negative [*P.*] *haemolytica* complex is most closely related to genus *Actinobacillus* as evaluated by the distances to the type species of the three old genera. The mean sequence difference between all pairs of sequences within all five clusters was 3.1 % (SD = 1.3). Based on the type strains of the species belonging to the genera *Actinobacillus* and *Pasteurella* as defined by DNA–DNA hybridizations (Mutters *et al.*, 1989) the mean 16S rRNA sequence difference within genus *Actinobacillus sensu stricto* was 5.0 % (SD = 2.3) and

Table 5. Mean differences of 16S rRNA sequences between the trehalose-negative [*P.*] *haemolytica* complex and reference strains within the family *Pasteurellaceae*

	[<i>P.</i>] <i>haemolytica</i> complex*	[<i>P.</i>] <i>trehalosi</i> †	<i>A. lignieresii</i> ^T	<i>P. multocida</i> ^T
[<i>P.</i>] <i>haemolytica</i> complex	3.1 (±1.3)			
<i>P. trehalosi</i> †	5.0 (±0.6)	0.9 (±0.4)		
<i>A. lignieresii</i> ^T	5.5 (±0.6)	6.3 (±0.1)	–	
<i>P. multocida</i> ^T	7.5 (±0.7)	6.0 (±0.1)	7.5	–
<i>H. influenzae</i> ^T	7.7 (±0.5)	7.2 (±0.2)	8.0	6.5

* Including all 16S rRNA clusters according to Figs 1 and 2.

† Including representatives of the four serotypes, NCTC 11550, NCTC 10641, NCTC 10624 and NCTC 10626.

Table 6. Matrix presentation of performed DNA-DNA hybridizations (D-values $\pm$ sd) between representatives of the trehalose-negative [*P.*] *haemolytica* complex and selected reference strains within the family *Pasteurellaceae*

Strain	Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1 NCTC 9380 ^T	[<i>P.</i>] <i>haem.</i> biogroup 1	100																										
2 265	[<i>P.</i>] <i>haem.</i> biogroup 1	91(7)	100																									
3 UT26	[<i>P.</i>] <i>haem.</i> biogroup 1	52(11)		100																								
4 P731	[<i>P.</i>] <i>haem.</i> biogroup 3A	71(7)		58(3)	100																							
5 P925 ^T	[<i>P.</i>] <i>haem.</i> biogroup 3B	63(5)			82(10)	100																						
6 UT18	[<i>P.</i>] <i>haem.</i> biogroup 3C	71(7)			86(7)	88(3)	100																					
7 P737	[<i>P.</i>] <i>haem.</i> biogroup 3G	54(8)		71(7)	64(8)		90(8)	100																				
8 P733	[<i>P.</i>] <i>haem.</i> biogroup 3H	69(9)		56(14)	44(8)	81(1)	95(5)	64(8)	100																			
9 W4672/1	[<i>P.</i>] <i>haem.</i> biogroup 3J								100																			
10 177 ^T	[<i>P.</i>] <i>haem.</i> biogroup 6	34(19)			24(13)			32(6)	27(1)		100																	
11 R19.2	[<i>P.</i>] <i>haem.</i> biogroup 7	35(15)									100																	
12 HPA102	[<i>P.</i>] <i>haem.</i> biogroup 8A	68(11)	78(5)				79(5)	74(4)	60(2)			100																
13 M14.4	[<i>P.</i>] <i>haem.</i> biogroup 8C	34(10)											100															
14 HPA98	[<i>P.</i>] <i>haem.</i> biogroup 8D	50(1)		83(3)			66(4)								100													
15 P730	[<i>P.</i>] <i>haem.</i> biogroup 9	29(9)		52(10)	62(6)	75(5)	86(5)	54(9)	55(9)		31(8)	82(3)				100												
16 HPA121	[<i>P.</i>] <i>haem.</i> biogroup 10	30(15)			25(7)			29(3)	26(8)		38(6)	68(4)		72(3)		18(17)	100											
17 UT27	[<i>P.</i>] <i>haem.</i> biogroup 10	42(4)		84(8)													9(13)	100										
18 P655	Taxon 15 biovar 1	0(10)		41(4)	18(7)			39	27		85(6)					27(9)			100									
19 HPA92 ^T	Taxon 18 biovar 1	63(3)		73(4)	60(7)		62(6)	64(5)	57(12)				52(1)		88(7)	59(7)			42(6)	100								
20 HPA109	Taxon 18 biovar 3 <i>xyf</i> ^T	66(1)																	19(11)		92(5)	100						
21 P411	Taxon 20 biovar 1	20(11)											35(3)									100						
22 P613	Taxon 20 biovar 1																						100					
23 BJ1680.3	Taxon 20 biovar 2																							100				
24 H39	Taxon 36										88(7)														100			
25 ATCC 49244 ^T	<i>P. granulosus</i>	32(12)		34(6)									34(5)								34(2)		81(6)	64(4)	96(6)		100	
26 NCTC 10624	[<i>P.</i>] <i>trehalosi</i>	18		28(4)				9(9)	0				15(6)			35			11		35(1)	18(7)					100	
27 NCTC 11408 ^T	<i>A. capsulatus</i>	21(11)																			27(4)	24(10)				34(8)		100

Fig. 3. Single-linkage clustering of DNA binding values within the [*P.*] *haemolytica* complex. Brackets indicate strains which cluster above 85% DNA binding. Area at 80–85% DNA binding is indicated by the box.

within genus *Pasteurella sensu stricto* was 7.0% (SD = 2.5). For comparison, the mean sequence difference between the five new species proposed in the present investigation was 2.7% (SD = 0.8). The mean sequence difference between one representative from each of the five 16S rRNA clusters (NCTC 9380^T, HPA 92^T, ATCC 49244^T, 177^T and HPA 121) was 3.4% (SD = 0.6).

G + C content

The G + C contents of the strains investigated are shown in Table 2. The values for the [*P.*] *haemolytica* complex ranged from 39.2–43.9 mol%. A correlation between the G + C content and the clusters outlined through the 16S rRNA sequence analysis was not observed.

DNA–DNA hybridizations

DNA–DNA hybridizations have been performed between 102 pairs of strains. The DNA binding values (D%) and the standard deviations of the experiments are presented as a matrix in Table 6. A dendrogram based upon single linkage clustering of the D-values is shown in Fig. 3.

The type strain of [*P.*] *haemolytica* (NCTC 9380^T) clustered above 70% with strains representing biogroups 3A, 3B, 3C, 3G, 3H, 8A, 9 and 10 of [*P.*] *haemolytica*, and strains representing biovars 1 and 3 of Bisgaard taxon 18. A DNA binding of 52% was observed with strain UT26, which shares the phenotypical characters of biogroup 1 (Angen *et al.*, 1997a).

