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Reclassification of Actinobacillus muris as Muribacter muris gen. nov. comb. nov.

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Reclassification of *Actinobacillus muris* as *Muribacter muris* gen. nov. comb. nov.

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The accession numbers of the 16S rRNA, *rpoB* and *infB* genes sequence of strains determined in the present study are KP278018 - KP278142 and KP664114 and KP664115 for *infB* gene sequences of strains HIM565_1 and 3996_85, respectively. The whole genome sequence of Ackerman80-443D^T is JWIZ00000000.

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Abstract

To reinvestigate the taxonomy of [*Actinobacillus*] *muris*, 474 strains mainly from mice and rats were characterized by phenotype and 130 strains selected for genotypic characterization by 16S

rRNA and partial *rpoB* gene sequencing. The type strain was further investigated by whole genome sequencing. Phylogenetic analysis of the DNA sequences showed one monophyletic group with intra group similarities of 96.7 % and 97.2 % for 16S rRNA and *rpoB* genes, respectively. The lowest 16S rRNA similarity to the closest related valid named taxon outside the group was 95.9 % to the type strain of [*Pasteurella*] *pneumotropica.* The closest related taxon based on *rpoB* sequence comparison was '*Haemophilus influenzae-murium*' with 88.4 %. A new genus, *Muribacter* is proposed based on a distinct phylogenetic position based on 16S rRNA and *rpoB* gene sequence comparisons with major divergence to the existing genera of *Pasteurellaceae*. The new genus includes the characteristics of [*Actinobacillus*] *muris* with the emendation that acid formation from (-)-D-mannitol is variable as well the hydrolysis of esculin while the α-glucosidase test is positive. There is no requirement for exogenously supplied nicotinamide adenine dinucleotide (V factor) for the majority of strains investigated, however, one strain was found positive. The major fatty acids of the type strain of *Muribacter muris* were $C_{14:0}$, $C_{14:0}$ 3OH/$C_{16:1}$ ISOI, $C_{16:1}$ ω7c and $C_{16:0}$ which is in line with most genera of *Pasteurellaceae*. The type strain of *Muribacter muris* is CCUG 16938^T (= NCTC 12432^T = ATCC 49577^T).

[*Actinobacillus*] *muris* was originally described based on 19 strains isolated from *cavum oris* of healthy mice with the provisional designation Bisgaard taxon 12 and the selection of a published reference strain (NCTC 12432^T) as type strain (Bisgaard, 1986) (Table S1). DNA-reassociation studies showed that [*Actinobacillus*] *muris* as a species was unrelated to other members of *Actinobacillus*, and further that [*Pasteurella*] *pneumotropica* and [*Actinobacillus*] *muris* were also unrelated to each other at the species level (Piechulla *et al*., 1985; Ryll *et al*., 1991). Phylogenetic analysis based on 16S rRNA sequence comparison documented a 'rodent group' within *Pasteurellaceae* including [*Pasteurella*] *pneumotropica* and [*Actinobacillus*] *muris* (Dewhirst *et al*., 1993) both taxa being unrelated to *Actinobacillus sensu stricto* and *Pasteurella sensu stricto*. Further investigations have confirmed that [*Actinobacillus*] *muris* is unrelated to *Actinobacillus sensu stricto* (Christensen & Bisgaard, 2004). Recently, a new genus, *Necropsobacter*, unrelated to [*Pasteurella*] *pneumotropica* and [*Actinobacillus*] *muris*, has been described that mainly included organisms from rodents (Christensen *et al*., 2011). In the 16S rRNA multiple alignment *Necropsobacter* had two characteristic deletions through pos. 203-206 and 213-216 (*Pasteurella multocida* acc. no. AY078999) compared with other members of *Pasteurellaceae* (Christensen *et al*., 2011). *Mesocricetibacter intestinalis* and *Cricetibacter osteomyelitidis* were recently described

