Comparative phylogenies of the housekeeping genes *atpD*, *infB* and *rpoB* and the 16S rRNA gene within the *Pasteurellaceae*

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Phylogenies of housekeeping gene and 16S rRNA gene sequences were compared to improve the classification of the bacterial family *Pasteurellaceae* and knowledge of the evolutionary relationships of its members. Deduced partial protein sequences of the housekeeping genes *atpD*, *infB* and *rpoB* were compared in 28, 36 and 28 representative taxa of the *Pasteurellaceae*, respectively. The monophyly of representatives of the genus *Gallibacterium* was recognized by analysis of all housekeeping genes, while members of *Mannheimia*, *Actinobacillus sensu stricto* and the core group of *Pasteurella sensu stricto* formed monophyletic groups with two out of three housekeeping genes. Representatives of *Mannheimia*, *Actinobacillus sensu stricto*, [*Haemophilus*] *ducreyi* and [*Pasteurella*] *trehalosi* formed a monophyletic unit by analysis of all three housekeeping genes, which was in contrast to the 16S rRNA gene-derived phylogeny, where these taxa occurred at separate positions in the phylogenetic tree. Representatives of the Rodent, Avian and Aphrophilus–*Haemophilus* 16S rRNA gene groups were weakly supported by phylogenetic analysis of housekeeping genes. Phylogenies derived by comparison of the housekeeping genes diverged significantly from the 16S rRNA gene-derived phylogeny as evaluated by the likelihood ratio test. A low degree of congruence was also observed between the individual housekeeping gene-derived phylogenies. Estimates on speciation derived from 16S rRNA and housekeeping gene sequence comparisons resulted in quite different evolutionary scenarios for members of the *Pasteurellaceae*. The phylogeny based on the housekeeping genes supported observed host associations between *Mannheimia*, *Actinobacillus sensu stricto* and [*Pasteurella*] *trehalosi* and animals with paired hooves.

The family *Pasteurellaceae* Pohl 1981 was originally proposed to accommodate existing genera and species on the basis of phenotypic and genotypic characteristics (Pohl, 1981) and has subsequently been shown to be monophyletic on the basis of rRNA–DNA hybridization and 16S rRNA gene sequence comparisons (De Ley *et al.*, 1990; Dewhirst *et al.*, 1992). Members of the family have been isolated from mucosal membranes of the respiratory, reproductive and alimentary tracts of healthy as well as diseased vertebrates (Bisgaard, 1993). Eight genera have now been named,

accommodating 57 named species. In spite of the naming of five genera during the last decade, the three ‘old’ genera, *Haemophilus*, *Actinobacillus* and *Pasteurella*, still seem to be polyphyletic. The existence of around 40 unnamed taxa is predominantly due to insufficient designation of type strains and lack of characters to separate them from existing named species. Other limitations include the limited number of strains available for some taxa and the lack of DNA–DNA hybridization results, which would permit distinct genotypes to be validated as new candidates for species.

16S rRNA gene sequence comparison has been the most beneficial tool so far to improve the classification of members of the *Pasteurellaceae*. Ninety-five of the species or species-like taxa have been characterized by 16S rRNA gene sequence analysis. In addition, phylogenetic inference based on 16S rRNA gene sequence comparison has been used for classification at the family level as well as for investigation of

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particular groups (Dewhirst *et al.*, 1993; Møller *et al.*, 1996; Angen *et al.*, 1999, 2003; Guettler *et al.*, 1999; Foster *et al.*, 2000; Christensen *et al.*, 2004; Olsen *et al.*, 2004).