NCTC 9380^T showed DNA binding of 91% with strain 265 from biogroup 1. Strain 265 was the most deviating strain within the ribotype cluster containing the type strain (Angen *et al.*, 1997c).

The strains representing biogroups 3A, 3B, 3C, 3G, 3H and 9 of [*P.*] *haemolytica* clustered above 85% by single linkage. Strain UT18 (biogroup 3C) showed DNA bindings at or above 86% with all other strains in this group against which it was tested. On the other hand, strains P731 (biogroup 3A) and P733 (biogroup 3H) only showed DNA binding of 44%. The group as such clustered with the type strain at a DNA binding level of 71%. Biogroup 8A (strain HPA102) showed high affiliation to this group with DNA binding values in the range 52–79%. All strains investigated in 16S rRNA cluster I (NCTC 9380^T, P731, P925, UT18, P733, P737, P730 and HPA102) were linked at DNA binding of 78%.

The two strains representing Bisgaard taxon 18 biovars 1 and 3 showed DNA binding of 92% and clustered with [*P.*] *haemolytica* biogroup 8D at 88%. Strain UT26 (biogroup 1) showed DNA binding of 83% with strain HPA98 ([*P.*] *haemolytica* biogroup 8D) and 84% with strain UT27 ([*P.*] *haemolytica* biogroup 10), respectively. All strains in 16S rRNA cluster II investigated (UT26, HPA92 and HPA98) were linked at DNA binding of 83%. The strains of 16S rRNA clusters I and II were linked at a DNA binding level of 70%.

The type strain of *P. granulomatis* and three strains representing Bisgaard taxon 20 biovars 1 (Ph13 and P411) and 2 (BJ1680.3) linked at a DNA binding level

Fig. 4. Plot of corresponding 16S rRNA sequence similarities and DNA binding values for 78 pairs of strains within the [*P.*] *haemolytica* complex. Vertical lines indicate the standard deviations of the DNA reassociation experiments.

of 86%. Additionally, 100% DNA binding was found between strain W4672/1 representing [*P.*] *haemolytica* biogroup 3J and *P. granulomatis*. This group represents taxa found in the 16S rRNA cluster III.

[*P.*] *haemolytica* biogroup 6, Bisgaard taxon 15 biovar 1 and Bisgaard taxon 36 were linked at 85% DNA binding. These taxa corresponded to those found in 16S rRNA cluster IV.

Three strains representing [*P.*] *haemolytica* biogroups 7, 8C and 10 showed DNA binding in the range 68–72%. Two of these strains (R19.2 and HPA121) were also investigated by 16S rRNA sequencing and were located in cluster V. Biogroup 10 of [*P.*] *haemolytica* was, however, genetically heterogeneous as DNA binding of only 9% was found between strains HPA121 and UT27.

Strains representing 16S rRNA clusters I + II, III, IV and V showed DNA binding at or below 42%. Approximately the same binding level was observed between representatives of these clusters and [*P.*] *trehalosi* and *A. capsulatus*.

Correlation between DNA binding levels and 16S rRNA sequence similarity

Fig. 4 shows a plot of 16S rRNA sequence similarities and the corresponding DNA binding levels between 78 pairs of strains. Two groups could be outlined in the plot. All pairs of strains having a sequence similarity below 97% had DNA binding of less than 40%. The separation between these groups was less distinct when the standard deviation of the DNA reassociations was considered (Fig. 4). A positive correlation between the 16S rRNA sequences similarity and DNA binding value was observed. Using linear regression, the correlation coefficient was 0.54. A plot of the sequence similarities against the DNA binding levels obtained

by single-linkage clustering separated the points of the plot in two clear groups as all y-values between 40 and 68% disappeared (data not shown), corresponding to the way the DNA binding data are presented in Fig. 3.

DISCUSSION

The present investigation aims at elucidating the taxonomic structure of the [*P.*] *haemolytica* complex by use of a polyphasic approach. Initially, a variety of strains received as [*P.*] *haemolytica*, *A. lignieresii* or as [*P.*] *haemolytica*-like from different parts of the world were characterized phenotypically. The test results were recorded in a quantitative way and the clusters outlined (Angen *et al.*, 1997a, b) showed a clear correlation with clusters found by ribotyping and MEE (Angen *et al.*, 1997c). In conjunction with the results of the present investigation, this gives a coherent and complementary picture of the taxonomic structure within the [*P.*] *haemolytica* complex. A summary of the results is given in Table 7. The clusters outlined through 16S rRNA analysis were almost identical to the lineages previously identified using MEE and ribotyping (Angen *et al.*, 1997c). Cluster I corresponds to MEE lineage A, cluster II to lineage B, cluster III to lineage F, cluster IV to lineage C, while cluster V corresponds to lineages D and E. Furthermore, DNA–DNA hybridizations have confirmed that taxa located in the different clusters (according to ribotyping, MEE or 16S rRNA sequencing) have high genetic affiliation. The overall correspondence noticed between results obtained using different methods allow us to draw conclusions from the polyphasic taxonomic investigation.

Taxonomic position within the family Pasteurellaceae

Maximum-likelihood analysis including the 16S rRNA sequences of the type strains of *A. lignieresii*, *P. multocida*, *H. influenzae*, [*P.*] *trehalosi* and *A. capsulatus* showed that these organisms were only distantly related to the trehalose-negative [*P.*] *haemolytica* complex (Fig. 2) underlining that a close affiliation to any of the recognized genera within the family Pasteurellaceae does not exist. The [*P.*] *haemolytica* complex, however, showed a higher affiliation to genus *Actinobacillus* than to genus *Pasteurella*, which is in accordance with previous investigations (Mraz, 1969; Mannheim, 1983; Mutters *et al.*, 1986; Dewhurst *et al.*, 1993; Davies *et al.*, 1996).

The trehalose-negative strains of the [*P.*] *haemolytica* complex which have been dealt with in the present investigation are clearly different from trehalose-positive, but phenotypically affiliated taxa ([*P.*] *trehalosi* and *A. capsulatus*) both by 16S rRNA sequencing and DNA–DNA hybridizations. Previously performed DNA hybridizations have indicated that [*Pasteurella*] *testudinis* might represent a genetic link between [*P.*] *trehalosi* and the trehalose-negative [*P.*] *haemolytica* complex (Mutters *et al.*,

Table 7. Summary of results from a polyphasic investigation of the taxonomic structure within the trehalose-negative [*Pasteurella*] *haemolytica* complex reclassified as *Mannheimia* gen. nov.