based on the characterization of strains isolated from hamsters (Christensen *et al*., 2014). In the present study a collection of *Pasteurellaceae* obtained from rodents was subjected to extended phenotypic characterization. Partial *rpoB* sequences were used to evaluate characters used for phenotypic identification and separation of taxa, and comparison of 16S rRNA sequences used to evaluate genotypic diversity mainly at the genus level. The study aimed to reclassify [*Actinobacillus*] *muris* away from *Actinobacillus sensu stricto* as a separate new monotypic genus, *Muribacter muris*, and further to investigate the diversity of this taxon which may lead to improved identification and consequently better understanding of epidemiology and clinical implications. Phenotypic diversity of [*Actinobacillus*] *muris* has been reported with respect to acid production from cellobiose, mannitol and salicin, hydrolysis of esculin, production of indole and urease activity (Nicklas, 2007), however, most biochemical profiles of *Actinobacillus muris* have never been mentioned in the literature, and some members of this taxon may have been misidentified (e.g., as *Pasteurella multocida*) or not identified at all. The current investigation shows that all members of the taxon are frequently found in colonies of laboratory mice.

We included the type strain of [*Actinobacillus*] *muris* and 473 additional isolates in the characterization (Table S1, S2). The strains were isolated during the years 1980 - 2014 and represented mainly mice and a few isolates from rats. Mice and rats sampling positive were received from other research institutions and universities or from commercial breeders of laboratory rodents, or bacterial isolates received from other diagnostic laboratories. In addition to laboratory mice and rats, wild rodents were trapped. In addition, mice and rats were bought from 10 different pet shops. Isolates were subjected to phenotypical characterization using 40 biochemical criteria examined by conventional tests as previously reported (Christensen *et al*., 2014). In these tests, acid formation from carbohydrates was tested in phenol red broth base (Difco Laboratories, Detroit, MI, USA) supplemented with 1 % of the respective carbohydrate and read after 2-3 days incubation at 37°C. All other reactions were read after 18-24 h incubation or as recommended by the author cited below. Hydrolysis of esculin was tested in esculin broth (Merck, Darmstadt, Germany). Urease, indole, and amino acid decarboxylase tests were performed as recommended by Kilian (1976). The requirement for growth factors was tested with filter paper disks containing 12.5 μg of NAD (Roche Diagnostics GmbH, Mannheim, Germany) or 25 μg hemin (Sigma, Chemical Co., St. Louis, MO, USA) on Mueller Hinton Agar (Heipha, Heidelberg, Germany). The ability to synthesize porphyrins from δ-aminolaevulinic acid was demonstrated under UV light in a dark room and by addition of

Kovac's indole reagent (Merck, Darmstadt, Germany) as described by Kilian (1976). Phenotypic characters shared by all strains investigated (some included for genus level separation, Table 1) were in accordance with the description of [*Actinobacillus*] *muris* (Bisgaard, 1986) except for variable reactions in acid formation from (-)-D-mannitol as well a variable reactions for indole, urease and hydrolysis of esculin. The α-glucosidase test (PNPG, 4-nitrophenyl-α-D-glucopyranoside) was found positive.

On the basis of phenotypic diversity, 16 biovars were identified (Table S2). We then selected isolates for further characterization by DNA sequencing from each biovar representing animals coming from different sources and different years (Table S1).

16S rRNA gene sequencing of 125 strains was performed as reported previously (Angen *et al*., 2003; Christensen *et al*., 2002). In addition, the reference strain of '*Haemophilus influenzae-murium*' was 16S rRNA gene sequenced since numerous ambiguous positions were present in the published sequence (GenBank accession number AF024530). Partial sequencing of the *rpoB* gene of 127 strains was performed according to previously described protocols (Korczak *et al*., 2004; Korczak & Kuhnert, 2008; Kuhnert *et al*., 2004). All GenBank accession numbers are listed with Table S1.

Searches for sequences in public databases were performed by BLAST (Altschul *et al*., 1997). Pairwise similarity was determined by the WATER program of EMBOSS (Rice *et al*. 2000). In addition to the 16S rRNA gene sequences determined in the current investigation, published sequences were included of [*Actinobacillus*] *muris*, '*Haemophilus influenzae-murium*' and taxon 17 of Bisgaard as well as type strains of type species of genera of *Pasteurellaceae* in addition to the type strain of [*Pasteurella*] *pneumotropica* (biovar Jawetz) and the reference strain of biovar Heyl of this species (Fig. 1).

