Multilocus sequence phylogenetic study of the genus *Haemophilus* with description of *Haemophilus pittmaniae* sp. nov.

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The phylogeny of human isolates of *Haemophilus* species was estimated based on partial sequences of four separate housekeeping genes. The clustering of each set of sequences was in accordance with speciation of the strains with few exceptions: of 108 gene fragments examined, only three appeared to have been subject to recombination events across the species barrier. Housekeeping gene similarity supported previous DNA–DNA hybridization data for the genus rather than the phylogeny inferred from 16S rRNA gene sequence comparison. The similarity of sequences of *Haemophilus parainfluenzae* with those of *Haemophilus influenzae* suggested preservation of the former species in the genus *Haemophilus*. Three strains representing a novel taxon were unique with respect to the four investigated gene loci. 16S rRNA gene sequence analysis suggested that this taxon belonged to the Parainfluenzae cluster. DNA–DNA hybridization data supported this generic placement. Nine strains of the novel taxon were available for analysis. They were distinct from representatives of all current species of the genus *Haemophilus* by conventional phenotypic characterization. Genotypic and phenotypic data show that the strains merit recognition as a novel species of *Haemophilus*. The name *Haemophilus pittmaniae* sp. nov. is proposed, with HK 85^T (=CCUG 48703^T=NCTC 13334^T) as the type strain.

INTRODUCTION

As delineation of the genus *Haemophilus* based on dependence on haemin (X-factor) or NAD (V-factor) has become obsolete, a major revision of the genus may be warranted. One strategy would be to adopt the hierarchical classification ‘that reflects the phylogeny of prokaryotes, as defined by 16S rRNA sequence analysis’ (Garrity *et al.*, 2003). If the *Pasteurellaceae* are to be divided into phylogenetically consistent monophyletic groups based on 16S

rRNA gene sequences, more than 20 genera will be needed to cover the family (Olsen *et al.*, 2004). Nomenclatural changes along these lines have been initiated and new genera include *Lonepinella* (Osawa *et al.*, 1995), *Mannheimia* (Angen *et al.*, 1999), *Phocoenobacter* (Foster *et al.*, 2000), *Gallibacterium* (Christensen *et al.*, 2003), *Histophilus* (Angen *et al.*, 2003) and *Volucribacter* (Christensen *et al.*, 2004).

Fifteen named *Haemophilus* species are listed in the latest edition of *Bergey’s Manual of Systematic Bacteriology* (Kilian, 2004). By 16S rRNA gene sequence comparison, the family *Pasteurellaceae* is divided into 21 rRNA clusters with named *Haemophilus* species dispersed among 10 of them (Olsen *et al.*, 2004). ‘*Haemophilus sensu stricto*’ (rRNA cluster 16) is composed of *Haemophilus influenzae*, *Haemophilus aegyptius* and *Haemophilus haemolyticus*, whereas the *Aphrophilus* cluster (13) encompasses *Haemophilus aphrophilus*, *Haemophilus paraphrophilus* and *Haemophilus segnis*, as well as *Actinobacillus actinomycetemcomitans*. *Haemophilus parainfluenzae*, *Haemophilus parasuis*, *Haemophilus felis*, *Haemophilus paracuniculus*, *Haemophilus haemoglobinophilus* and *Haemophilus ducreyi* are placed in separate clusters (2, 8, 9, 11, 17 and 19, respectively) and *Haemophilus paragallinarum* is grouped with other taxa isolated

Published online ahead of print on 24 September 2004 as DOI 10.1099/ijs.0.63325-0.

Abbreviations: *adk*, adenylate kinase gene; *infB*, translation initiation factor 2 gene; *pgi*, glucose-6-phosphate isomerase gene; *recA*, recombinase A gene.

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are AJ289631, AJ289636, AJ289639, AJ289640, AJ289644, AJ289650, AJ289653, AJ289659, AJ289664–AJ289671, AJ289675, AJ289677, AJ289679–AJ289681, AJ289685, AJ289688, AJ289690, AJ289694, AJ290754, AJ290755, AJ290758, AJ295746, AJ438112, AJ585268–AJ585341, AY597040 and AY736136–AY736145.

