Genetic relationships among avian isolates classified as *Pasteurella haemolytica*, 'Actinobacillus salpingitidis' or *Pasteurella anatis* with proposal of *Gallibacterium anatis* gen. nov., comb. nov. and description of additional genomospecies within *Gallibacterium* gen. nov.

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Bacteria of the avian [*Pasteurella haemolytica*]–'Actinobacillus salpingitidis' complex have been associated with different pathological conditions in birds, among which salpingitis and peritonitis in chickens of layer type seem to dominate. The aim of this study was to classify these bacteria by comparison of 37 strains tentatively classified as biovars of the avian [*P. haemolytica*]–'A. salpingitidis' complex or as *Pasteurella anatis*. PFGE, AFLP and plasmid profiling showed that strains representing different biovars were genotypically different. Phylogenetic analysis of 22 strains characterized by 16S rRNA gene sequence comparison showed that strains classified as biovars 5, 8 and 9 were closely related to the suggested type strain of 'A. salpingitidis' (98.4–99.9 % similarity), whereas the remaining strains classified in 12 biovars or as *P. anatis* were closely related to the type strain of *P. anatis* (98.1–100 % similarity). The two groups were related at 95.7–97.1 % similarity. The closest similarity outside this group was 94.6 %, between biovar 15 and Bisgaard taxon 3. DNA–DNA hybridization was performed with 34 strains and showed binding above 85 % for strains of biovars 5 and 8, including the suggested type strain of 'A. salpingitidis'. Two strains of *P. anatis* (F 149^T and F 279) were closely related at 79 % DNA binding to 27 strains of biovars 1, 3, 4, 11, 12, 17–20, 22 and 24. A new genus, *Gallibacterium* gen. nov., is proposed to include the avian [*P. haemolytica*]–'A. salpingitidis'–*P. anatis* complex, since these taxa form a monophyletic unit with similarities above 95 % on the basis of 16S rRNA sequence comparison and they are unrelated to other genera of the family *Pasteurellaceae* Pohl 1981. The new genus consists of Gram-negative, non-motile, rod-shaped or pleomorphic bacteria. The bacteria are catalase-, oxidase- and phosphatase-positive. Nitrate is reduced and acid is produced without gas formation from glycerol, (–)D-ribose, (+)D-xylose, (–)D-mannitol, (–)D-fructose, (+)D-galactose, (+)D-glucose, (+)D-mannose, sucrose and raffinose. The genus *Gallibacterium* can be separated from other genera of *Pasteurellaceae* by differences in catalase, symbiotic growth, haemolysis, urease, indole, acid production from (+)D-xylose, (–)D-mannitol, (–)D-sorbitol, (+)D-mannose, maltose, raffinose and dextrin and ONPG and PNPG tests. *Pasteurella anatis* Mitters *et al.* 1985 is transferred to the new genus as *Gallibacterium anatis* gen. nov., comb. nov. Genomospecies 1 of *Gallibacterium* is proposed to include the former biovars 5 and 8 of the avian [*P. haemolytica*]–'A. salpingitidis' complex. The type strain of *Gallibacterium anatis* is F 149^T (= ATCC 43329^T = NCTC 11413^T) and the reference strain of *Gallibacterium* genomospecies 1 is CCM 5974.

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Abbreviation: ITS, internal transcribed spacer.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences determined in this investigation are AF228001–AF228018, AY038519 and AY038592–AY038594, as listed in Table 1.

A DNA–DNA binding matrix for the strains included in this paper is available as supplementary material in IJSEM Online (<http://ijs.sgmjournals.org/>).

INTRODUCTION

Haemolytic *Pasteurella*-like bacteria associated with salpingitis and peritonitis in layers were first reported by Kjos-Hansen (1950). Similar organisms were later classified as '*Pasteurella salpingitidis*' by Kohlert (1968). Mráz *et al.* (1976) suggested that these organisms should be reclassified as '*Actinobacillus salpingitidis*' on the basis of their phenotypic features. Several papers have subsequently been published on avian isolates identified as either [*Pasteurella haemolytica*] or '*A. salpingitidis*' (Asnani & Pathak, 1975; Bisgaard, 1977; Bisgaard & Dam, 1981; Édes *et al.*, 1994; Greenham & Hill, 1962; Hacking & Pettit, 1974; Harbourne, 1962; Harry, 1962; Hinz, 1969; Janetschke & Risk, 1970; Matthes *et al.*, 1969; Mráz *et al.*, 1976; Nicolet & Fey, 1965; Shaw *et al.*, 1990). These organisms seem to constitute part of the normal upper-respiratory-tract flora of chickens (Bisgaard, 1977), but are also associated with pathological lesions, including salpingitis with or without peritonitis, septicaemia, pericarditis, hepatitis, respiratory tract lesions and enteritis. In addition to chickens, these organisms have also been isolated from turkeys, web-footed birds, pigeons, pheasants, partridges and psittacine birds (Bisgaard, 1993).

Bisgaard (1982) reported similar phenotypic characters for '*A. salpingitidis*' and avian *P. haemolytica*-like strains. Strains tentatively designated taxon 1 by Bisgaard (1982) and subsequently classified as *Pasteurella anatis* by Mutters *et al.* (1985) were also reported to be phenotypically related to '*A. salpingitidis*' and avian *P. haemolytica*-like organisms. In contrast to *P. anatis*, however, the avian [*P. haemolytica*]-'*A. salpingitidis*' complex was found to show major variations in many of the characters examined. DNA-DNA hybridizations performed by Piechulla *et al.* (1985) showed that groups of organisms named avian *P. haemolytica*-like and '*A. salpingitidis*' seem to form a common DNA relatedness cluster that was linked to the genus *Actinobacillus* by 48% DNA relatedness. DNA binding at the genus level was, however, not observed between taxon 1 of Bisgaard (*P. anatis*) and '*A. salpingitidis*' and *P. haemolytica*-like organisms. Subsequent DNA-rRNA hybridizations confirmed that these organisms belong to the family *Pasteurellaceae* Pohl 1981, but they did not cluster with any of the seven rRNA branches outlined (De Ley *et al.*, 1990).

According to 16S rRNA gene sequence analysis, the proposed type strain of '*A. salpingitidis*' clustered with *P. anatis*. Although deeply branching, these organisms formed a monophyletic group (cluster 3D) with Bisgaard taxa 2 and 3 and *Pasteurella langaaensis* (Dewhirst *et al.*, 1993) confirming previous studies, which showed that Bisgaard taxa 2 and 3 are only distantly related to the avian [*P. haemolytica*]-'*A. salpingitidis*' complex (Bisgaard *et al.*, 1993). In addition, [*P. haemolytica*] from ruminants, pigs, rabbits and hares has recently been shown to represent a new genus, *Mannheimia*, containing several species (Angen *et al.*, 1999).

The present uncertain taxonomic situation of the avian

P. haemolytica-'*A. salpingitidis*'-like organisms is undesirable, as it makes interpretation of studies on these organisms difficult and it may hinder progress in studies on epidemiology and pathogenesis. In the present investigation, selected strains were initially characterized by PFGE, AFLP, internal transcribed spacer (ITS) and plasmid profiling to obtain more information about their genotypic relationships. Subsequently, strains representing existing biovars of the avian [*P. haemolytica*]-'*A. salpingitidis*' complex and selected strains of *P. anatis* were characterized by sequencing of 16S rRNA genes and by DNA-DNA hybridization. Based on the results obtained, a proposal for a revised taxonomy is presented.

