

# Reclassification of *Pasteurella gallinarum*, [*Haemophilus*] *paragallinarum*, *Pasteurella avium* and *Pasteurella volantium* as *Avibacterium gallinarum* gen. nov., comb. nov., *Avibacterium paragallinarum* comb. nov., *Avibacterium avium* comb. nov. and *Avibacterium volantium* comb. nov.

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This paper describes a phenotypic and genotypic investigation of the taxonomy of [*Haemophilus*] *paragallinarum*, *Pasteurella gallinarum*, *Pasteurella avium* and *Pasteurella volantium*, a major subcluster within the avian 16S rRNA cluster 18 of the family *Pasteurellaceae*. An extended phenotypic characterization was performed of the type strain of [*Haemophilus*] *paragallinarum*, which is NAD-dependent, and eight NAD-independent strains of [*Haemophilus*] *paragallinarum*. Complete 16S rRNA gene sequences were obtained for one NAD-independent and four NAD-dependent [*Haemophilus*] *paragallinarum* strains. These five sequences along with existing 16S rRNA gene sequences for 11 other taxa within avian 16S rRNA cluster 18 as well as seven other taxa from the *Pasteurellaceae* were subjected to phylogenetic analysis. The analysis demonstrated that [*Haemophilus*] *paragallinarum*, *Pasteurella gallinarum*, *Pasteurella avium* and *Pasteurella volantium* formed a monophyletic group with a minimum of 96.8% sequence similarity. This group can also be separated by phenotypic testing from all other recognized and named taxa within the *Pasteurellaceae*. As both genotypic and phenotypic testing support the separate and distinct nature of this subcluster, the transfer is proposed of *Pasteurella gallinarum*, [*Haemophilus*] *paragallinarum*, *Pasteurella avium* and *Pasteurella volantium* to a new genus *Avibacterium* as *Avibacterium gallinarum* gen. nov., comb. nov., *Avibacterium paragallinarum* comb. nov., *Avibacterium avium* comb. nov. and *Avibacterium volantium* comb. nov. The type strains are NCTC 1118<sup>T</sup> (*Avibacterium gallinarum*), NCTC 11296<sup>T</sup> (*Avibacterium paragallinarum*), NCTC 11297<sup>T</sup> (*Avibacterium avium*) and NCTC 3438<sup>T</sup> (*Avibacterium volantium*). Key characteristics that separate these four species are catalase activity (absent only in *Avibacterium paragallinarum*) and production of acid from galactose (negative only in *Avibacterium paragallinarum*), maltose (negative only in *Avibacterium avium*) and mannitol (negative in *Avibacterium gallinarum* and *Avibacterium avium*).

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The GenBank/EMBL/DBJ accession numbers for the 16S rRNA gene sequences of [*Haemophilus*] *paragallinarum* strains reported in this study are AY498867–AY498871.

Strain details and sequence accession numbers are given as supplementary material in IJSEM Online.

Members of the family *Pasteurellaceae* are commonly isolated from birds – both wild and domestic (Bisgaard, 1993). The use of 16S rRNA gene sequencing has revealed that many of these avian members of the *Pasteurellaceae* form phylogenetically related clusters (Dewhirst *et al.*, 1993; Olsen *et al.*, 2004), with Dewhirst *et al.* (1993) recognizing avian clusters 3A, 3D and 7. More recently, the avian 3A and

3D clusters of Dewhirst *et al.* (1993) have been retermed avian 16S rRNA cluster 18 (Olsen *et al.*, 2004). Cluster 7 of Dewhirst *et al.* (1993) is now termed 16S rRNA cluster 21, also known as the 'Testudinis' cluster (Olsen *et al.*, 2004).

The most recent taxonomic study has indicated that avian 16S rRNA cluster 18 of Olsen *et al.* (2004) consists of at least 11 species or species-like taxa (Christensen *et al.*, 2003b). Within avian 16S rRNA cluster 18, the new genus *Gallibacterium*, which incorporates organisms once known as avian [*Pasteurella haemolytica*], '*Actinobacillus salpingitidis*' and *Pasteurella anatis*, has recently been proposed (Christensen *et al.*, 2003a). Similarly, Bisgaard taxon 33 has been reclassified as *Volucribacter psittacida* and *V. amazonae* (Christensen *et al.*, 2004b).

The other major subcluster within avian 16S rRNA cluster 18 consists of a collection of four named species that are well known to veterinary bacteriologists – [*Haemophilus*] *paragallinarum*, *Pasteurella gallinarum*, *Pasteurella avium*, *Pasteurella volantium* – as well as the unnamed taxon *Pasteurella* sp. A. While all of the named species are routinely encountered in the investigation of upper respiratory tract disease in birds, only [*Haemophilus*] *paragallinarum* is widely regarded as a primary pathogen, being the causative agent of infectious coryza, an economically important disease of chickens (Blackall *et al.*, 1997). While not universally accepted as a primary pathogen, there are a number of publications reporting on the role of *Pasteurella gallinarum* as a pathogen in infections of chickens (Bock *et al.*, 1977; Droual *et al.*, 1992a, b; Mohan *et al.*, 2000; Mushin *et al.*, 1977; Terzolo *et al.*, 1980; Yadav *et al.*, 1977) and turkeys (Bisgaard *et al.*, 2005). The other species within this subgrouping of 16S rRNA cluster 18, *Pasteurella avium* and *Pasteurella volantium*, are regarded as part of the normal microbiota of the upper respiratory tract of chickens (Bisgaard, 1993; Mutters *et al.*, 1985a). In the current study, based on phenotypic properties as well as the results of the sequencing of 16S rRNA genes, we propose a revised taxonomy that accommodates these four species within a single genus.

