Classification of the taxon 2 and taxon 3 complex of Bisgaard within *Gallibacterium* and description of *Gallibacterium melopsittaci* sp. nov., *Gallibacterium trehalosifermentans* sp. nov. and *Gallibacterium salpingitidis* sp. nov.

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This investigation was based on 23 isolates from several European countries collected over the past 30 years, and included characterization of all isolates. Published data on amplified fragment length polymorphism typing of isolates representing all biovars as well as protein profiles were used to select strains that were then further characterized by polyamine profiling and sequencing of 16S rRNA, *infB*, *rpoB* and *recN* genes. Comparison of 16S rRNA gene sequences revealed a monophyletic group within the avian 16S rRNA group of the *Pasteurellaceae*, which currently includes the genera *Avibacterium*, *Gallibacterium* and *Volucrobacter*. Five monophyletic subgroups related to *Gallibacterium anatis* were recognized by 16S rRNA, *rpoB*, *infB* and *recN* gene sequence comparisons. Whole-genome similarity between strains of the five subgroups and the type strain of *G. anatis* calculated from *recN* sequences allowed us to classify them within the genus *Gallibacterium*. In addition, phenotypic data including biochemical traits, protein profiling and polyamine patterns clearly indicated that these taxa are related. Major phenotypic diversity was observed for 16S rRNA gene sequence groups. Furthermore, comparison of whole-genome similarities, phenotypic data and published data on amplified fragment length polymorphism and protein profiling revealed that each of the five groups present unique properties that allow the proposal of three novel species of *Gallibacterium*, for which we propose the names *Gallibacterium melopsittaci* sp. nov. (type strain F450^T = CCUG 36331^T = CCM 7538^T), *Gallibacterium trehalosifermentans* sp. nov. (type strain 52/S3/90^T = CCUG 55631^T = CCM 7539^T) and *Gallibacterium salpingitidis* sp. nov. (type strain F150^T = CCUG 15564^T = CCUG 36325^T = NCTC 11414^T), a novel genomospecies 3 of *Gallibacterium* and an unnamed taxon (group V). An emended description of the genus *Gallibacterium* is also presented.

Organisms associated with salpingitis and peritonitis in ducks were first reported as atypical *Actinobacillus*

Abbreviation: AFLP, amplified fragment length polymorphism.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *infB*, *rpoB* and *recN* gene sequences determined in this study are detailed in Supplementary Table S1.

Details of strains and sequence accession numbers, whole-genome similarity values and a comparison with DNA–DNA reassociation results and phylogenetic trees based on *infB*, *rpoB* and *recN* gene sequences are available as supplementary material with the online version of this paper.

lignieresii by Bisgaard (1975). Comparative investigations of avian-associated members of the *Pasteurellaceae* including additional isolates of atypical *Actinobacillus lignieresii* obtained from ducks, geese and pigeons subsequently resulted in classification of these organisms as taxa 2 and 3 based upon differences in production of acid from (+)-L-arabinose and dulcitol (Bisgaard, 1982).

A total of 35 strains isolated from the respiratory tract, liver, heart and spleen of pigeons and different species of the Psittaciformes were reported by Beichel (1986). Nineteen of these were reinvestigated by Bisgaard *et al.*

(1999) and biotyped as described previously (Bisgaard, 1993). Considering these examinations, the host spectrum of these organisms was extended to include galliform birds like partridges and pheasants (Bisgaard, 1993). The importance of taxa 2 and 3 in salpingitis in web-footed birds was subsequently confirmed by Bisgaard (1995). On the basis of DNA–DNA hybridizations, these taxa were shown to form a large, distinct group which seems to represent a new genus with several species within the family *Pasteurellaceae* Pohl 1981 (Piechulla *et al.*, 1985). However, full-matrix DNA–DNA hybridizations were not carried out.

De Ley *et al.* (1990) performed hybridizations between labelled rRNA from seven representative members of the family *Pasteurellaceae* and 53 strains of the *Pasteurellaceae* and showed that strain HIM 730-3 (=F420) of taxon 2/3 (according to Bisgaard *et al.*, 1993 and not taxon 2 as listed by De Ley *et al.*, 1990) and strain NCTC 11412 of taxon 3 biovar 2 clustered with different nodes of the rRNA branches outlined, indicating major genetic diversity within taxa 2 and 3.

Different polyamine patterns were obtained with a duck isolate of taxon 2 biovar 1 (F150^T) and a parakeet isolate of taxon 3 biovar 1 (F450^T) according to Busse *et al.* (1997). Both isolates had profiles different from those of the newly established genera *Gallibacterium* (Christensen *et al.*, 2003a) and *Avibacterium* (Blackall *et al.*, 2005) of avian origin. Comparison of phenotypes defined by protein profiling with species/groups previously established by DNA–DNA hybridization, carbohydrate profiling and biovar typing showed that the best correlation existed between DNA–DNA hybridization and biovar typing (Bisgaard *et al.*, 1993). A correlation between results obtained from DNA–DNA hybridizations and protein profiling was not observed. Protein profiling, however, indicated a connection between protein profiles and hosts of isolation (Bisgaard *et al.*, 1993). Host-related bacterial lineages of the taxon 2 and 3 complex have subsequently been demonstrated by amplified fragment length polymorphism (AFLP) typing (Bojesen *et al.*, 2007).