METHODS

Bacterial strains and phenotypic characterization. The selection of strains was based upon previous investigations including phenotypic tests and DNA-DNA hybridizations (Bisgaard, 1982; Piechulla *et al.*, 1985). Based upon differences in acid production from (+)L-arabinose, (+)D-xylose, *m*-inositol, (−)D-sorbitol, maltose, trehalose and dextrin, a total of 24 different biovars has been recorded. However, only strains representing 15 biovars have been kept or survived and were consequently available for the present investigation. In addition to strains of avian origin, strains of bovine origin tentatively classified as *P. anatis* were included (Tables 1 and 2).

PFGE. PFGE was carried out as described by Ojeniyi *et al.* (1991) with digestion of DNA of 19 strains by *SalI* or *XbaI* (Table 1).

Plasmid analysis. Analysis of presence and size-distribution of plasmids was performed with 25 strains according to Kado & Liu (1981) and Sorensen *et al.* (1991) (Table 1).

AFLP. With a few modifications, DNA extraction and AFLP analysis were performed as described recently (Kokotovic *et al.*, 2000). Briefly, DNA was extracted from cell pellets of 19 strains (Table 1) grown in heart infusion broth (Difco). DNA solutions were digested simultaneously with *BglII* and *BspDI* and ligation to adaptors was performed before amplification of the modified restriction fragments. The non-selective *BglII* primer was labelled at the 5'-end with 6-carboxyfluorescein and the non-selective *BspDI* primer was unlabelled. Amplification products were detected on an ABI 377 automated sequencer (PE Biosystems). Fragment size determination and pattern analysis were done by using GeneScan 3.1 fragment analysis software (Applied Biosystems). Only AFLP profiles comprising fragments in the size range 50–500 bp were considered for numerical analysis. Numerical analysis was performed on data files comprising both bacterial AFLP profiles and internal size standards by the use of the program Bionumerics 2.0 (Applied Maths). Normalized AFLP fingerprints were compared using the Dice similarity coefficient and clustering analysis was performed by UPGMA. All isolates were analysed by carrying out the entire procedure at least twice.

Sequencing of 16S rRNA genes. Bacteria were cultured overnight in brain/heart infusion broth (Difco). DNA was extracted as described previously (Leisner *et al.*, 1999). PCR amplification was performed as described by Vogel *et al.* (1997). Oligonucleotides for both PCR amplification and sequencing were synthesized according to sequences and 16S rRNA positions given by Dewhirst *et al.* (1989) and Paster & Dewhirst (1988). In addition, the primer 26r (5'-CAGGGCATCCACCGT) located on the 23S rRNA

Table 1. Bacterial strains investigated and tests performed with the avian [*P. haemolytica*]-*A. salpingitidis*-*P. anatis* complex. Strains are listed according to our proposed classification.

Strain	Animal	Country*	Biovar	Lesion-associated isolation	16S rRNA accession no.	PFGE	AFLP	Plasmid	DNA-hybrid
<i>Gallibacterium anatis</i> gen. nov., comb. nov.									
10672/6 Salp.	Chicken	DK	1	+	AF228008	+	+	+	+
Gerl. 646/s2/89	Chicken	G	1	+				+	+
1307/89	Budgerigar	G	1	+					+
12158/5 Salp.	Chicken	DK	3	+	AF228009	+	+	+	+
36961/Sv15	Chicken	DK	3	–				+	+
36961/Sv4	Chicken	DK	3	–					+
10672/9 Salp. (=F 114)	Chicken	DK	4	+	AF228004	+	+	+	+
Gerl. 2396/79 (=F 465)	Chicken	G	4	+				+	+
5821/88	Parrot	G	4	+					+
Gerl. 2737/S3/89	Turkey	G	11	+				+	+
36961/Sv7	Chicken	DK	11	–	AF228011	+	+	+	+
BJ 3453.2	Cow	B	12	+	AF228013	+	+	+	+
42447 Liver 2	Chicken	DK	12	+			+	+	+
2962/88	Budgerigar	G	12	+					+
IPDH 697/78	Chicken	G	15	+	AF228007	+	+	+	+
Gerl. 3348/80 (=F 219)	Goose	G	17	+	AF228005	+	+	+	+
Gerl. 3076/88	Duck	G	17	+				+	+
BK 3387.2	Cow	B	18	–	AF228012	+	+	+	+
Gerl. 444/s5/89	Budgerigar	G	18	+		+		+	+
13251/1 (=F 106)	Goose	DK	18	–					+
20558/3kl.	Goose	DK	19	–	AF228010	+	+	+	+
CCM 5995 (=CCUG 23138 =Mraz 26b/65A)	Chicken	Cz	20	Unknown	AF228003	+	+	+	+
Gerl. 4224/88	Avian	G	22	+	AF228014	+	+	+	+
Gerl. 220/s1/89	Avian	G	24	Unknown	AF228006	+	+	+	+
146/89	Parrot	G	UC†	+					+
B 96/20	Bovine	B	UC	+	AY038592				
B 96/24	Bovine	B	UC	+	AY038593				+
Hom.3	Bovine	B	UC	+	AY038594				+
Hom.32	Bovine	B	UC	Unknown					
Hom.33	Bovine	B	UC	+					
<i>P. anatis</i> F 149 ^T (=ATCC 43329 ^T =CCUG 15563 ^T =NCTC 11413 ^T)	Duck	DK	UC	–	AF228001, M75054	+	+	+	+
F 279 (=CCUG 26455)	Duck	DK	UC	–	AF228002	+	+	+	+
<i>Gallibacterium genomospecies 1</i>									
CCM 5975‡ (=CCUG 23137 =Mraz 558g/71C)	Chicken	Cz	5	–	AF228016	+	+	+	+
Gerl. 2740/89	Pigeon	G	5	+	AF228018	+	+	+	+
' <i>A. salpingitidis</i> ' CCM 5974 (=CCUG 23139 =Mraz 556/71B)	Chicken	Cz	8	+	AF228015, L06077	+	+	+	+
Gerl. 3191/88	Chicken	G	8	+	AY038519				+
<i>Gallibacterium genomospecies 2</i>									
CCM 5976 (=Mraz 573a/71D)	Chicken	Cz	9	+	AF228017	+	+	+	+

*B, Belgium; Cz, Czech Republic; DK, Denmark; G, Germany.

†UC, Phenotypic classification not performed at the biovar level, see Table 2.

‡All CCM strains listed were removed from the collection in 1990 but have been maintained by the authors.

Table 2. Key characters for separation of the biovars of the avian [*P. haemolytica*]-*A. salpingitidis*' complex

Characters are scored as: +, positive within 1–2 days; (+), positive within 3–14 days; –, negative within 14 days; w, weak reaction.