The bacteria used in this study consisted of the type strain of [*Haemophilus*] *paragallinarum*, NCTC 11296<sup>T</sup>, plus the three reference strains for the Page serotyping scheme for [*Haemophilus*] *paragallinarum*, strains 0083, 0222 and Modesto, respectively representing serovars A, B and C (Page, 1962). In addition, eight isolates of the NAD-independent form of [*Haemophilus*] *paragallinarum* (Mouahid *et al.*, 1992), termed [*Haemophilus*] *paragallinarum* biovar 2 as suggested by Mutters *et al.* (1989), were included.

Phenotypic characterization of the type strain of [*Haemophilus*] *paragallinarum* and the eight strains of [*Haemophilus*] *paragallinarum* biovar 2 was performed according to Bisgaard *et al.* (1991). For the type strain of [*Haemophilus*] *paragallinarum*, all media were supplemented with 1% chicken serum and 0.0025% NAD (reduced form).

16S rRNA gene sequencing of [*Haemophilus*] *paragallinarum* biovar 2 strain SA7177 was performed as reported previously (Angen *et al.*, 1998). For the type strain of [*Haemophilus*] *paragallinarum* and the three Page serovar reference strains of [*Haemophilus*] *paragallinarum*, the 16S rRNA gene was sequenced as described previously (Blackall *et al.*, 2001).

In addition to the five sequences determined in the present study, GenBank was searched for highly similar 16S rRNA gene sequences. The selected sequences included 11 taxa of the *Pasteurellaceae* that are isolated from birds and which fall within the avian 16S rRNA cluster 18 of Olsen *et al.* (2004) as recently extended by Christensen *et al.* (2003b). A further seven taxa of the *Pasteurellaceae* that are either isolated from birds or represent the major 16S rRNA clusters within the family were included (Olsen *et al.*, 2004). The strains of [*Haemophilus*] *paragallinarum* and other members of the avian 16S rRNA cluster 18 used in the 16S rRNA gene sequencing are listed in the supplementary material in IJSEM Online.

Pairwise comparisons for similarity were performed by BESTFIT (GCG, Wisconsin Sequence Analysis Package). The alignment was constructed by PILEUP (GCG) and included the region between *Escherichia coli* positions 64 and 1391 of the *rnnB* gene, with 1246 positions left after removal of ambiguous positions and 218 distinct data patterns analysed. Maximum-likelihood analysis including bootstrap analysis was performed by fastDNAmL (Olsen *et al.*, 1994) run on a Linux 7.2-compatible server. The analysis was run with a transition/transversion ratio of 1.5 and rate heterogeneities with 35 rates were introduced into the fastDNAmL analysis by DNARates 1.1 (<http://geta.life.uiuc.edu/~gary/programs/DNARates/>). Parsimony and neighbour-joining analysis were performed by PHYLIP (Felsenstein, 1995) on a Digital UNIX computer.

All eight strains of [*Haemophilus*] *paragallinarum* biovar 2 as well as the type strain, representing biovar 1, were non-motile, Gram-negative rods that were catalase-negative, oxidase-positive and fermented glucose without gas production. Strain CCUG 12835<sup>T</sup> showed satellitic growth, while the biovar 2 strains did not. All nine strains did not cause haemolysis of bovine red blood cells and were negative in the Simmons' citrate, acid mucate, malonate, methyl red and Voges-Proskauer tests. The strains were alanine aminopeptidase-positive and reduced nitrate, without gas production, and did not possess urease, arginine dihydrolyase, lysine decarboxylase, ornithine decarboxylase, phenylalanine deaminase or gelatinase. Strain CCUG 12835<sup>T</sup> did not show phosphatase activity, while the eight biovar 2 strains did. All nine strains did not hydrolyse Tween 20 or Tween 80 and all failed to grow on MacConkey agar. The strains all produced acid from D-mannitol, D-sorbitol, D-fructose, D-mannose and sucrose. No acid was produced by any strain from glycerol, *meso*-erythritol, adonitol, D-arabitol, xylitol, L- or D-arabinose, L-xylose, dulcitol, *meso*-inositol, D-fucose, D-galactose, L-rhamnose, L-sorbose,

cellobiose, lactose, D-melibiose, trehalose, D-melezitose, raffinose, D-glycogen, inulin, aesculin, amygdalin, arbutin, gentiobiose, salicin, D-turanose or  $\beta$ -N-CH<sub>3</sub>-glucosamid. The biovar 2 strains produced acid from D-ribose, D-xylose and L-fucose, while the biovar 1 strain CCUG 12835<sup>T</sup> did not. Strain CCUG 12835<sup>T</sup> produced acid from maltose and dextrin, while the eight biovar 2 strains did not. None of the strains were positive for  $\beta$ -galactosidase,  $\beta$ -glucosidase,  $\alpha$ -fucosidase,  $\alpha$ -galactosidase,  $\alpha$ -glucosidase,  $\beta$ -glucuronidase,  $\alpha$ -mannosidase or  $\beta$ -xylosidase activity.

