Reclassification of Bisgaard taxon 33, with proposal of *Volucribacter psittacida* gen. nov., sp. nov. and *Volucribacter amazonae* sp. nov. as new members of the *Pasteurellaceae*

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A total of 25 strains isolated from parrots, budgerigars, parakeets (Psittaciformes) and a chicken, mostly associated with respiratory disease or septicaemia, were classified as a new genus, *Volucribacter* gen. nov., within the family *Pasteurellaceae*, on the basis on unique phenotypic characteristics and clear monophyly as determined by 16S rRNA gene sequence comparison. Comparison of 16S rRNA gene sequences from six strains showed at least 98.8% similarity and the closest similarity outside the genus was found to Bisgaard taxon 34 and to *Pasteurella avium*, at 94.6 and 94.5%, respectively. Phenotypes that separate the new genus from other genera of the *Pasteurellaceae* included at least two characters. The genus includes two species, *Volucribacter psittacida* sp. nov. and *Volucribacter amazonae* sp. nov., corresponding to the two biovars previously outlined, underlining that most isolates have been obtained from psittacine birds. The two species can be separated by fermentation of meso-inositol, (–)-L-fucose, maltose and dextrin and a positive ONPG test. The type strains for *Volucribacter psittacida* and *Volucribacter amazonae* are respectively Gerl. 236/81^T (=CCUG 47536^T=DSM 15534^T) and 146/S8/89^T (=CCUG 47537^T=DSM 15535^T).

High density of pet birds under confined rearing facilities might change the parasite–host balance and result in disease. In a previous investigation, it was found that some organisms isolated from these birds could not be classified as any named species and consequently they remained as unnamed taxa of the *Pasteurellaceae* (Bisgaard *et al.*, 1999). Accurate diagnosis of bacteria isolated from diseased pet birds is a prerequisite for disease treatment and prevention, in addition to health improvement. Especially with members of the *Pasteurellaceae*, identification has remained a problem, because many groups are weakly characterized or are at most classified as unnamed taxa. This might result in uncertain or misleading identification (Bisgaard, 1993; Christensen *et al.*, 2002b, 2003b).

Twelve strains, all originating from psittacine bird species, were shown to represent a new taxon tentatively named Bisgaard taxon 33 on the basis of phenotypic

characterization and ribotyping (Bisgaard *et al.*, 1999). Ribotyping of selected strains showed that taxon 33 strains formed a separate cluster (Bisgaard *et al.*, 1999). The original publication included isolates obtained from lesions of lungs, trachea, air sac, liver, spleen and eyes of parrots. Since this publication, 13 additional strains have been obtained and investigated and been found to be both phenotypically and genotypically similar to taxon 33.

The present study aimed at a final classification of Bisgaard taxon 33, with the proposal of a new genus within the *Pasteurellaceae*.

Phenotypic characterization

In addition to the 12 strains already characterized phenotypically, nine of which were ribotyped in the previous publication (Bisgaard *et al.*, 1999), 13 further strains were phenotyped according to Bisgaard *et al.* (1991). The total number of strains investigated originated from parrots (15), budgerigars (7), parakeets (2) and a chicken. With the exception of a single isolate, all isolates were obtained from lesions, mostly from the respiratory tract or septicaemia. Isolates have so far only been obtained in European countries, including Belgium, Denmark and Germany.

Published online ahead of print on 28 November 2003 as DOI 10.1099/ijs.0.02797-0.

The GenBank/EMBL/DDBJ accession numbers for the 16S rDNA sequences of strains Gerl. 236/81^T, 146/S8/89^T and B96/5 are AY216868–AY216870 and those for strains 94, 101 and 103 are AF487722–AF487724, respectively.

Common phenotypic characteristics of the 25 strains classified with Bisgaard taxon 33 and proposed as *Volucribacter* gen. nov. included positive reactions for methyl red, nitrate, porphyrin and acid production without gas from (–)-D-ribose, (–)-D-fructose, (+)-D-galactose, (+)-D-glucose, (+)-D-mannose, sucrose and raffinose, whereas negative reactions were observed for symbiotic growth, urease, ornithine decarboxylase, α -glucosidase (PNPG; 4-nitrophenyl α -D-glucopyranoside) and fermentation of (–)-D-mannitol, (–)-D-sorbitol and trehalose. A full list of the 81 phenotypic characters investigated is given with the description of the genus.