Phylogenetic trees of the *Pasteurellaceae* based on maximum-likelihood analysis of 16S rRNA gene sequences have recently been published (Angen *et al.*, 2003; Christensen & Bisgaard, 2003, 2004; Christensen *et al.*, 2003b). To aid in comparison in the present study, such analysis has been included (Fig. 1). The tree was constructed according to Christensen *et al.* (2003b) and the 'multiple outgroup' approach was used to determine the phylogeny of the family. Thirteen monophyletic groups and 13 monotypic taxa were identified by maximum-likelihood analysis of 16S rRNA gene sequences, with reasonably good correspondence to the neighbour-joining tree published by Olsen *et al.* (2004). The groups are summarized briefly below to allow further comparison to the phylogenetic analysis of housekeeping genes. Whenever possible, the names of the groups of Olsen *et al.* (2004) have been adopted. The core group of *Pasteurella sensu stricto*, the *Mannheimia*, *Actinobacillus sensu stricto*, Rodent and Parasuis groups were identified as monophyletic. Recently, *Histophilus somni* was proposed as a new monotypic genus (Angen *et al.*, 2003), and formed a group with the *Succinogenes* group related to the *Seminis* group. The *Haemophilus* and *Aphrophilus* groups were identified and included within a monophyletic group. The Avian group included taxon 33 of Bisgaard (proposed as *Volucrobacter*; Christensen *et al.*, 2004) in addition to the genus *Gallibacterium* (Christensen *et al.*, 2003a), avian members of *Pasteurella sensu stricto* and three unnamed taxa of Bisgaard. The Testudinis group formed the outgroup to which *Phocoenobacter uteri* and [*Haemophilus*] *parainfluenzae* were closely related. The Rossii group was recognized, but left Bisgaard taxon 7 monotypic. The two species [*Actinobacillus*] *scotia* and [*Actinobacillus*] *delphinicola* formed the group Delphinicola and *Pasteurella langaaensis* formed the group Langaa with [*Pasteurella*] *caballi*. The six remaining taxa, [*Haemophilus*] *ducreyi*, [*Haemophilus*] *felis*, *Lonepinella koalarum*, [*Pasteurella*] *bettyae*, [*Pasteurella*] *trehalosi* and taxon 5 of Bisgaard, could not be allocated to any of the monophyletic groups, whereas Taxon 16 of Bisgaard was found to be closely related to *Mannheimia*. The major drawback with phylogenetic inference based on 16S rRNA gene sequence comparison is insufficient separation with certain species (Fox *et al.*, 1992; Stackebrandt & Goebel, 1994) despite this being the most valuable unit for classification. Analysis of the so-called housekeeping genes has been suggested to avoid this problem, which will also base phylogenetic inference on a number of genes. These genes have been selected with regard to properties suitable for phylogenetic inference, such as evolutionary conservation and low selection pressure.

DNA sequence comparison of housekeeping genes has been used for phylogenetic investigation of a few groups of the *Pasteurellaceae* (Hedegaard *et al.*, 2001; Petersen *et al.*, 2001; Angen *et al.*, 2003; Korczak *et al.*, 2004; Nørskov-Lauritsen

et al., 2004). At the domain level, informational genes branched more deeply than operational ones (Rivera *et al.*, 1998; Garcia-Vallvé *et al.*, 2000). For this reason, both informational and operational genes, both referred to as housekeeping genes, were selected for evaluation of the consistency of 16S rRNA gene-based phylogeny in the present investigation. The gene *atpD* encodes the β -subunit of the ATP synthase and has been used for phylogenetic investigation of the domain *Bacteria* and the genus *Salmonella* (Ludwig *et al.*, 1993; Christensen & Olsen, 1998). The *infB* gene encodes the translation initiation factor 2, which is essential for the initiation of protein synthesis in prokaryotes (Laalami *et al.*, 1991). The region compared encodes some of the most conserved and essential parts of the gene, including the G-domain and the ribosome-binding sites (March & Inouye, 1985). DNA sequence comparison of *infB* has been used for phylogenetic investigation of the *Enterobacteriaceae* (Hedegaard *et al.*, 1999), the genus *Haemophilus* (Hedegaard *et al.*, 2001) and members of *Actinobacillus* (Nørskov-Lauritsen *et al.*, 2004). DNA sequence comparison of *rpoB*, encoding the β -subunit of RNA polymerase, was used for classification by Mollet *et al.* (1997), used for the classification of *Histophilus somni* (Angen *et al.*, 2003) and recently for phylogenetic analysis of the *Pasteurellaceae* (Korczak *et al.*, 2004). The gene codons 509–680 (according to the *Escherichia coli* numbering) were chosen to represent a variable part of the gene (Klenk & Zillig, 1994; Mollet *et al.*, 1997). The genes *atpD*, *infB* and *rpoB* chosen for the present study are genetically unlinked, as indicated by the fact that they are dispersed over at least 14 kbp on the genomic sequences of *Haemophilus influenzae* and *Pasteurella multocida* available from public databases (<http://www.ncbi.nlm.nih.gov>).