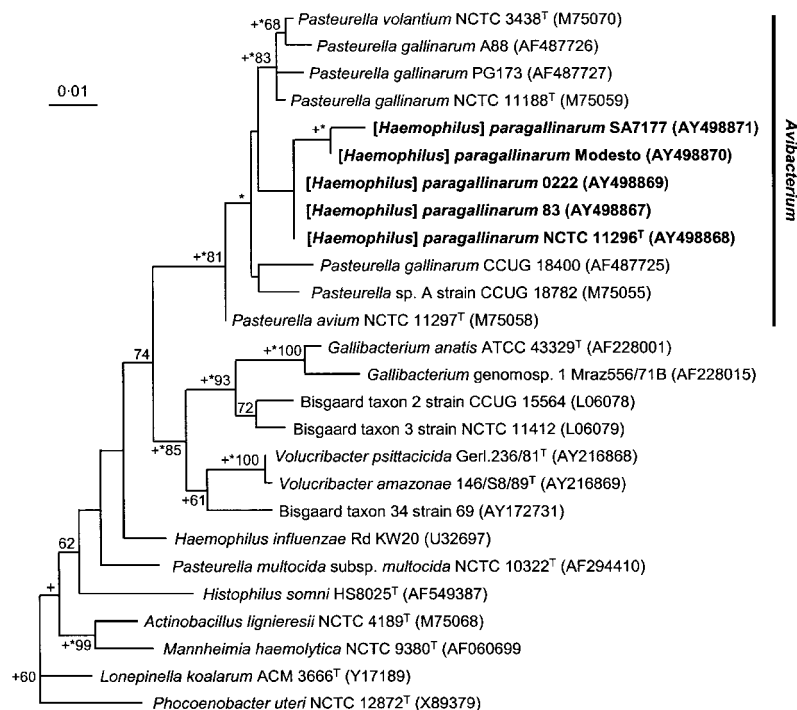
16S rRNA gene sequences were obtained in the region covering *E. coli* positions 28–1491 for four strains of [*Haemophilus*] *paragallinarum* biovar 1 (NCTC 11296<sup>T</sup>, 0083, 0222 and Modesto) and one strain of [*Haemophilus*] *paragallinarum* biovar 2 (SA7177). The 16S rRNA gene sequences of the three strains NCTC 11296<sup>T</sup>, 0083 and 0222 were identical when ambiguous positions were removed.

The similarity of 16S rRNA gene sequences of the type strain and strains 0222 and 0083 of [*Haemophilus*] *paragallinarum* to strain Modesto was 98.8%, while the similarities between these strains of biovar 1 and strain SA7177 of biovar 2 were between 98.9 and 97.7%. The highest similarities between strains of [*Haemophilus*] *paragallinarum* and other taxa of the *Pasteurellaceae* were obtained to the species *Pasteurella gallinarum*, *Pasteurella volantium* and *Pasteurella avium* and to *Pasteurella* sp. A, with between 96.8 and 98.1% similarity. Similarities between strains of [*Haemophilus*] *paragallinarum* and

strains of *Gallibacterium* ranged between 92.7 and 94.8% and similarities of between 92.2 and 93.8% were obtained to *Volucrobacter* and the unnamed taxa 2, 3 and 34 of Bisgaard. The highest similarities to other members of the Avian group were 94.8% to strain IPDH 697/78 of *Gallibacterium anatis* and 94.9% to strain 101 of *V. psittacida*. Outside the Avian group (which includes the genera *Avibacterium*, *Gallibacterium* and *Volucrobacter* and taxa 2, 3 and 34 of Bisgaard), the highest similarity obtained was between the type strain of [*Haemophilus*] *paragallinarum* and the type strain of *Actinobacillus capsulatus*, at 94.7%.

The phylogenetic analysis was performed with 24 sequences, including three from the present investigation; [*Haemophilus*] *paragallinarum* strains NCTC 11296<sup>T</sup>, Modesto and SA7177. Maximum-parsimony analysis resulted in 25 trees with identical numbers of steps. The phylogenetic analysis shown in Fig. 1 shows that the clade containing [*Haemophilus*] *paragallinarum* and the taxa *Pasteurella gallinarum*, *Pasteurella volantium*, *Pasteurella avium* and *Pasteurella* sp. A was monophyletic.

The family *Pasteurellaceae* has been studied extensively by molecular methods such as DNA–DNA hybridization, rRNA–DNA hybridization and 16S rRNA gene sequence analysis in recent years (Angen *et al.*, 1999, 2003; Christensen *et al.*, 2003a; De Ley *et al.*, 1990; Dewhirst *et al.*, 1993; Mutters *et al.*, 1989). These studies have clearly shown that the original three genera of *Haemophilus*, *Actinobacillus* and *Pasteurella* were polyphyletic and have resulted in the



**Fig. 1.** Phylogenetic relationships based upon maximum-likelihood analysis of 16S rRNA gene sequences of members of the genus *Avibacterium* gen. nov. and members of representative genera in the family *Pasteurellaceae*. Support for specified nodes obtained in bootstrap analysis 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 shown in bold. Bar, 0.01 evolutionary distance.

establishment of six new genera: *Gallibacterium*, *Histophilus*, *Mannheimia*, *Lonepinella*, *Phocoenobacter* and *Volucribacter* (Angen *et al.*, 1999, 2003; Christensen *et al.*, 2003a, 2004b; Foster *et al.*, 2000; Osawa *et al.*, 1995). The studies on these new genera, particularly *Gallibacterium* (Christensen *et al.*, 2003a) and *Volucribacter* (Christensen *et al.*, 2004b), have highlighted that the other members of the avian 16S rRNA cluster 18 within the *Pasteurellaceae* represent another group of taxa that needs reclassification.