Characters that are variable within the genus are listed with Table 1 and allow two biovars to be distinguished. Biovar 1, including 22 strains, is negative for the ONPG (o-nitrophenyl D-galactopyranoside) test and does not produce acid from (–)-L-fucose, maltose or dextrin, whereas biovar 2, including three strains, is positive for these characters.

Biovar 2 was defined on the basis of three phenotypic variants. Strain 146/S8/89^T can be described as a *meso*-inositol-, ONPG-, maltose-, melibiose-, dextrin- and α -galactosidase-positive variant of *Volucribacter*, while strain

Table 1. Phenotypic variation observed for isolates of *Volucribacter* gen. nov. (Bisgaard taxon 33)

Strains examined: *V. psittacidica* sp. nov., 22 strains isolated from parrot, budgerigar, parakeet and chicken in Belgium, Denmark and Germany; *V. amazonae* sp. nov., three strains isolated from parrot and parakeet in Belgium, Denmark and Germany. Characters are scored as: +, 90% or more of strains positive within 1–2 days; (+), 90% or more of strains positive within 3–14 days; –, fewer than 10% of strains positive within 14 days; d, 11–89% of strains positive. Numbers of strains positive for particular characters are given in parentheses.

Character	<i>V. psittacidica</i> (biovar 1)	<i>V. amazonae</i> (biovar 2)
Catalase	d (11)	+
Oxidase	d (17)	d (2)
Phosphatase	+ /weak	+
Glycerol	(+) (21)	d (2)
(+)-L-Arabinose	–	d (1)
(–)-D-Arabinose	– (1)	d (1)
(+)-D-Xylose	– (1)	d (1)
<i>meso</i> -Inositol	– (1)	+ /(+)
(–)-L-Fucose	–	(+)
Lactose	–	d (1)
ONPG	–	+
Maltose	–	+
(+)-D-Melibiose	–	d (2)
Dextrin	–	+ /(+)
α -Galactosidase	– (1)	d (2)

B96/5 represents an (+)-L-arabinose-, *meso*-inositol-, ONPG, maltose- and dextrin-positive variant. Finally, strain 64 can be regarded as a (+)-D-xylose-, *meso*-inositol-, lactose-, ONPG-, maltose-, melibiose-, dextrin- and α -galactosidase-positive variant.

The existence of two biovars indicated the existence of two species within *Volucribacter*, as also indicated by the lower 16S rRNA gene sequence similarities between the biovars than within them and the low degree of DNA–DNA hybridization (see below).

The phenotypic characters indicate that *Volucribacter* is a member of the *Pasteurellaceae* as defined by Olsen *et al.* (2004) and previously suggested by Bisgaard *et al.* (1999). Between two and four phenotypic characters separate the genus *Volucribacter* from other genera of the *Pasteurellaceae* (Table 2). Data for the other genera and genus-like groups of *Pasteurellaceae* were obtained from Angen *et al.* (1999) (*Mannheimia*), Mutters *et al.* (2004) (*Pasteurella sensu stricto*), Osawa & Stackebrandt (2004) (*Lonepinella*), Foster & Collins (2004) (*Phocoenobacter*), Kilian (2004) (*Haemophilus sensu stricto*), Christensen *et al.* (2003a) (*Gallibacterium*), Angen *et al.* (2003) (*Histophilus somni*) and Christensen & Bisgaard (2004) (*Actinobacillus sensu stricto*). Only differences in (–)-D-mannitol and α -glucosidase separate taxon 33 from *Gallibacterium*. However, most isolates of *Gallibacterium* are β -haemolytic and trehalose-positive, while taxon 33 is negative. At least three phenotypic characters separate taxon 33 from other existing genera containing avian taxa. Separation from possible candidates for new genera containing avian taxa, including *Pasteurella sensu stricto* of cluster 18 (Olsen *et al.*, 2004), [*Haemophilus*] *paragallinarum* and the taxon 2 and 3 complex of Bisgaard, has to await separate investigations.

