Phylogeny of the family Pasteurellaceae based on rpoB sequences

Bożena Korczak,¹ Henrik Christensen,² Stefan Emler,³ Joachim Frey¹ and Peter Kuhnert¹

¹Institute of Veterinary Bacteriology, University of Bern, CH-3012 Bern, Switzerland
²Department of Veterinary Microbiology, The Royal Veterinary and Agricultural University, DK-1870 Frederiksberg C, Denmark
³SmartGene GmbH, PSE-C EPFL-Ecublens, CH-1015 Lausanne, Switzerland

Sequences of the gene encoding the β-subunit of the RNA polymerase (rpoB) were used to delineate the phylogeny of the family Pasteurellaceae. A total of 72 strains, including the type strains of the major described species as well as selected field isolates, were included in the study. Selection of universal rpoB-derived primers for the family allowed straightforward amplification and sequencing of a 560 bp fragment of the rpoB gene. In parallel, 16S rDNA was sequenced from all strains. The phylogenetic tree obtained with the rpoB sequences reflected the major branches of the tree obtained with the 16S rDNA, especially at the genus level. Only a few discrepancies between the trees were observed. In certain cases the rpoB phylogeny was in better agreement with DNA–DNA hybridization studies than the phylogeny derived from 16S rDNA. The rpoB gene is strongly conserved within the various species of the family of Pasteurellaceae. Hence, rpoB gene sequence analysis in conjunction with 16S rDNA sequencing is a valuable tool for phylogenetic studies of the Pasteurellaceae and may also prove useful for reorganizing the current taxonomy of this bacterial family.

The family Pasteurellaceae Pohl 1981 currently comprises 57 named bacterial species which have been isolated from man and various animals. The three genera Haemophilus, Actinobacillus and Pasteurella which originally formed the family have recently been joined by new genera, the most prominent being Mannheimia, which contains the species Mannheimia haemolytica (formerly Pasteurella haemolytica) (Angen et al., 1999). Three genera are currently formed by single species, these are Phocoenobacter uteri (Foster et al., 2000), Lornipinella koalarum (Oswa et al., 1995) and Histophilus somni (Angen et al., 2003). The latter genus has been proposed to include the three species incertae sedis ‘Haemophilus somni’, ‘Haemophilus aegi’ and ‘Histophilus ovis’. Finally, Christensen et al. (2003a) have described the genus Gallibacterium, which currently consists of one species and one genomospecies.

Members of the Pasteurellaceae are generally isolated from mucosal membranes and tissue of birds, turtles and mammals, including man. They show a strong host association and have probably co-evolved with their corresponding hosts (Bisgaard, 1993). While most of the species are commensals, there are a few that act as pathogens (Olsen et al., 2004). These include the human pathogens Haemophilus influenzae, which causes neonatal meningitis and otitis media, and Actinobacillus actinomycetemcomitans, which causes juvenile periodontitis. Important animal diseases are caused, for example, by M. haemolytica (shipping fever of cattle), Pasteurella multocida (atrophic rhinitis in swine and fowl cholera) and Actinobacillus pleuroneumoniae (pleuropneumonia in pigs). In many of the pathogenic species of Pasteurellaceae, specific toxins belonging to the RTX toxin family are found (Frey & Kuhnert, 2002). These toxins are often associated with specifically pathogenic isolates and seem to determine to some extent the host range of the pathogen (Lally et al., 1994; Kuhnert et al., 2003b). They are mainly absent in non-pathogenic strains.

The taxonomy of Pasteurellaceae is not well settled and is under continuous revision. The taxonomy has involved mainly phenotypic characterization, including the determination of polyamine patterns (Olsen et al., 2004; Busse et al., 1997). A few thorough studies have also been carried out at the genotypic level. Whole genome DNA–DNA hybridizations were carried out some time ago on Pasteurellaceae.
(Mutters et al., 1984, 1985) and genetic relatedness was investigated by DNA–rRNA hybridization (De Ley et al., 1990). Dewhirst et al. (1992, 1993) investigated the phylogenetic relationships within the family by 16S rDNA sequence comparisons and a full phylogenetic tree has been published recently (Christensen et al., 2003b). Hedegaard et al. (2001) used infB sequences to investigate in more detail the genetic relationships of the genus Haemophilus. However, other phylogenetic marker genes are needed to clarify the taxonomy within the Pasteurellaceae, to provide additional tools for genetic identification and to give insight into the evolution of this group of bacteria. The rpoB gene has been used successfully for the elaboration of phylogenetic relationships in several groups of bacteria, and normally has a higher discriminatory power than 16S rDNA sequences (Mollet et al., 1997; Dahllof et al., 2000). Besides the use of partial rpoB sequences for delineating phylogenetic relationships (Mollet et al., 1998; Taillardat-Bisch et al., 2003) the gene has also been applied for improving diagnostics (Drcourt & Raoult, 2002; Ko et al., 2003). Recently, the usefulness of rpoB for the description of Histophilus somni was shown (Angen et al., 2003). In this paper we describe the phylony of the entire family of Pasteurellaceae based on a universal amplification and sequencing approach of rpoB.