Taxa are given according to Angen *et al.* (1997a, b). Ribotype cluster and MEE lineage according to Angen *et al.* (1997c). 16S rRNA cluster according to Fig. 1. According to Fig. 3, a DNA homology group is defined as strains clustering at a DNA binding level $\geq 85\%$ by single linkage, thereby representing the same species; two groups of strains clustering above 60% are boxed. UC, Unclustered; –, not investigated.

Taxon	Ribotype cluster	MEE lineage	16S rRNA cluster	DNA homology group
[<i>P.</i>] <i>haemolytica</i> biogroup 1	1	A1	I	<i>M. haemolytica</i>
[<i>P.</i>] <i>haemolytica</i> biogroup 3A–3H and 9	4–11	A3–A6	I	<i>M. glucosida</i>
[<i>P.</i>] <i>haemolytica</i> biogroup 8A	2	A2	I	UC
[<i>P.</i>] <i>haemolytica</i> biogroup 8D	12	B1	II	<i>M. ruminalis</i>
Bisgaard taxon 18 biovars 1 and 3	13 + 14	B*	II*	<i>M. ruminalis</i>
[<i>P.</i>] <i>haemolytica</i> biogroup 1 (strain UT26)	UC	B	II	UC
[<i>P.</i>] <i>haemolytica</i> biogroup 10†	UC	B	–	UC
Bisgaard taxon 15 biovars 1 and 2	15 + 16	C1 + C2‡	IV‡	<i>M. varigena</i> ‡
[<i>P.</i>] <i>haemolytica</i> biogroup 6	15	C1 + C3	IV	<i>M. varigena</i>
Bisgaard taxon 36	15	–	–	<i>M. varigena</i>
Bisgaard taxon 20 biovars 1 and 2	18 + 19	F1 + F2	III§	<i>M. granulomatis</i>
[<i>P.</i>] <i>haemolytica</i> biogroup 3J	3 + 19	–	–	<i>M. granulomatis</i>
<i>Pasteurella granulomatis</i>	3	F3	III	<i>M. granulomatis</i>
[<i>P.</i>] <i>haemolytica</i> biogroup 10	17	D + E	V	UC
[<i>P.</i>] <i>haemolytica</i> biogroup 7	17	D	V	UC
[<i>P.</i>] <i>haemolytica</i> biogroup 8C	17	D	–	UC
[<i>P.</i>] <i>haemolytica</i> biogroup 8B and 9	17	D	–	–

* Only taxon 18 biovar 1 investigated.

† 3 out of the 10 investigated strains from biogroup 10 of [*P.*] *haemolytica* found in these clusters.

‡ Only taxon 15 biovar 1 investigated.

§ Only taxon 20 biovar 1 investigated.

|| *meso*-Inositol and NPG-negative strains of biogroup 9.

1986). 16S rRNA sequencing has, however, not confirmed this observation (Dewhirst *et al.*, 1993). Further studies on the taxonomic relationships among these taxa are clearly indicated.

Presently great uncertainty seems to exist as to the taxonomic structure within the family *Pasteurellaceae* (Bisgaard, 1995). The delineation of the genera *Pasteurella sensu stricto*, *Actinobacillus sensu stricto* and *Haemophilus sensu stricto* has been based on DNA binding values, species showing more than 50% DNA binding having been included in the same genus. In 1989, 12 species were listed as *Pasteurella sensu stricto* while 10 species were listed as *Actinobacillus sensu stricto* and five species as *Haemophilus sensu stricto* (Mutters *et al.*, 1989). According to the International Committee on Systematic Bacteriology Ad hoc committee on approaches to taxonomy within the *Proteobacteria*, genera should consist of taxa having a common phylogenetic origin and they should also be identified by phenotypic characters (Murray *et al.*, 1990). Phenotypic separation of genera within the family *Pasteurellaceae* has, however, been difficult under the present conditions where the composition of

genera has been based upon DNA–DNA hybridizations (Mutters *et al.*, 1989). In recent publications new species within the family *Pasteurellaceae* tend to be allocated to *Actinobacillus* or *Pasteurella* more or less arbitrarily (Bisgaard, 1995), making phenotypic delineation between the genera increasingly difficult. Analysis of 16S rRNA sequences within the family (Dewhirst *et al.*, 1993) further complicated the taxonomic situation, as it was shown that the three genera delineated by DNA–DNA hybridization did not seem to represent separate phylogenetic lineages.

According to Dewhirst *et al.* (1993) accepted species of *Pasteurella sensu stricto* were located in three different 16S rRNA clusters: 3B (six species, including the type species *P. multocida*), 3A (four species) and 3D (two species). The 16S rRNA sequences of accepted *Pasteurella* spp. within clusters 3A, 3B and 3D differ on average by 2.4 (SD = 0.5), 4.4 (SD = 1.6) and 10%, respectively. Cluster 3A only contains taxa isolated from avian species whereas most of the taxa found in cluster 3B are isolated from mammals. However, phenotypic separation of clusters 3A and 3B is not

Table 8. Phenotypic characters separating the genera within the family *Pasteurellaceae*

Mannheimia results refer to Table 9. *Actinobacillus* data based on type strains of *A. equuli*, *A. suis*, *A. ureae*, *A. pleuropneumoniae*, *A. hominis*, *A. lignieresii* and Bisgaard taxon 8, 9 and 11. *Pasteurella* data based on type strains of *P. multocida*, *P. dagmatis*, *P. stomatis*, *P. canis*, *P. avium* and *Pasteurella* sp. B. *Haemophilus* data based on *H. influenzae*, *H. aegyptius*, *H. intermedius* and [*H.*] *haemolyticus*. *Lonepinella* data according to Osawa *et al.* (1995).
+, Only positive reactions; —, only negative reactions; d, + or —.

Test	<i>Mannheimia</i>	<i>Actinobacillus</i>	<i>Pasteurella</i>	<i>Haemophilus</i>	<i>Lonepinella</i>
Haemolysis	d	d	—	d	—
V-factor dependency	—	d	d	+	—
X-factor dependency	—	—	—	+	—
VP, 37 °C	—	—	—	—	+
Urease	—	+	d	d	—
Ornithine decarboxylase	d	—	d	d	—
Indole	—*	—	+†	d	—
L-Arabinose	d	d	—	—	d
Glucosides‡	d	d	—	—	d
Mannitol	+	d	d	—	—
D-Mannose	—	d	+	d	Not known
D-Melibiose	—*	d	—	—	d
meso-Inositol	d	—	—	—	—
D-Sorbitol	d	—§	d	—	Not known
Trehalose	—	d	d	—	Not known
ONPF (α-fucosidase)	d	—	—	—	Not known

* Deviating strains occur.

† *P. avium* indole-negative.

‡ Aesculin, amygdalin, arbutin, cellobiose, gentiobiose, salicin and NPG (α-glucosidase).