16S rRNA gene sequence comparison

Six strains were selected for 16S rRNA gene sequencing (Table 3). 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). PCR-amplified fragments were purified on Microspin columns (Pharmacia Biotech) and cycle-sequenced (Thermo Sequenase fluorescent-labelled primer cycle-sequencing kit; Amersham) on an ALF sequencer (Pharmacia Biotech) using fluorescein-labelled primers.

Sequences of between 1434 and 1468 bp were obtained. The accession numbers for the strains are listed in Table 3. Searches for 16S rRNA sequences were performed by FASTA and BLAST by use of the Wisconsin sequence analysis package (GCG, Madison). Pairwise comparisons for similarity were performed by BESTFIT (GCG). The four strains Gerl. 236/81^T, 94, 101 and 103 of biovar 1 showed 99.2–99.9% similarity, whereas the two strains B96/5 and 146/S8/89^T of biovar 2 showed 99.9% 16S rRNA similarity. The lowest

Table 2. Phenotypic characters that allow separation of *Volucribacter* gen. nov. from other accepted genera of the family Pasteurellaceae

Genera: 1, *Mannheimia*; 2, *Actinobacillus sensu stricto*; 3, *Pasteurella sensu stricto* cluster 12; 4, *Pasteurella sensu stricto* cluster 18; 5, *Lonepinella*; 6, *Phocoenobacter*; 7, *Haemophilus sensu stricto* (*H. influenzae*, *H. haemolyticus* and *H. aegyptius*); 8, *Gallibacterium*; 9, *Histophilus*; 10, *Volucribacter* gen. nov. Characters are scored as in Table 1; W, weakly positive; ND, no data.

Character	1	2	3	4	5	6	7	8	9	10
Symbiotic growth	—	— ^{a*}	—	+ ^b	—	—	+	—	—	—
Methyl red (37 °C)	—	—	—	—	—	ND	ND	W	ND	+
Voges-Proskauer (37 °C)	—	—	—	—	d	+	ND	—	—	—
Urease	—	+	— ^c	—	—	—	+	—	—	—
Ornithine decarboxylase	d	—	d	— ^d	—	—	d	—	—	—
Indole	—	—	+	—	—	—	d	—	+	—
Phosphatase	+	+	+	+	—	+	+	+	+	+ / W
Acid production from:										
Dulcitol	—	—	d	—	+	—	—	—	ND	—
(–)-D-Mannitol	+	+ ^e	d	d	—	—	—	+	ND	—
(+)-D-Galactose	+	+	+	+	+	—	+	+	—	+ / (+)
(+)-D-Glucose (with gas)	—	—	—	—	—	ND	d	—	ND	—
(+)-D-Mannose	—	d	+	+	+	—	—	+	ND	+
(+)-L-Rhamnose	—	—	—	—	d	—	—	—	ND	—
Sucrose	+	+	+	+	d	—	—	+	—	+
Trehalose	—	d	d	+	—	—	—	d	—	—
α-Glucosidase (PNPG)	—	d	+	+	—	ND	—	+	ND	—

*Variable results are indicated by: *a*, *Actinobacillus pleuropneumoniae* biovar 1 positive; *b*, *Pasteurella gallinarum* negative; *c*, *Pasteurella dagmatis* positive; *d*, *Pasteurella volantium* might be positive; *e*, *Actinobacillus suis* negative.

similarity observed between biovar 1 and 2 strains was 98.8 %, between strains 94 and B96/5, while the highest similarity (99.5 %) was observed between strains 103 and 146/S8/89^T. Strain Gerl. 236/81^T showed 99.2–99.9 % similarity to biovar 1 strains and 99.4 % similarity to biovar 2 strains. Such high 16S rRNA similarity between species is not unusual for members of the Pasteurellaceae, where 16S rRNA similarities of 99.7 % between type strains of *Pasteurella gallinarum* and *Pasteurella volantium*, as well as between *Actinobacillus lignieresii* and *Actinobacillus*

pleuropneumoniae, corresponded to 72 % DNA reassociation (Pohl *et al.*, 1983; Muters *et al.*, 1985), and 99.6 % 16S rRNA similarity between the type strains of *Actinobacillus suis* and *Actinobacillus equuli* corresponded to 66 % DNA reassociation (Christensen *et al.*, 2002a). The highest 16S rRNA similarity outside the genus *Volucribacter* was found to the type strain of *Pasteurella avium* (accession no. M75058; 94.5 %) for strain 101 and to strain 69 of Bisgaard taxon 34 (accession no. AY172731; 94.6 % similarity) for strain 94.