A table showing the origins of human isolates of *Haemophilus pittmaniae* sp. nov. is available as supplementary material in IJSEM Online.

from birds in the Avian cluster (18). *Haemophilus paraphrohaemolyticus* is located in a so-called Porcine cluster (3), adjacent to the Parainfluenzae cluster (Olsen *et al.*, 2004); *Haemophilus parahaemolyticus* belongs to the same cluster (see below).

Some caution should be exercised before a classification based solely on 16S rRNA gene sequences is adopted. With respect to the genus *Haemophilus*, the problems are particularly related to *H. parainfluenzae* and to some extent to the parahaemolyticus group (*H. parahaemolyticus* and *H. paraphrohaemolyticus*). For reasons of clarity, the term 'cluster' is reserved to denote *Pasteurellaceae* 16S rRNA gene sequence clusters as defined in the latest version of *Bergey's Manual* (Olsen *et al.*, 2004). Comprehensive DNA–DNA hybridization studies have indicated a generic placement of *H. parainfluenzae* within the boundaries of the genus *Haemophilus* (Burbach, 1987; Mitters *et al.*, 1989). DNA–DNA hybridization also indicates that the parahaemolyticus group takes up an intermediate position between '*Haemophilus sensu stricto*' and other parts of the family *Pasteurellaceae* (Burbach, 1987). By investigating 66 isolates of *Haemophilus* and related bacteria, it has been shown previously that partial *infB* sequencing supports delineation of the genus *Haemophilus* obtained by DNA–DNA hybridization (Hedegaard *et al.*, 2001).

The present study was undertaken to investigate further the structure of the genus *Haemophilus* by examining the phylogeny of three other housekeeping genes, the adenylate kinase gene (*adk*), the glucose-6-phosphate isomerase gene (*pgi*) and the recombination protein gene (*recA*). Apart from *H. ducreyi*, which is unrelated to *H. influenzae* by DNA–DNA hybridization (Casin *et al.*, 1985) and only distantly related (93.6%) by 16S rRNA gene sequence analysis (Olsen *et al.*, 2004), representatives of all *Haemophilus* species primarily associated with man were examined.

METHODS

Bacterial strains and phenotypic characterization. Sequences from 28 representatives of *Haemophilus* species and related bacteria were examined in the study: *H. influenzae* HK 389^T, *H. aegyptius* HK 367^T, *H. haemolyticus* HK 386^T and HK 676 (=CCUG 49488), *H. parainfluenzae* HK 409^T, HK 23 (=CCUG 49489), HK 102 (=CCUG 49490) and HK 755 (=CCUG 49491) (of biotypes I, II, III and V, respectively), a novel *Haemophilus* taxon (subcluster 2C in Hedegaard *et al.*, 2001) HK 85^T (=CCUG 48703^T), HK 847 (=CCUG 48704) and P1525 (=CCUG 48706), *H. parahaemolyticus* HK 385^T and HIM 570-6 (=CCUG 49512), *H. paraphrohaemolyticus* HK 411^T and P1300 (=CCUG 49492), *Actinobacillus lignieresii* P151^T, *Actinobacillus pleuropneumoniae* ATCC 27088^T, *H. aphrophilus* P801^T and RH01 (=CCUG 49493), *H. paraphrophilus* HK 415^T and HK 83 (=CCUG 49494), *H. segnis* HK 316^T, HK 321 (=CCUG 49495) and HK 701 (=CCUG 18088), *A. actinomycetemcomitans* CCUG 13227^T and HK 1651 (=CCUG 49496), and *Pasteurella multocida* P801^T and Pm70. Strains were selected from our previous study of *infB* sequences (Hedegaard *et al.*, 2001) and supplemented with the type strain of *A. lignieresii*, the additional *H. aphrophilus* isolate (RH01) and Pm70, a fully sequenced isolate of *P. multocida* (May *et al.*, 2001).