§ Bisgaard taxon 9 late positive.

easy. All members of cluster 3A are indole-negative, whereas the members of cluster 3B with the exception of *Pasteurella avium* biovar 2 (isolated from calf pneumonia) are indole-positive. Furthermore, the two biovars of *P. avium* are found in clusters 3A and 3B, respectively, even though DNA–DNA hybridizations have shown 88% DNA binding between the two biovars (Mutters *et al.*, 1985). However, only a single strain from *P. avium* biovar 2 has been investigated by 16S rRNA sequencing and DNA–DNA hybridization. Considering the discrepancies observed, these results ought to be reinvestigated, including more strains from each biovar before taxonomic conclusions can be drawn.

Most of the species classified as *Actinobacillus sensu stricto* (Mutters *et al.*, 1989) were located in cluster 4A according to Dewhirst *et al.* (1993). The exceptions were *A. capsulatus* and Bisgaard taxon 5 which were located in clusters 3C and 4B, respectively. However, these species can phenotypically be separated from the other species in *Actinobacillus sensu stricto*. *Actinobacillus capsulatus* is sorbitol-positive, while Bisgaard taxon 5 ferments meso-inositol and is urease-negative. The mean sequence difference within genus *Actinobacillus sensu stricto* (as defined by DNA–DNA

hybridizations) is 5.0% (SD = 2.3), which is considerably lower than that found for *Pasteurella sensu stricto* (7.0%, SD = 2.5). By excluding *A. capsulatus* and Bisgaard taxon 5 the mean sequence difference within genus *Actinobacillus* is only 3.1% (SD = 0.7).

16S rRNA has not been sequenced for all members of *Haemophilus sensu stricto* (as defined by DNA–DNA hybridizations). Consequently, calculation of sequence similarities has not been performed for this genus.

Based upon a combination of chemotaxonomic and physiological properties and genetic characterization, including determination of G + C content of DNA and 16S rRNA analysis, a new genus and species of the family *Pasteurellaceae* Pohl 1981, *Lonepinella koalarum*, was proposed (Osawa *et al.*, 1995) and subsequently validated (Osawa *et al.*, 1996). Following the same lines and in agreement with current recommendations in defining genera (Murray *et al.*, 1990) it is possible to identify and separate the [*P.*] *haemolytica* complex from existing genera based upon 16S rRNA sequence analysis and the phenotypical characters shown in Table 8.

The [*P.*] *haemolytica* complex can be separated from genus *Pasteurella* by not producing acid from D-

mannose and from a redefined genus *Actinobacillus* (excluding Bisgaard taxon 5) by being urease-negative and from genus *Haemophilus* (including *H. influenzae*, *H. aegyptius*, [*H.*] *intermedius* and *H. haemolyticus*) by being mannitol-positive. A negative Voges–Proskauer reaction separates the [*P.*] *haemolytica* complex from the new genus *Lonepinella*. Additional characters for separation of genera are listed in Table 8.

The trehalose-negative [*P.*] *haemolytica* complex has a common root relative to other strains within the family *Pasteurellaceae* according to the maximum-likelihood analysis, indicating that they have a common phylogenetic origin. In the bootstrap analysis, however, the five clusters were monophyletic in only 33% of the repeats. Representatives of the five 16S rRNA clusters were also investigated by Davies *et al.* (1996) using the neighbour-joining method and a larger number of strains within *Pasteurellaceae*. The trehalose-negative [*P.*] *haemolytica* complex also formed a separate cluster in that analysis, increasing the confidence in the results obtained during the present investigation. In addition, the mean 16S rRNA sequence differences between representatives of the five clusters outlined for [*P.*] *haemolytica* in the present investigation was lower than that of the existing genera *Actinobacillus sensu stricto* and *Pasteurella sensu stricto*.

DNA binding levels of three of the five 16S rRNA clusters are lower than what was previously used to define genus boundaries within the family *Pasteurellaceae* (Mutters *et al.*, 1989). Only strains representing clusters I and II show DNA binding values above 50%. This might, however, be altered if additional isolates of [*P.*] *haemolytica*-like organisms are investigated. The correlation between DNA–DNA reassociation and 16S rRNA sequence analysis was discussed by Fox *et al.* (1992) and Stackebrandt & Goebel (1994). According to these investigations, organisms that have less than 97% sequence homology will not reassociate to more than 60%, no matter which hybridization method is used. Additionally, 16S rRNA sequencing was not found to be sufficient for a reliable species delineation. Using the spectrophotometric method, 85% DNA binding has been found to correspond to well-defined species within the family *Pasteurellaceae* (Mutters *et al.*, 1989). According to Fig. 4, no clear relationship could be observed between the DNA binding value used for species delineation and the corresponding 16S rRNA sequence similarities within the [*P.*] *haemolytica* complex. Strains having less than 97% sequence homology had DNA binding below 40% (Fig. 4). However, the standard deviations of the DNA hybridization experiments were quite high and made the separation of the two 'groups' less clear-cut. Using single-linkage clustering on DNA binding values exaggerates these differences. This is reflected in Fig. 3 where strains belonging to 16S rRNA clusters III–V and [*P.*] *trehalosi* apparently were equally distantly related to [*P.*] *haemolytica sensu stricto*. However, a realistic image of the true distances and interrelationships based on DNA hybridization is only

possible when a complete matrix of DNA binding values is available, which is unrealistic to obtain when a large number of strains is involved. Consequently, 16S rRNA sequencing has been found to be more appropriate for determining phylogenetic relationships above species level (Murray *et al.*, 1990). However, exact limits for genus delineation based upon 16S rRNA sequence similarity has not yet been proposed as these probably differ depending on which group of organisms is being investigated (Murray *et al.*, 1990).

In this investigation the present recommendations have been followed, relying on DNA binding values for species delineation (Wayne *et al.*, 1987) and 16S rRNA sequences for genus delineation (Murray *et al.*, 1990). Furthermore, we regard the presence or the absence of phenotypic coherency among taxa as a deciding factor in the delineation of both species and genera (Wayne *et al.*, 1987; Murray *et al.*, 1990). As already mentioned, the trehalose-negative [*P.*] *haemolytica* complex can be phenotypically separated from the other genera of the family. Furthermore, the strains within the five 16S rRNA clusters were found phenotypically affiliated to such an extent that phenotypic delineation between some of the clusters is almost impossible, e.g. between clusters I and III and clusters IV and V. Separation of the five clusters into different genera therefore conflicts with current recommendations (Murray *et al.*, 1990). A new genus, *Mannheimia* gen. nov., is therefore proposed for all taxa of the trehalose-negative [*P.*] *haemolytica* complex.