Genome sequencing of the type strain of [*Actinobacillus*] *muris* was done by Illumina Hiseq 2000 and reads were assembled by CLC Genomic Workbench version 7.5. Automatic annotation was performed by RAST http://rast.nmpdr.org/ (Overbeek *et al*., 2014). The GC % is 43.7 % determined by whole genome sequencing. The GC mol % was previously reported as 46.9 % determined by the

DNA renaturation method (Piechulla *et al*., 1985) and the difference may be related to different methodologies applied.

The genome could be assembled to 2684001 nt on 148 contigs., and 2810 coding sequences were identified by RAST, 1376 of which could be associated with a known function based on database search included with RAST.

Multiple alignments of DNA sequences were constructed by ClustalX2 (Larkin *et al.*, 2007). Columns with gaps were trimmed out of the multiple alignment by use of Bioedit (Hall, 1999). Phylogenetic analysis of the 16S rRNA and *rpoB* gene sequences were carried out by neighbour joining using Jukes-Cantor correction and included calculation of bootstrap support. MEGA6 (Tamura *et al*., 2011) was used for graphical representation of trees. Two sequences were excluded from the 16S rRNA gene sequence based multiple alignments since they were too short (Table S1). The multiple alignment included at least 1173 nt. of the remaining strains

The 16S rRNA gene sequence based phylogenetic comparisons documented a monophyletic group of strains isolated from rodents including the type strain of [*Actinobacillus*] *muris* (Fig. 1, Fig. S1). The group to be referred as *Muribacter* in the following. The lowest similarity within the group was 96.7 %. This is slightly below the normal lower limit of 16S rRNA gene sequence similarity within a species (Stackebrandt & Goebel, 1994), however, all members of the taxon were related in a continuum and neither geno- or phenotypical differences justified a separation into more species. The highest similarity outside the group was found to the reference strain (HIM565-1) of '*Haemophilus influenzae-murium*' with 96.4 % to strain 1999096011 of *Muribacter* which is below the recognized 97 % 16S rRNA gene sequence threshold for species separation (Stackebrandt & Goebel, 1994). Characterization of '*Haemophilus influenzae-murium*' was not the aim of this paper and since this taxon is unrelated at the species level to *Muribacter muris*, it is left out of the current taxonomic treatment. The 16S rRNA gene similarity between the type strain of [*Actinobacillus*] *muris* and the type strain of [*Pasteurella*] *pneumotropica* (biovar Jawatz) was only 94.8 % which is below the similarity between most genera within *Pasteurellaceae* of around 95 % (Christensen *et al*., 1997).

In the 16S rRNA gene sequence multiple alignment all strains of *Muribacter* had a characteristic deletion of five nt. in the region 211-217 (numbering according to *Pasteurella multocida* acc. no. AY078999) which may be utilized for identification purpose since the signature is a a slightly different location compared to *Necropsobacter rosorum* (pos. 203-206 and 213-216) (Christensen *et al*., 2011)

The type strain of [*Pasteurella*] *pneumotropica* (biovar Jawatz) was used as outgroup for the *rpoB* phylogeny (Fig. S2). The *rpoB* sequences were identical for Ackerman80-443D ^{T} and 24 other strains (group I). Another large group included 25 strains with identical *rpoB* sequences (group VIII) (Table S1). Eight other groups and a singleton could be identified as well. Strain 2005150026 (group XI) diverged from the other groups, however, analysis of *rpoB* still showed high similarity within *Muribacter muris* of 97.2 -100%. The closest related taxon outside the *Muribacter muris* group based on *rpoB* sequence comparison was '*Haemophilus influenzae-murium*' with 88.4 % which is at the lower range of similarity between species of *Pasteurellaceae* within a range of 91 - 99 % (Bisgaard *et al*., 2012; Korczak *et al.*, 2014). The 16S rRNA gene sequence similarity between '*Haemophilus influenzae-murium*' and *Muribacter muris* was also below the species level and further taxonomic investigation will show if '*Haemophilus influenzae-murium*' belongs to *Muribacter* eventually as a genomospecies.