The origins of the nine isolates described as a novel species (*H. pittmaniae* sp. nov.) are listed in a table available as supplementary material in IJSEM Online. NAD dependence was assessed by V-factor tablets from Rosco Diagnostics placed on inoculated brain heart infusion agar plates. IgA1 protease activity was analysed as described previously (Kilian *et al.*, 1979). Activities of proline arylamidase and γ -glutamyl transferase were tested with the API NH system (bioMérieux). Activities of acid and alkaline phosphatases plus leucine arylamidase were tested with API ZYM (bioMérieux). Other phenotypic tests were carried out as described previously (Kilian, 1976). Detection of adenylate kinase activity by multilocus enzyme electrophoresis was performed according to Selander *et al.* (1986). The biotypes of *H. parainfluenzae* were defined as described by Kilian (2003). Activity of β -lactamase was evaluated by a chromogenic assay (PCN; Becton-Dickinson).

DNA sequencing. Housekeeping genes *adk*, *pgi* and *recA* were selected from the multilocus sequence typing scheme of *H. influenzae* (Meats *et al.*, 2003). The genes were identified in *Escherichia coli* (GenBank accession nos AE000153, X15196 and AE005498), *H. influenzae* Rd KW20 (NC_000907), *P. multocida* PM70 (AE004439) and *A. actinomycetemcomitans* HK 1651 [unfinished genome by the Actinobacillus Genome Sequencing Project (B. A. Roe, F. Z. Najjar, A. Gillaspay, S. Clifton, T. Ducey, L. Lewis and D. W. Dyer) at <http://www.genome.ou.edu/act.html>]. From alignments of the individual genes, the following degenerate primers were designed (nt numbering with reference to *E. coli*): *adk*.34f, 5'-GGIAAAGGIACWCARGC-ICARTT; *adk*.610r, 5'-CTTCCACTTTTCKYGTMCCTGTC; *pgi*.838f, 5'-GATGGIAAAGAYGTIATGCC; *pgi*.1331r, 5'-GCTGACCAYAA-IGARTAACG; *recA*.54f, 5'-GARAACAATTTGGKAAAGGC; and *recA*.617r, 5'-TTACCRAACATMACRCCIAT (I, deoxyinosine; W, A or T; R, G or A; K, G or T; Y, C or T; M, A or C). The primers cover approximately the same internal fragments of the genes as used in the multilocus sequence typing scheme of *H. influenzae*.

DNA was released from cells as previously described (Hedegaard *et al.*, 2001) and 1 μ l was amplified in Ready-to-go vials (Pharmacia) with 15 pmol forward and 15 pmol reverse primers in a total volume of 25 μ l. After an initial denaturation step at 94 °C for 5 min, 30 cycles (94 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min) were run. Amplicons were purified on silica-gel columns (QIAquick PCR purification kit) and cycle sequenced from both ends using the PCR primers and a ThermoSequenase dye terminator sequencing kit (Pharmacia Biotech). Sequencing reactions were analysed on an ABI 377 DNA sequencer.

Near-full-length 16S rRNA gene sequences were amplified with the primers (numbering with reference to *E. coli*, sequence accession number J01695) 16S.8f (5'-AGAGTTTGATYMTGGCT; Angen *et al.*, 1999) and 16S.1513r (5'-TACGGYTACCTTGTACGACT; Dewhirst *et al.*, 1989). Purified products were sequenced as described above using the PCR primers and the internal primers described by Paster & Dewhirst (1988) and Dewhirst *et al.* (1989). Fragments of 1450–1454 bp (corresponding to nt 25–1478 of *E. coli* 16S rRNA) were used for phylogenetic analysis.

Sequences were aligned as described previously (Nørskov-Lauritsen *et al.*, 2004). Phylogenetic analysis and construction of dendrograms were carried out using MEGA version 2.1 (Kumar *et al.*, 2001).

DNA–DNA hybridization. DNA was isolated using a French pressure cell (ThermoSpectronic) and was purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). DNA–DNA hybridization was carried out at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany, as described by De Ley *et al.* (1970), with the modifications described by Huß *et al.* (1983) and Escara & Hutton (1980), using a model 2600 spectrophotometer equipped with a model 2527-R

thermoprogrammer and plotter (Gilford Instrument Laboratories). Renaturation rates were computed with the TRANSFER.BAS program of Jahnke (1992).