Strain	Biovar	Production of acid without gas formation from:						
		(+)L-Arabinose	(+)D-Xylose	m-Inositol	(-)D-Sorbitol	Maltose	Trehalose	Dextrin
10672/6	1	–	+	+	+	+	+	+
Gerl. 646/s2/89	1	–	+	(+)	+	+	+	+
1307/89	1	–	(+)	(+)	+	+	+	+
12158/5Salp.	3	–	+	+	+	+	–	+
36961/Sv4	3	–	+	(+)	+	+	–	+
36961/Sv15	3	–	+	(+)	+	+	–	+
10672/9Salp.	4	–	+	+	+	–	+	(+)
Gerl. 2396/79	4	–	+	+	+	–	+	(+)
5821/88	4	–	+	+	+	–	+	(+)
CCM 5975	5	–	+	–	–	+	+	(+)
Gerl. 2740/89	5	–	+	–	–	+	+	(+)
CCM 5974	8	+	+	–	–	+	+	+
Gerl. 3191/88	8	+	+	–	–	+	+	+
CCM 5976	9	–	+	+	–	+	–	+
36961/Sv7	11	–	+	+	+	–	–	w
Gerl.2737/s3/89	11	–	+	+	+	–	–	(+)
BJ3453.2	12	–	+	+	–	–	+	–
42447liver2	12	–	(+)	+	–	–	+	(+)
2962/88	12	–	+	+	–	–	+	–
IPDH 697/78	15	–	–	+	+	–	–	–
Gerl. 3348/80	17	–	+	+	–	–	–	–
Gerl. 3076/88	17	–	+	+	–	–	–	–
BK3387.2	18	–	+	–	–	–	+	(+)
Gerl. 444/s5/89	18	–	+	–	–	–	+	(+)
13251/1	18	–	+	–	–	–	+	(+)
20558/3kl.	19	–	+	–	–	–	–	–
CCM 5995	20	–	–	–	–	–	+	–
Gerl. 4224/88	22	–	+	–	+	–	–	–
Gerl. 220/s1/89	24	–	+	+	–	+	+	+
F 149 ^T	<i>Pa</i> *	–	+	–	–	–	+	–
F 279	<i>Pa</i>	–	+	–	–	–	+	–
B96/24	' <i>Pa</i> '	–	+	+	–	–	+	–
Hom.3	' <i>Pa</i> '	–	+	+	–	–	+	–
B96/20	' <i>Pa</i> '	–	+	–	+	–	+	–
Hom.32	' <i>Pa</i> '	–	+	–	+	–	+	–
Hom.33	' <i>Pa</i> '	–	+	–	+	–	+	–
146/89	UCT†	–	–	(+)	–	+	–	+

**Pa*, *P. anatis*. Non-haemolytic bovine and anatine isolates not previously subjected to biovar typing.

†UC, Unclassified. Non-haemolytic, negative for (–)D-mannitol and positive for (+)D-melibiose.

gene was used for PCR amplification just as the primers 37f (5'-GGCTCAGRWYGAACGC) and 1540r (5'-GGAGGTGWTCARCCGC), both complementary to the 16S rRNA gene sequence, were used for sequencing. PCR-amplified fragments were purified on Microspin columns (Amersham Pharmacia Biotech) and cycle-sequenced using the Thermo Sequenase fluorescent-labelled primer cycle-sequencing kit (Amersham Pharmacia Biotech) on an ALF Sequencer (Pharmacia Biotech) using fluorescein-labelled primers.

Analysis of sequence data. Searches for 16S rRNA sequences in public databases were performed by FASTA and BLAST (Wisconsin

sequence analysis package, Genetics Computer Group). Sequences were aligned manually to the *Escherichia coli* *rrnB* DNA sequence and to the consensus sequence (Lane, 1991). Maximum-likelihood analysis including bootstrap analysis was performed by fast DNAML (Felsenstein, 1981, 1995; Olsen *et al.*, 1994). Parsimony and neighbour-joining phylograms were computed by PHYLIP (Felsenstein, 1995). The phylogenetic analysis was performed with 16S rRNA sequences of the avian [*P. haemolytica*]-*A. salpingitidis*-*P. anatis* complex, Bisgaard taxa 2 and 3 and *Pasteurella gallinarum* with 1457 nt, of which 1338 could be analysed after removal of ambiguous positions.

ITS analysis. The 16S–23S rRNA ITS sequence was amplified by three nested primer sets with four strains. The primer for reverse amplification was located on the *rrl* gene with similarity to the *E. coli rrrB* positions 115–130 (23S–130, 5'-GGGTTTCCCCATTCGG) (Christensen *et al.*, 2000b). The primers for forward amplification of the ITS region were located on the *rrs* gene at *E. coli rrrB* positions 1525–1541 (16S–1525, 5'-GGTTGGATCACCTCCTT) (Christensen *et al.*, 2000b), 1485–1504 (16S1, 5'-TGGGGTGAAGTCGTAACAAG; Privitera *et al.*, 1998) and 1337–1353 (1337f, 5'-GGAATCGCTAGTAATCG; Fussing *et al.*, 1998). Fragments generated with the three primer sets are 149, 189, 337 bp longer than the 16S–23S ITS. PCR amplification and denaturing PAGE were performed as described in Christensen *et al.* (1999). Overhangs with the 16S and 23S rRNA genes were subtracted from fragment lengths and peak intensities were represented as relative values. Mean lengths of ITS fragments were calculated for each strain.

DNA–DNA hybridization. DNA–DNA hybridizations were performed by the spectrophotometric and micro-well methods according to Mitters *et al.* (1985) and Christensen *et al.* (2000a), respectively.

RESULTS

PFGE and plasmid analysis

PFGE showed that 19 strains (Table 1) representing 15 biovars of the avian [*P. haemolytica*]-*A. salpingitidis*' complex and *P. anatis* were distantly related, since no common fragments for all strains were present after digestion with either *Sa*I or *Xba*I (data not shown). Plasmids were not found in nine strains, while 16 strains contained plasmids of different sizes varying between 1.9 and 14.5 kb (Table 3). A total of 21 different plasmid sizes was demonstrated. Single isolates contained from one to four different plasmids (Table 3). The diversity in plasmid profiles confirmed that the strains were genotypically different.

Table 3. Plasmids and size distribution in strains of the avian [*P. haemolytica*]-*A. salpingitidis*-*P. anatis* complex

Strain	Plasmids (size in kb)
CCM 5975	2.2, 14.5
10672/6 Salp.	3.2, 4.8
12158/5 Salp.	4.5, 8.2
36961/Sv7	3.3, 4.8
BJ 3453.2	3.7, 5.6
IPDH 697/78	3.6, 4.0, 5.1, 6.6
Gerl. 3348/80	1.9
20558/3kl.	2.6, 3.3, 4.0
CCM 5995	6.6
Gerl. 220/S1/89	3.9, 4.6, 7.6
42447 Liver 2	3.7, 5.9
Gerl. 3076/88	2.0, 3.2
36961/Sv15	3.3, 4.6
Gerl. 444/S5/89	8.2
Gerl. 646/S2/89	6.6
F 279	3.7, 4.3

AFLP

The fingerprints of the strains studied consisted of 29–55 fragments. The similarity between repeated analyses of the same strain was 97.7 % (SD 1.9 %) for the 19 strains (Fig. 1). All strains were considered genotypically different, as less than 88 % similarity was observed (Fig. 1), confirming results obtained by PFGE and plasmid profiling. AFLP confirmed the results obtained by 16S rRNA phylogenetic analysis and DNA–DNA hybridization (see below), with a deep cluster of four strains including the former proposed type strain of '*A. salpingitidis*' (72 % similarity) and a main cluster of 15 strains including the type strain of *P. anatis* (54 % similarity) (Fig. 1).

16S rRNA sequence comparison

16S rRNA sequences were obtained in the region covering *E. coli* positions 28–1481 for 17 strains and positions 28–1550 for strains F 149^T and F 279. For strain Hom.3, the 16S rRNA sequence covered positions 28–1491. With strains B 96/20 and B 96/24, the 5'-region of the 16S rRNA was polymorphic between operons so that the sequence

Fig. 1. Dendrogram (UPGMA) of AFLP similarities (Dice coefficient) between selected strains of the avian [*P. haemolytica*]-*A. salpingitidis*-*P. anatis* complex. The similarity of repeated analysis is given in the right-hand column.

could not be determined precisely and 16S rRNA sequences were obtained from *E. coli* positions 73 and 55, resulting in approximate lengths of 1417 and 1436 bp.