Phylogenetic analysis by 16S rRNA gene sequence comparison has shown that taxon 2 biovar 1 (represented by NCTC 11414^T) and taxon 3 biovar 2 (represented by CCUG 15565^T, wrongly referred to as CCUG 15563 by Dewhirst *et al.*, 1993) cluster together, forming subcluster 3D of Dewhirst, together with strains later classified as *Gallibacterium* and [*Pasteurella*] *langaaensis* (Dewhirst *et al.*, 1993). In a subsequent investigation including avian taxa, Bisgaard taxon 2 and taxon 3 formed a separate entity within the so-called avian cluster (Christensen *et al.*, 2003b). A single isolate from septicaemia in a budgerigar classified as trehalose-positive taxon 3 biovar 1 showed only 94.5% 16S rRNA gene sequence similarity to biovar 2 of taxon 3, underlining the heterogeneity of this complex group (Christensen *et al.*, 2003b).

The accumulated evidence outlined above underlines the present uncertain taxonomic situation of the taxon 2 and

taxon 3 complex of avian origin and the lack of unambiguous diagnostic tests associated with these organisms, which makes interpretation of studies difficult and prevents progress in understanding their epidemiology and pathogenesis. For the same reasons, the aims of the present investigation were to clarify the taxonomic position(s) of the taxon 2 and 3 complex.

In the present study, a subset of 23 strains representing existing biovars characterized by AFLP profiling (Bojesen *et al.*, 2007) were investigated by phylogenetic analysis of the 16S rRNA, *rpoB*, *infB* and *recN* gene sequences. Moreover, *recN* gene sequences were used for estimation of whole-genome similarity to allow comparison with previously published DNA–DNA hybridizations. Selected strains were additionally characterized by polyamine profiling. Clusters outlined by Bojesen *et al.* (2007) and shown not to belong to *Gallibacterium sensu stricto* were excluded from the present paper, and these taxa will be described separately.

Bacterial strains

A total of 23 strains representing AFLP clusters 1, 4, 6 and 7 identified by Bojesen *et al.* (2007) were investigated (Table 1). Compared with the study of Bojesen *et al.* (2007), isolate 5436/S6/89 in the current study appeared as 5436/S6/88, and 52/S3/90 in the current study appeared as 52/90. These strains were listed incorrectly by Bojesen *et al.* (2007).

Sequencing of 16S rRNA, *rpoB*, *infB* and *recN* genes

16S rRNA gene sequencing of 13 strains was performed as reported previously (Angen *et al.*, 2003; Christensen *et al.*, 2002) in Copenhagen yielding at least 1296 bp of the 16S rRNA gene sequence. In addition, 18 strains were sequenced in Bern as previously reported by Kuhnert *et al.* (2002), generating 1360 bp of the 16S rRNA gene sequence. Eight strains were sequenced independently in both laboratories to allow for comparison of variation (Supplementary Table S1, available in IJSEM Online). One of these was reported in a previous study (Christensen *et al.*, 2003b). Duplicated 16S rRNA gene sequences from eight strains (Supplementary Table S1) showed between 100 and 99.6% similarity (F150^T and F151), resulting from ambiguous positions due to multiple rRNA operons.

Sequencing of *rpoB* and *infB* genes was performed according to protocols described previously (Korczak *et al.*, 2004; Kuhnert *et al.*, 2004). In addition, *recN* gene sequences were used as a representative target for determining whole-genome similarity as described by Kuhnert & Korczak (2006), including 1400 bp of the gene. Whole genome similarity values were calculated based on the formula $SI_{\text{genome}} = -1.30 + 2.25 SI_{\text{recN}}$ of Zeigler (2003), where SI_{genome} is the predicted DNA sequence identity shared by the genomes and SI_{recN} is the sequence similarity between *recN* orthologues.

Table 1. Strains of Bisgaard taxa 2 and 3 subjected to 16S rRNA, *infB*, *rpoB* and *recN* gene sequencing and classified within *Gallibacterium*

Strain	16S rRNA group	Taxon	Biovar	AFLP cluster	Host	Lesion
<i>Gallibacterium melopsittaci</i> sp. nov.						
F450 ^T * (=CCUG 36331 ^T =HIM778-5 ^T =CCM 7538 ^T)	I	3	1	6	Parakeet	Septicaemia
F417 (=HIM698-6)	I	2/3	5	6	Parakeet	Septicaemia
3077/89	I	2/3	5	6	Budgerigar	Septicaemia
1596/S4/90	I	3	9	6	Budgerigar	Septicaemia
HPA129	I	3	1	6	Budgerigar	Septicaemia
F292 (=CCUG 36332)	I	3	1	6	Parakeet	Salpingitis
5436/S6/89	I	3	1	6	Budgerigar	Septicaemia
F420* (=HIM730-3)	I	2/3	1	6	Parakeet	Septicaemia
F416* (=HIM778-3)	I	2	4	6	Parakeet	Septicaemia
<i>Gallibacterium trehalosifermentans</i> sp. nov.						
52/S3/90 ^T (=CCUG 55631 ^T =CCM 7539 ^T)	II	2/3	5 Tre ⁺	6	Budgerigar	Septicaemia
B7/99/1	II	3	1 Tre ⁺	6	Budgerigar	Septicaemia
<i>Gallibacterium genomospecies</i> 3						
F151* (=CCUG 15565 =NCTC 11412)	III	3	2	1	Duck	Salpingitis
3565/S2/89* (=CCUG 36330)	III	2	5	1	Pigeon	Septicaemia
F448* (=CCUG 36333)	III	3	2	1	Parakeet	Rhinitis
F298	III	3	4	1	Duck	Septicaemia
19060/1120*	III	3	6	1	Pigeon	Pneumonia
59/S3/89*	III	3	7	1	Turkey	Septicaemia
282/S4/90*	III	2/3	2	1	Budgerigar	Septicaemia
<i>Gallibacterium salpingitidis</i> sp. nov.						
F150 ^T * (=CCUG 15564 ^T =CCUG 36325 ^T =NCTC 11414 ^T)	IV	2	1	4	Duck	Salpingitis
18469/18*	IV	2	3	4	Duck	Salpingitis
BK1010/1	IV	3	5	4	Cow	Pericarditis
19987/2	IV	2	4	4	Goose	Salpingitis and peritonitis
<i>Gallibacterium</i> V						
39199/1L*	V	3	3	7	Pheasant	Pneumonia