Table 3. Strains of *Volucribacter* gen. nov. investigated by 16S rRNA gene sequence comparison

Strain	Source*	Country	Organ of isolation	Lesion	16S rRNA gene accession no.
<i>V. psittacida</i> (biovar 1)					
Gerl. 236/81 ^T (=HIM 698-7/8 ^T = CCUG 47536 ^T = DSM 15534 ^T)	Parakeet	Germany	Liver, heart	Septicaemia	AY216868
101	African grey parrot	Germany	Salivary gland	Purulent inflammation	AF487723
94	Fischer's love bird	Germany	Lung	Pneumonia	AF487722
103	Budgerigar	Germany	Trachea	No macroscopic lesions	AF487724
<i>V. amazonae</i> (biovar 2)					
146/S8/89 ^T (=CCUG 47537 ^T = DSM 15535 ^T)	African grey parrot	Germany	Liver, heart	Septicaemia	AY216869
B96/5	Parakeet	Belgium	No information	No information	AY216870

*African grey parrot (*Psittacus erithacus*), Fischer's love bird (*Agapornis fischeri*) and budgerigar (*Melopsittacus undulatus*) (Bisgaard *et al.*, 1999).

Phylogenetic analysis

The alignment was constructed by PILEUP (GCG) and included the region between *Escherichia coli* positions 64 and 1391 of *rrnB*, with 1283 positions left after removal of ambiguous positions and 184 distinct data patterns analysed. The alignment was in accordance with the secondary structure mask of Lane (1991). Maximum-likelihood analysis including bootstrap analysis was performed by fastDNAmL (Olsen *et al.*, 1994) run on a Linux 7.2-compatible server with a transition/transversion ratio of 1.5. Parsimony and neighbour-joining analysis were performed by PHYLIP (Felsenstein, 1995).

The high 16S rRNA gene sequence similarity between the strains of taxon 33 proposed as *Volucribacter* was reflected in the phylogenetic comparison, where the representatives of the taxon formed a distinct monophyletic group supported by a bootstrap value of 99 % (Fig. 1). The phylogenetic analysis shown in Fig. 1 was based on 16S rRNA gene sequences of type strains of the genera of *Pasteurellaceae*. The topology shown in Fig. 1 was identical when data were analysed by maximum-parsimony and neighbour-joining analysis. The distinct phylogenetic position of *Volucribacter* (Bisgaard taxon 33) with reference to all members of the family *Pasteurellaceae* has been shown previously (Christensen *et al.*, 2003b). The genus was related to representatives of other members of the ‘avian’ cluster (Olsen *et al.*, 2004), including members of *Pasteurella sensu stricto* (Mutters *et al.*, 1989) isolated from birds, the new genus *Gallibacterium* (Christensen *et al.*, 2003a), [*Haemophilus*] *paragallinarum* and taxa 2, 3 and 34 of Bisgaard (Christensen *et al.*, 2003b). In conclusion, the

Fig. 1. Phylogenetic relationships between the taxa of *Pasteurellaceae* based on maximum-likelihood analysis of 16S rRNA sequences with special regard to the newly proposed genus *Volucribacter* and members of type species of genera of *Pasteurellaceae*. Support for monophyletic groups by bootstrap analysis is indicated as percentages. Branch lengths have been drawn according to the scale bar (evolutionary distance), which is related to the number of nucleotide substitutions between the sequences and the time since the taxa diverged.

proposed genus *Volucribacter* was unrelated to representatives of type species and 16S rRNA clusters (Olsen *et al.*, 2004) of *Pasteurellaceae*.