The database search showed only three published sequences for which the similarity to sequences of the avian [*P. haemolytica*]-*A. salpingitidis*' complex was higher than 95 %. These included *P. anatis* ATCC 43329^T (accession no. M75054), *A. salpingitidis* CCUG 23139 (L06077) and the 16S rRNA sequence of strain CCUG 28018 (=F 114) (AF224310), classified as *A. cf. salpingitidis*, which showed 99.9 % similarity to strain 10672/9Salp. Differences were due to inter-operon polymorphisms or sequencing errors, since both strains represent the same isolate, 10672/9Salp. The greatest similarities to representatives outside the group were 94.6, 94.5 and 93.9 %, between strain IPDH 697/78 and the 16S rRNA sequences of Bisgaard taxon 3 strain CCUG 15563 (L06079), *Pasteurella avium* NCTC 11297^T (M75058) and Bisgaard taxon 2 strain CCUG 15564 (L06078). The sequence of the previously suggested type strain of *A. salpingitidis*, CCM 5974, diverged by five positions from the published sequence for this strain (L06077). For *P. anatis*, the published sequence of the type strain ATCC 43329^T (M75054) diverged by three bases. These differences were assumed to be related to differences between *rrs* operons and sequencing errors, and only sequences of the present study were included for phylogenetic analysis. The similarity between the type strain and strain F 279 of *P. anatis* was 99.7 %. The 16S rRNA sequences of the bovine strains Hom.3, B 96/20 and B 96/24, classified with *P. anatis*, showed high similarity to the type strain of *P. anatis* (98.1–99.7 %), underlining that these organisms might exist in bovine as well as avian hosts.

The phylogenetic analysis based on the sequences of the avian [*P. haemolytica*]-*A. salpingitidis*-*P. anatis* complex and published sequences for *A. salpingitidis*, *P. anatis* and Bisgaard taxa 2 and 3 is shown on Fig. 2, with *P. gallinarum* as an outgroup. *P. anatis* and the avian [*P. haemolytica*]-*A. salpingitidis*' complex formed a monophyletic unit related to Bisgaard taxa 2 and 3. The avian [*P. haemolytica*]-*A. salpingitidis*' complex, however, formed two monophyletic groups. Group 1 included the previously suggested type strain of *A. salpingitidis* and four additional strains (Fig. 2). The monophyly of this group was supported strongly by a high bootstrap value of 100 % of the maximum-likelihood analysis, as well as by parsimony and neighbour-joining analysis. The sequences of these strains showed 98.4–99.9 % similarity. The remaining strains of the avian [*P. haemolytica*]-*A. salpingitidis*' complex and the strains of *P. anatis* included formed another monophyletic group (group 2) supported by a bootstrap value of 71 % and neighbour-joining and parsimony analysis. Within this group, a large monophyletic group included the type strain of *P. anatis* and nine more strains (Fig. 2). The 16S rRNA similarities of group 2 ranged from 98.1 %, between Hom.3 and B 96/20, to 100 % between the following pairs, CCM 5995 and 12158/

5Salp., 12158/5Salp. and F 149^T, BJ 3453.2 and F 149^T and BJ 3453.2 and 12158/5Salp., with a mean similarity of 99.2 % of all strains in group 2. The similarity between groups 1 and 2 (*A. salpingitidis*-like and *P. anatis*-like) was 95.7–97.1 %.

ITS

The four strains analysed shared a fragment with mean length of 258 bp. Three strains shared another fragment, with a mean length of 501 bp, whereas the fourth strain showed a reduced length of this fragment (454 bp). One strain (Hom.33) possessed a third fragment, the length of which was intermediate (Table 4).

DNA–DNA hybridization

Fig. 3 shows a neighbour-joining dendrogram of 17 strains hybridized in a full matrix, including results published by Piechulla *et al.* (1985). A full DNA–DNA binding matrix is available as supplementary material in IJSEM Online (<http://ijs.sgmjournals.org/>). The three strains CCM 5974, Gerl. 2740/89 and CCM 5975 were linked to each other at 78–100 % DNA binding. The highest binding of strain CCM 5976 was to CCM 5975 and Gerl. 2740/89, with 75 %. This group corresponds to 16S rRNA group 1, while the remaining strains were included in another group corresponding to 16S rRNA group 2 (see Fig. 2).

To relate the remaining strains not hybridized in a full matrix to these groups, a single linkage dendrogram was constructed based on all DNA binding data available for the strains of the avian [*P. haemolytica*]-*A. salpingitidis*-*P. anatis* complex (Fig. 3). The *P. anatis*-like cluster included 29 strains, 13 of which were hybridized in a complete matrix. All of these strains were linked to each other at 79–100 % DNA binding; however, single DNA binding values as low as 31 % were observed. Three strains, referred to below as *Gallibacterium* genomospecies 1, were linked at 87 %; however, within this group, binding as low as 48 % was observed.

DISCUSSION

Taxonomic problems within the *Pasteurellaceae* have mainly been related to difficulties in the biochemical characterization of these bacteria, since representatives of many groups have been difficult to cultivate or inert to biochemical characterization. For several groups, host range and disease manifestations are not constant, also contributing to difficulties in classification (Mutters *et al.*, 1989; Bisgaard, 1993). The application of molecular biological methods, especially DNA–DNA hybridization and 16S rRNA sequence analysis, has contributed to an improved classification (Mutters *et al.*, 1989; Dewhirst *et al.*, 1993). The three original genera, *Pasteurella*, *Actinobacillus* and *Haemophilus*, have been found to be polyphyletic and, within the past decade, three new genera *Lonepinella*, *Mannheimia* and *Phocoenobacter* have been proposed

Fig. 2. Phylogenetic relationships between the avian [*P. haemolytica*]-*A. salpingitidis*-*P. anatis* complex based on maximum-likelihood analysis of 16S rRNA sequences. The significance for specific nodes obtained in bootstrap analysis ($n = 100$) is indicated for values higher than 50%. Nodes supported in phylogenetic trees obtained by neighbour-joining and parsimony methods are respectively indicated by + and *. Strains sequenced in the present study are in bold. Bar, 0.01 evolutionary distance.

Table 4. 16S-23S rRNA ITS fragment lengths and relative distributions in the avian [*P. haemolytica*]-*A. salpingitidis*-*P. anatis* complex

Fragment lengths are given as means $\pm$ SD ($n = 3$).

Strain	Fragment lengths (bp)	Relative distribution
CCM 5974	267 $\pm$ 11, 454 $\pm$ 2	0.53:0.47
F 149 ^T	258 $\pm$ 13, 492 $\pm$ 2	0.56:0.47
Hom.32	249 $\pm$ 23, 507 $\pm$ 5	0.52:0.50
Hom.33	249 $\pm$ 23, 279 $\pm$ 15, 506 $\pm$ 4	0.38:0.17:0.46

(Osawa *et al.*, 1995; Angen *et al.*, 1999; Foster *et al.*, 2000). Although outlining of 'true' monophyletic groups of *Pasteurella*, *Actinobacillus* and *Haemophilus* is in progress, many taxa still need reclassification, as demonstrated in the present study, where the new genus *Gallibacterium* is proposed to include taxa previously included within both *Actinobacillus* and *Pasteurella*.