The strains represented by the three serovars and two biovars of [*Haemophilus*] *paragallinarum* analysed by 16S rRNA gene sequence comparison formed a single subgroup within the Avian 16S rRNA-group of *Pasteurellaceae* together with the species *Pasteurella gallinarum*, *Pasteurella avium*, *Pasteurella volantium* and the unnamed species A of *Pasteurella*, showing between 96.8 and 98.1% similarity. The monophyly of the species [*Haemophilus*] *paragallinarum* as well as *Pasteurella gallinarum* and *Pasteurella* sp. A was unsupported by bootstrap, parsimony and neighbour-joining analysis. The species *Pasteurella gallinarum* was even polyphyletic by maximum-likelihood analysis. The explanation for this is probably that 16S rRNA gene sequence comparison is insufficient to resolve all species. This issue is well known for all species (Stackebrandt & Goebel, 1994; Christensen *et al.*, 2002a). A similar comb-like topology has previously been shown for some taxa of *Mannheimia* (Blackall *et al.*, 2001) and *Actinobacillus equuli* subsp. *equuli* and subsp. *haemolyticus* (Christensen *et al.*, 2002a).

These results have confirmed earlier findings that the species [*Haemophilus*] *paragallinarum*, *Pasteurella gallinarum*, *Pasteurella volantium* and *Pasteurella avium* comprise a monophyletic group within the family *Pasteurellaceae* (Christensen *et al.*, 2003b; Dewhirst *et al.*, 1992, 1993). While the 16S rRNA gene phylogenetic tree shown in Fig. 1 was generated by maximum-likelihood analysis, the monophyletic group that comprises the new genus *Avibacterium* was also supported by the phylograms generated by neighbour-joining and maximum-parsimony analysis (not shown). This support by all three analytical methods indicates a very high likelihood for the grouping. It has been proposed that new genera should be capable of being recognized from existing genera on the basis of both 16S rRNA gene sequence analysis and phenotypic data (Murray *et al.*, 1990). As there is clear support by 16S rRNA gene sequence analysis and an existing knowledge of distinguishing phenotypic characters (see Tables 1 and 2), we propose that the four species should be housed within a separate genus for which we propose the name *Avibacterium* gen. nov.

At the genus level, *Avibacterium* is clearly phenotypically distinguishable from all other genera in the family *Pasteurellaceae* except the genus *Gallibacterium* (Table 1). All members of the genus *Gallibacterium* do not show symbiotic growth, while most members of the genus *Avibacterium* show symbiotic growth (Table 1). Also, most isolates of

the genus *Gallibacterium* show strong haemolysis on blood agar (Christensen *et al.*, 2003a), allowing a further clear differentiation of these haemolytic forms of *Gallibacterium* from the non-haemolytic genus *Avibacterium*. However, the non-haemolytic forms of *Gallibacterium*, i.e. *G. anatis* biovar *anatis*, are not distinguishable at the genus level from the NAD-independent forms of *Avibacterium* (Table 1). Inclusion of speciation-level identification tests (Table 2) allows all members of the genus *Avibacterium* to be clearly distinguished from all members of the genus *Gallibacterium*. Table 2 presents phenotypic properties established in this study as well as other studies in which strains have been confidently identified on the basis of genetic methods (Bisgaard *et al.*, 2005; Christensen *et al.*, 2002b, 2003b; Mutters *et al.*, 1985a). Additional phenotypic data for *Avibacterium paragallinarum* comb. nov. has been included from the studies of Blackall & Reid (1982) and Blackall *et al.* (1989), as all the strains of *Avibacterium* ([*Haemophilus*] *paragallinarum* in these studies have been subsequently reconfirmed as *Avibacterium* ([*Haemophilus*] *paragallinarum* by PCR (Chen *et al.*, 1996) and/or have been serotyped by the Page scheme (Page, 1962). In addition to the differences shown in Table 2, differences in polyamine patterns can be used for separation between *Gallibacterium* and *Avibacterium* (Busse *et al.*, 1997), just as crossed immunoelectrophoresis of antigens clearly separated the genera (Schmid *et al.*, 1991).

The significance of V-factor dependency in the taxonomy of *Haemophilus* species and related organisms was discussed by Niven & O'Reilly (1990). A number of species within the *Pasteurellaceae* that contain both NAD-dependent and NAD-independent strains have been reported, e.g. *Actinobacillus pleuropneumoniae*, which causes pleuropneumonia in swine (Pohl *et al.*, 1983), *Avibacterium* ([*Haemophilus*] *paragallinarum* (Bragg *et al.*, 1993; Mouahid *et al.*, 1992), *Haemophilus parainfluenzae*, which can cause pneumonia and meningitis in humans (Gromkova & Koornhof, 1990), and *Haemophilus ducreyi*, which causes the sexually transmitted disease chancroid in humans (Windsor *et al.*, 1993). To date, there have been no extensive studies on the variations, if any, between the 16S rRNA gene sequences of NAD-dependent and NAD-independent strains within these species. Hence, it is not possible to compare the level of sequence variation we found between the NAD-dependent and NAD-independent *Avibacterium* ([*Haemophilus*] *paragallinarum* strains with comparable data from other species.