16S rRNA gene sequence based phylogenetic analysis confirmed four of the groups (I, III, V, VI) indentified by *rpoB* gene sequence analysis, whereas six groups were further subdivided in the 16S rRNA gene sequence analysis into two or three (II, VIII) subgroups. The singleton (XI) was also recognized by 16S rRNA sequences based phylogenetic analysis (Fig. S1, Fig. S2, Table S1)**.**

Sequence based comparison indicated that strain R002094 belongs to *Muribacter muris.* The strain was classified as a variant of biovar 6, phenotypically related with taxon 17 of Bisgaard and with *Pasteurella dagmatis*, however, the 16S rRNA sequence of the reference strain of Bisgaard taxon 17 (CCUG 17206; acc. no. AY362902) only showed 94.0 % similarity with *Muribacter muris*. Strain 33696Asv8 classified as biovar 1 which also represents Bisgaard taxon 26 biovar 4 (non-haemolytic) was recently excluded from the new species *Actinobacillus anseriformium* which was based on the classification of taxon 26 of Bisgaard (Bisgaard & Christensen, 2012). Strain 33696Asv8 shared *rpoB* sequence with the type strain of *Muribacter muris* and obviously was

unrelated to *Actinobacillus anseriformium* (Bisgaard & Christensen, 2012). Strain 3996-85 obtained from a mouse in the USA and phenotypically classified as biovar 15 is also known as taxon 27 of Bisgaard (Bisgaard, 1993). This taxon belonged to genotype group VIII which included three other biovars (Table S3). Phenotypically, taxon 27 did not fit completely to any of the 16 biovar categories (Table S2). It can be considered as a variant of biovar 15 (Actino 12 rib pos) which is phosphatase positive and arbutin and PNPF (2-nitrophenyl-α-L-fucopyranoside) α-fucosidase-test negative. Isolates classified with taxon 27 have been obtained from mice and a rat in the USA (Bisgaard, unpublished data).

Biovar 4 matched the monophyletic group VI identified by *rpoB* and 16S rRNA gene sequence comparison. This biovar could be separated in at least two characters from the other groups (Table S2). The lowest 16S rRNA gene sequence similarity within the group was 98.6 % (2004455011, 1995048012) and the similarity to other members of *Muribacter muris* was from 96.8 % (2010503011 Past22; 2013141011 VIII Past21 xyl neg) up to 98.6 % (1995048012; 1993159022 II Actino 12). The combination of monophyly, unique phenotype and divergence in 16S rRNA similarity make the taxon a candidate for a subspecies of *Muribacter muris*. However, the taxon has no particular properties with respect to host, associated site of isolation or disease compared to other member of *Muribacter muris* and there is currently no need to classify the taxon at subspecies level.

The other biovars were para- or polyphyletic since they included more than one monophyletic group identified by *rpoB* and 16S rRNA (Table S2, Table S3). Isolate 2012062093 of biovar Past HW was NAD dependent but otherwise identical to growth factor-independent strains. This shows that *Muribacter muris* includes both V-factor dependent and –independent isolates.

Partial *infB* gene (translation initiation factor 2) sequencing was performed for a few strains as described by Christensen *et al.* (2004 b). The analysis documented 98.1 % similarity between the type strain (acc. no. EU350935) and strain 3996-85 of biovar 15 (Bisgaard taxon 27) in *Muribacter muris* genotypic group VIII (KP664115). Partial *infB* gene sequence based relationship between the type strain of *Muribacter muris* and strain HIM565-1 of '*Haemophilus influenzae-murium*' (KP664114) and the type strain of [*Pasteurella*] *pneumotropica* (biovar Jawetz) (acc. no. AJ438124) only demonstrated 84.5 and 80.2 % similarity, respectively. Comparable level of partial *infB* gene sequence similarity between genera of *Pasteurellaceae* range from 83 to 85 %

(*Frederiksenia* and *Actinobacillus*) (Korczak & Kuhnert, 2008; Korczak *et al.*, 2014) thus confirming the genus level classification of *Muribacter muris*.