The aim of the study was to compare phylogenies of housekeeping gene sequences with the 16S rRNA gene sequence-based phylogeny in order to improve the classification of the bacterial family *Pasteurellaceae* and to obtain information about the evolutionary relationships of its members.

DNA sequencing and phylogenetic analysis

Sequencing of *atpD* was performed according to Petersen *et al.* (2001) with modification of the oligonucleotide primers in order to sequence the different taxa investigated (Table 1). The sequencing of a fragment of the *infB* gene was performed as reported by Nørskov-Lauritsen *et al.* (2004) with modified oligonucleotide primers (Table 1). The partial *rpoB* sequence was determined according to Korczak *et al.* (2004) using oligonucleotide primers listed in Table 1. Bacteria were cultured overnight in brain heart infusion broth (Difco) or blood agar base supplemented with 5% bovine blood and DNA was extracted or colonies lysed by boiling and used directly for PCR. PCR amplification of *atpD* and *infB* was performed according to the standard conditions of 35 cycles of 30 s denaturation at 94 °C, 120 s annealing at 55 °C and 60 s extension at 72 °C. PCR amplification of *rpoB* was performed as described by Korczak *et al.* (2004) with 35 cycles of 30 s denaturation

Fig. 1. Phylogeny of the family Pasteurellaceae based on maximum-likelihood analysis of 16S rRNA gene sequences. Monophyletic groups supported by the 'multiple outgroup method' are indicated with bold lines. Support for monophyletic groups by bootstrap analysis is indicated numerically as numbers out of 100. Nodes supported by neighbour-joining and maximum-parsimony analysis are indicated by + and *, respectively. Numbered clusters refer to Olsen *et al.* (2004).

Table 1. Oligonucleotide primers used for partial sequencing of *atpD*, *infB* and *rpoB* genes of members of *Pasteurellaceae*

DNA sequences are written in the 5' to 3' direction and numbers refer to the 3' position of the comparable *Escherichia coli* DNA sequences of the genes. Primers used for initial PCR amplification are underlined. Several versions of each sequencing primer were designed to cover the ambiguities found in different members of *Pasteurellaceae*.

Target	Forward primer(s)	Reverse primer(s)	Modifications based on
<i>atpD</i>	<u>GCTCGTCAAGCAAGTATYAC</u> -(−128)	<u>CTARYRCTTCRTC</u> GATDGWAC-1343	Petersen <i>et al.</i> (2001)
	YGCAHTDGGWWCATCDG-181	GWACCDKTA ^{AA} HACTTCHGC-1222	
	AAGYTAYGAAGAACAAC-362	GTTCTTSWCCMACRAC-1045	
	TAYRAYGTTGCWCGTGGTG-1080		
<i>infB</i>	<u>CCTGACTAYATTCGTAAAGC</u> -1241	<u>GTAGCAACCGGACCACGACCTTTAT</u> -1727	Hedegaard <i>et al.</i> (2001); Nørskov-Lauritsen <i>et al.</i> (2004)
<i>rpoB</i>	<u>GCAGTGAAAGARTTCTTTGGTTC</u> -1523	<u>GTTGCATGTTIGIACCCAT</u> -2041	Korczak <i>et al.</i> (2004)

at 94 °C, 30 s annealing at 54 °C and 30 s extension at 72 °C. PCR-amplified fragments were purified on Microspin columns (Amersham Pharmacia Biotech) and cycle-sequenced (Thermo Sequenase fluorescent labelled primer cycle sequencing kit; Amersham Pharmacia Biotech) on an ALF Sequencer (Pharmacia Biotech) using fluorescein-labelled primers for *atpD* and *infB* or cycle-sequenced on an ABI3100 with the dRhodamine kit according to protocols described in the Applied Biosystems Chemistry Guide for *rpoB*.