*Total protein profiles characterized by Bisgaard *et al.* (1993).

Searches for sequences in public databases were performed by BLAST (Altschul *et al.*, 1997). Multiple alignments were constructed by CLUSTAL_X (Thompson *et al.*, 1997). Phylogenetic analysis of the 16S rRNA, *infB*, *rpoB* and *recN* gene sequences was carried out by neighbour joining using Jukes–Cantor correction in Bionumerics v. 4.5 (Applied Maths). Both individual and combined trees were constructed.

16S rRNA, *rpoB*, *infB* and *recN* gene sequence-based phylogenetic analysis of strains previously classified with the taxon 2 and 3 complex of Bisgaard was performed with all strains sequenced in the present study as well as published sequences of all members of *Gallibacterium* and related strains. The results documented that the strains investigated formed a monophyletic group within the *Pasteurellaceae*, closely related to *Gallibacterium* and unrelated to other genera including *Avibacterium* (Fig. 1 and Supplementary Fig. S1). To provide a simplified overview of the phylogenetic results, only strains included in the cluster following analysis of each of the 16S rRNA,

rpoB, *infB* and *recN* gene sequences are shown on Fig. 1 and Supplementary Fig. S1. More detailed phylogenetic comparisons performed by maximum-likelihood analysis including bootstrap analysis were also performed by fastDNAm1 (Felsenstein, 1995; Olsen *et al.*, 1994) and supported conclusions drawn from the above-mentioned neighbour-joining analysis (data not shown).

Nine strains (F420, F292, F450^T, HPA129, F416, F417, 5436/S6/89, 1596/S4/90 and 3077/89) clustered together (group I) in the 16S rRNA gene sequence-derived tree, showing similarities between 99.5 % (F420 to F450^T) and 100 % (Fig. 1). These strains also clustered together in the *rpoB*, *infB* and *recN* sequence-derived trees (Supplementary Fig. S1).

Two strains (52/S3/90^T and B7/99/1) showed 16S rRNA gene sequence similarities of 98.3 % and formed a second cluster (group II). These strains also grouped together in the *rpoB*, *infB* and *recN* trees (except for B7/99/1; *recN* not sequenced) (data not shown). Phylogenies shown in Fig. 1

Fig. 1. Phylogenetic relationships between strains of *Gallibacterium* and type species of genera of the *Pasteurellaceae* based on 16S rRNA gene sequences. Jukes–Cantor correction was applied for the distance matrix and the neighbour-joining method was used for tree construction in Bionumerics. Bootstrap values from 500 experiments are given as percentages at branches. Bar, 2% sequence divergence. *, Sequence available at <http://www.genome.ou.edu>.

and Supplementary Fig. S1 are based on strains analysed by sequencing all four genes, which explains why group II contains only one strain. Groups I and II were closely related, and demonstrated 16S rRNA gene sequence similarities between 96.2% (F420 and B7/99/1) and 96.9% (F416 and B7/99/1).

Four strains (F151, 3565/S2/89, F448, 59/S3/89) formed a third group (group III) in the 16S rRNA gene sequence-derived tree, showing a maximum of 99.3% similarity, as observed between strain F448 and 3565/S2/89. Bootstrap support of 100% was found for group III (Fig. 1). Group III was also recognized by phylogenetic analysis of housekeeping genes (Supplementary Fig. S1).

According to 16S rRNA gene sequence-based analysis, the four strains F150^T, BK1010/1, 18469/18 and 19987/2 formed group IV, with at least 99.2% similarity (F150^T and 18469/18) (only two strains are shown in Fig. 1). The group was linked to strain 39199/1L (group V) by 16S rRNA gene sequence similarities between 96.4% (18469/18) and 96.6% (BK1010/1). Groups IV and V were supported by a bootstrap value of 100%. Groups IV and V were also recognized as separate groups by phylogenetic analysis of housekeeping gene sequences (Supplementary Fig. S1).

In 16S rRNA as well as housekeeping gene-based phylogenetic analyses, 16S rRNA groups I–V and *Gallibacterium* always formed a monophyletic unit (Fig. 1 and Supplementary Fig. S1). The lowest similarity of 16S

rRNA gene sequences for these taxa was 91.9% (3191/88 of *Gallibacterium* genomospecies 1 with 18469/18 of group IV). Most genera of the *Pasteurellaceae* are related at below approximately 95% 16S rRNA gene sequence similarity. Establishing a new genus based only upon 16S rRNA groups I–V increases the overall minimum similarity to 93.2% (F450^T of group I – 39199/1L of group V). However, phenotypic separation of this group from *Gallibacterium* seems impossible (see Table 3).