Phenotypic characters shared by all strains investigated and included for genus level separation are listed in Table 1. *Muribacter* can be phenotypically separated from the existing genera of *Pasteurellaceae* in at least two characters. Fatty acids were investigated by Culture Collection, University of Göteborg (CCUG). Major fatty acids of the type strain of *Muribacter muris* are $C_{14:0}$, $C_{14:0}$ 3OH/$C_{16:1}$ ISOI, $C_{16:1}$ ω7c and $C_{16:0}$ (Table S4). No obvious differences were found to type strains of 12 other genera of *Pasteurellaceae* that were available for comparison.

Description of *Muribacter* gen. nov.

Muribacter (Mu.ri.bac' ter. L.n. mus, muris the mouse, N.L. masc n. bacter (derived from bactrum) rod, N.L. masc. n. Muribacter rod from mice).

The description is based on *Actinobacillus muris* (Bisgaard, 1986) with the following emendations. Reactions of urease and indole are variable. Acid formation from (-)-D-mannitol is variable as well as the hydrolysis of esculin. The α-glucosidase test (PNPG, 4-nitrophenyl-α-D-glucopyranoside) is positive. There is no requirement for X factor. Major fatty acids of the type strain of *Muribacter muris* are $C_{14:0}$, $C_{14:0}$ 3OH/$C_{16:1}$ ISOI, $C_{16:1}$ ω7c and $C_{16:0}$. The GC % is 43.7 % of the type strain of the type species determined by whole genome sequencing.

The type species is *Muribacter muris.*

Description of *Muribacter muris* sp. nov.

Basonym: *Actinobacillus muris* Bisgaard 1986. The desciption of the species is according to (Bisgaard, 1986) with the additions that acid formation from (+)-D-xylose, meso-inositol, (+)-D-ribose, (+)-D-melibiose, cellobiose and salicin are variable. Acid is formed from trehalose, but one negative strain has been observed. Acid is usually not formed from sorbitol, but four strains have been observed positive. Some phenotypes show a weak haemolysis or CAMP reaction (eg. genotypic group VIII). The ONPG (o-nitro-phenyl-D-galactopyranoside) β-galactosidase-test is variable as well as tests for β-glucosidase, α-fucosidase and β-glucuronidase, whereas the β-xylosidase test is negative. The bacteria have mainly been isolated from mice but also from rats.

The type strain is Ackerman80-443D^T (= NCTC 12432^T = CCUG 16938^T = ATCC 49577^T) isolated a from mouse uterus with the phenotypic properties originally reported by Bisgaard (1986).

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legends for figures

Fig. 1. Phylogenetic relationships between the type strain of *Muribacter muris* and genera of *Pasteurellaceae* as well as some reference taxa based on neighbour joining analysis of 16S rRNA sequences. Support for monophyletic groups by bootstrap-analysis are indicated as numbers out of 100. The scale bar represents sequence variation considering the model for nucleotide substitution (Jukes & Cantor) and tree-shape used in the neighbour joining analysis.

Table 1. Phenotypic separation of *Muribacter* gen. nov. from the existing genera of *Pasteurellaceae.*

1, *Muribacter* gen. nov.; 2, *Mesocricetibacter* (Christensen *et al*., 2014); 3, *Cricetibacter* (Christensen *et al*., 2014); 4, *Haemophilus sensu stricto* (Kilian, 2005; Norskov-Lauritsen *et al*., 2005; Winslow *et al*., 1917; Zinnemann & Biberstein, 1974); 5, *Actinobacillus sensu stricto* (Brumpt, 1910; Christensen & Bisgaard, 2004); 6, *Lonepinella* (Osawa *et al*., 1995); 7, *Mannheimia* (Angen *et al*., 1999); 8, *Pasteurella sensu stricto* (Trevisan, 1887; Mutters *et al*., 1985; Christensen & Bisgaard, 2006); 9, *Phocoenobacter* (Foster *et al*., 2000); 10, *Gallibacterium* (Bisgaard *et al*., 2009); 11, *Volucribacter* (Christensen *et al*., 2004 a); 12, *Histophilus* (Angen *et a*l., 2003); 13, *Avibacterium* (Blackall *et al*., 2005); 14, *Nicoletella* (Kuhnert *et al*., 2004); 15, *Bibersteinia* (Blackall *et al*., 2007); 16, *Aggregatibacter* (Norskov-Lauritsen & Kilian, 2006); 17, *Basfia* (Kuhnert *et al*., 2010); 18, *Chelonobacter* (Gregersen *et al*., 2009); 19, *Necropsobacter* (Christensen *et al*., 2011); 20, *Bisgaardia* (Foster *et al*., 2011); 21, *Otariodibacter* (Hansen *et al*., 2012); 22, *Frederiksenia* (Bisgaard & Mutters, 1986; Korczak *et al*., 2014); 23, *Vespertiliibacter* (Mühldörfer *et al*., 2014).