This study confirms previous findings by phylogenetic analysis of 16S rRNA sequences and rRNA-DNA hybridizations (De Ley *et al.*, 1990; Dewhirst *et al.*, 1993) that bacteria of the avian [*P. haemolytica*]-*A. salpingitidis*

Single-linkage dendrogram

Neighbour-joining dendrogram

Fig. 3. Genotypic relationships between selected strains of the avian [*P. haemolytica*]-*A. salpingitidis*-*P. anatis* complex as measured by DNA-DNA hybridization, illustrated with single-linkage (left) and neighbour-joining (right) cluster analysis based on percentages of DNA binding. Numbers on the single-linkage dendrogram refer to actual DNA binding percentages.

complex represent true members of the *Pasteurellaceae*. Additional 16S rRNA sequence comparison and DNA-DNA hybridizations showed that the avian [*P. haemolytica*]-*A. salpingitidis* complex should be classified as a separate genus within the *Pasteurellaceae* Pohl 1981. The new genus is proposed to be named *Gallibacterium* gen. nov. The present investigations also showed that *P. anatis* is unrelated to other species of *Pasteurella*, including *Mannheimia haemolytica* (formerly *Pasteurella haemolytica*; Angen *et al.*, 1999), just as *A. salpingitidis* is unrelated to other species of *Actinobacillus*, including *Actinobacillus sensu stricto* (Christensen *et al.*, 2002).

The avian [*P. haemolytica*]-*A. salpingitidis*-*P. anatis* complex formed two monophyletic groups by 16S rRNA sequence comparison and the same groups could be identified by AFLP analysis and DNA hybridizations. Strains were related either to the previously suggested type strain of *A. salpingitidis* or to *P. anatis*. Strains classified in biovars 5, 8 and 9 were related to *A. salpingitidis*, while the remaining biovars were related to *P. anatis*. The similarity between the two groups was 96–97% by 16S rRNA sequence comparison. In addition, a group of bovine strains tentatively classified with *P. anatis* was related to the *P. anatis* group at 98–100% 16S rRNA similarity.

The 16S rRNA phylogenetic tree was based on maximum-likelihood analysis, which was found to be most accurate for phylogenetic inference (Yang *et al.*, 1994); however, comparison was made to phylograms generated by neighbour-joining and parsimony methods, since these tests behave better than the maximum-likelihood method on some occasions (Hillis *et al.*, 1994). Nodes supporting the genus-like group of the avian [*P. haemolytica*]-*A. salpingitidis*-*P. anatis* complex were supported by all three methods, suggesting a very high likelihood for the grouping. A unique 258 bp ITS fragment, not found in other members of the *Pasteurellaceae* investigated (Fussing *et al.*, 1998; Gu *et al.*, 1998; Leys *et al.*, 1994; Privitera *et al.*, 1998), was present in four strains representing the avian [*P. haemolytica*]-*A. salpingitidis*-*P. anatis* complex, supporting the unique genotypic nature of the group.

Five genotyping methods were used during different parts of the investigation. PFGE was used to verify that isolates used in the taxonomic analysis were genotypically diverse. This information could not be used further for classification since not even a single fragment was shared among all strains. Analysis of plasmids further confirmed that isolates were unrelated; however, this information was used for descriptive purposes only, since plasmid content has not

been recommended for classification (Gürtler & Mayall, 2001; Stackebrandt *et al.*, 2002). 16S rRNA sequence comparison was used to confirm the monophyly of the new genus proposed and to identify species-like groups. ITS typing confirmed the unique genotype of the genus and might be used for identification in future studies. AFLP and DNA–DNA hybridization were used to genotype the isolates according to the whole-genome approach (e.g. Gürtler & Mayall, 2001) and to identify groups at the level of genomospecies. Within the dendrograms constructed by the two methods, similar groups of related strains could be identified as defined by phylogenetic analysis of 16S rRNA sequences; however, the correlation between the percentage similarity determined by AFLP (Dice) and percentages of DNA binding determined by DNA–DNA hybridization was poor (data not shown). It was impossible to define a ‘cut-off’ value of species similarity based on AFLP data, as with the DNA–DNA hybridization method (see below). The present study showed that AFLP might represent an alternative to DNA–DNA hybridization for genotypic separation of species, as suggested previously by Stackebrandt *et al.* (2002); however, the comparison has to be performed at the level of groups defined as clusters or monophyletic units. For example, if species have been defined by DNA–DNA hybridization, AFLP can be used to classify novel isolates with the species-like groups.

Variability of the methods used for genotyping might be related to variation with different cultures of the same isolate. Such variation has never been reported for members of the *Pasteurellaceae*, and the high similarity obtained by AFLP with repeated analysis of the same isolates had a good or even better reproducibility compared with previous work (Tenover *et al.*, 1994; Huys *et al.*, 1996; Duim *et al.*, 1999; On & Harrington, 2000), suggesting low variation between different cultures of the same isolates as well as low experimental error. For some strains, there was minor variation between the fingerprints. Various reasons have been suggested to contribute to variation. Gel-to-gel variation, faintly stained bands, doublet bands representing two or more fragments of approximately the same size and variations in peak height due to differences in PCR efficiency may all influence the final result (Tenover *et al.*, 1995). In this study, repeated analysis of strain F 279 on different gels resulted in 93.7 % similarity between fingerprints, although both runs showed the same number of bands at nearly the same positions. The low band-position tolerance of 0.5 % used in the comparison of fragments may give similarities lower than 100 % for identical fingerprints. The comparison of restriction patterns remains, in part, a subjective process that cannot be reduced totally to rigid algorithms (Tenover *et al.*, 1995). Despite the minor variations, the AFLP results support the phylogenetic relationships suggested with 16S rRNA and DNA–DNA hybridizations techniques.

The methodological variation in relation to DNA–DNA hybridization was discussed recently (Christensen *et al.*,

2000a) and data in the present study represent at least repetitions where strains used for driver and tracer had been reversed.

DNA–DNA hybridization showed that the bacteria of the avian [*P. haemolytica*]–‘*A. salpingitidis*’–*P. anatis* complex were related at the genus level. The closest relationships of this new genus *Gallibacterium* were found to *Pasteurella multocida*, *P. langaaensis*, *Actinobacillus* sp. and *Pasteurella trehalosi*, with 54, 51, 48 and 45 % DNA binding (Piechulla *et al.*, 1985). Strains previously classified as biovars 1, 3, 4, 11, 12, 15, 17–20, 22 and 24 of the avian [*P. haemolytica*]–‘*A. salpingitidis*’ complex and avian and bovine isolates of *P. anatis* were linked at a DNA binding level of 79 % or higher, forming a single species, for which the name *Gallibacterium anatis* gen. nov., sp. nov. is proposed. Biovar 9 (CCM 5976) was linked to *Gallibacterium* at a DNA binding level of 75 % and might represent a separate species. Three strains (CCM 5974, CCM 5975 and Gerl. 2740/89) representing biovars 5 and 8 of the former avian [*P. haemolytica*]–‘*A. salpingitidis*’ complex showed 87 % or more DNA binding. However, phenotypic separation of this group was not possible at the species level and the group is considered to represent a novel genomospecies of *Gallibacterium* with phenotypic similarities to those of *G. anatis*. A single strain, Gerl. 3191/88, representing biovar 8, however, showed only 65 % DNA binding with CCM 5974, which also belongs to biovar 8. The final classification of biovars 5, 8 and 9 remains unsolved and must await the analysis of more strains, including extended phenotypic characterization and DNA hybridizations. However, 16S rRNA and AFLP data and complete DNA matrix data from *G. anatis* seem to indicate the existence of only one additional genomospecies.

The limits of DNA binding of 79–87 % within isolates of the species-like group of *Gallibacterium* are within the limits of the family. A study of *Actinobacillus sensu stricto* showed a limit for separation of species of around 80 % (Christensen *et al.*, 2002), which is slightly lower than for species of the genus *Mannheimia* and for other species of the family, for which a limit of 85 % has been proposed (Angen *et al.*, 1999; Mutters *et al.*, 1989).