There was a trend towards *Gallibacterium anatis* forming an outgroup to groups I–V (Fig. 1 and Supplementary Fig. S1). Phylogenetic analysis of translated housekeeping gene sequences confirmed the analysis performed at the 16S rRNA gene sequence level, although 16S rRNA groups I–V could not always be separated (data not shown).

Whole-genome similarity values calculated from *recN* sequences of strains investigated and type species of the genera *Gallibacterium*, *Volucrobacter* and *Avibacterium* appear in Supplementary Table S2. All strains representing 16S rRNA gene sequence clusters I–V showed similarity values of 0.42, 0.40, 0.36–0.37, 0.38–0.39 and 0.36, respectively, with the type strain of the type species *G. anatis*. Whole-genome similarity values calculated from *recN*, however, showed values of just 0.00–0.06 with the type species of *Avibacterium* and *Volucrobacter*.

Whole-genome similarity values calculated from *recN* sequences confirmed the existence of phylogenetic groups

outlined by comparison of housekeeping and 16S rRNA gene sequences (Supplementary Table S2). Whole-genome-derived similarity values based on *recN* ranging between 0.93 and 0.95 were observed for the nine strains making up group I, while values observed between group I and groups II–V ranged between 0.42 and 0.62, suggesting classification within the same genus. Similarity values observed between group II and the other four groups ranged between 0.42 and 0.62. Strains representing group III showed whole-genome similarities ranging between 0.89 and 0.93, while values with groups I–II and IV–V varied between 0.39 and 0.52. A whole-genome similarity of 0.87 was observed for the two strains representing group IV, similarity values with the other groups ranging between 0.45 and 0.50. Finally, the single strain representing group V showed similarity values with groups I–IV ranging from 0.39 to 0.49. In summary, *G. anatis* and all five groups are linked by whole-genome similarity values above 0.40 (Supplementary Table S2).

Phenotypic characteristics

Due to major within-group variations, genotypic groupings as stated above were not confirmed by the polyamine patterns (Table 2). Similar variations were observed for established species like *G. anatis* (Table 2). Mean percentages of the total amount of polyamines calculated for the single polyamines, however, seem to indicate that 1,3-diaminopropane is the dominant polyamine in some

strains of group III and the strain of group V. The predominant compound spermidine and large amounts of putrescine were common to the two representatives of group I. Large amounts of either 1,3-diaminopropane or cadaverine were also detected. The single representative of group IV exhibited the major compounds putrescine and spermidine. Like the type strain of *G. anatis* and two other strains of this species, strains of group III and V showed a polyamine pattern with three predominant compounds, 1,3-diaminopropane, putrescine and spermidine. However, the content of putrescine can be quite small, as found in strain CCM 5974 of *Gallibacterium* genomospecies 1.

All strains investigated were Gram-negative, non-motile at 22 and 37 °C and fermentative in Hugh & Leifson's medium with glucose. Negative reactions were obtained in growth on Simmons' citrate, growth with KCN and the Voges–Proskauer reaction at 37 °C and H₂S was not produced in triple-sugar iron (TSI) medium. Gas was not produced from nitrate and none of the strains produced arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, phenylalanine deaminase, gelatinase or pigment. Symbiotic growth and haemolysis of bovine blood were not observed and the isolates did not hydrolyse Tween 20 or 80. All isolates were positive for the porphyrin, methyl red and phosphatase tests, reduced nitrate and produced alanine aminopeptidase. None of the isolates investigated produced acid from *meso*-erythritol, adonitol, (–)-L-xylose, (+)-D-fucose, (+)-L-rhamnose, (–)-L-sorbose, maltose, cellobiose, (+)-melezitose, (+)-glycogen,

Table 2. Polyamine patterns of strains investigated

Values are given as $\mu\text{mol (g dry weight)}^{-1}$, with percentages of the total given in parentheses. tr, Trace [$\text{less than } 0.05 \mu\text{mol (g dry weight)}^{-1}$]; –, not detected. Abbreviations: DAP, 1,3-diaminopropane; PUT, putrescine; CAD, cadaverine; SPD, spermidine; HSPD, *sym*-homospermidine; SPM, spermine. None of the strains contained tyramine or *sym*-norspermidine.