Martin *et al.* (2001) have recently shown that a single plasmid-encoded gene, *nadV*, from *Haemophilus ducreyi* is responsible for the occurrence of NAD independence in *Haemophilus ducreyi* and can confer NAD independence on NAD-dependent strains of both *Actinobacillus pleuropneumoniae* and *Haemophilus influenzae*. While the explanation for the NAD independence of *Avibacterium* ([*Haemophilus*] *paragallinarum* biovar 2 strains has not been elucidated, the work of Martin *et al.* (2001) suggests that NAD

**Table 1.** Key characters for differentiation of genera within the family *Pasteurellaceae*

Genera: 1, *Avibacterium* gen. nov.; 2, *Pasteurella*; 3, *Actinobacillus*; 4, *Haemophilus*; 5, *Lonepinella*; 6, *Mannheimia*; 7, *Phocoenobacter*; 8, *Gallibacterium*; 9, *Histophilus*; 10, *Volucrobacter*. Data based on Angen *et al.* (1999, 2003), Bisgaard & Muters (1986), Blackall & Reid (1982), Blackall *et al.* (1989), Christensen & Bisgaard (2003, 2004), Christensen *et al.* (2003a, b, 2004a, b), Muters *et al.* (1985a, b) and this study. Characters are scored as: +, 90% or more of strains positive within 1–2 days; (+), 90% or more of strains positive within 3–14 days; –, less than 10% of strains positive within 14 days; d, 11–89% of strains positive; w, weakly positive.

| Characteristic           | 1               | 2              | 3              | 4  | 5   | 6 | 7  | 8   | 9  | 10  |
|--------------------------|-----------------|----------------|----------------|----|-----|---|----|-----|----|-----|
| Catalase                 | d               | +              | +              | +  | –   | + | –  | +   | +  | +   |
| Symbiotic growth         | + <sup>a*</sup> | – <sup>b</sup> | – <sup>c</sup> | +  | –   | – | –  | –   | –  | –   |
| Voges–Proskauer, 37 °C   | –               | –              | –              | ND | d   | – | +  | –   | –  | –   |
| Urease                   | –               | – <sup>d</sup> | +              | +  | –   | – | –  | –   | –  | –   |
| Ornithine decarboxylase  | – <sup>e</sup>  | d              | –              | d  | –   | d | –  | –   | –  | –   |
| Indole                   | –               | +              | –              | d  | –   | – | –  | –   | +  | –   |
| Phosphatase              | + <sup>f</sup>  | +              | +              | +  | –   | + | +  | +   | +  | +/w |
| Growth on MacConkey agar | –               | –              | –              | ND | –   | d | –  | d   | ND | –   |
| Acid from:               |                 |                |                |    |     |   |    |     |    |     |
| D-Arabinose              | d               | d              | –              | ND | (+) | d | ND | (+) | ND | –   |
| Dulcitol                 | –               | d              | –              | –  | +   | – | –  | –   | ND | –   |
| meso-Inositol            | – <sup>g</sup>  | –              | –              | –  | –   | d | –  | d   | ND | d   |
| D-Mannitol               | d               | d              | + <sup>h</sup> | –  | –   | + | –  | +   | ND | –   |
| D-Fructose               | +               | +              | +              | –  | +   | + | ND | +   | –  | +   |
| D-Mannose                | +               | +              | d              | –  | +   | – | –  | +   | ND | +   |
| Maltose                  | d               | – <sup>i</sup> | +              | +  | +   | d | –  | d   | –  | d   |
| Sucrose                  | +               | +              | +              | –  | d   | + | –  | +   | –  | +   |
| Dextrin                  | d               | – <sup>i</sup> | +              | ND | (+) | d | ND | d   | ND | d   |
| Glycosides               | –               | –              | d              | –  | +   | d | d  | –   | ND | –   |
| α-Fucosidase             | –               | –              | –              | –  | –   | d | ND | –   | ND | –   |
| α-Glucosidase            | d               | +              | –              | –  | –   | – | ND | +   | ND | –   |
| β-Glucuronidase          | –               | –              | –              | –  | +   | – | ND | –   | ND | –   |

\*Discrepant results are indicated by: a, *Avibacterium gallinarum* negative, some isolates of *Avibacterium paragallinarum* also negative (biovar 2); b, *Pasteurella multocida* might be positive; c, *Actinobacillus pleuropneumoniae* biovar 1 positive; d, *Pasteurella dagmatis* positive; e, *Avibacterium volantium* can be positive; f, *Avibacterium paragallinarum* biovar 1 might be negative; g, *Avibacterium gallinarum* might be positive; h, *Actinobacillus suis* negative; i, *Pasteurella dagmatis* and *Pasteurella* sp. B positive.

independence may be a very minor component of the overall genome.