Phylogenetic analysis of the housekeeping gene sequences determined in the present study was performed on the translated DNA sequences due to high variation in DNA sequences (strain numbers and accession numbers are available as supplementary material in IJSEM Online, including published sequences deposited in public databases). The deduced sequences of *atpD*, *infB* and *rpoB* were compared in 28, 36 and 28 representative taxa of the *Pasteurellaceae*, respectively.

Sequences were aligned with PILEUP (Wisconsin sequence analysis package, GCG). Positions with gaps in the alignments were excluded in the phylogenetic analysis. In the protein alignment of *atpD*, one amino acid insertion was found in [*Actinobacillus*] *rossii*, [*Actinobacillus*] *succinogenes*, [*Pasteurella*] *caballi* and taxon 14 of Bisgaard compared with the other taxa investigated. With the *infB* protein alignment, a single amino acid was found to be deleted in sequences of *Pasteurella multocida*, [*A.*] *succinogenes*, *L. koalarum* and taxon 7 of Bisgaard compared with the other taxa investigated. With the *rpoB* protein alignment, a single amino acid was deleted in *Mannheimia* compared with the other taxa investigated. Phylogenetic analysis was performed by PROTML as included in MOLPHY version 2.3b3 (<http://ftp.ism.ac.jp:8000/ISMLIB/MOLPHY>) using the PAM substitution matrix. Parsimony analysis was performed by PROTPARS with standard settings and bootstrap analysis as included with PHYLIP (Felsenstein, 1995). Neighbour-joining analysis was performed with NEIGHBOR after construction of the distance matrix with PROTDIST using the PAM substitution matrix (PHYLIP). To allow consensus

comparison between phylogenetic trees derived from housekeeping gene and 16S rRNA gene sequences, new 16S rRNA gene alignments were constructed that included exactly the taxa analysed in the respective housekeeping gene sequence alignments. Majority rule consensus comparisons between housekeeping gene-derived and 16S rRNA gene sequence-based trees were performed by CONSENSE (PHYLIP).

The phylogeny obtained by alignment of the region 12–428 (*E. coli* numbering) of the deduced *atpD* protein sequences corresponded to the 16S rRNA gene phylogeny in that the *Mannheimia*, core *Pasteurella sensu stricto*, Rodent and *Gallibacterium* groups could be recognized. In addition, the two members of the *Haemophilus* and *Aphrophilus* groups formed a monophyletic unit (Fig. 2a, Table 2). The inclusion of [*Haemophilus*] *ducreyi* with the taxa of *Actinobacillus sensu stricto* and the close relationship of this group to [*Haemophilus*] *parasuis*, [*Pasteurella*] *trehalosi* and *Mannheimia* within a large monophyletic group supported by high bootstrap values was unrecognized by 16S rRNA gene phylogenetic analysis (Fig. 2a, Table 2).

Phylogenetic analysis of the alignment of the region 436–561 (*E. coli* numbering) of the deduced *infB* protein sequence allowed members of the *Actinobacillus sensu stricto*, Avian *Pasteurella* and *Gallibacterium* groups to be recognized as monophyletic. Members of the 16S rRNA gene *Haemophilus* and *Aphrophilus* groups formed monophyletic units, but were not united in one large group. The deduced *infB* protein sequence was identical for the type strains of *Haemophilus influenzae* and *Haemophilus haemolyticus*, for [*Haemophilus*] *segni*s and [*Haemophilus*] *parainfluenzae*, for [*Haemophilus*] *aphrophilus* and [*Haemophilus*] *paraphrophilus*, for the two taxa of *Gallibacterium* and for the three serotypes of *Actinobacillus pleuropneumoniae* available from the NCBI (<http://www.ncbi.nlm.nih.gov>). The relationships between *Pasteurella multocida* and Bisgaard taxon 33 (*Volucribacter*; Christensen *et al.*, 2004) and between *Gallibacterium* and the two strains of taxon 14 of Bisgaard were not recognized in the 16S rRNA gene-derived phylogeny (Fig. 2b, Table 2). As with the *atpD*-derived phylogeny, a monophyletic group of members of *Mannheimia*,

Fig. 2. Phylogenetic relationships between representative taxa of the *Pasteurellaceae* based on parsimony analysis of partially deduced protein sequences of the genes *atpD* (a), *infB* (b) and *rpoB* (c). Support for monophyletic groups by bootstrap analysis is indicated as numbers out of 100. Nodes recognized in maximum-likelihood and neighbour-joining analysis are indicated by * and +, respectively. Monophyletic groups recognized by 16S rRNA gene phylogenetic analysis are shown by filled circles when recognized in the full 16S rRNA gene tree and by open circles when recognized in the partial 16S rRNA gene tree based on the same taxa analysed for housekeeping gene sequences.