16S rRNA cluster I

[*P.*] *haemolytica* biogroup 1 ([*P.*] *haemolytica sensu stricto*) was found as a separate and genetically homogeneous group by all methods used. Biogroup 1 included the type strain of [*P.*] *haemolytica* and reference strains of serotypes 1, 2, 5–9, 12–14 and 16. The most divergent member of ribotype cluster 1 (strain 265) (Table 7) showed DNA binding of 91% with the type strain, indicating that all strains investigated from this biogroup belong to the same species. The only exception was strain UT26, which was found to deviate by all methods. It only showed DNA binding with the type strain of 52% and consequently represents a different species. However, none of the phenotypic tests used so far could separate this strain from the other strains of biogroup 1.

It should be noted that none of the biogroup 1 strains fermented L-arabinose (Table 9) even though they are referred to as 'biotype A'. For decades there has been contradictory reports as to whether or not [*P.*] *haemolytica* ferments L-arabinose. It is difficult to explain this simply as a result of differences in media and incubation conditions. We have found that if strains within the [*P.*] *haemolytica* complex ferment L-arabinose, 96.5% do so reproducibly within 2 d, the remaining strains being positive within 7 d. Negative strains are negative also after 14 d incubation (Angen

Table 9. Phenotypic separation of species and taxa within genus *Mannheimia*, corresponding to the trehalose-negative [*Pasteurella*] *haemolytica* complex

Tests were performed according to the methods of Angen *et al.* (1997a). Table is based upon strains investigated by ribotyping and MEE by Angen *et al.* (1997c). Previous classifications: *M. haemolytica* was [*P.*] *haemolytica* biogroup 1; *M. glucosida* was [*P.*] *haemolytica* biogroups 3 and 9; *M. granulomatis* was *P. granulomatis*, [*P.*] *haemolytica* biogroup 3J and Bisgaard taxon 20 biovars 1 and 2; *M. ruminalis* biovar 1 was Bisgaard taxon 18 biovars 1 and 3; *M. ruminalis* biovar 2 was [*P.*] *haemolytica* biogroup 8D; *M. varigena* biovar 1 was Bisgaard taxon 15/biovar 1, Bisgaard taxon 36 and [*P.*] *haemolytica* biogroup 6; *M. varigena* biovar 2 was Bisgaard taxon 15/biovar 2. +, Strong or delayed positive (for fermentations: positive d 1–7); (+), weak or very weak positive (for fermentations: positive 8–14 d); –, negative; d, + or –. Common characters for all biovars of the species *M. glucosida*, *M. ruminalis* and *M. varigena* are boxed. Origins: B, bovine; O, ovine; P, porcine; L, leprine; D, deer.

Test	Species/taxon...	<i>M. haemolytica</i>	<i>M. glucosida</i>										<i>M. granulomatis</i>		<i>M. ruminalis</i>		<i>M. varigena</i>		Unnamed taxa within <i>Mannheimia</i>					
			A	B	C	D	E	F	G	H	I		1	2	1	2	8A	8B	8C	7	9*	10†		
Biovar...																								
Haemolysis	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	–	–	–	d	d	(+)	(+)	(+)	d	(+)	d			
Omithine decarboxylase	–	–	+	+	+	+	–	–	–	+	–	–	–	+	–	–	–	–	–	+	–			
L-Arabinose	–	–	+	–	+	–	–	–	–	–	–	–	–	+	+	–	–	–	–	+	+			
D-Sorbitol	+	+	+	+	+	+	+	+	+	+	+	+	d	–	–	–	–	–	–	+	+			
D-Xylose	+(+)	+	+	+	+	+	+	+	+	+	d	d	–	+	+	+	–	+	+	+	+			
Maltose	+	+	+	+	+	+	+	+	+	+	d	–	+	d	d	+	+	+	+	+	+			
Dextrin	+	+	+	+	+	+	+	+	+	+	d	–	d	d	d	+	+(+)	+(+)	+(+)	+	d			
Glucosides	–	–	+	+	+	+	+	+	+	–	d	–	–	d¶	–	–	–	–	–	–	–			
Gentiobiose	–	–	+	+	–	+	+	+	+	–	d	–	–	–	–	+	–	–	–	–	–			
NPG (β-glucosidase)	–	–	+	+	+	+	+	+	+	+	+	–	–	d	d	–	–	–	–	–	–			
meso-Inositol	d	+	+(+)	+	+(+)	+(+)	+(+)	+(+)	+(+)	+	d	–	–	d	–	(+)	+	+	d	–	d			
ONPF (α-fucosidase)	+	+(+)	+(+)	+	+	+(+)	–	+(+)	+(+)	+	–	–	–	d	–	+	–	–	–	–	d			
ONPX (β-xylosidase)	d	+	+	–	–	–	–	–	–	d	(+)	–	–	–	+	–	d	–	d	–	d			
ONPG (β-galactosidase)	d	+	+	+	+	+	+	+	+	+	d	+	+	d	+	–	+	+	+	+	+			
Indole	–	–	–	–	–	–	–	–	–	–	–	–	–	d	–	–	+(+)	+(+)	d	–	(+)			
D-Melibiose	–	–	–	–	–	–	–	–	–	–	–	–	–	d	–	–	–	–	–	–	–			
Origin of isolates	BO	O	O	O	O	O	O	O	O	O	BLD	O	BO	BP	P	O	B	B	BO	B	BO			
No. of strains	30										28	12		51	5	2	3	7	3		8			

* *meso*-Inositol- and NPG-negative strains of [*P.*] *haemolytica* biogroup 9, the *meso*-inositol and NPG-positive strains reclassified as *M. glucosida* biovar 1.

† Consisting of two genetically distinct groups that could not be phenotypically separated.

‡ Arabinose-positive strains of Bisgaard taxon 18 exist but their taxonomic allocation is still undecided.

§ One strain negative.

|| Amygdalin, arbutin, aesculin (acid), cellobiose and salicin.

¶ Most strains negative for all glucosides; the strains that ferment glucosides also ferment D-melibiose.

et al., 1997a). Furthermore, we have shown that L-arabinose is a reliable taxonomic marker correlating with genetic groups identified by ribotyping, MEE, 16S rRNA sequencing and DNA–DNA hybridization. L-Arabinose-positive strains, however, exist within the [*P.*] *haemolytica* complex, but they are genetically remote from [*P.*] *haemolytica sensu stricto*. Reports on isolation of [*P.*] *haemolytica* strains fermenting L-arabinose might therefore represent one of the L-arabinose-positive taxa within the [*P.*] *haemolytica* complex (biogroups 3, 6, 7, 9 and 10). On the other hand, all reference 'biotype A' serotypes (except serotype 11) have been found to belong to biogroup 1, and it is at present an open and intriguing question whether these serotypes can be found within other taxa as well. Previous investigations have shown that some strains representing serotypes 11 and 14 might belong to biogroup 6 and some serotype 9 strains in biogroup 8 (Bisgaard & Mutters, 1986). A pig isolate belonging to serotype 8 has also been reported (Biberstein & Thompson, 1966). These observations question whether serotyping represents a reliable method for allocating strains to [*P.*] *haemolytica sensu stricto*. *Mannheimia haemolytica* comb. nov. is proposed as a new name for [*P.*] *haemolytica* biogroup 1.