DNA–DNA hybridizations

DNA–DNA hybridizations were performed according to the microwell method described by Christensen *et al.* (2000). The DNA reassociation between strains Gerl. 236/81^T and 101 of biovar 1 was 94 % (Table 4). DNA reassociation between strain Gerl. 236/81^T from biovar 1 and 146/S/89^T from biovar 2 was only 47 %, underlining the presence of two species. The DNA reassociation results published by Piechulla *et al.* (1985) were at most 24 % to other taxa investigated, as observed between Gerl. 236/81^T and three strains of taxa 2 and 3 of Bisgaard. Only 6 % DNA reassociation was found between Gerl. 236/81^T and the type strain of *Pasteurella gallinarum*. A value of 70 % DNA reassociation between strain Gerl. 236/81^T and strain Gerlach 3348/80 (= F 219) of *Gallibacterium* was observed by Piechulla *et al.* (1985). This result was subsequently reinvestigated and found to be only 11 % (Christensen *et al.*, 2003a). A value of 76 % DNA reassociation was found between Gerl. 236/81^T and a V-factor-requiring strain, Sittich 1, obtained from a parakeet (Piechulla *et al.*, 1985). Further information is unfortunately not available for this strain. A group of *Pasteurella avium*-like organisms obtained from the respiratory tract of psittacine birds was reported by Beichel (1986). The single isolate (103) included in the present investigation and meeting the phenotypic characters of taxon 33 showed DNA reassociation values between 24 and 30 % with *Pasteurella multocida*, *Pasteurella stomatis*, *Pasteurella avium* and *Gallibacterium anatis* and between 18 and 44 % with Bisgaard taxon 16 (Stenzel, 1992).

The name *Volucribacter* gen. nov. is suggested for taxon 33, since the strains were isolated exclusively from birds. The names *Volucribacter psittacidica* sp. nov. and *Volucribacter amazonae* sp. nov. are suggested with reference to the hosts being members of the Psittaciformes. The strains of the new genus investigated all (except one) originated from psittacine birds, in which they were almost all associated with pathological conditions. Further studies are required,

Table 4. DNA–DNA hybridization results obtained with strains of *Volucribacter* gen. nov.

DNA from test strains was hybridized with DNA from strain Gerl. 236/81^T (biovar 1). Numbers in parentheses are SD (*n*=8).

Strain	DNA–DNA relatedness (%)
Biovar 1, strain 101	94 (17)
Biovar 2, strain 146/S/89 ^T	47 (9)
<i>Gallibacterium</i> genomospecies 1, strain CCM 5974	9 (9)
<i>Gallibacterium anatis</i> , strain Gerlach 3348/80	10 (12)

however, in order to elucidate the host relations and pathogenicity of this genus.

Description of *Volucribacter* gen. nov.

Volucribacter (Vo.lu.cri.bac'ter. L. fem. n. *volucris* bird; N.L. masc. n. *bacter* rod; N.L. masc. n. *Volucribacter* rod-shaped bacterium of birds).

Gram-negative rods forming surface colonies on blood agar that are circular and slightly raised with an entire margin. The surface of the colonies is smooth, shiny and opaque with a greyish tinge. Colonies are 1.0–1.5 mm in diameter after aerobic incubation at 37 °C for 24 h. After incubation for 48 h, a diameter of 2.0 mm might be reached. The consistency is buttery and colonies do not adhere to the agar surface. Growth on bovine blood agar is not accompanied by haemolysis. A greenish colour is sometimes associated with heavy growth. (+)-D-Glucose is catabolized fermentatively in Hugh and Leifson's medium and porphyrin, methyl red (37 °C), nitrate reduction and alanine aminopeptidase tests are positive within 1–2 days. (–)-D-Fructose, (+)-D-glucose, (+)-D-mannose and sucrose are fermented within 1–2 days, while (–)-D-ribose, (+)-D-galactose and raffinose are positive after 3 or more days. The phosphatase test is positive, but weak reactions might occur. Within 14 days of incubation, motility at 37 and 22 °C is negative, and tests for symbiotic growth, growth on Simmons' citrate, malonate base, H₂S formation in TSI, KCN growth, Voges–Proskauer at 37 °C, gas from nitrate, urease, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, phenylalanine deaminase, indole formation, gelatinase, hydrolysis of Tween 20 or 80, MacConkey growth, pigment, formation of gas from (+)-D-glucose, α -fucosidase, α -glucosidase, β -glucuronidase, α -mannosidase and β -xylosidase are all negative. Acid is not formed from mucate, *meso*-erythritol, adonitol, (+)-D-arabitol, xylitol, (–)-L-xylose, dulcitol, (–)-D-mannitol, (–)-D-sorbitol, (+)-D-fucose, (+)-L-rhamnose, (–)-L-sorbose, trehalose, cellobiose, (+)-D-melezitose, (+)-D-glycogen, inulin, aesculin, amygdalin, arbutin, gentiobiose, salicin, (+)-D-turanose or *N*-methyl β -glucosamide. Variable characters include catalase, oxidase, glycerol, (+)-L-arabinose, (–)-D-arabinose, (+)-D-xylose, *meso*-inositol, (–)-L-fucose, lactose, ONPG, maltose, (+)-D-melibiose, dextrin and α -galactosidase. The bacteria have been isolated from parrots, budgerigars, parakeets and a chicken, mainly showing respiratory tract or septicemic lesions. The type species is *Volucribacter psittacida*.