Based on sequence comparison analysis we have chosen the primers Pasrpob-L (5’-GCAGTGAAAAGARTTTCTTG-GTTC) and RpoR-R (5’-GTTGCATGTGTTNACCACCAT) for amplification of a 560 bp fragment from the rpoB gene, corresponding to positions 1501–2059 of the Escherichia coli rpoB gene. PCR amplification was performed in 50 µl containing 20 pmol each primer, 1 mM dNTPs, Taq buffer and 2.5 U Taq polymerase (Roche Life Sciences). Template DNA was added as purified genomic DNA, as crude lysate or directly from bacterial colonies. Cycling conditions on a GeneAmp PCR System 9700 (Applied Biosystems) were 3 min denaturation at 94 °C, followed by 35 cycles at 94 °C for 30 s, 54 °C for 30 s and 72 °C for 30 s. A final extension step at 72 °C for 7 min was included to finish the PCR amplification. After purification of the PCR product with a HighPure PCR purification kit (Roche Life Sciences), about 20–30 ng PCR product was used for sequencing with the BigDye Terminator cycle sequencing kit (Applied Biosystems) using Pasrpob-L and RpoR-R as sequencing primers. Sequencing products were analysed on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems) and edited using the program Sequencher (GeneCode, Ann Arbor, MI, USA). In parallel to the rpoB sequences, the 16S rDNA sequence of each strain was determined as described previously (Kuhnert et al., 1996, 2002), in order to confirm the species identity and improve available sequences. If the 16S rDNA sequence was better than the one publicly available (i.e. unknown bases were resolved) it was deposited as a new entry in GenBank. The strains used in this study and the accession numbers of the corresponding rpoB and 16S rDNA sequences are given in the figures. Phylogenetic analysis was done using the program BioNumerics version 3.0 (Applied Maths). The primers used for amplifying and sequencing the partial rpoB gene from Pasteurellaceae proved to be universal for this family of bacteria, since all strains analysed so far gave amplification products.

Phylogenetic trees of the 16S rDNA and the corresponding rpoB sequences of 72 Pasteurellaceae strains, including E. coli as an outgroup, were drawn and are represented in Figs 1 and 2, respectively. Corresponding clusters observed are labelled according to Olsen et al. (2004). Overall, there was a good correlation when comparing the two trees with a congruency analysis in the BioNumerics software, with a correlation of r=0.80. The main clusters were well recognized within the rpoB tree. There were, however, a few clusters where discrepancies were observed and these are marked in grey in Fig. 2.

Within the cluster of Pasteurella sensu stricto, both rpoB and 16S rDNA trees show a similar separation of Pasteurella multocida subsp. septica from the other two subspecies. The location of Haemophilus felis is very similar in the 16S rDNA and rpoB trees, suggesting a close relationship to the type species Pasteurella multocida. Besides these two observations, the trees differ concerning the clustering of the other Pasteurella species. The mammalian species Pasteurella canis, Pasteurella dagmatis and Pasteurella stomatis cluster in the 16S rDNA tree close to the type species of the genus Pasteurella but they form a separate cluster in the rpoB tree.

Pasteurella trehalosi is closely related to Mannheimia phenotypically as well as in the 16S rDNA tree. However, the rpoB tree separates this species from the Mannheimia cluster and places it in close proximity to Haemophilus parasuis/Actinobacillus indolicus (Parasuis group). The two subclusters observed in the 16S-rDNA-derived tree within Mannheimia are also represented in the rpoB tree. Strain P737 shows a full match with the Mannheimia glucosida type strain in the rpoB tree, which is not the case in the 16S rDNA tree, where this strain is located differently but still in the same subcluster. The full match of the rpoB sequences confirms its designation as M. glucosida, which was based on DNA–DNA hybridization initially (Angen et al., 1999).