The available DNA–DNA hybridization data of Mouahid *et al.* (1992), who reported 89% DNA binding between strain ATCC 29545<sup>T</sup> and the biovar 2 strain SA 4461, indicate that the biovar 1 and 2 forms of *Avibacterium* (*[Haemophilus]*) *paragallinarum* can be accommodated in one species. The DNA fingerprinting work of Mifflin *et al.* (1995) has provided evidence that the South African NAD-independent isolates of *Avibacterium* (*[Haemophilus]*) *paragallinarum* are clonal, indicating that the DNA–DNA hybridization data for strain SA 4461 are applicable for all of the biovar 2 strains in this study. As well, all South African biovar 2 isolates of *Avibacterium* (*[Haemophilus]*) *paragallinarum* tested to date have yielded a positive

reaction for the *[Haemophilus]* *paragallinarum*-specific PCR (Chen *et al.*, 1996; Mifflin *et al.*, 1999). Furthermore, all of the South African biovar 2 isolates that have been serotyped (Mifflin *et al.*, 1995) have been allocated to the existing Page serotyping scheme, which was originally created using typical biovar 1 strains (Page, 1962). Hence, there are a number of separate bodies of evidence – the DNA–DNA hybridization data of Mouahid *et al.* (1992), the clonality of the South African biovar 2 isolates as shown by Mifflin *et al.* (1995), the positive reaction in the *[Haemophilus]* *paragallinarum* PCR (Chen *et al.*, 1996; Mifflin *et al.*, 1999), the ability to assign the South African isolates of biovar 2 to the existing *[Haemophilus]* *paragallinarum* serotyping scheme (Mifflin *et al.*, 1995) and the 16S rRNA gene sequencing results of the current study – that all support the inclusion of the biovar 2

**Table 2.** Key characters for the differentiation of taxa within the genera *Avibacterium* gen. nov. and *Gallibacterium*

Data based on Blackall & Reid (1982), Blackall *et al.* (1989), Christensen *et al.* (2002a, 2003a), Muters *et al.* (1985a) and this study. All species are Gram-negative and non-motile. All species reduce nitrate, are oxidase-positive and ferment glucose. Most isolates of *Avibacterium paragallinarum* require an enriched CO<sub>2</sub> (5–10%) atmosphere and most will show improved growth in the presence of 5–10% chicken serum. Most isolates of *Avibacterium gallinarum* show improved growth in an enriched CO<sub>2</sub> (5–10%) atmosphere. Characters are scored as: +, 90% or more of strains positive within 1–2 days; (+), 90% or more of strains positive within 3–14 days; –, less than 10% of strains positive within 14 days; d, 11–89% of strains positive; w, weakly positive. Entries in square brackets indicate the reaction of the type strain.

| Characteristic           | <i>A. gallinarum</i> | <i>A. paragallinarum</i> | <i>A. volantium</i> | <i>A. avium</i> | <i>Avibacterium</i><br>sp. A | <i>G. anatis</i>        |                              |
|--------------------------|----------------------|--------------------------|---------------------|-----------------|------------------------------|-------------------------|------------------------------|
|                          |                      |                          |                     |                 |                              | Biovar<br><i>anatis</i> | Biovar<br><i>haemolytica</i> |
| Catalase                 | +                    | – (0/51)                 | +                   | +               | +                            | +                       | +                            |
| Symbiotic growth         | –                    | + [+] (8/100)            | +                   | +               | +                            | –                       | –                            |
| Haemolysis               | –                    | – (0/100)                | –                   | –               | –                            | –                       | +                            |
| Ornithine decarboxylase  | –                    | – (0/9)                  | d [–] (2/10)        | –               | –                            | –                       | –                            |
| Growth on MacConkey agar | –                    | – (0/9)                  | –                   | –               | –                            | d [+] (4/5)             | d (13/24)                    |
| Acid from:               |                      |                          |                     |                 |                              |                         |                              |
| Glycerol                 | + [–] (61/64)        | – (0/9)                  | –*                  | –*              | –*                           | +/(+)                   | +/(+)                        |
| L-Arabinose              | –                    | – (0/100)                | –                   | –               | +                            | –                       | –                            |
| D-Arabinose              | d [–] (28/64)        | – (0/9)                  | [+]*                | –*              | –*                           | –                       | d (13/23)                    |
| D-Xylose                 | d [–] (29/64)        | – [–] (8/100)            | d [+] (5/10)        | d [–] (2/3)     | d [–] (2/5)                  | +                       | d (22/25)                    |
| <i>meso</i> -Inositol    | d [–] (49/64)        | – (0/100)                | –                   | –               | –                            | d [–] (2/7)             | d (19/25)                    |
| D-Mannitol               | –                    | + [(+)] (96/100)         | +                   | –               | d [+] (3/5)                  | +                       | +                            |
| D-Sorbitol               | – (1/64)             | + [(+)] (9/9)            | d [–] (4/10)        | –               | –                            | d [–] (3/7)             | d (13/25)                    |
| L-Fucose                 | (+) [w] (62/64)      | d [–] (8/9)              | [+]*                | –*              | –*                           | –                       | d (12/23)                    |
| D-Galactose              | +                    | – (0/100)                | +                   | +               | +                            | +                       | +                            |
| Lactose                  | d [–] (24/64)        | – (0/100)                | d [–] (5/10)        | –               | –                            | d [+] (4/5)             | d (20/24)                    |
| ONPG                     | d [–] (48/64)        | – (0/9)                  | +                   | –               | d [–] (2/5)                  | +                       | +                            |
| Maltose                  | +                    | d [(+)] (71/100)         | +                   | –               | d [–] (2/5)                  | –                       | d (8/25)                     |
| Trehalose                | +                    | – (0/100)                | +                   | +               | +                            | +                       | d (14/25)                    |
| Raffinose                | + [(+)] (61/64)      | – (0/100)                | –*                  | –*              | (+)*                         | +                       | +                            |
| Dextrin                  | +                    | d [(+)] (1/9)            | +*                  | –*              | –*                           | –                       | d (17/25)                    |
| α-Glucosidase            | +                    | – (0/51)                 | +                   | +               | +                            | +                       | +                            |
| Number of strains†       | 64                   | 100 <sup>a</sup>         | 10                  | 3               | 5                            | 7 <sup>b</sup>          | 25 <sup>b</sup>              |

\*Data from type/reference strain only (M. Bisgaard, unpublished data).