RESULTS

Comparison of individual housekeeping gene fragments

The relationship of the study strains based on comparison of *adh* sequences showed a core group of *Haemophilus*

encircling the influenzae group (*H. influenzae*, *H. aegyptius* and *H. haemolyticus*), the heterogeneous species *H. parainfluenzae* and the novel taxon *H. pittmaniae* sp. nov. (Fig. 1b). These species were more related to strains of both *Pasteurella* and *Actinobacillus sensu stricto* than to the *Aphrophilus* cluster (Fig. 1b and Table 1). It was not possible to amplify an *adh* fragment in isolates of the parahaemolyticus group and these four strains are not included in the neighbour-joining dendrogram based on *adh* sequences. The type strain of *H. parahaemolyticus* contains detectable adenylate kinase activity, as evaluated

Fig. 1. Neighbour-joining dendrograms based on partial sequences of separate housekeeping genes. Type strains are emphasized in bold. Nucleotide sequence accession numbers are given, except for HK 1651, for which no sequence accession numbers are yet available (see Methods). Evolutionary distances are given in terms of the number of base substitutions weighted by the Kimura two-parameter model. Values to the left of nodes are bootstrap replications (%) supporting the node. Bars, 5 substitutions per 100 nt. (a) Translation initiation factor 2 gene, *infB* (453 nt); (b) adenylate kinase gene, *adh* (453 nt); (c) glucose-6-phosphate isomerase gene, *pgi* (393 nt); and (d) recombinase A gene, *recA* (447 nt).

Table 1. Sequence similarity (%) of four housekeeping gene fragments of the type strain of *H. influenzae* with selected groups of *Pasteurellaceae*

Sequence group (number of strains)	Gene fragment			
	<i>infB</i>	<i>adk</i>	<i>pgi</i>	<i>recA</i>
<i>H. aegyptius</i> , <i>H. haemolyticus</i> (3)	95–98	95–99	93–95	93–99
<i>H. parainfluenzae</i> (4)	87–89	86–88	78–80	81–82
<i>H. pittmaniae</i> sp. nov. (3)	87	81	74	81
Parahaemolyticus group (4)	85–86	ND	79–81	72–74
<i>A. lignieresii</i> , <i>A. pleuropneumoniae</i> (2)	81	79–80	75	71–72
<i>P. multocida</i> (2)	79–80	76–77	77	77–78
Aphrophilus cluster (9)	79–82	73–76	69–74	75–76
<i>E. coli</i> (1)	65	60	73	63

ND, Not determined.

by multilocus enzyme electrophoresis (result not shown), and the failure of the amplification must be ascribed to a primer mismatch.

The relationship inferred from an alignment of *pgi* sequences was somewhat different (Fig. 1c). The sequence carried by *E. coli* was sufficiently unique to place this species as an outgroup, despite the larger number of nucleotide substitutions observed between *H. influenzae* and some members of the Aphrophilus cluster than between *H. influenzae* and *E. coli* (Table 1). The Aphrophilus cluster (*H. aphrophilus*, *H. paraphrophilus*, *H. segnis* and *A. actinomycetemcomitans*) was located on a separate and distant branch, which is similar to the phylogeny inferred from the other housekeeping genes. However, the rest of the sequence groups, including representatives of *Pasteurella* and *Actinobacillus*, showed similarity with *H. influenzae* at a level of 75–80 % (Table 1) and appeared to branch at equal depths in the dendrogram (Fig. 1c). Only moderate bootstrap values supported the new constellation of sequence groups.

Using *recA* sequence comparisons, the outline of the family inferred from *infB* and *adk* analysis was recognized. However, the *P. multocida* strains were more closely related to the influenzae group using *recA* than they were using *infB* and *adk* sequences (Table 1) and took up a different position in the dendrogram. The type strain of *H. aphrophilus* (accession no. AJ585333) clustered with *H. segnis* rather than with the other representatives of *H. aphrophilus* and *H. paraphrophilus*; the *recA* locus of this strain has most likely been subject to a recombination event (Fig. 1d).