In agreement with the genotypic divergence observed by 16S rRNA sequence comparison and DNA–DNA hybridization, a higher putrescine content was found in cells walls of biovars 1 and 4 of the avian [*P. haemolytica*]–‘*A. salpingitidis*’–*P. anatis* complex than for the previously suggested type strain of ‘*A. salpingitidis*’, representing biovar 8 (Busse *et al.*, 1997). It was further concluded that the heterogeneity of polyamines in cell walls of 16S rRNA cluster 3D of Dewhirst *et al.* (1993) (‘*A. salpingitidis*’, *P. anatis*, *P. langaaensis* and Bisgaard taxa 2 and 3) indicated the presence of more genera. This is in agreement with the distant relationship between the avian [*P. haemolytica*]–‘*A. salpingitidis*’ complex and taxa 2 and 3 found by SDS-PAGE (Bisgaard *et al.*, 1993) and the 16S rRNA sequence comparison of the present study. The

classification of taxa 2 and 3 as well as reclassification of *P. langaaensis* must await further studies.

Due to lack of information on biovars in previous reports, the sources of organisms described as *G. anatis* and genomospecies of *Gallibacterium* in the present paper are based upon strains characterized in our laboratory only. *G. anatis* has been isolated from chickens (287 strains), ducks (24), geese (19), cattle (14), budgerigars (7), psittacine birds (5), turkeys (4), partridges (3), pigs (2) and guinea fowl (1), while four isolates were of unknown source. Strains of *G. anatis* have been obtained from lesions in addition to normal mucosal membranes. *Gallibacterium* genomospecies 1 (biovars 5 and 8) has been isolated from chickens (8 strains) and a pigeon. Biovars 5, 8 and 9 have only been obtained from lesions.

Differentiation of members of *Gallibacterium* gen. nov. from related taxa

Members of *Gallibacterium* can be separated from other genera of *Pasteurellaceae* by differences in catalase, symbiotic growth, haemolysis, urease, indole, acid production without gas from (+)D-xylose, (–)D-mannitol, (–)D-sorbitol, (+)D-mannose, maltose, raffinose and dextrin and *o*-nitrophenyl α -D-glucopyranoside (ONPG) and *p*-nitrophenyl α -D-glucopyranoside (PNPG) tests. Members of *Gallibacterium* are (+)D-mannitol-positive, while *P. gallinarum* strains are negative. *Gallibacterium* are either non-haemolytic and negative for growth on (–)D-sorbitol, maltose and dextrin or haemolytic and variable for these characters, while *P. gallinarum* is non-haemolytic, (–)D-sorbitol-negative and maltose- and dextrin-positive. *Gallibacterium* is negative for symbiotic growth and positive in raffinose fermentation, while *Pasteurella volantium* shows symbiotic growth and a negative reaction for raffinose fermentation. *P. avium* shows symbiotic growth and is negative for acid production from (–)D-mannitol, while *Gallibacterium* is (–)D-mannitol-positive. *Gallibacterium* is catalase- and (+)D-xylose-positive while *P. langaaensis* and *Lonepinella koalarum* are negative for these characters. *Phocoenobacter uteri* is also catalase-negative and (–)D-mannitol-negative, while *Gallibacterium* is (–)D-mannitol-positive. *Gallibacterium* is ONPG-positive and indole-negative, whereas the members *P. multocida*, *Pasteurella canis*, *Pasteurella stomatis* and *Pasteurella dagmatis* of *Pasteurella* 16S rRNA cluster 3B (Dewhirst *et al.*, 1993) are indole-positive and ONPG-negative. *Gallibacterium* is urease-negative and PNPG-positive whereas genuine members of *Actinobacillus* (see Christensen *et al.*, 2002) are urease-positive and PNPG-negative. *Gallibacterium* is positive for (+)D-mannose and PNPG while *Mannheimia* is negative for these characters (Angen *et al.*, 1999). *Gallibacterium* is urease-negative and ONPG-positive while genuine members of *Haemophilus* (16S rRNA clusters 1B and 1C; Dewhirst *et al.*, 1993) are urease-positive and ONPG-negative.

Description of *Gallibacterium* gen. nov.

Gallibacterium (Gal'li.bac.te'ri.um. L. n. *gallus* chicken; N.L. n. *bacterium* rod; N.L. n. *Gallibacterium* bacterium of chicken).

A member of the family *Pasteurellaceae* (Pohl 1979) Pohl 1981 that includes the avian [*P. haemolytica*]–'A. *salpingitidis*'–*P. anatis* complex. The phenotypic description is based on Bisgaard (1982) and characterization of subsequent isolations.

Gram-negative, non-motile, rod-shaped or pleomorphic with cells occurring singly and in pairs. Colonies on bovine blood agar are mostly strongly β -haemolytic, greyish, non-transparent, but eventually translucent at the periphery, with a butyrous consistency, smooth and shiny, circular, raised with an entire margin and 1.0–2.0 mm in diameter after 24–48 h at 37 °C. Endospores are not formed. Growth is mesophilic and facultatively anaerobic or microaerophilic. Catalase-, oxidase- and phosphatase-positive. Nitrate is reduced. The reaction in Hugh–Leifson medium with (+)D-glucose is fermentative. Porphyrin and alanine aminopeptidase tests are positive. Acid is formed without gas from glycerol, (–)D-ribose, (+)D-xylose, (–)D-mannitol, (–)D-fructose, (+)D-galactose, (+)D-glucose, (+)D-mannose, sucrose and raffinose. ONPG and PNPG tests are positive. Negative in symbiotic growth, Simmons citrate, mucate-acid, malonate-base, H₂S/tri-sugar iron (TSI), growth in the presence of KCN, Voges–Proskauer at 37 °C and urease. Negative tests are further observed with arginine dehydrolase, lysine decarboxylase, ornithine decarboxylase, phenylalanine deaminase, indole, gelatinase and Tweens 20 and 80. Pigment is not formed. Acid is not produced from *m*-erythritol, adonitol, (+)D-arabitol, xylitol, (–)L-xylose, dulcitol, (+)D-fucose, (+)L-rhamnose, (–)L-sorbose, cellobiose, (+)D-melibiose, (+)D-melezitose, (+)D-glycogen, inulin, aesculin, amygdalin, arbutin, gentiobiose, salicin, (+)D-turanose or β -N-CH₃-glucosamid. Reactions for *p*-nitrophenyl β -D-glucopyranoside (NPG), *o*-nitrophenyl α -L-fucopyranoside (ONPF), α -galactosidase, *p*-nitrophenyl β -D-glucopyranosiduronic acid (PGUA), α -mannosidase and *o*-nitrophenyl β -D-xylanopyranoside (ONPX) are also negative. Variations are observed in the methyl red reaction at 37 °C, growth on MacConkey agar and acid production from (+)L-arabinose, (–)D-arabinose, *m*-inositol, (–)D-sorbitol, (–)L-fucose, lactose, maltose, trehalose and dextrin. The type species is *Gallibacterium anatis*.

Description of *Gallibacterium anatis* (Mutters *et al.* 1985) comb. nov.

Basonym: *Pasteurella anatis* Mutters *et al.* 1985.

Isolates are haemolytic, (–)D-arabinose- and (–)L-fucose-positive (biovar *haemolytica*, former biovars 1, 3, 4, 11, 12, 15, 17–20, 22, 24 of the avian [*P. haemolytica*]–'A. *salpingitidis*' complex) or non-haemolytic, trehalose-positive and (–)D-arabinose-, (–)L-fucose-, maltose- and

Table 5. Phenotypic characters that separate taxa of *Gallibacterium* gen. nov.