Character	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**
Catalase	+	-	-	d	d*	-	+*	+	-	d	d	-	d	+	d	d	-	+	+	+	+	+	+
Oxidase	+	+	+	+*	d*	-	d	d	+	d	d	+	+	+	d	-	+	+	+	+	+	+	+
X-factor requirement †	-	-	-	+*	-*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
V-factor requirement †	-	-	-	+*	d*	-	-	-	-	-	-	-	d	-	-*	d	-	-	-	-	-	-	+
Alkaline phosphatase	-	W	+	+	+	-	+	+	+	+	+	+	d	d	+	+	+	+/w	+	+	+ *	+	+
Methyl red	-	W	W	nd	-	nd	nd	-	nd	+	+	nd	-	nd	-	nd	+	nd	+	nd	nd	-	+
Voges Proskauer	-	-	-	-*	-*	+*	-	-*	+	-	-	-	-	nd	-	nd	-	nd	-*	-	+	-	-
Hydrolysis of Tween 80	+	-	-	nd	nd	nd	nd	-	nd	-	-	nd	-	nd	nd	nd	nd	nd	-	nd	nd	nd	nd
Acid from																							
(+)-L-arabinose	-	-	+	nd	d*	+*	d	d*	nd	d	d	nd	d	-	-	-	-*	+	+	nd	+	-*	+
Dulcitol	-	-	+	-*	-	nd	-*	d*	-*	d*	-	nd	-	-	-	nd	-*	+	+	nd	-	-	-
(-)-D-ribose	+	+	+	+/ w	nd	nd	nd	+	nd	d	(+)	nd	d	nd	+	nd	nd	+	+	d	nd	+	nd
(+)-D-mannose	+	+	+	-*	d*	nd	-	+*	-*	+	+	nd	+	-	+	d	+	+	+	+	-	+	+*
Maltose	+	-	+	d	+*	nd	d*	d*	-*	d*	d	-	d	-	+	+	+	+	+	+*	+	+	-
(-)-D-sorbitol	-	+	+	-*	d*	nd	d	d*	-	d	-	-*	d	-	+	-	-	-	-	+	-	-	-
Sucrose	+	+	+	d	+*	d*	+*	+*	-*	+	+	-	+	-	+	d	+	+	+*	+	-	+	-
Trehalose	+	-	-	-*	d	nd	-	d	-*	d	-	-	d	-	+	d	+	+	+	+	+	d*	d*
Dextrin	-	-	+	nd	+	nd	d	d	nd	d	d	nd	d	nd	+	nd	+	+	+	nd	nd	+	nd
Arbutin	+	-*	-	nd	d	nd	d	-	nd	-	-	nd	-	nd	d	nd	nd	nd	-	nd	nd	nd	nd
Growth on MacConkey agar	-	+	-	nd	+	-*	nd	d	-	d	-	nd	-	-	w	nd	nd	nd	nd	-	-*	nd	nd
α-glucosidase PNPG (o-nitrophenyl-a-D-Glucopyranoside)	+	-	-	-	d*	nd	nd	d*	nd	d	-	nd	d	nd	d	nd	-	-	+	-	nd	w	d
GC mol %	43.7	47.5-48.7	41.9-42.0	39 *	35.5-43.7 *	37.5 *	39.2	37.7-43.9	41.5	39.9 – 42.3	40.8	nd	44.2 - 47	nd	42.6	42 - 44	42 .5	47.2	52.5	39.5	36.2	43.5	38.2

Characters are scored as: +, 90 % or more of the strains positive within 1-2 days; -, less than 10 % of the strains are positive within 14 days; d, 11-89 % of the strains are positive; w, weak positive. All tests performed at 37ºC. nd, no data available.

