International Journal of Systematic and Evolutionary Microbiology (2006), **56**, 2135–2146 **DOI** 10.1099/ijs.0.64207-0

Reclassification of *Actinobacillus actinomycetemcomitans*, *Haemophilus aphrophilus*, *Haemophilus paraphrophilus* and *Haemophilus segnis* as *Aggregatibacter actinomycetemcomitans* gen. nov., comb. nov., *Aggregatibacter aphrophilus* comb. nov. and *Aggregatibacter segnis* comb. nov., and emended description of *Aggregatibacter aphrophilus* to include V factor-dependent and V factor-independent isolates

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The aim of this study was to reinvestigate the relationships and the generic affiliations of the species *Actinobacillus actinomycetemcomitans*, *Haemophilus aphrophilus*, *Haemophilus paraphrophilus* and *Haemophilus segnis*. The nicotinamide phosphoribosyltransferase gene (*nadV*) conferring V factor-independent growth was identified in *Haemophilus aphrophilus*. The gene encodes a polypeptide of 462 amino acids that shows 74·5 % amino acid sequence identity to the corresponding enzyme from *Actinobacillus actinomycetemcomitans*. Ten isolates of *Haemophilus paraphrophilus* all carried a *nadV* pseudogene. DNA from *Haemophilus aphrophilus* was able to transform *Haemophilus paraphrophilus* into the NAD-independent phenotype. The transformants carried a full-length *nadV* inserted in the former locus of the pseudogene. The DNA–DNA relatedness between the type strains of *Haemophilus aphrophilus* and *Haemophilus paraphrophilus* was 77 %. We conclude that the division into two species *Haemophilus aphrophilus* and *Haemophilus paraphrophilus* is not justified and that *Haemophilus paraphrophilus* should be considered a later heterotypic synonym of *Haemophilus aphrophilus*. Forty strains of *Actinobacillus actinomycetemcomitans*, *Haemophilus aphrophilus* and *Haemophilus segnis* were investigated by multilocus sequence analysis. The 40 strains form a monophyletic group clearly separate from other evolutionary lineages of the family *Pasteurellaceae*. We propose the transfer of *Actinobacillus actinomycetemcomitans*, *Haemophilus aphrophilus* and *Haemophilus segnis* to a new genus *Aggregatibacter* gen. nov. as *Aggregatibacter actinomycetemcomitans* comb. nov. (the type species; type strain ATCC 33384^T = CCUG 13227^T = CIP 52.106^T = DSM 8324^T = NCTC 9710^T), *Aggregatibacter aphrophilus* comb. nov. (type strain ATCC 33389^T = CCUG 3715^T = CIP 70.73^T = NCTC 5906^T) and *Aggregatibacter segnis* comb. nov. (type strain HK316^T = ATCC 33393^T = CCUG 10787^T = CCUG 12838^T = CIP 103292^T = NCTC 10977^T). The species of the genus *Aggregatibacter* are independent of X factor and variably dependent on V factor for growth *in vitro*.

Abbreviations: NMN, nicotinamide mononucleotide; NR, nicotinamide riboside; RFLP, restriction fragment length polymorphism.

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are DQ223269–DQ223325, as detailed in Supplementary Table S1.

Details of the strains included in this study, including sequence accession numbers, detailed DNA–DNA hybridization results and details of *nadV* PCR results with selected strains are available as supplementary material in IJSEM Online.

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INTRODUCTION

'*Bacterium actinomycetem comitans*' was described by Klinger (1912) as coccobacillary bacteria isolated together with *Actinomyces* from actinomycotic lesions of man. It was reclassified as *Actinobacillus actinomycetemcomitans* by Topley & Wilson (1929) and as *Haemophilus actinomycetemcomitans* by Potts *et al.* (1985). The species has attracted attention because of its association with localized aggressive periodontitis (Slots & Ting, 1999; Haubek *et al.*, 2001). Six serotypes, a–f, have been described. The serological specificity is defined by six structurally and antigenically distinct *O*-polysaccharide components of their respective lipopolysaccharide molecules (Page *et al.*, 1991; Perry *et al.*, 1996a, b; Kaplan *et al.*, 2001).

Haemophilus aphrophilus was described by Khairat (1940) as a novel species of *Haemophilus* isolated from a case of infective endocarditis. The bacterium qualified for membership of the genus *Haemophilus* because of its X factor-dependence described by Khairat (1940). Successive investigations failed to demonstrate this character, but the generic affiliation was supported by the subsequent description of a closely related V factor-dependent species, *Haemophilus paraphrophilus* (Zinnemann *et al.*, 1968). *H. aphrophilus* and *H. paraphrophilus* are members of the indigenous flora of the biofilms that form on tooth surfaces of man, but are occasionally isolated from cases of cerebral abscesses or infective endocarditis (King & Tatum, 1962; Duel *et al.*, 1991; Darras-Joly *et al.*, 1997).