Strains representing biogroups 3A–3H and 9 clustered above 85 % DNA binding, indicating that they should be regarded as biovars of the same species (Mutters *et al.*, 1989), for which the name *Mannheimia glucosida* sp. nov. is proposed. They represent, however, a quite heterogeneous group. The different biovars (see Table 9) have been found to correspond to related, but distinct, MEE and ribotype clusters (Angen *et al.*, 1997c), and in Fig. 1 considerable diversity in 16S rRNA sequences is reflected. The low values observed in the bootstrap analysis indicate that the branching pattern within cluster I is quite unstable and consequently should be interpreted with caution. Previous DNA–DNA hybridizations only included representatives of biogroups 3A, 3G, 3H and 9 (Mutters *et al.*, 1986), and their low DNA binding values indicated that they represented different species. However, by including strains representing additional phenotypic and genetic variation we found that biogroups 3B and 3C link the different sub-groups together as one DNA homology group. This underlines the importance of including strains representing as much as possible of the genetic variation and that analysis based upon a restricted data matrix might lead to biased conclusions. Mutters *et al.* (1986) reported that strain P733

(biogroup 3H) showed DNA binding of 85 % with the type strain of [*P.*] *haemolytica*. As this was in conflict with the taxonomic structure previously outlined through analysis of MEE and ribotype data (Angen *et al.*, 1997c), this hybridization was repeated. These investigations showed DNA binding of 69 % (mean of 10 experiments using two different DNA preparations), and we have confidence in this value due to the consistency obtained with the other methods applied.

Based upon 16S rRNA sequencing results, strains investigated by Davies *et al.* (1996) representing serotype 11 and untypable group 3 (UG3) probably belong to this DNA homology group as well. None of these strains had a sequence identical to strains that we investigated, but the UG3 strains are clearly affiliated to biogroups 3H and 9 according to Fig. 1. This is supported by the high values observed in the bootstrap analysis. All strains investigated within this putative DNA homology group have been isolated from sheep (except a bovine strain, UT23, from Davies' UG3), and the typable strains all belonged to serotype 11. Serotype 11 was suggested as a third taxon of [*P.*] *haemolytica* by Frederiksen (1973). More recent investigations have shown that serotype 11 differs from the other serotypes in a number of virulence factors (Murray *et al.*, 1992; Burrows *et al.*, 1993; Lee *et al.*, 1994), supporting its separation as a new species. Davies *et al.* (1997) investigated the population structure of [*P.*] *haemolytica* using MEE. Here a clear separation between serotype 11 and UG3 (by Davies *et al.* called the 'A11 taxon') and the other 'A' serotypes of [*P.*] *haemolytica* was demonstrated.

Biogroup 8A of [*P.*] *haemolytica* also seems to represent a new species which is highly affiliated to the aforementioned groups. According to phenotypic tests, ribotyping and MEE, biogroup 8A was the taxon showing the highest similarity to [*P.*] *haemolytica sensu stricto* (Angen *et al.*, 1997a, c). However, by 16S rRNA sequencing it was found most affiliated to biogroup 3. The strains of biogroup 8A have all been isolated from the rumen or mouth of sheep. It differs from biogroups 1 and 3 by not fermenting D-sorbitol and from biogroup 3 by not fermenting glucosides. Due to the limited number of strains isolated so far, no formal species name is proposed for this taxon.

16S rRNA cluster II

Biogroup 8D of [*P.*] *haemolytica*, Bisgaard taxon 18, three strains of biogroup 10 of [*P.*] *haemolytica* and strain UT26 of biogroup 1 showed high affiliations both by ribotyping and by MEE. Representatives of biogroup 8D (HPA98) and Bisgaard taxon 18 (HPA92 and HPA109) showed DNA binding at or above 88 %, which qualifies their allocation to the same species, for which the name *Mannheimia ruminantis* sp. nov. is proposed. The two strains representing Bisgaard taxon 18 were located in different clusters by ribotyping (Table 7). In these ribotype clusters also represen-

tatives of taxon 18 biovars 2 and 4 (both are arabinose-positive taxa) were loosely connected, indicating that all biovars of taxon 18 might belong to the same species. Additional DNA-DNA hybridizations including biovars 2 and 4 of taxon 18 should, however, be performed before firm conclusions can be drawn.

Strain UT26 which shares the phenotypical characters of biogroup 1 showed DNA binding of 83 % with *M. ruminantis* and DNA binding of 84 % with strain UT27 (biogroup 10 of [*P.*] *haemolytica*). The taxonomic allocation of these strains is still undecided. Strain UT27 was also investigated by Davies *et al.* (1996), who classified it in the untypable group 1 (UG1). Another UG1 strain was found by Davies *et al.* (1996) to have a 16S rRNA sequence identical to [*P.*] *haemolytica* biogroup 1. Our findings indicate that UG1 is genetically heterogeneous as strain UT27 only had DNA binding of 42 % with [*P.*] *haemolytica sensu stricto*.

16S rRNA cluster III

Bisgaard taxon 20 biovars 1 and 2, [*P.*] *haemolytica* biogroup 3J and *P. granulomatis* were linked above 85 % DNA binding (Fig. 3) and should consequently be regarded as the same species, *Mannheimia granulomatis* comb. nov. Taxon 20 represents strains isolated from conjunctivitis and bronchopneumonia in hares and rabbits (Devriese *et al.*, 1991), in addition to a single isolate from a deer. *P. granulomatis* represents strains isolated from skin granulomas in cattle in Brazil (Ribeiro *et al.*, 1989; Riet-Correa *et al.*, 1992). The taxonomic proximity between taxon 20 and *P. granulomatis* was supported by MEE (Table 7). In addition, ribotyping indicated that biogroup 3J of [*P.*] *haemolytica* (representing strains isolated from cattle and deer) was affiliated to both of these groups. This observation was confirmed by DNA-DNA hybridization.