Table 2. Congruence between phylogenies of the deduced protein sequences of housekeeping genes and 16S rRNA gene monophyletic groups of *Pasteurellaceae*

16S rRNA gene monophyletic group*	Taxa (n)	Host(s)	Congruence with 16S rRNA gene monophyletic groups†		
			<i>atpD</i>	<i>infB</i>	<i>rpoB</i>
<i>Gallibacterium</i>	2	Birds	MP	MP	MP
Core <i>Pasteurella sensu stricto</i> (12)	7	Mammals, birds	MP	ND	MP
<i>Actinobacillus sensu stricto</i> (1)	12	Mammals, birds	—	MP	MP
<i>Mannheimia</i> (4)	6	Ruminants, pigs, leporidae	MP	ND	MP
Avian <i>Pasteurella sensu stricto</i>	4	Birds	ND	MP	ND
Aphrophilus (13)	4	Man	ND	MP	ND
<i>Haemophilus</i> (16)	3	Man	ND	MP	ND
Aphrophilus– <i>Haemophilus</i> (13, 16)	7	Man	MP	—	—
Rodent (15)	5	Rodents	MP	—	—
Avian (18)	11	Birds	—	—	—
<i>Histophilus</i> – <i>Succinogenes</i> (14)	4	Ruminants	—	—	—
<i>Histophilus</i> – <i>Succinogenes</i> – <i>Seminis</i> (14, 20)	7	Ruminants, pigs, guinea pigs, horses	—	—	—

*Clusters with both names and numbers have been adopted from Olsen *et al.* (2004) and unnumbered groups refer to Christensen *et al.* (2003b) (see Fig. 1).

†MP, Monophyly documented by maximum-likelihood, maximum-parsimony and neighbour-joining analysis methods; ND, not determined because group was represented by fewer than two taxa; —, monophyly not documented.

[*Haemophilus*] *ducreyi*, *Actinobacillus sensu stricto*, [*Haemophilus*] *parasuis* and [*Pasteurella*] *trehalosi* was also recognized (Fig. 2b).

Phylogenetic analysis of the alignment of the region 509–680 (*E. coli* numbering) of the deduced *rpoB* protein sequence allowed recognition of the core *Pasteurella sensu stricto*, *Gallibacterium*, *Actinobacillus sensu stricto* and *Mannheimia* 16S rRNA gene groups, whereas members of *Haemophilus*–Aphrophilus, Rodent and Avian groups did not form monophyletic units (Fig. 2c, Table 2). Identical protein sequences were observed for the two taxa of *Mannheimia*, the two taxa of *Gallibacterium* and for [*Pasteurella*] *pneumotropica* and taxon 17 of Bisgaard. The 16S rRNA gene monophyletic groups recognized by housekeeping gene sequence analysis were recognized by all three phylogenetic methods (Figs 2a–c, Table 2); however, the recognition of these groups should be taken with caution since some of the groups were represented by only a few taxa (Fig. 2). Further investigation is necessary in order to document fully the monophyly of these groups.

Comparison of the deduced protein sequences of all three housekeeping genes *atpD*, *infB* and *rpoB* (end-to-end) was possible for 23 taxa with 707 positions. The 16S rRNA gene groups *Gallibacterium*, *Actinobacillus sensu stricto* and Rodent were recognized (Fig. 3). Strong support was found for the group with *Mannheimia*, *Actinobacillus sensu stricto*, [*Haemophilus*] *ducreyi* and [*Pasteurella*] *trehalosi*. The two members of the Rodent group and *Haemophilus influenzae* were closely related to this group. In addition, *Gallibacterium* and taxon 14 of Bisgaard formed