The V factor-dependent *Haemophilus segnis* is a member of the human oral flora (Kilian, 1976) and has only rarely been implicated in disease (Bangsborg *et al.*, 1988; Lau *et al.*, 2004).

The generic affiliation of these species has been disputed. '*Bacterium actinomycetem comitans*' was transferred to the genus *Actinobacillus* despite a weak resemblance to *Actinobacillus lignieresii*, the type species of the genus *Actinobacillus*. According to Cowan (1974), the bacterium was placed in this genus because 'neither Topley nor Wilson could think where to put it'. King & Tatum (1962) described the close phenotypic similarity of *Actinobacillus actinomycetemcomitans* with *H. aphrophilus*, and *Actinobacillus actinomycetemcomitans* was subsequently reassigned to the genus *Haemophilus* (Potts *et al.*, 1985). This transfer did not give rise to a satisfactory classification. *Actinobacillus actinomycetemcomitans* is unrelated to *Haemophilus influenzae*, the type species of the genus *Haemophilus*, and the reclassification of *Actinobacillus actinomycetemcomitans* was not favoured by the ICSB Subcommittee on *Pasteurellaceae* and related organisms (Frederiksen, 1987). However, the statement 'is not favoured', nomenclaturally, does not affect the formal validity of the proposed transfer, and *Haemophilus actinomycetemcomitans* and *Actinobacillus actinomycetemcomitans* are both legitimate names for the same species.

H. aphrophilus is discriminated from *H. paraphrophilus* by the V factor-dependence of the latter. The two species are highly related as estimated by both phenotypic and genotypic methods. The overall DNA similarity between them has been examined by DNA hybridization in several studies and by different methods (Tanner *et al.*, 1982; Potts *et al.*, 1986; Tønjum *et al.*, 1990). The reported DNA relatedness values are in conflict with the criteria for designation as separate species, if the suggested threshold value of 70 % is accepted (Wayne *et al.*, 1987).

Members of the family *Pasteurellaceae* are incapable of synthesizing nicotinamide adenine dinucleotide (NAD) *de novo* and acquire this essential nutrient from their environment as either NAD or a limited number of precursors. Extracellular pyridine nucleotides are degraded to nicotinamide riboside (NR) and internalized and NR is converted to NAD via nicotinamide mononucleotide (NMN) (Cynamon *et al.*, 1988; Martin *et al.*, 2001). Some species are capable of synthesizing NMN from nicotinamide and phosphoribosyl pyrophosphate in a reaction catalysed by nicotinamide phosphoribosyltransferase (EC number 2.4.2.12). Ample nicotinamide is present in complex media, and such isolates are therefore independent of exogenously supplied V factor (NAD, NMN or NR). The presence or absence of a single enzyme, encoded by the gene *nadV*, may thus be the sole feature that determines the requirement for V factor (Niven & O'Reilly, 1990), and V factor-dependent and -independent biovars have been documented for *Actinobacillus pleuropneumoniae* (Pohl *et al.*, 1983), *Haemophilus parainfluenzae* (Gromkova & Koornhof, 1990) and *Avibacterium paragallinarum* (Mouahid *et al.*, 1992). In *Haemophilus ducreyi*, the *nadV* gene encoding nicotinamide phosphoribosyltransferase is located on a plasmid capable of conferring NAD independence on *H. influenzae* (Windsor *et al.*, 1993; Martin *et al.*, 2001).

Phylogenetic analysis of DNA sequences from *Actinobacillus actinomycetemcomitans*, *H. aphrophilus*, *H. paraphrophilus* and *H. segnis* indicates a relationship at the genus level. When individual housekeeping genes are compared, a monophyletic origin of the four species with a DNA sequence similarity in excess of 85 % is usually observed (Hedegaard *et al.*, 2001; Nørskov-Lauritsen *et al.*, 2005). When the phylogenetic analysis is based on 16S rRNA gene sequences from 114 members of the family *Pasteurellaceae*, representatives of *Actinobacillus actinomycetemcomitans*, *H. aphrophilus*, *H. paraphrophilus* and *H. segnis* are the sole members of rRNA cluster 13 (Olsen *et al.*, 2005).

The present study was initiated to establish a phylogenetically coherent classification of the group with speciation supported by genotypic data. The genetic basis of the V factor-dependent phenotype was investigated. A multilocus sequence approach was used to study the relationship of 40 representatives of the four species in question. A novel genus encompassing three species is proposed.