Relationships based on *infB* sequences from our previous investigation (Hedegaard *et al.*, 2001) are shown (Fig. 1a) for comparison with those for the other housekeeping genes. The outline of the family inferred from *adk* and *recA* sequence comparison is supported by *infB* analysis. However, the three strains of *H. segnis* are in two separate groups, probably because of a recombination event affecting the ancestor of the type strain and strain HK 701.

Multilocus phylogeny

The sequenced fragments of the separate housekeeping genes from each strain were joined together and phylogenetic analysis was performed. The concatenated sequence comprised 1746 nt for most of the strains, but only 1293 nt for representatives of the parahaemolyticus group, because of the lack of *adk* sequences. For *E. coli* and *P. multocida*, a triplet deletion in *infB* resulted in a concatenated sequence of 1743 nt. A dendrogram constructed by the neighbour-joining method is shown in Fig. 2(a). The three representatives of *H. segnis* coalesced into a single group, although the aberrant result of *infB* sequencing was discernible in the dendrogram and in the moderate bootstrap value of 79 % (Fig. 2a). Also, the type strain of *H. aphrophilus* was located next to its kin, despite the atypical *recA* sequence.

Based on a multilocus sequence comparison of the investigated taxa, *H. parainfluenzae* and *H. pittmaniae* sp. nov. are the species most closely related to the influenzae group (*H. influenzae*, *H. aegyptius* and *H. haemolyticus*). The Aphrophilus cluster is as separate from the type species of the genus *Haemophilus* as are the type species of *Actinobacillus* and *Pasteurella*. Multilocus sequence phylogenetic analysis could not support conservation of the parahaemolyticus group within the genus ‘*Haemophilus sensu stricto*’ (Fig. 2a), although this comparison is restricted to three genes.

Fig. 2(b) shows the phylogenetic comparison based on near-full-length 16S rRNA gene sequences. The 16S rRNA gene sequence of the type strain of *H. parahaemolyticus*, available in databases as M75073, is based on an incorrect strain (Hedegaard *et al.*, 2001) and therefore both the type strain (HK 385^T) and *H. parahaemolyticus* strain HIM 570-6 (sequence accession nos AJ295746 and AJ290758, respectively) were sequenced. The 1466 nt 16S rRNA gene sequence of HK 385^T determined by us showed 96 % similarity with the deposited sequence of the type strain of *H. paraphrohaemolyticus* (M75076). However, it showed 99 % similarity with strain MCCM 00189 (AF224283),

Fig. 2. (a) Neighbour-joining tree based on concatenated sequences from the four separate housekeeping genes *infB*, *adh*, *pgi* and *recA* (1746 nt, except for the parahaemolyticus group, where *adh* was not amplified resulting in a total of 1293 nt). Bar, 5 substitutions per 100 nt (weighted by the Kimura two-parameter model). (b) Neighbour-joining tree based on 1450–1454 nt 16S rRNA gene sequences from type strains. Nucleotide sequence accession numbers are given. Bar, 1 substitution per 100 nt (calculation performed with even-weighted characters ignoring gaps and ambiguous positions). Brackets delineate numbered 16S rRNA gene clusters of Olsen et al. (2004).

classified as *Actinobacillus pleuropneumoniae*, which is in the Porcine cluster of the *Pasteurellaceae* according to Olsen et al. (2004). This level of similarity (99%; 1456/1466 nt) is also seen between HK 385^T and strain HIM 570-6 and suggests incorrect identification of strain MCCM 00189.

The phylogeny of the 16S rRNA gene in the family *Pasteurellaceae* (Olsen et al., 2004) is shown in Fig. 2(b), with the *Aphrophilus* cluster (13) being most closely related to '*Haemophilus sensu stricto*' (rRNA cluster 16) and *H. parainfluenzae* taking up a more distant position in the dendrogram. The 1468 nt 16S rRNA gene sequence of strain HK 85^T (type strain of *H. pittmaniae* sp. nov.) showed 95% similarity with the type strain of *H. parainfluenzae* and was located in the *Parainfluenzae* cluster (2).