Description of *Volucribacter psittacida* sp. nov.

Volucribacter psittacida (psit.ta.ci'ci.da. L. masc. n. *psittacus* the parrot; L. masc./fem. suffix n. *-cida* killer; N.L. masc. n. *psittacida* killer of parrots).

Colony and cellular morphology and phenotypic reactions are identical to those given in the genus description. In

addition, the species is positive or weakly positive in reactions for phosphatase. Late positive reactions are observed with glycerol, while negative reactions are observed with (+)-L-arabinose, (–)-D-arabinose, (+)-D-xylose, *meso*-inositol, (–)-L-fucose, lactose, maltose, (+)-D-melibiose, dextrin, α -galactosidase and ONPG. Variable reactions are observed for catalase and oxidase. The species can be separated from *V. amazonae* by the lack of fermentation of *meso*-inositol, (–)-L-fucose, maltose and dextrin and the negative ONPG reaction. Plasmids were not found in the type strain (procedure reported by Christensen *et al.*, 2003a).

The type strain is Gerl. 236/81^T (=CCUG 47536^T=DSM 15534^T), obtained from a parakeet with septicemia and received from H. Gerlach, Munich, Germany, in 1981. The characterization was based on 22 strains from three different countries representing isolates mostly obtained from lesions in parrots, budgerigar, parakeets and a chicken. The DNA G+C content of strain 103 was 39.9 mol%, while the genome mass was 1.4×10^9 Da (Stenzel, 1992). The G+C content of strain Gerl. 236/81^T was 40.8 mol% and the genome mass 2.4×10^9 Da (Piechulla *et al.*, 1985).

Description of *Volucribacter amazonae* sp. nov.

Volucribacter amazonae (a.ma.zo.na'e. N.L. gen. sing. n. *amazonae* of parrots of the genus *Amazona*).

Colony and cellular morphology and other phenotypic reactions are identical to those given in the genus description; in addition, the species is positive for catalase, phosphatase and ONPG and ferments *meso*-inositol, (–)-L-fucose, maltose and dextrin. Variable reactions are observed for production of acid from glycerol, (+)-L-arabinose, (–)-D-arabinose, (+)-D-xylose, lactose and (+)-D-melibiose and the α -galactosidase test. The species can be separated from *V. psittacida* by fermentation of *meso*-inositol, (–)-L-fucose, maltose and dextrin and positive reaction for ONPG.

Three strains of the species isolated from parrots and parakeet have been characterized. The type strain, 146/S8/89^T (=CCUG 47537^T=DSM 15535^T), was originally isolated from an African grey parrot (*Psittacus erithacus*) with septicemia by H. Gerlach, Munich, Germany, in 1989.

Acknowledgements

Pia Mortensen, Pia Katrine Nielsen and Kristian Petersen are thanked for excellent technical assistance. Professor H. G. Trüper, Department of Microbiology, Universität Bonn, and Dr J. P. M. Euzéby, National Veterinary School of Toulouse, are thanked for careful help with the Latin names. This project was financed by the Danish Agricultural and Veterinary Research Council grant number 9702797.

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