†All strains tested except where marked as: *a*, not all strains examined for all tests; *b*, all strains examined for all tests except where indicated.

strains within the species *Avibacterium* ([*Haemophilus*] *paragallinarum*).

Recently, biovar 2 isolates of [*Haemophilus*] *paragallinarum* have been identified in Mexico (García *et al.*, 2004). While these biovar 2 isolates were not available for the current study, the Mexican isolates have been shown to be positive in the [*Haemophilus*] *paragallinarum*-specific PCR and have been allocated to existing Page serovars (García *et al.*, 2004). This is strong evidence that these new Mexican biovar 2 isolates of [*Haemophilus*] *paragallinarum* are also typical of the species *Avibacterium paragallinarum*.

In the initial description of *Avibacterium* (*Pasteurella*)

*avium*, two biovars were recognized, with biovar 1 being NAD-dependent and associated with chickens while biovar 2 was NAD-independent and associated with cattle (Mutters *et al.*, 1985a). There has been continuing confusion about *Pasteurella avium* biovar 2, with Miflin & Blackall (2001) reporting that these biovar 2 isolates gave a positive reaction with a *Pasteurella multocida*-specific PCR based on the 23S rRNA gene. In recent work, an extended genotypic study has shown conclusively that *Pasteurella avium* biovar 2 is, in fact, *Pasteurella multocida* (Christensen *et al.*, 2004a). Hence, our study has only involved the avian, NAD-dependent forms of *Avibacterium* (*Pasteurella*) *avium*.

The proposed new genus contains four well-characterized

species: *Avibacterium gallinarum* comb. nov., *Avibacterium paragallinarum* comb. nov., *Avibacterium avium* comb. nov. and *Avibacterium volantium* comb. nov. In addition, the taxon once called *Pasteurella* sp. A is clearly a member of the genus and is best termed *Avibacterium* sp. A. Phenotypic differentiation of the members of the new genus from *Gallibacterium* is possible but relies on a few key characteristics such as catalase, NAD dependence, haemolysis, ONPG reaction and fermentation of glycerol, D-mannitol, D-galactose and maltose (Table 2). The differentiation of these organisms has always relied upon a few key characteristics, as has been highlighted in the traditional veterinary texts and laboratory manuals (Rhoades & Rimler, 1991; Rimler *et al.*, 1998).

The existence of *Avibacterium paragallinarum* and *Avibacterium gallinarum* within a single genus does raise the possibility of some confusion. There has been a long tradition within the genus *Haemophilus* of the use of the prefix 'para' to highlight that such a species is like an existing species but differs in growth factor requirements. This was the case for avian haemophilic organisms – with many publications from the 1960s to the 1980s referring to two species as the cause of infectious coryza – '*Haemophilus gallinarum*' and *Avibacterium* [*Haemophilus*] *paragallinarum* (Blackall & Yamamoto, 1989). These two species differ in growth factor requirements – '*Haemophilus gallinarum*' being X- and V-factor dependent and [*Haemophilus*] *paragallinarum* being V-factor dependent (Blackall & Yamamoto, 1989). While no valid strains of '*Haemophilus gallinarum*' exist, experimental work has shown that isolates of [*Haemophilus*] *paragallinarum* mistakenly appear to be X- and V-factor dependent when tested using the limited methodologies of the 1930s to 1950s (Blackall & Yamamoto, 1989). Hence, while it is not possible to be totally certain, it appears that the organism '*Haemophilus gallinarum*' never actually existed but was a mischaracterized *Avibacterium* ([*Haemophilus*] *paragallinarum*) (Blackall & Yamamoto, 1989). There is a possibility that some bacteriologists may assume the *Avibacterium gallinarum* is a renamed '*Haemophilus gallinarum*' or that species *Avibacterium paragallinarum* and *Avibacterium gallinarum* reflect the existence of species with V-factor and X- and V-factor dependency, respectively. We accept this risk of confusion as a reasonable compromise: the only alternative would be to propose a new species name for *Avibacterium paragallinarum*, since *Avibacterium gallinarum* was reported first and consequently has priority according to the rules of nomenclature. We do not feel that this renaming is justifiable.

### Description of *Avibacterium* gen. nov.

*Avibacterium* (Av'i.bac.te'ri.um. L. pl. n. *aves* birds; N.L. neut. n. *bacterium* rod; N.L. neut. n. *Avibacterium* bacterium of birds).

A member of the family *Pasteurellaceae* as defined by Olsen *et al.* (2004). Gram-negative, non-motile, rod-shaped