Strain	16S rRNA group	Polyamine content					
		DAP	PUT	CAD	SPD	HSPD	SPM
<i>G. melopsittaci</i> sp. nov.							
F416	I	10.5 (19.9)	15.2 (28.8)	0.1 (0.2)	23.5 (44.6)	–	3.4 (6.5)
F450 ^{T*}	I	0.2 (0.5)	7.7 (21.7)	8.5 (24.1)	18.0 (50.7)	–	1.1 (3.1)
<i>G. trehalosifermentans</i> sp. nov. 52/S3/90 ^T	II	34.9 (73.9)	0.9 (1.9)	0.1 (0.2)	9.5 (20.1)	–	1.8 (3.8)
<i>Gallibacterium</i> genomospecies 3							
F151	III	19.0 (28.1)	27.9 (41.2)	0.1 (0.1)	19.2 (28.4)	–	1.5 (2.2)
F448	III	24.2 (40.1)	18.2 (30.2)	0.1 (0.2)	16.1 (26.7)	–	1.7 (2.8)
3565/S2/89	III	19.3 (27.8)	29.6 (42.7)	–	17.9 (25.8)	–	2.5 (3.6)
59/S3/89	III	33.0 (51.5)	8.9 (13.9)	–	19.8 (30.9)	0.2 (0.3)	2.2 (3.4)
<i>G. salpingitidis</i> sp. nov. F150 ^T	IV	0.4 (0.7)	37.6 (61.7)	–	20.2 (33.2)	0.2 (0.3)	2.5 (4.1)
<i>Gallibacterium</i> sp. 39199/1L	V	32.6 (48.6)	12.7 (18.9)	0.1 (0.1)	19.0 (28.3)	–	2.7 (4.0)
<i>G. anatis</i>							
CCUG 15563 ^{T*}		50.8 (56.0)	22.5 (24.8)	0.1 (0.1)	14.1 (15.5)	–	3.3 (3.6)
10672/6*		38.7 (72.2)	4.9 (9.2)	–	8.9 (16.6)	–	1.1 (2.0)
F114*		75.1 (75.8)	8.3 (8.4)	tr	11.6 /11.7	–	4.1 (4.1)
<i>Gallibacterium</i> genomospecies 1		40.8 (68.6)	0.9 (1.5)	0.4 (0.6)	14.3 (24.0)	–	3.1 (5.2)
CCM 5974*							

*Data from Busse *et al.* (1997).

Table 3. Phenotypic characters that separate the strains investigated

Taxa: 1, *G. melopsittaci* sp. nov.; 2, *G. trehalosifermentans* sp. nov.; 3, *G. salpingitidis* sp. nov.; 4, *Gallibacterium* genomospecies 3; 5, *Gallibacterium* group V; 6, *G. anatis* (data from Christensen *et al.*, 2003a). Characters are scored as: +, all strains positive within 1–2 days; (+), all strains positive within 3–14 days; –, all strains negative after 14 days; w, weakly positive; d, some strains positive while others are negative (number of strains positive/number of strains tested).

Character	1	2	3	4	5	6
Catalase	d 5/9	d 1/2	+	d 6/7	+	+
Oxidase, TMPD	d 3/9	d 1/2	+	d 4/7	+	+
Urease	–	–	d 2/4	d 3/7	+	–
Indole	–	–	–	d 1/7	–	–
Growth on MacConkey agar	d 5/9	–	+	d 2/7	+	d
Acid production from:						
Mucate	d 4/9	–	+	d 2/7	+	–
Glycerol	(+)	d 1/2	(+)	d 6/7	(+)	+
(+)-D-Arabitol	–	–	+	d 3/6	+	–
Xylitol	–	–	w	–	–	–
(+)-L-Arabinose	d 2/9	–	+	d 2/7	–	–
(–)-D-Arabinose	d 1/9	d 1/2	(+)	d 2/6	(+)	d
(–)-D-Ribose	+/(+)	+/(+)	+	d 5/6	+	+
(+)-D-Xylose	d 8/9	+	+	+/(+)	–	+
Dulcitol	d 3/9	d 1/2	+	d 1/7	–	–
(–)-D-Sorbitol	d 8/9	+	d 1/4	d 5/7	+	d
(–)-L-Fucose	d 1/9	+/(+)	+	d 3/6	+	d
Lactose	+/(+)	–	(+)	d 6/7	+	d
ONPG	+	–	+	+	+	+
(+)-Melibiose	+/(+)	+/(+)	d 1/2	–	+	–
Trehalose	–	+	–	–	–	d
Raffinose	+	+	+/(+)	d 6/7	+	+
Dextrin	–	–	–	–	–	d
Activity of:						
α -Galactosidase	+	+	d 1/2	–	+	–
α -Glucosidase (PNPG)	–	+	–	–	–	+
β -Xylosidase (ONPX)	+ 7/7	d 1/2	+	d 2/6	–	–

inulin, aesculin, amygdalin, arbutin, gentiobiose, salicin, (+)-turanose or β -N-CH₃-glucosamid. Negative reactions were also observed for β -glucosidase (NPG), α -fucosidase (ONPF), β -glucuronidase (PGUA) and α -mannosidase. Likewise, none of the strains produced gas from (+)-D-glucose. All strains produced acid from *myo*-inositol, (–)-D-mannitol, (–)-D-fructose, (+)-D-galactose, (+)-D-glucose, (+)-D-mannose and sucrose. Variable reactions were observed for the remaining tests investigated (Table 3).

According to Table 3, one to nine phenotypic characters separate clusters I–V defined according to genotypic characters as stated above. Major phenotypic diversity was observed for 16S rRNA gene sequence groups I and III, for which 10 and 17 characters showed different reactions, respectively, complicating phenotypic separation of the genotypic groups.