DNA–DNA hybridization and homogeneity of the novel taxon

DNA–DNA hybridization experiments were performed to disclose further the position of the novel taxon. The DNA relatedness between strain HK 85^T and strain HK 847 was ~80%, which is well above the threshold value of 70% that has been suggested for the definition of bacterial species (Wayne et al., 1987). Of the type strains tested, HK 85^T was most closely related to *H. parainfluenzae* (Table 2).

Nine isolates of *H. pittmaniae* sp. nov. were available for

investigation. The species appeared relatively homogeneous, with an *infB* sequence similarity in excess of 97% (based on 453 nt) and a 16S rRNA gene sequence similarity in excess of 99% (380 nt) (alignments not shown; see supplementary material available in IJSEM Online regarding strain origin and individual nucleotide sequence accession numbers). Phenotypic characteristics are given in the species description.

Near-full-length 16S rRNA gene sequences of *H. pittmaniae* strains HK 85^T and P1525 have been deposited under accession numbers AJ290755 and AJ290754, respectively.

Table 2. DNA–DNA relatedness between *H. pittmaniae* sp. nov. HK 85^T and selected strains of the *Pasteurellaceae*

Strain	DNA–DNA relatedness (%)
<i>H. pittmaniae</i> HK 847	80 ± 3*
<i>A. lignieresii</i> P151 ^T	22
<i>P. multocida</i> P427 ^T	22
<i>H. aphrophilus</i> HK 372 ^T	19
<i>H. parahaemolyticus</i> HK 385 ^T	22
<i>H. influenzae</i> HK 389 ^T	29
<i>H. parainfluenzae</i> HK 409 ^T	34

*Mean ± SD of two experiments.

DISCUSSION

The degenerate primers employed for partial sequencing of the four housekeeping genes were able to recognize the target genes in the 28 study strains, except for the four representatives of the parahaemolyticus group, where a probable mismatch prevented successful amplification of the *adh* gene fragment. A total of 108 gene fragments was thus available for analysis.

The sequences obtained were divided into separate and distinct sequence groups. The grouping of sequences followed speciation of the strains with few exceptions. As previously shown for *H. segnis*, the *infB* gene fragment in this species can belong to either of two separate sequence groups (Hedegaard *et al.*, 2001). An aberrant result was obtained with the *recA* gene fragment of *H. aphrophilus* P801^T, which was related to the sequences obtained from representatives of *H. segnis* rather than *H. aphrophilus* and *H. paraphrophilus* (Fig. 1d). In all other cases, the grouping of sequences was in accordance with speciation of the strains, although some sequence groups enclosed more than one species.

The *Haemophilus* species isolated from man and dependent on both the X- and V-factor (the influenzae group: *H. influenzae*, *H. aegyptius* and *H. haemolyticus*) are closely related. In fact, the differences between four representatives of the species *H. parainfluenzae* were larger than those between four representatives of the three species in the influenzae group (Fig. 2a; calculation not shown). The close relationship of the species of the influenzae group is supported by DNA–DNA hybridization data (Burbach, 1987; Casin *et al.*, 1986) and 16S rRNA gene sequence similarity analysis (Olsen *et al.*, 2004).

The interrelationship between *A. actinomycescomitans*, *H. aphrophilus*, *H. paraphrophilus* and *H. segnis* has previously been demonstrated by DNA–DNA hybridization (Potts *et al.*, 1986), rRNA gene sequence analysis (Olsen *et al.*, 2004) and *infB* sequence comparison (Hedegaard *et al.*, 2001). The vicinal position of these species was emphasized by the sequence similarities of three additional housekeeping gene fragments analysed in this study. A controversy, however, exists about the generic placement of the group. A proposed transfer of *A. actinomycescomitans* to the genus *Haemophilus* (Potts *et al.*, 1985) is not supported due to the low level of relatedness with the type species (Kilian, 2004). Early comparisons of 16S rRNA gene sequences in the family Pasteurellaceae placed *A. actinomycescomitans*, *H. aphrophilus*, *H. paraphrophilus* and *H. segnis* together with *H. influenzae* (Dewhirst *et al.*, 1992). However, after inclusion of 114 16S rRNA gene sequences, this delineation of the genus *Haemophilus* was found to be polyphyletic and separate clusters were created for '*Haemophilus sensu stricto*' (rRNA cluster 16) and the Aphrophilus cluster (13) (Olsen *et al.*, 2004). Results from the housekeeping gene sequencing are unequivocal. The sequences carried by members of the Aphrophilus cluster