G. anatis comb. nov. biovar *haemolytica* represents biovars 1, 3, 4, 11, 12, 15, 17–20, 22 and 24 of the [*P. haemolytica*]-*A. salpingitidis* complex. Genomospecies 1 represents biovars 5 and 8 and genomospecies 2 represents biovars 8 and 9. Characters are scored as: +, $\geq 90\%$ of strains positive within 1–2 days; (+), $\geq 90\%$ of strains positive within 3–14 days; –, $< 10\%$ of strains positive within 14 days; d, 11–89% of strains positive.

Character	<i>G. anatis</i> bv. <i>haemolytica</i>	<i>G. anatis</i> bv. <i>anatis</i>	<i>Gallibacterium</i> genomospecies 1	<i>Gallibacterium</i> genomospecies 2
Haemolysis	+	–	+	+
Production of acid from:				
(–)D-Arabinose	(+)	–	(+)	d
(+)L-Arabinose	–	–	d	d
<i>m</i> -Inositol	d	d	–	d
(–)D-Sorbitol	d	d	–	–
(–)L-Fucose	(+)	–	+/(+)	d
Maltose	d	–	+	+
Trehalose	d	+	+	d
Dextrin	d	–	+/(+)	+

dextrin-negative (biovar *anatis*, former *P. anatis*) (Table 5). The G+C content ranges from 39.9 to 42.6 mol% and the genome size from 1.6 to 2.1 GDa (Mutters *et al.*, 1985; Piechulla *et al.*, 1985). The type strain is strain F 149^T (=ATCC 43329^T =NCTC 11413^T), isolated from the intestinal tract of a duck.

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REFERENCES

- Angen, Ø., Mutters, R., Caugant, D. A., Olsen, J. E. & Bisgaard, M. (1999). Taxonomic relationships of the [*Pasteurella*] *haemolytica* complex as evaluated by DNA–DNA hybridizations and 16S rRNA sequencing with proposal of *Mannheimia haemolytica* gen. nov., comb. nov., *Mannheimia granulomatis* comb. nov., *Mannheimia glucosida* sp. nov., *Mannheimia ruminalis* sp. nov. and *Mannheimia varigena* sp. nov. *Int J Syst Bacteriol* **49**, 67–86.
- Asnani, P. J. & Pathak, P. N. (1975). Bacteriological status of respiratory tract of normal fowl. *Indian J Anim Health* **14**, 25–29 (cited in *Vet Bull* **45**, 892, abstract 6772, 1975).
- Bisgaard, M. (1977). Incidence of *Pasteurella haemolytica* in the respiratory tract of apparently healthy chickens and chickens with infectious bronchitis. Characterisation of 213 strains. *Avian Pathol* **6**, 285–292.
- Bisgaard, M. (1982). Isolation and characterization of some previously unreported taxa from poultry with phenotypical characters related to *Actinobacillus*- and *Pasteurella* species. *Acta Pathol Microbiol Immunol Scand B* **90**, 59–67.
- Bisgaard, M. (1993). Ecology and significance of *Pasteurellaceae* in animals. *Zentbl Bakteriol* **279**, 7–26.
- Bisgaard, M. & Dam, A. (1981). Salpingitis in poultry II. Prevalence, bacteriology, and possible pathogenesis in egg-laying chickens. *Nord Vetmed* **33**, 81–89.
- Bisgaard, M., Brown, D. J., Costas, M. & Ganner, M. (1993). Whole cell protein profiling of *Actinobacillus*-like strains classified as taxon 2 and taxon 3 according to Bisgaard. *Zentbl Bakteriol* **279**, 92–103.
- Busse, H.-J., Bunka, S., Hensel, A. & Lubitz, W. (1997). Discrimination of members of the family *Pasteurellaceae* based on polyamine patterns. *Int J Syst Bacteriol* **47**, 698–708.
- Christensen, H., Jørgensen, K. & Olsen, J. E. (1999). Differentiation of *Campylobacter coli* and *C. jejuni* by length and DNA sequence of the 16S–23S rRNA internal spacer region. *Microbiology* **145**, 99–105.
- Christensen, H., Angen, Ø., Mutters, R., Olsen, J. E. & Bisgaard, M. (2000a). DNA–DNA hybridization determined in micro-wells using covalent attachment of DNA. *Int J Syst Evol Microbiol* **50**, 1095–1102.
- Christensen, H., Møller, P. L., Vogensen, F. K. & Olsen, J. E. (2000b). 16S to 23S rRNA spacer fragment length polymorphism of *Salmonella enterica* at subspecies and serotype levels. *J Appl Microbiol* **89**, 130–136.
- Christensen, H., Bisgaard, M., Angen, Ø. & Olsen, J. E. (2002). Final classification of Bisgaard taxon 9 as *Actinobacillus arthritidis* sp. nov. and recognition of a novel genomospecies for equine strains of *Actinobacillus lignieresii*. *Int J Syst Evol Microbiol* **52**, 1239–1246.
- De Ley, J., Mannheim, W., Mutters, R. & 7 other authors (1990). Inter- and intrafamilial similarities of rRNA cistrons of the *Pasteurellaceae*. *Int J Syst Bacteriol* **40**, 126–137.
- Dewhirst, F. E., Paster, B. J. & Bright, P. L. (1989). *Chromobacterium*, *Eikenella*, *Kingella*, *Neisseria*, *Simonsiella*, and *Vitreoscilla* species comprise a major branch of the beta group *Proteobacteria* by 16S ribosomal ribonucleic acid sequence comparison: transfer of *Eikenella* and *Simonsiella* to the family *Neisseriaceae* (emend.). *Int J Syst Bacteriol* **39**, 258–266.
- Dewhirst, F. E., Paster, B. J., Olsen, I. & Fraser, G. J. (1993). Phylogeny of the *Pasteurellaceae* as determined by comparison of 16S ribosomal ribonucleic acid sequences. *Zentbl Bakteriol* **279**, 35–44.