Reassessment of *Gallibacterium* and outline of novel species

A subset of 60 isolates of the taxon 2 and 3 complex of Bisgaard representing all biovars of the taxon 2 and 3

complex (53 isolates) and seven related isolates demonstrating atypical phenotypes were characterized previously by AFLP typing (Bojesen *et al.*, 2007). Seven major AFLP clusters were identified using maximum-parsimony and bootstrap analyses. In the present study, seven, four, 11 and a single strain representing AFLP clusters 1, 4, 6 and 7, respectively, of Bojesen *et al.* (2007) were characterized by sequencing of 16S rRNA, *rpoB*, *infB* and *recN* genes and by analysis of polyamines. Phylograms observed with neighbour-joining and maximum-likelihood analyses of the four genes investigated confirmed the existence of clusters identified by AFLP except for AFLP cluster 6. Two strains, 52/S3/90 and B7/99/1, branching deeply with the other strains of AFLP cluster 6, made up a separate group in phylogenies based upon 16S rRNA gene sequence and housekeeping genes.

16S rRNA gene sequence similarities within and between groups I–V clearly indicate the existence of novel species which should be classified within the same genus. 16S rRNA gene sequence groups I–V formed a separate cluster with the *G. anatis* complex and 16S rRNA gene sequence similarities of 93.2–94.8% were observed with the type

strain of *G. anatis*. These observations confirm previous investigations on inter- and intrafamilial similarities of rRNA cistrons of the *Pasteurellaceae* by De Ley *et al.* (1990), who demonstrated that strain F151 (16S rRNA gene sequence group III) and strain CCM 5974 of *Gallibacterium* genomospecies 1 and strain 3348/80 of *G. anatis* were rooted at the base of the seven rRNA branches reported, while strain F420 (16S rRNA gene sequence group I) and *G. anatis* strain F114 were rooted at the common root of five rRNA branches.

Whole-genome similarity values calculated from *recN* sequences within the family *Pasteurellaceae* have previously indicated similarity values of approximately 0.40 and above for species classified within the same genus (Kuhnert & Korczak, 2006; Kuhnert *et al.*, 2007). *G. anatis* and all strains investigated representing 16S rRNA gene sequence clusters I–V are linked by similarity values above 0.40 and should be classified within the same genus. In addition, phenotypic data including biochemical traits (Christensen *et al.*, 2003a), protein profiling (Bisgaard *et al.*, 1993) and polyamine patterns (Table 2) clearly indicate that these taxa are related.

DNA–DNA hybridizations between strains classified with 16S rRNA gene sequence group I have previously included strains F450^T, F420, F417 and F416 (Piechulla *et al.*, 1985). Strains F417 and F450^T showed 91 % reassociation, while repetition showed only 51 % DNA reassociation, underlining the uncertainty of the method. Only 71 % DNA reassociation was observed between strains F417 and F420 belonging to group I in the current investigation, while strains F420 and F416 of the same group showed 75 % DNA reassociation (Supplementary Table S3).

Assuming strain F283 might replace F150^T (group II), since 100 % DNA reassociation was observed between these strains (Piechulla *et al.*, 1985), and the observed 95 % DNA reassociation between F416 (group I) and 19987/2 (group IV) is incorrect, DNA reassociation between 16S rRNA gene sequence group I (F416, F420) and groups III (F151, F448) and IV (F150^T, 19987/2) varied between 74 % (F283–F416) and 20 % (F420–F448), which is below the values normally observed for strains belonging to the same species of the *Pasteurellaceae*.

Kuhnert & Korczak (2006) proposed that sequence comparison of the three genes *recN*, *rpoA* and *thdF* could replace DNA–DNA hybridizations. They showed that even sequencing of a single gene, *recN*, might serve this purpose. With two recently described species of the *Pasteurellaceae* (*Actinobacillus capsulatus* and *Avibacterium endocarditis*), a correlation between DNA–DNA hybridization results and *recN* similarity was demonstrated (Bisgaard *et al.*, 2007; Kuhnert *et al.*, 2007), and full matrices of *recN* similarity have extended the comparison based on the more limited DNA reassociation data. In the current investigation, the same approach has been followed, in that published DNA reassociation data have been complemented by full-matrix comparison of *recN* sequence similarity data.

DNA reassociation observed for group I (65–75 %; Supplementary Table S3) is slightly lower than the normal range of 80–85 % for species of the family (Christensen *et al.*, 2007). For 16S rRNA gene sequence group I, both AFLP results (Bojesen *et al.*, 2007) and whole-genome similarity values calculated from *recN* sequences of strains investigated (Supplementary Tables S2 and S3) clearly support the establishment of a novel species, for which the name *Gallibacterium melopsittaci* sp. nov. is proposed. Although phenotypically diverse (Tables 1 and 3), this species can be separated from both *G. anatis* (Christensen *et al.*, 2003a) and the other 16S rRNA gene sequence groups outlined here (Table 3). Finally, this group also represents a well-defined group (phenon 1) according to protein profiling, as demonstrated previously by Bisgaard *et al.* (1993).

Gallibacterium 16S rRNA gene sequence group II represents a unique group of bacteria which can be separated from both previously reported taxa and the other groups investigated. Both 16S rRNA gene sequence comparisons (Fig. 1) and whole-genome similarity values calculated from *recN* sequences (Supplementary Table S2) show clearly that this group should be classified as a novel species of *Gallibacterium*, *Gallibacterium trehalosifermentans* sp. nov., which, according to Table 3, can be separated from other taxa of *Gallibacterium*.