or pleomorphic with cells occurring singly and in pairs or short chains depending upon the growth stage. Colonies on sheep-blood agar are non-haemolytic, greyish, non-transparent, but eventually translucent at the periphery, with a butyrous consistency, smooth and shiny, circular and raised with an entire margin. Some isolates show symbiotic growth. Major differences are consequently observed in the size of colonies after 24 h incubation (pinpoint up to almost 2 mm in diameter). Pigment production is variable. Endospores are not formed. Growth is mesophilic and facultatively anaerobic or microaerophilic. The oxidase reaction is positive and nitrate is reduced. The reaction in Hugh–Leifson medium with D-glucose is fermentative without gas production. Porphyrin and alanine aminopeptidase tests are positive. Negative reactions occur for Simmons' citrate, mucate-acid, malonate-base, H<sub>2</sub>S/trisugar iron (TSI), growth in the presence of KCN, Voges–Proskauer, methyl red and urease tests. Negative tests are further observed with arginine dehydrolase, lysine decarboxylase, phenylalanine deaminase, indole, gelatinase and Tween 20 and 80 hydrolysis. Acid is formed from D-fructose, D-mannose and sucrose. Acid is not produced from *meso*-erythritol, adonitol, 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  $\beta$ -N-CH<sub>3</sub>-glucosamid. Reactions for  $\beta$ -glucosidase (NPG),  $\alpha$ -fucosidase (ONPF),  $\alpha$ -galactosidase,  $\beta$ -glucuronidase (PGUA),  $\alpha$ -mannosidase and  $\beta$ -xylosidase (ONPX) are also negative. No growth occurs on MacConkey agar. Variable reactions occur for catalase, phosphatase, ornithine decarboxylase and ONPG and PNPG tests and the production of acid from glycerol, D-arabitol, xylitol, L-arabinose, D-arabinose, D-ribose, D-xylose, *meso*-inositol, D-mannitol, D-sorbitol, L-fucose, D-galactose, lactose, maltose, trehalose, raffinose and dextrin. The DNA G + C content ranges from 44.2 to 47 mol%. The type species is *Avibacterium gallinarum*, originally described as *Pasteurella gallinarum* by Hall *et al.* (1955).

### Description of *Avibacterium gallinarum* (Hall *et al.* 1955) comb. nov.

Basonym: *Pasteurella gallinarum* Hall, Heddleston, Legenhausen and Hughes 1955.

Growth on blood agar is non-symbiotic, with most isolates producing a greyish-yellow pigment and showing a more even colony development if incubated under 5–10% CO<sub>2</sub>. Catalase and phosphatase reactions are positive. ONPG test is variable. Acid is produced from D-ribose, D-galactose, maltose, trehalose and dextrin. Acid is not produced from L-arabinose, D-mannitol or D-sorbitol. Acid production from glycerol, D-arabitol, D-arabinose, D-xylose, *meso*-inositol, L-fucose, lactose and raffinose is variable.

The type strain is NCTC 11188<sup>T</sup> (= ATCC 13361<sup>T</sup> = CCUG 12391<sup>T</sup> = CIP 102676<sup>T</sup>). This strain, originally isolated from the sinus of a chicken, has a genome mass of

$1.6 \times 10^9$  Da and a DNA G+C content of 43.6 mol% (Piechulla *et al.*, 1985).

**Description of *Avibacterium paragallinarum* (Biberstein and White 1969) comb. nov.**

Basonym: *Haemophilus paragallinarum* Biberstein and White 1969.

Most isolates show symbiotic growth and a requirement for 5–10% CO<sub>2</sub> for growth in early *in vitro* passages. Catalase reaction is negative. Phosphatase reaction is variable while the ONPG test is negative. Acid is produced from D-mannitol and D-sorbitol. No acid is produced from glycerol, D-arabitol, L-arabinose, D-arabinose, *meso*-inositol, D-galactose, lactose, trehalose or raffinose. The production of acid from D-ribose, D-xylose, L-fucose, maltose and dextrin is variable.

The type strain, NCTC 11296<sup>T</sup> (=ATCC 29545<sup>T</sup>=CIP 103453<sup>T</sup>), represents biovar 1, which is NAD-dependent, with a genome mass of  $1.7 \times 10^9$  Da and a DNA G+C content of 42.2 mol% (Mouahid *et al.*, 1992).

**Description of *Avibacterium avium* (Hinz and Kunjara 1977) comb. nov.**

Basonym: *Haemophilus avium* Hinz and Kunjara 1977.

Growth on blood agar is symbiotic. Catalase and phosphatase reactions are positive. ONPG test is negative. Acid is produced from D-galactose and trehalose. Acid is not produced from L-arabinose, D-arabinose, *meso*-inositol, D-mannitol, D-sorbitol, lactose or maltose. Acid production from D-xylose is variable.

The type strain is NCTC 11297<sup>T</sup> (=ATCC 29546<sup>T</sup>=CCUG 12833<sup>T</sup>=CIP 100919<sup>T</sup>). The genome mass and DNA G+C content are  $1.9 \times 10^9$  Da and 44.7 mol%, respectively (Mutters *et al.*, 1985a).

**Description of *Avibacterium volantium* (Mutters *et al.* 1985a) comb. nov.**

Basonym: *Pasteurella volantium* Mutters, Piechulla, Hinz and Mannheim 1985.

Growth on blood agar is symbiotic. A yellowish pigment is produced by some isolates. Catalase, phosphatase and ONPG reactions are positive. Acid is produced from D-ribose, D-mannitol, D-galactose, maltose, trehalose and dextrin. Acid is not produced from glycerol, D-arabitol, L-arabinose, *meso*-inositol or raffinose. Acid production from D-arabinose, D-xylose, D-sorbitol, L-fucose and lactose is variable.

The type strain is NCTC 3438<sup>T</sup> (=ATCC 14385<sup>T</sup>=CCUG 3713<sup>T</sup>=CIP 102677<sup>T</sup>). The genome mass and DNA G+C content are  $1.5 \times 10^9$  Da and 43.8 mol%, respectively (Mutters *et al.*, 1985a).

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