- Duim, B., Wassenaar, T. M., Rigter, A. & Wagenaar, J. (1999). High-resolution genotyping of *Campylobacter* strains isolated from poultry and humans with amplified fragment length polymorphism fingerprinting. *Appl Environ Microbiol* **65**, 2369–2375.
- Édes, I., Fodor, L. & Glávits, R. (1994). Bacteriological characterization and pathological properties of *Pasteurella haemolytica* strains isolated from muscovy ducks. *Magy Allatorv Lapja* **48**, 23–26.
- Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *J Mol Evol* **17**, 368–376.
- Felsenstein, J. (1995). PHYLIP (Phylogeny Inference Package) version 3.57c. Distributed by the author. Department of Genetics, University of Washington, Seattle, USA.
- Foster, G., Ross, H. M., Malnick, H., Willems, A., Hutson, R. A., Reid, R. J. & Collins, M. D. (2000). *Phococobacter uteri* gen. nov., sp. nov., a new member of the family Pasteurellaceae Pohl (1979) 1981 isolated from a harbour porpoise (*Phocoena phocoena*). *Int J Syst Evol Microbiol* **50**, 135–139.
- Fussing, V., Paster, B. J., Dewhirst, F. E. & Poulsen, L. K. (1998). Differentiation of *Actinobacillus pleuropneumoniae* strains by sequence analysis of 16S rRNA and ribosomal intergenic regions, and development of a species specific oligonucleotide for *in situ* detection. *Syst Appl Microbiol* **21**, 408–418.
- Greenham, L. W. & Hill, T. J. (1962). Observations on an avian strain of *Pasteurella haemolytica*. *Vet Rec* **74**, 861–863.
- Gu, X. X., Rossau, R., Jannes, G., Ballard, R., Laga, M. & Van Dyck, E. (1998). The *rrs* (16S)–*rml* (23S) ribosomal intergenic spacer region as a target for the detection of *Haemophilus ducreyi* by a heminested-PCR assay. *Microbiology* **144**, 1013–1019.
- Gürtler, V. & Mayall, B. C. (2001). Genomic approaches to typing, taxonomy and evolution of bacterial isolates. *Int J Syst Evol Microbiol* **51**, 3–16.
- Hacking, W. C. & Pettit, J. R. (1974). *Pasteurella hemolytica* in pullets and laying hens. *Avian Dis* **18**, 483–486.
- Harbourne, J. F. (1962). A haemolytic cocco-bacillus recovered from poultry. *Vet Rec* **74**, 566–567.
- Harry, E. G. (1962). A haemolytic coccobacillus recovered from poultry. *Vet Rec* **74**, 640.
- Hillis, D. M., Huelsenbeck, J. P. & Cunningham, C. W. (1994). Application and accuracy of molecular phylogenies. *Science* **264**, 671–677.
- Hinz, K.-H. (1969). *Bakteriologische Befunde bei Erkrankung der Atmungsorgane von Junghennen*, pp. 713–718. Beograd: 9th Congress of the World Veterinary Poultry Association.
- Huys, G., Coopman, R., Janssen, P. & Kersters, K. (1996). High-resolution genotypic analysis of the genus *Aeromonas* by AFLP fingerprinting. *Int J Syst Bacteriol* **46**, 572–580.
- Janetschke, P. & Risk, G. (1970). Über gehäuftes Auftreten von *Pasteurella hämolytica* beim Haushuhn in Syrien. *Monatsh Vetmed* **25**, 23–27.
- Kado, C. I. & Liu, S. T. (1981). Rapid procedure for detection and isolation of large and small plasmids. *J Bacteriol* **145**, 1365–1373.
- Kjos-Hansen, B. (1950). Oviduct peritonitis in hens due to pathogenic 'cloacal bacteria'. *Nord Vetmed* **2**, 523–531.
- Kohlert, R. (1968). Untersuchungen zur Ätiologie der Eileiterentzündung beim Huhn. *Monatsh Vetmed* **23**, 392–395.
- Kokotovic, B., Bolske, G., Ahrens, P. & Johansson, K. (2000). Genomic variations of *Mycoplasma capricolum* subsp. *capripneumoniae* detected by amplified fragment length polymorphism (AFLP) analysis. *FEMS Microbiol Lett* **184**, 63–68.
- Lane, D. (1991). 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–147. Edited by E. Stackebrandt & M. Goodfellow. New York: Wiley.
- Leisner, J. J., Pot, B., Christensen, H., Rusul, G., Olsen, J. E., Wee, B. W., Muhamad, K. & Ghazali, H. M. (1999). Identification of lactic acid bacteria from chili bo, a Malaysian food ingredient. *Appl Environ Microbiol* **65**, 599–605.
- Leys, E. J., Griffen, A. L., Strong, S. J. & Fuerst, P. A. (1994). Detection and strain identification of *Actinobacillus actinomycetem-comitans* by nested PCR. *J Clin Microbiol* **32**, 1288–1294.
- Matthes, D., Löfger, H.-Ch. & Schubert, H. J. (1969). Enzootische Auftreten der *Pasteurella haemolytica* beim Huhn. *Dtsch Tierärztl Wochenschr* **76**, 94–95.
- Mráz, O., Vladík, P. & Boháček, J. (1976). Actinobacilli in domestic fowl. *Zentbl Bakteriolog Hyg I Abt Orig A* **236**, 294–307.
- Mutters, R., Ihm, P., Pohl, S., Frederiksen, W. & Mannheim, W. (1985). Reclassification of the genus *Pasteurella* Trevisan 1887 on the basis of deoxyribonucleic acid homology, with proposals for the new species *Pasteurella dagmatis*, *Pasteurella canis*, *Pasteurella stomatis*, *Pasteurella anatis*, and *Pasteurella langaa*. *Int J Syst Bacteriol* **35**, 309–322.
- Mutters, R., Mannheim, W. & Bisgaard, M. (1989). Taxonomy of the group. In *Pasteurella & Pasteurellosis*, pp. 3–34. Edited by C. Adlam & J. M. Rutter. London: Academic Press.
- Nicolet, J. & Fey, H. (1965). Role de la *Pasteurella hémolytica* dans la salpingite de la poule. *Schweiz Arch Tierheilkd* **107**, 329–334.
- Ojienyi, B., Høiby, N. & Rosdahl, V. T. (1991). Genome fingerprinting as a typing method used on polyagglutinable *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. *APMIS* **99**, 492–498.
- Olsen, G. J., Matsuda, H., Hagstrom, R. & Overbeek, R. (1994). fastDNAmL: a tool for construction of phylogenetic trees of DNA sequences using maximum likelihood. *Comput Appl Biosci* **10**, 41–48.
- On, S. L. & Harrington, C. S. (2000). Identification of taxonomic and epidemiological relationships among *Campylobacter* species by numerical analysis of AFLP profiles. *FEMS Microbiol Lett* **193**, 161–169.
- Osawa, R., Rainey, F., Fujisawa, T., Lang, E., Busse, H. J., Walsh, T. P. & Stackebrandt, E. (1995). *Lonepinella koalarum* gen. nov., sp. nov., a new tannin-protein complex degrading bacterium. *Syst Appl Microbiol* **18**, 368–373.
- Paster, B. J. & Dewhirst, F. E. (1988). Phylogeny of campylobacters, wolliellas, *Bacteroides gracilis*, and *Bacteroides ureolyticus* by 16S ribosomal ribonucleic acid sequencing. *Int J Syst Bacteriol* **38**, 56–62.
- Piechulla, K., Bisgaard, M., Gerlach, H. & Mannheim, W. (1985). Taxonomy of some recently described avian *Pasteurella/Actinobacillus*-like organisms as indicated by deoxyribonucleic acid relatedness. *Avian Pathol* **14**, 281–311.
- Privitera, A., Rappazzo, G., Sangari, P., Giannino, V., Licciardello, L. & Stefani, S. (1998). Cloning and sequencing of a 16S/23S ribosomal spacer from *Haemophilus parainfluenzae* reveals an invariant, mosaic-like organisation of sequence blocks. *FEMS Microbiol Lett* **164**, 289–294.
- Shaw, D. P., Cook, D. B., Maheswaran, S. K., Lindeman, C. J. & Halvorson, D. A. (1990). *Pasteurella haemolytica* as a co-pathogen in pullets and laying hens. *Avian Dis* **34**, 1005–1008.
- Sorensen, M., Brown, D. J., Bisgaard, M., Hansen, H. C. & Olsen, J. E. (1991). Plasmid profiles of *Salmonella enterica* serovar Berta isolated from broilers in Denmark. *APMIS* **99**, 609–614.
- Stackebrandt, E., Frederiksen, W., Garrity, G. M. & 10 other authors (2002). Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *Int J Syst Evol Microbiol* **52**, 1043–1047.

Tenover, F. C., Arbeit, R., Archer, G. & 7 other authors (1994). Comparison of traditional and molecular methods of typing isolates of *Staphylococcus aureus*. *J Clin Microbiol* **32**, 407–415.

Tenover, F. C., Arbeit, R. D., Goering, R. V., Mickelsen, P. A., Murray, B. E., Persing, D. H. & Swaminathan, B. (1995). Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* **33**, 2233–2239.

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

Yang, Z. (1994). Statistical properties of the maximum likelihood method of phylogenetic estimation and comparison with distance matrix methods. *Syst Biol* **43**, 329–342.