* not part of formal genus description.

† X-factor, referring to the dependence on haemin for growth *in vitro* and V-factor related to dependence on NAD (or related substances) for growth *in vitro*.

Figure
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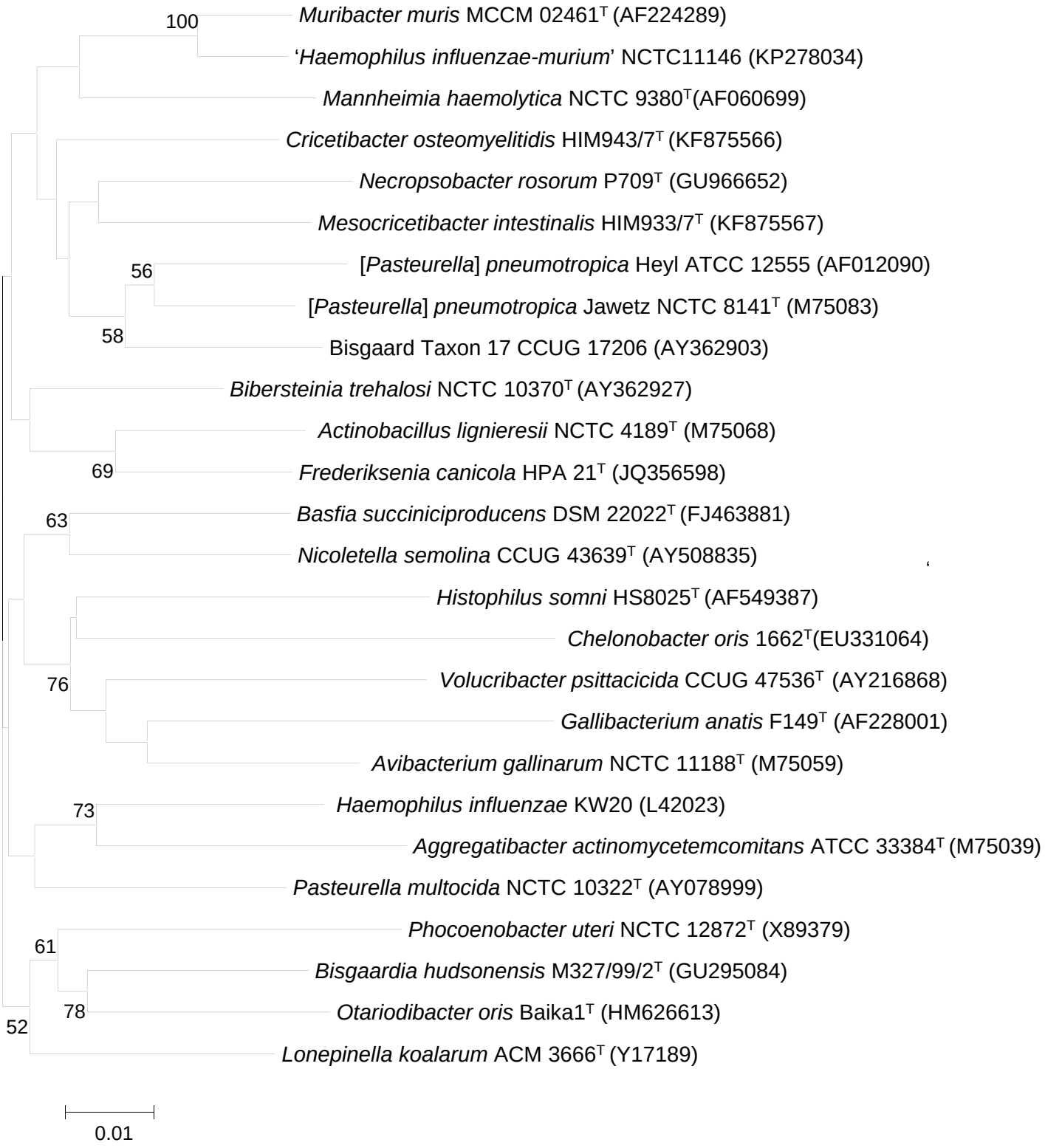
Fig. 1. 16S



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