For *Gallibacterium* group III, DNA–DNA hybridization results exist for strains F151 and F448, which showed 81 % DNA reassociation (Piechulla *et al.*, 1985), while only 25 % DNA reassociation was observed between F151 and F450^T (16S rRNA gene sequence group I) and 20 % DNA reassociation between F448 and F420 (16S rRNA gene sequence group I). Finally, 25 % DNA reassociation was observed between strain F283 (100 % DNA reassociation to F150^T of 16S rRNA gene sequence group IV) and strain F418, which clustered with strain F448 of group III in AFLP (Bojesen *et al.*, 2007) (Supplementary Table S3). Combination of previous AFLP results (Bojesen *et al.*, 2007), the present sequence results (Fig. 1) and whole-genome similarities (Supplementary Table S2) clearly shows that 16S rRNA gene sequence group III should be classified as a novel species of *Gallibacterium*. However, *Gallibacterium* 16S rRNA gene sequence group III can not be separated clearly from the other taxa by biochemical traits, in particular *Gallibacterium salpingitidis* (Table 3). The group is unique according to protein profiling (phenon 2b=pigeon isolates; phenon 2c=various avian species; phenon 4=parakeet), as stated previously by Bisgaard *et al.* (1993). The colony morphology for this group named as *Gallibacterium* genomospecies 3 is similar to that of other species of *Gallibacterium* and the *Pasteurellaceae* in general. Haemolysis and adherence to the agar are not observed. The reference strain F151 (=CCUG 15565 =NCTC 11412) was isolated from salpingitis in a duck and is catalase-positive and oxidase-negative. Weak acid production is observed from mucate. Urease is produced and weak growth is observed on MacConkey agar. Strains are indole-negative. Acid is

produced from glycerol, (+)-D-arabitol, (–)-D-arabinose, dulcitol and (–)-L-fucose. β -Xylosidase (ONPX) is not produced. Differences that separate *Gallibacterium* genomospecies 3 from the novel species *G. melopsittaci* and *G. trehalosifermentans* (Table 3) are stated in the descriptions of these species. Due to major phenotypic variation observed for *Gallibacterium* genomospecies 3, only the difference in acid formation from xylitol separates this species from *G. salpingitidis*, while differences in acid production from (+)-D-xylose and (+)-melibiose and α -galactosidase activity separate *Gallibacterium* genomospecies 3 from *Gallibacterium* group V. Characters given in the description of *G. melopsittaci* in addition to α -glucosidase (PNPG) separate *Gallibacterium* genomospecies 3 from *G. anatis*. Strains of *Gallibacterium* genomospecies 3 have been obtained from different lesions in pigeons, ducks, turkeys and psittacine birds.

Two of the four strains classified with 16S rRNA gene sequence group IV have been characterized previously by DNA–DNA hybridization (Piechulla *et al.*, 1985). Strain F283, which clustered with F150^T by AFLP typing (Bojesen *et al.*, 2007), also showed 100% DNA reassociation with this strain, while 74 and 51% DNA reassociation was observed with strains F416 and F420, respectively, and 45% DNA reassociation with F417, all of which represent 16S rRNA gene sequence group I. Surprisingly, 95% DNA reassociation measured with a standard deviation of 1.0% was observed between F416 and 19987/2 (Supplementary Table S3), which clearly represent distinct genetic groups (Fig. 1 and Supplementary Table S2) and protein profiles (Bisgaard *et al.*, 1993), although they represent the same biovar (Table 1). For the same reason, the DNA–DNA reassociation value stated probably represents an error. Combined with the present findings, which clearly show that 16S rRNA gene sequence group IV represents a distinct group both genetically (Fig. 1 and Supplementary Table S2) and phenotypically, it is suggested that this group of bacteria is classified as a novel species of *Gallibacterium*, *Gallibacterium salpingitidis* sp. nov. Phenotypic characters that separate this species from other species are given in Table 3. Several phenotypic characters separate *Gallibacterium* 16S rRNA gene sequence group IV from *G. anatis* (Christensen *et al.*, 2003a). In addition, protein profiling might be used for separation of the suggested species (Bisgaard *et al.*, 1993).

Finally, group V represented by strain 39199/1L showed a maximum of 96.6% 16S rRNA gene sequence similarity with related taxa and probably represents a novel species. In addition, whole-genome similarity values calculated from *recN* sequences clearly demonstrate that this taxon, which has so far only been obtained from partridges and pheasants (Bisgaard, 1993), should be classified as a separate species of *Gallibacterium*. Two strains also represent a distinct phenon (3a) by protein profiling (Bisgaard *et al.*, 1993) and AFLP (Bojesen *et al.*, 2007), just as phenotypic characters separate these organisms from *G. anatis* (Christensen *et al.*, 2003a) as well as other species of

Gallibacterium (Table 3). Since only two strains have been investigated, the species is not yet named and will be referred to as *Gallibacterium* group V.