Within the genus Actinobacillus sensu stricto, two distinct subclusters can be observed with rpoB sequences, which is not the case in the 16S rDNA tree. One subcluster is formed by A. pleuropneumoniae, Actinobacillus lignieresi, Actinobacillus arthritidis and the two Actinobacillus genomospecies. The second subcluster within Actinobacillus sensu stricto is formed by the species Actinobacillus suis, Actinobacillus equuli, Actinobacillus ureae, Actinobacillus hominis and Actinobacillus capsulatus. The presence of A. capsulatus in the cluster of Actinobacillus sensu stricto was most striking and, therefore, two field isolates from rabbits were included in the tree to support this observation. Moreover, the A. capsulatus type strain NCTC 11408 T received from another culture collection showed 100% sequence identity to CCUG 12396 T, confirming the phylogenetic position of this species in the rpoB tree. This branching is in contrast to
Fig. 1. Phylogenetic tree of the family Pasteurellaceae based on 16S rDNA sequences. The species names, strain numbers and accession numbers are given. Sequences determined in this study are indicated by asterisks. Bootstrap values of 100 replications are indicated at branches. The scale bar represents percentage of sequence divergence. Clusters formed are indicated at the right. The numbers in parentheses correspond to the clusters in Olsen et al. (2004).

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Fig. 2. Phylogenetic tree of the family Pasteurellaceae based on partial rpoB sequences. The species names, strain numbers and accession numbers are given. Sequences determined in this study are indicated by asterisks. Bootstrap values of 100 replications are indicated at branches. The scale bar represents percentage of sequence divergence. Clusters formed are indicated at the right. The numbers in parentheses correspond to the clusters in Olsen et al. (2004) and are shown in grey, where differences from the 16S rDNA tree can be observed.
the 16S rDNA phylogeny. However, it strongly supports earlier statements by Mutters et al. (1989) that this species should be included in the genus *Actinobacillus*. This is a further indication that the *rpoB* gene sequence can be used as a valuable tool for a solid taxonomy, especially at the genus level, as has been shown when analysing the type species of the genera (Angen et al., 2003). Phylogenetic discrimination between the two species *A. suis* and *A. equuli* was not evident in the *rpoB* tree or in the 16S rDNA phylogeny. In addition, we have shown recently that the RTX toxins of these two species are different, which permits easy differentiation between the haemolytic *A. suis* and *A. equuli* (Berthoud et al., 2002; Kuhnert et al., 2003a, b). The non-haemolytic species *Actinobacillus minor* settles on a distinct branch near the *Actinobacillus sensu stricto* cluster in both trees. The two haemolytic strains of a newly discovered *Actinobacillus* species tentatively designated *Actinobacillus porcinsillarum*, which phenotypically resembles *A. pleuropneumoniae*, showed a closer relationship to *A. minor* with the *rpoB* gene than with the 16S rDNA. Phylogenetically, *A. minor* and *A. porcinsillarum* cannot be separated as two species. However, phenotypically the latter is differentiated from *A. minor* since it is haemolytic due to the secretion of ApxII, and *A. minor* is, by definition, non-haemolytic (Möller et al., 1996; Gottschalk et al., 2003). Because of this, and also the problems these strains cause in bacterial and serological diagnosis of porcine pleuropneumonia, where they might be misidentified as *A. pleuropneumoniae* leading to grave consequences, isolates of *A. porcinsillarum* deserve particular attention (Gottschalk et al., 2003).

There was a difference in the phylogenetic position of *Actinobacillus succinogenes* between the two trees. Whereas in the 16S rDNA tree this species is on a branch with *Histophilus somni*, it clustered with Bisgaard taxon 5 in the *rpoB* tree.