are quite different from those of the influenzae group in all four gene loci (Fig. 1). In the comparison of the concatenated sequences, representatives of the Aphrophilus cluster were less related to *H. influenzae* than to any of the other investigated taxa, including type species of the genera *Pasteurella* and *Actinobacillus* (Fig. 2). Consequently, species of the Aphrophilus cluster do not belong to the genus *Haemophilus*.

The specific status and generic placement of the parahaemolyticus group (*H. parahaemolyticus* and *H. paraphrohaemolyticus*) have also been a subject of discussion. Kilian (1976) questioned the specific assignment of *H. parahaemolyticus* on the basis of an extended phenotypic investigation. The only characteristic that distinguished this species from biotype III of *H. parainfluenzae* was haemolytic activity and this was considered unstable. Results of DNA–DNA hybridization experiments, however, verified the specific status of the parahaemolyticus group, although the separation into two species was not investigated in detail (Burbach, 1987). Further differentiating phenotypic characters have been searched for, and fermentation of mannose, production of β -galactosidase and the presence of IgA1 protease have been shown to differentiate *H. parainfluenzae*, *H. parahaemolyticus* and *H. paraphrohaemolyticus* (Kilian, 2004).

The confused identity of strains subjected to 16S rRNA gene sequencing has complicated the issue. In the comprehensive comparison of 16S rRNA gene sequences by Olsen *et al.* (2004), *H. parahaemolyticus* is included in *Actinobacillus sensu stricto*, whereas *H. paraphrohaemolyticus* is located in the so-called Porcine cluster (rRNA cluster 3), together with a strain (MCCM 00189) labelled *A. pleuropneumoniae* and two strains of *Actinobacillus minor*. However, as mentioned above, the 16S rRNA gene sequence of the type strain of *H. parahaemolyticus* available in the databases as M75073 is not based on the correct strain (Hedegaard *et al.*, 2001). The 16S rRNA gene sequence of the type strain of *H. parahaemolyticus* determined in this study showed 96% similarity to the type strain of *H. paraphrohaemolyticus* (accession no. M75076); this difference supports the existence of separate species in the parahaemolyticus group. Moreover, the 16S rRNA gene sequence of the two representatives of *H. parahaemolyticus* determined in this study showed 99% similarity to that of strain MCCM 00189 (accession no. AF224283) of 16S rRNA cluster 3. This suggests that accession number AF224283 represents an isolate of *H. parahaemolyticus* and that the so-called Porcine cluster (rRNA cluster 3) of Olsen *et al.* (2004) is composed mainly of human bacterial species.

In addition to their different growth factor requirements, *H. influenzae* and *H. parainfluenzae* differ in their ability to ferment a number of carbohydrates (Kilian, 1976). Results from DNA–DNA hybridization studies, however, support placement of *H. parainfluenzae* in the genus *Haemophilus* (Burbach, 1987; Mutters *et al.*, 1989). It was therefore unexpected when a markedly different phylogeny was

inferred from 16S rRNA gene sequence analysis. In the initial report on 54 representative strains of species in the family *Pasteurellaceae*, *H. parainfluenzae* was located as an outgroup to the entire family (Dewhirst *et al.*, 1992). In the study by Olsen *et al.* (2004), the *Parainfluenzae* cluster (encompassing the species *H. parainfluenzae* only) has been included as one of 21 clusters in the family, but at a position quite distant from '*Haemophilus sensu stricto*'. With respect to housekeeping gene similarity, the heterogeneous group of *H. parainfluenzae* is related to *H. influenzae*, the type species of the genus *Haemophilus*. When a phylogenetic tree was constructed on the basis of a concatenated sequence of four genes, *H. parainfluenzae*, *H. pittmaniae* sp. nov. and the *influenzae* group constituted a monophyletic lineage supported by a bootstrap value of 91 % (Fig. 2).