a group. Pairwise consensus comparisons between the three housekeeping gene-derived trees showed only 19% (*atpD*–*infB*, *atpD*–*rpoB*) to 29% (*infB*–*rpoB*) of all nodes to be conserved. In comparison, nodes supported by all three phylogenetic methods with bootstrap values higher than 50% accounted for around 40% of all nodes in the trees shown in Figs 2 and 3. In all three pairs (*atpD*–*infB*, *atpD*–*rpoB*, *infB*–*rpoB*), the two taxa of *Gallibacterium*, [*Pasteurella*] *pneumotropica* and taxon 17 of Bisgaard and [*Haemophilus*] *ducreyi* and *A. pleuropneumoniae* formed common nodes. When conserved nodes were counted between the housekeeping gene and 16S rRNA gene trees in Fig. 2(a–c), similarly low percentages of conserved nodes were observed (19–26%). Based on these approximate consensus comparisons, the congruence between individual housekeeping gene trees was no better than between these and the 16S rRNA gene tree.

In summary, the phylogeny derived from the partially sequenced housekeeping genes supported the 16S rRNA gene-derived phylogeny with respect to the Testudinis group as an outgroup and confirmed the existence of *Gallibacterium*, *Mannheimia* and *Actinobacillus sensu stricto*. The core group of *Pasteurella sensu stricto* was confirmed by monophyly of the subspecies of *Pasteurella multocida* and *Pasteurella canis* (see also Petersen *et al.*, 2001). The *Haemophilus* and Aphrophilus groups were confirmed by partial *infB* sequence comparison, except for the inclusion of [*Haemophilus*] *parainfluenzae* (see also Hedegaard *et al.*, 2001) (Table 2).

The 16S rRNA gene groups Rodent and Avian were only occasionally recognized by housekeeping gene-derived

The likelihood ratio test was used to test whether alternative topologies were supported by significantly high logarithms (ln) of likelihood values (Huelsenbeck & Chandall, 1997).

It was surprising to observe the conservation of members of *Mannheimia*, *Actinobacillus sensu stricto*, [*Haemophilus*] *ducreyi*, [*Pasteurella*] *trehalosi* and [*Haemophilus*] *parasuis* within a monophyletic group by housekeeping gene analysis. With the exception of [*Haemophilus*] *ducreyi* and a few species of *Actinobacillus sensu stricto* and *Mannheimia*, these taxa are mainly associated with artiodactyls (animals

with paired hooves), and RTX-type toxins have been found in three of them (*Mannheimia*, *Actinobacillus sensu stricto* and [*Pasteurella*] *trehalosi*; Frey & Kuhnert, 2002). The housekeeping gene-derived phylogeny justifies the classification of *Mannheimia* and *Gallibacterium*. Further support for groups like *Actinobacillus sensu stricto*, the core group of *Pasteurella sensu stricto* (Olsen *et al.*, 2004) and the split of the Avian group into different genera such as *Gallibacterium* and the new genus proposed as *Volucribacter* (Christensen *et al.*, 2004) was also obtained. The split of the Somnus group was previously justified with formation of the new monotypic genus *Histophilus* (Angen *et al.*, 2003).

The limitations in order to achieve congruence of housekeeping gene- and 16S rRNA gene-derived phylogenies question the appropriateness of further investigations with the deeper branchings. The 16S rRNA gene-derived phylogeny showed good congruence with whole genome-based phylogeny (Henz *et al.*, 2003) and might still be best suited for the deeper branching levels. Using *gyrB* sequence-based phylogenetic analysis with the *Enterobacteriaceae*, congruence was obtained at the species level but not at deeper levels (Dauga, 2002). Comparisons of housekeeping genes are probably best suited for separation and analysis at the species and genus level and could serve as an alternative to DNA–DNA hybridizations (Zeigler, 2003). The analysis of housekeeping genes is a promising approach capable of contributing to the ongoing revision and improvement of the taxonomy of the *Pasteurellaceae*. Further investigations should focus on analysis of the whole gene sequences of *infB* and *rpoB* in order to confirm the regions most suitable for phylogenetic analysis. Analysis of more housekeeping genes from both the informational and the operational groups should also be performed.

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