Species of the genus *Haemophilus* clustered in a similar way to that described for the phylogeny of the genus based on *infB* sequences (Hedegaard et al., 2001). The phylogenetic identity between *Haemophilus influenzae* and *Haemophilus aegyptius* was reflected by a 100% sequence match of the *rpoB* gene sequence between the two. For *rpoB* there was a similar close relationship between the species *Haemophilus parainfluenzae* and the type species, as observed with *infB*, in contrast to the 16S rDNA phylogeny. Also, the clustering of *Haemophilus aphrophilus* with *A. actinomycetemcomitans*, which is quite distant from the type species of the genus, reflects the *infB* phylogeny and is in contrast to the 16S rDNA branching, which places the two in close proximity to the type species *Haemophilus influenzae*. *Haemophilus parasuis*, which like *Haemophilus haemoglobinophilus* does not belong to *Haemophilus sensu stricto*, clusters in both the *rpoB* and the 16S rDNA tree with *A. indolicus*. Several strains of *Haemophilus parasuis* were included in our study. They represent the two 16S rDNA clusters observed within this species (Dewhirst et al., 1992; P. Kuhnert, unpublished observation). These two clusters are represented by the two reference strains Bakos A9 and Bakos B26 (Morozumi & Nicolet, 1986). Both strains also cluster in the *rpoB* tree and the Bakos A9 strain is on the same branch in both trees as the *Haemophilus parasuis* type strain, whereas *A. indolicus* clusters tightly with the Bakos B26 strain in the *rpoB* tree. The sequence of *Haemophilus ducreyi* indicated a separate branch in the *rpoB* tree, which was similar to the *infB* phylogeny (Hedegaard et al., 2001).

The genus *Gallibacterium* in both trees is clearly part of the avian cluster. This also holds true for the species *Pasteurella avium*, *Pasteurella volantium* and *Pasteurella gallinarum*, which show a very tight phylogenetic relationship in both trees on a separate branch different from the type species of the genus *Pasteurella*. In both trees, Bisgaard taxon 33 forms a distinct branch within the avian cluster. However, in the *rpoB* tree it is accompanied by Bisgaard taxon 5, which clusters with *A. succinogenes* in the 16S rDNA tree.

The Seminis cluster formed by *Pasteurella aerogenes*, *Pasteurella mairii* and *A. seminis* is also conserved in the *rpoB* and 16S rDNA trees. We described the Pax RTX toxin recently, in a subset of *Pasteurella aerogenes* strains causing swine abortion (Kuhnert et al., 2000). Pax-positive strains cause problems in phenotypic identification and are often identified as *Pasteurella mairii*, which contains a variant of the Pax toxin (P. Kuhnert, unpublished results). However, we found a 100% sequence match between the Pax-negative type strain of *Pasteurella aerogenes* and the Pax-positive field strain JF1319 and both are clearly separated from *Pasteurella mairii* in both trees. Therefore, the *rpoB* sequence together with the 16S rDNA would help to reorganize the *Pasteurella aerogenes* complex, which needs to be separated, probably into a new genus. *Pasteurella aerogenes* is phenotypically closely related to *Actinobacillus rossii*. However, this is on a distant branch together with *Actinobacillus porcinus* in the 16S rDNA tree as well as in the *rpoB* tree. In the latter, these two porcine species cluster with *Pasteurella caballi* and *Pasteurella langaoensis*, a phylogenetic relationship that is at odds with that found with 16S rDNA.

The similarity matrix of the *rpoB* sequences for all the strains investigated in this study is available as supplementary material in IJSEM Online. Given that sequence divergences of the 16S rDNA are approximately 6–7% between the genera of the family, we can draw a threshold value for the *rpoB* gene sequences analysed in this study at 10–12% between the genera.

In conclusion, we found that the resolution of the *rpoB* gene within the family *Pasteurellaceae* is generally greater than that of the 16S rDNA sequence but still does not allow the investigation of very closely related species. There was a good correlation between 16S rDNA- and *rpoB* gene-based phylogenies within the family *Pasteurellaceae*, even though discrepancies were observed for certain species.
However, the topology of the rpoB tree is in a better agreement with the DNA–DNA hybridization results and seems to dissect the genera of the family better than the 16S rDNA tree does, as shown, for example, by the clustering of A. capsulatus within the Actinobacillus sensu stricto group in the rpoB tree. Therefore, the rpoB gene not only complements the 16S rDNA phylogeny but will actually be a powerful tool for the revision of the taxonomy of Pasteurellaceae. Moreover, by describing a universally applicable amplification and sequencing approach for the partial rpoB gene sequence determination of Pasteurellaceae species, we generated a powerful tool for DNA-sequence-based identification. Generating parallel databases for 16S rDNA and rpoB gene sequences, as is currently being done on the SmartGene IDNS platform (www.smartgene.ch), will further help to clarify the phylogeny of Pasteurellaceae and help in the reclassification of this family. The inclusion of further housekeeping genes like infB, atpD and others, resulting in a multi-locus-sequencing approach, will certainly help to achieve this aim (Christensen et al., 2004).

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References