The novel taxon demonstrated in this study is sufficiently distinct from all named taxa by analysis of sequences of 16S rRNA and selected housekeeping genes, DNA–DNA hybridization and phenotypic traits to warrant specific recognition. The spectrum of sugars used was similar to that observed for *H. parainfluenzae*, but the novel taxon was negative in the tests used for biotyping *H. parainfluenzae* (production of indole, urease and ornithine decarboxylase). Strains of *H. parainfluenzae* that are negative in the three biotyping tests have recently been demonstrated (biotype V; Hedegaard *et al.*, 2001). The presence of haemolytic activity serves to discriminate the novel taxon from *H. parainfluenzae* biotype V. Haemolytic activity has been described for some isolates of *H. parainfluenzae* biotype II and IV (Hedegaard *et al.*, 2001; Kilian, 2003). Negative reactions in the tests used for biotyping *H. parainfluenzae* are characteristic of both *H. paraphrophilus* and *H. segnis*. Apart from the lack of haemolysis, the former can be differentiated from the novel taxon by its ability to produce acid from lactose, the latter by its weak fermentative capability.

The questionable generic affiliation of *H. parainfluenzae* affects the naming of the novel taxon. It is most closely related to *H. parainfluenzae* by phenotype, comparison of both 16S rRNA and housekeeping gene sequences (Figs 1 and 2) and DNA–DNA hybridization data (Table 2). It is considered that removal of *H. parainfluenzae* from the genus *Haemophilus* cannot be supported by the available data and it is proposed that the novel taxon be maintained in the genus *Haemophilus* as *H. pittmaniae* sp. nov.

Description of *Haemophilus pittmaniae* sp. nov.

Haemophilus pittmaniae (pitt.ma'ni.æ. N.L. gen. fem. n. *pittmaniae* of Pittman, named in honour of Margaret Pittman for her substantial contributions to *Haemophilus* research).

Non-motile, facultatively anaerobic, Gram-negative, small, pleomorphic rods, with occasional long, filamentous forms. Colonies on chocolate agar are greyish white and reach a

diameter of 1–2 mm after 24 h at 35 °C. A distinct β -haemolytic zone is produced around the colonies on horse or sheep blood agar. Depends on V-factor for growth on brain heart infusion agar plates, but is capable of growth on blood plates due to release of V-factor from lysed blood cells. Positive in the porphyrin test, negative or weakly positive in catalase and oxidase tests. Negative in the indole and urease tests. Acid is produced from D-glucose, D-fructose, sucrose, D-mannose, D-galactose and maltose. A small amount of gas is produced from glucose. Acid is not produced from lactose, D-xylose, D-mannitol, D-sorbitol, sorbose, melibiose, inulin, aesculin or amygdalin. Negative in lysine and ornithine decarboxylase and arginine dihydrolase tests. Negative for IgA1 protease. Produces β -galactosidase (ONPG), alkaline phosphatase, acid phosphatase and leucine arylamidase, but not β -glucosidase (NPG), α -glucosidase (PNPG), β -glucosaminidase (GNAC), β -glucuronidase (PGUA) or α -fucosidase (ONPF). Of the nine strains analysed, six are positive for γ -glutamyl transferase (GGT) activity and one is positive for β -lactamase activity.

The type strain is HK 85^T (=CCUG 48703^T=NCTC 13334^T), isolated from human saliva. The species is part of the normal flora of the oral mucous membranes of man. It is an opportunistic pathogen and has been isolated from various sites of infection, including blood and bile.

ACKNOWLEDGEMENTS

Professor Dr Hans G. Trüper is thanked for etymological assistance. Our sequencing facility was a donation from the Velux Foundation.

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