Emended description of the genus *Gallibacterium* Christensen *et al.* 2003

All strains investigated are Gram-negative, non-motile at 22 and 37 °C and fermentative in Hugh & Leifson's medium with glucose. Negative reactions are obtained in Simmons' citrate, malonate base, KCN growth and the Voges–Proskauer test at 37 °C and H₂S is not produced in TSI. Gas is not produced from nitrate and none of the strains produce arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, phenylalanine deaminase, gelatinase or pigment. Symbiotic growth is not observed and haemolysis of bovine blood is observed only for *Gallibacterium anatis* biovar haemolytica. Isolates do not hydrolyse Tween 20 or 80. All isolates are positive for the porphyrin, methyl red and phosphatase tests, reduce nitrate and produce alanine aminopeptidase. Acid is produced from (–)-D-mannitol, (–)-D-fructose, (+)-D-galactose, (+)-D-glucose, mannose and sucrose. None of the members of the genus produce acid from *meso*-erythritol, adonitol, (–)-L-xylose, (+)-D-fucose, (+)-L-rhamnose, (–)-L-sorbose, cellobiose, (+)-melezitose, (+)-glycogen, inulin, aesculin, amygdalin, arbutin, gentiobiose, salicin, (+)-turanose or β -N-CH₃-glucosamid. Negative reactions are observed for β -glucosidase (NPG), α -fucosidase (ONPF), β -glucuronidase (PGUA) and α -mannosidase. None of the strains produce gas from (+)-D-glucose. Compared with the original description of *Gallibacterium* (Christensen *et al.*, 2003a), activities of catalase and oxidase and acid production from glycerol and D-ribose should be regarded as variable following the inclusion of the species described below. The type species is *Gallibacterium anatis* (Christensen *et al.*, 2003a).

Description of *Gallibacterium melopsittaci* sp. nov.

Gallibacterium melopsittaci (me.lo.psit.ta'ci. N.L. masc. gen. n. *melopsittaci* of *Melopsittacus*, a zoological name for a genus of parakeet, from which the type strain was isolated).

Isolates on blood agar form regular, circular, slightly raised, non-haemolytic colonies with an entire margin. Colonies are smooth, shiny and non-transparent with a greyish colour. After 24 h aerobic incubation at 37 °C, colonies are 1.0–2.0 mm in diameter. Adherence to the agar is not observed and the consistency of the colonies is unguent-like. All isolates meet the phenotypic characters of the genus as stated above. The type strain is catalase- and oxidase-negative, and acid is not produced from mucate. Weak growth is observed on MacConkey agar. Acid is produced from (+)-D-xylose and (–)-D-sorbitol, but not from (+)-L-arabinose, (–)-D-arabinose, dulcitol or (–)-L-

fucose. Differences in acid production from lactose and trehalose and β -galactosidase (ONPG) and α -glucosidase (PNPG) activities separate *Gallibacterium melopsittaci* from *Gallibacterium trehalosifermentans*, while differences in acid production from (+)-melibiose and α -galactosidase, (+)-D-arabitol and xylitol, and urease and (+)-D-arabitol and β -xylosidase (ONPX) separate *Gallibacterium melopsittaci* from *Gallibacterium* genomospecies 3, *Gallibacterium salpingitidis* and *Gallibacterium* group V, respectively. Isolates have been obtained from septicaemia of parakeets and budgerigars and salpingitis in a parakeet.

The type strain, F450^T (=CCUG 36331^T =CCM 7538^T), was isolated from septicaemia in a parakeet.

Description of *Gallibacterium trehalosifermentans* sp. nov.

Gallibacterium trehalosifermentans (tre.ha.lo'si.fer.men'tans. N.L. n. *trehalosum* trehalose; L. v. *fermento* to ferment; N.L. part. adj. *trehalosifermentans* trehalose-fermenting).

Colony morphology is similar to that of other species of *Gallibacterium* and members of the *Pasteurellaceae* in general. Haemolysis and adherence to the agar are not observed. The type strain is catalase- and oxidase-positive. Acid is produced from glycerol, (–)-D-arabinose and dulcitol. β -Xylosidase is not produced. Differences in at least four characters separate the species from the other novel species reported in this study (Table 3). Differences in β -galactosidase (ONPG) and α -galactosidase activity separate the species from *Gallibacterium anatis*. Strains have been isolated from septicaemia in budgerigars.

The type strain, 52/S3/90^T (=CCUG 55631^T =CCM 7539^T), was isolated from septicaemia in a budgerigar.

Description of *Gallibacterium salpingitidis* sp. nov.

Gallibacterium salpingitidis (sal.pin.gi'ti.dis. N.L. fem. gen. n. *salpingitidis* from an inflammation of the ovarian tube).

Colony morphology is similar to that observed for other species of *Gallibacterium* and members of the *Pasteurellaceae* in general. Haemolysis and adherence to the agar are not observed. The type strain is urease-positive and negative for acid production from (–)-D-sorbitol. Five phenotypic characters separate the species from *Gallibacterium* group V (Table 3). Differences that separate the species from the other species reported in this study are stated in the descriptions of those species. Finally, differences in acid production from (+)-L-arabinose, trehalose and dulcitol in addition to α -glucosidase activity and haemolysis separate the species from *Gallibacterium anatis* (Christensen *et al.*, 2003a). Strains have been obtained from reproductive tract lesions in web-footed birds and pericarditis in a cat. To avoid confusion with regard to the name, the names '*Pasteurella salpingitis*' and '*Actinobacillus salpingitidis*', not included on the Approved

Lists of Bacterial Names (Skerman *et al.*, 1980), refer to *Gallibacterium* genomospecies 1 and 2 described by Christensen *et al.* (2003a), whereas *Gallibacterium salpingitidis* refers to taxon 2 of Bisgaard (1982).

The type strain, F150^T (=CCUG 15564^T =CCUG 36325^T =NCTC 11414^T), was isolated from salpingitis in a duck.

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