16S rRNA cluster IV

16S rRNA cluster IV branched into two lines (Fig. 1). One branch included two strains representing Bisgaard taxon 15 while the other included biogroup 6 of [*P.*] *haemolytica* and Davies' untypable group 4 (UG4). UG4 and biogroup 6 obviously represent the same taxon as UG4 strain PH360 has a 16S rRNA sequence identical to strain 177 of biogroup 6, while the other UG4 strain (UT35) previously has been classified as biogroup 6 by Bisgaard & Mutters (1986). Both taxa had been isolated from the respiratory tract and intestines, taxon 15 from pigs and biogroup 6 from ruminants, mostly cattle. Some of the isolates have been associated with pneumonia and septic conditions. Previous investigations have shown that some of the taxon 15 strains have a ribotype identical with certain biogroup 6 strains (Angen *et al.*, 1997c). Furthermore, it is impossible to separate biogroup 6 of [*P.*] *haemolytica* and taxon 15 biovar 1 by the 79 phenotypic tests

used so far (Angen *et al.*, 1997a, b), the only difference between the two taxa being the species from which they were isolated. DNA–DNA hybridizations showed that biogroup 6 and Bisgaard taxon 15 biovar 1 had DNA binding of 85% which supports their inclusion in the same species for which the name *Mannheimia varigena* sp. nov. is proposed. Bisgaard taxon 36 had DNA binding of 88% to biogroup 6 thereby confirming the high genetic affiliation between these groups found by ribotyping (Angen *et al.*, 1997c).

Taxon 15 biovar 2 has not been investigated by 16S rRNA sequencing and DNA–DNA hybridization, but a previous investigation has shown that some strains from the two biovars of taxon 15 have identical ribotypes (Angen *et al.*, 1997c), indicating that Bisgaard taxon 15 biovar 2 also should be included in *M. varigena* and that differences in ornithine decarboxylase have no taxonomic significance within this species. However, phenotypic separation between these strains and biogroup 7 of [*P.*] *haemolytica* has not been possible (Table 9), the only difference being the origin of isolation.

16S rRNA cluster V

Cluster V included representatives of biogroups 7 and 10 of [*P.*] *haemolytica* and Davies' UG5. Correspondingly, DNA binding values between 68 and 72% were found between biogroups 7, 8C and 10. The taxonomic relatedness between these taxa is further supported by observations based upon ribotyping and MEE (Table 7). Biogroup 10 was found to be heterogeneous by DNA–DNA hybridization as strains UT27 and HPA121 only had DNA binding of 9%. The separation of biogroup 10 into two genetically distinct groups is in accordance with the results obtained by analysis of ribotypes and MEE (Angen *et al.*, 1997c). However, none of the phenotypic tests used so far have been able to separate these two groups. Furthermore, ribotyping and MEE indicated that biogroups 8B, 12 and the β -glucosidase and meso-inositol-negative strains of biogroup 9 are affiliated to biogroups 7 and 10. Further DNA–DNA hybridizations are necessary to draw conclusions about the taxonomic interrelationships between these groups, and new species are consequently not proposed for taxa within 16S rRNA cluster V at the present stage.

Description of *Mannheimia* gen. nov.

Mannheimia (Mann.heim'i.a. L. fem. n. *Mannheimia* named in tribute to Walter Mannheim, a German microbiologist whose research has improved our understanding of the taxonomy of the family *Pasteurellaceae*).

Mannheimia is a new genus within the family *Pasteurellaceae* Pohl (1979) 1981. Consists of Gram-negative, non-motile rods or coccobacilli. Endospores are not formed. Growth is mesophilic and facultatively

anaerobic or microaerophilic. Glucose is fermented without gas production. Oxidase reaction is normally positive but might be variable. Alkaline phosphatase test is positive and nitrate is reduced. Simmon's citrate test and arginine dihydrolase test are negative and there is no fermentation of adonitol or L-sorbose. All strains ferment mannitol. Urease reaction is negative, trehalose and D-mannose are not fermented. The phenotypic characters separating genus *Mannheimia* from the other genera of *Pasteurellaceae* are given in Table 8. The type species is *Mannheimia haemolytica* (*Pasteurella haemolytica* Newsom and Cross 1932).

Description of *Mannheimia haemolytica* comb. nov. (*Pasteurella haemolytica* Newsom and Cross 1932)

Mannheimia haemolytica (haem.o.ly'ti.ca. Gr. n. *haima* blood; Gr. *lyt-* adjectival form of verb *lyo* loosen, dissolve; Gr. adjectival suff. *-ikos* Latinized as *-icus*, fem. *-ica*; M.L. adj. *haemolytica* referring to the haemolysis seen on blood agar).

Corresponds to [*P.*] *haemolytica* biogroup 1 (Bisgaard & Mitters, 1986). Cells are Gram-negative, non-motile small rods and coccobacilli. Colonies are smooth and greyish on blood agar and are 1–2 mm in diameter after 24 h incubation. Most strains show a characteristic β -haemolysis on bovine blood agar. Biochemical characteristics important for characterization are listed in Table 9 and under the description of the genus *Mannheimia*. D-sorbitol, D-xylose, maltose and dextrine are fermented. No strains ferment L-arabinose or glucosides. Strains are negative for ornithine decarboxylase and NPG (β -glucosidase) and positive for ONPF (α -fucosidase). Isolated from pneumonia in cattle and sheep and from septicaemia in lambs and mastitis in ewes. Some of the serotypes are probably part of the resident microflora of the upper respiratory tract of ruminants (Frank, 1989; Gilmour & Gilmour, 1989). A total of 17 serotypes have been described within [*P.*] *haemolytica*, among which serotypes 3, 4, 10 and 15 now are classified as [*Pasteurella*] *trehalosi*. Representatives of serotypes 1, 2, 5, 6, 7, 8, 9, 12, 13, 14 and 16 are found in *Mannheimia haemolytica*. Serotype 11 is reclassified as *Mannheimia glucosida*. The type strain is NCTC 9380^T (= J. A. Watt 1266A&B^T = CCUG 12392^T) isolated from sheep in the UK in 1956. The type strain represents serotype 2, is ONPX- (β -xylosidase) and ONPG-negative and shows a late meso-inositol fermentation. The G + C content of the DNA of the type strain is 43.6 mol %.

Description of *Mannheimia granulomatis* comb. nov. (*Pasteurella granulomatis* Ribeiro *et al.* 1990)

Mannheimia granulomatis (gran.nu.lo'ma.tis. L. dim. n. *granulum* a small grain; Gr. suff. *-oma* a swelling or tumour; M.L. n. *granuloma* a granuloma; M.L. n. *granulomatis* of a granuloma).

Cells are Gram-negative, non-motile small rods and coccobacilli. Colonies are smooth and greyish on

blood agar and are 1–2 mm in diameter after 24 h incubation. No strains were haemolytic on bovine blood agar, but they have been reported as haemolytic using ovine blood (Ribeiro *et al.*, 1989). All strains are negative for ornithine decarboxylase and ONPF and positive for NPG and fermentation of D-sorbitol. Strains are variable in fermentation of D-xylose, maltose, dextrin, glucosides and *meso*-inositol. Further biochemical characteristics are listed in Table 9 and under the description of the genus *Mannheimia*. The species includes the following taxa: *Pasteurella granulomatis*, isolated from bovine panniculitis (Ribeiro *et al.*, 1989, 1990; Riet-Correa *et al.*, 1992), Bisgaard taxon 20 biovars 1 and 2 isolated from bronchopneumonia and conjunctivitis in leprine species and deer (Devriese *et al.*, 1991; Angen *et al.*, 1997b), and [*P.*] *haemolytica* biogroup 3J isolated from deer and the oral cavity of cattle (Angen *et al.*, 1997a). The type strain is ATCC 49244^T (= 26^T), isolated from bovine panniculitis in Brazil, previously classified as *Pasteurella granulomatis* (Ribeiro *et al.*, 1989). The type strain is ONPG-positive and ferments maltose, dextrin, glucosides and *meso*-inositol, but not D-xylose. The G + C content of the type strain is 39.2 mol %.

Description of *Mannheimia glucosida* sp. nov.

Mannheimia glucosida (glu.co'si.da. Gr. adj. *glukus* sweet; L. adj.-forming suff. *-os* rich in; L. adj.-forming suff. *-id(us)*, fem. *-ida*; M.L. adj. *glucosida* pertaining to glucosides, which are fermented by almost all biovars).

Corresponds to Frederiksen's third taxon of [*P.*] *haemolytica* (Frederiksen, 1973), classified as [*P.*] *haemolytica* biogroups 3 and 5 by Bisgaard & Mutters (1986) and reclassified as [*P.*] *haemolytica* biogroups 3A–3H by Angen *et al.* (1997a). The species also includes NPG and *meso*-inositol-positive strains of [*P.*] *haemolytica* biogroup 9 (Bisgaard & Mutters, 1986). *M. glucosida* is correspondingly listed as biovars A–I in Table 9. Cells are Gram-negative, non-motile small rods. Colonies are smooth and greyish on blood agar and are 1–2 mm in diameter after 24 h incubation. All strains are haemolytic on bovine blood agar. All strains ferment D-sorbitol, D-xylose, maltose and dextrin. ONPG and NPG tests are positive for all biovars. The biovars are separated by differences in ornithine decarboxylase, ONPX, ONPF and fermentation of L-arabinose and glucosides. Further biochemical characteristics are listed in Table 9 and under the description of the genus *Mannheimia*. Typable strains within *M. glucosida* all belong to serotype 11 (Angen *et al.*, 1999). All strains investigated have been isolated from sheep, although isolates of serotype 11 have been reported from cattle (Quirie *et al.*, 1986). This group of bacteria is normally not associated with disease conditions and probably represents part of the resident microflora in the upper respiratory tract (Biberstein & Gills, 1962). The type strain is P925^T (numbering according to SSI

Strain Collection = CCUG 38457^T), originally received as B558 from Moredun Research Institute. It was isolated from ovine lung in UK, and belongs to biovar B and serotype 11 (Angen *et al.*, 1997a). The G + C content of the type strain is 41.6 mol %.

Description of *Mannheimia ruminalis* sp. nov.

Mannheimia ruminalis (ru.mi.na'lis. L. n. *rumen* stomach, esp. first stomach of ruminants; L. adj.-forming suff. *-alis*; M.L. adj. *ruminalis* referring to ruminants, from which the bacteria have been isolated).

Cells are Gram-negative, non-motile, non-haemolytic small rods or coccobacilli. Colonies are smooth and greyish on blood agar and are 1–2 mm in diameter after 24 h incubation. All strains are negative for ornithine decarboxylase, NPG, ONPF and ONPX and do not ferment L-arabinose, *meso*-inositol or glucosides (Table 9). For further biochemical characteristics, reference is made to the description of the genus *Mannheimia*. *M. ruminalis* consists of two distinct phenotypic groups. Biovar 1 consists of strains which do not ferment maltose and dextrin and show variable reactions in fermentation of D-sorbitol and D-xylose and correspond to Bisgaard taxon 18 biovars 1 and 3 (Bisgaard *et al.*, 1986; Angen *et al.*, 1997b). Biovar 2 corresponds to [*P.*] *haemolytica* biogroup 8D (Angen *et al.*, 1997a). All strains have been isolated from the rumen of sheep or cattle and were not associated with disease conditions. The strains have previously been described as *A. lignieresii* (Phillips, 1961, 1964, 1966). The type strain is HPA92^T (numbering according to RVAU Strain Collection = CCUG 38470^T), originally isolated as strain S1/15/6 from rumen of sheep in UK by J. E. Phillips, previously classified as Bisgaard taxon 18 biovar 1 (Bisgaard *et al.*, 1986). The type strain belongs to biovar 1 and does not ferment D-sorbitol and D-xylose.

Description of *Mannheimia varigena* sp. nov.

Mannheimia varigena (va.ri'ge.na. L. adj. *varia* varied; L. adj.-forming element *-gena* originating from; L. adj. *varigena* of different origin, referring to the isolation of strains of this species both from ruminants and from pigs).

Cells are Gram-negative, non-motile, small rods or coccobacilli. Colonies are smooth and greyish on blood agar and are 1–2 mm in diameter after 24 h incubation. Most strains are haemolytic on bovine blood agar. All strains ferment L-arabinose and D-xylose but not D-sorbitol. Most strains do not ferment glucosides, those fermenting glucosides also ferment D-melibiose. Most strains are ornithine decarboxylase-positive (biovar 1) and correspond to [*P.*] *haemolytica* biogroup 6, Bisgaard taxon 36 and Bisgaard taxon 15 biovar 1. Ornithine decarboxylase-negative strains (biovar 2) were isolated from pigs and correspond to

Bisgaard taxon 15 biovar 2. Strains of *M. varigena* have been isolated from pneumonia, mastitis and septicaemia in cattle as well as from the oral cavity, rumen and intestines (Angen *et al.*, 1997a). Isolates from pigs have been associated with septicaemia, enteritis or pneumonia and have also been isolated from the upper respiratory tract (Bisgaard, 1984; McLaughlin *et al.*, 1991; Angen *et al.*, 1997b). The type strain is 177^T (= CCUG 38462^T), isolated from bovine pneumonia in Western Germany, previously classified as [*P.*] *haemolytica* biogroup 6 (Bisgaard & Mutters, 1986). This strain is indole-negative, ONPG and ONPF-positive, NPG-negative, ferments maltose and meso-inositol, but not dextrin. The G + C content of the DNA of the type strain is 41.7 mol %.

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