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METHODS

Bacterial strains, phenotypic tests and isolation of DNA. Ten strains each of *Actinobacillus actinomycetemcomitans* (strains 1–10), *H. aphrophilus* (strains 11–20), *H. paraphrophilus* (strains 21–30) and *H. segnis* (strains 31–40) were investigated. Seven strains served to outline the family *Pasteurellaceae*: the type strains of the type species of the three classical genera *Actinobacillus* (*Actinobacillus lignieresii*, RNA cluster 1 of Olsen *et al.*, 2005), *Pasteurella* (*Pasteurella multocida*, RNA cluster 12) and *Haemophilus* (*H. influenzae*, RNA cluster 16), the type strain and an additional isolate (ATCC 29242) of *Haemophilus parainfluenzae* (RNA cluster 2) and the type strains of *Pasteurella aerogenes* and *Pasteurella pneumotropica* (RNA clusters 14 and 15). Data on strain identity, origin and individual nucleotide sequence accession numbers are listed in Supplementary Table S1 available in IJSEM Online.

Carbohydrate fermentation tests, oxidase (spot) test and assessment of hydrogen sulphide emission using lead acetate paper were carried out as described by Kilian (1976). V factor-dependence was tested with tablets from Rosco Diagnostics placed on inoculated brain heart infusion agar plates. For detection of porphyrin synthesis from δ-aminolaevulinic acid, a small loopful (approx. 1 µl) of the test strain was suspended in 50 µl 2 mM δ-aminolaevulinic acid/80 µM $MgSO_4$/0·1 M phosphate buffer, pH 6·9. After incubation for 4 and 24 h at 35 °C, 15 µl was placed on plastic film on a UV light board and photographed. For PCR, DNA was isolated in the presence of a resin as described previously (Hedegaard *et al.*, 2001). When genomic DNA was used in transformation assays or subjected to restriction endonuclease digestion, purified DNA was released from the cells using MagNA Pure Compact (Roche).

DNA transformation. *H. paraphrophilus* was transformed to V factor-independent growth using a semi-quantitative plate assay (Tønjum *et al.*, 1985). Colonies (24 h old) were suspended in 1 ml brain heart infusion broth (10^7–10^{10} c.f.u. ml^{-1}) and an aliquot of 10 µl was plated on 5 % horse blood agar (9 cm diameter plates). Ten microlitres crude *H. aphrophilus* genomic DNA was added immediately after plating and allowed to stay in contact with the cells during the incubation (no DNase treatment). Single colonies (indicating NAD-independent growth) were subcultured twice on 5 % blood agar plates before analysis of the *nadV* gene. DNA sterility and the number of c.f.u. per 10 µl in serial 10-fold dilutions of the test strain suspension were assessed in parallel with each experiment.

***nadV* sequencing.** The nicotinamide phosphoribosyltransferase genes from *P. multocida* PM70 (GenBank accession no. NC_002663; gene PM0999), *Actinobacillus actinomycetemcomitans* HK 1651 (http://www.oralgen.lanl.gov/; gene AA02355) and *Haemophilus somnus* strain 2336 (GenBank accession no. NZ_AACJ010 00048; locus_tag = "Haso02002086") were aligned and a number of degenerate primers were designed. Primers *nadV* 82f and *nadV* 1184r (Table 1) were able to amplify a product of approximately 1100 nt from the 10 strains of *H. aphrophilus*. Sequencing of the amplicon from the type strain revealed 66 % nucleotide sequence similarity with the *nadV* sequence from *Actinobacillus actinomycetemcomitans*.

The 3′ end of the *nadV* gene and flanking DNA was identified by inverse PCR (Andersen *et al.*, 2001). An aliquot containing 0·5 µg genomic DNA was digested with *Ngo*MIV, which recognizes nucleotides 687–692 in the *nadV* gene (*Actinobacillus actinomycetemcomitans* numbering). After heat-inactivation of enzyme and purification of DNA using QIAEX II particles (Qiagen), DNA fragments were diluted in 500 µl buffer and ligated overnight at 16 °C with 400 U T4 DNA ligase. DNA was concentrated by binding to QIAEX II particles and circularized DNA containing the 3′ end of the *nadV* gene was PCR-amplified using primers *nadV* 764r and *nadV* 1165f. The resulting amplicon of approximately 3000 nt was sequenced by primer walking. Because of the resemblance between the regions downstream of *nadV* in *H. aphrophilus* and *Actinobacillus actinomycetemcomitans* (see below), a similar resemblance was anticipated for the region upstream of *nadV*. Aspartokinase I (*thrA*) is located 5′ of *nadV* in *Actinobacillus actinomycetemcomitans*, and a successful attempt to amplify the 5′ end of *nadV* and flanking DNA was carried out using primers *thrA* 419r and *nadV* 424r. Primers used for the amplification and sequencing of the *nadV* region are detailed in Table 1.

Housekeeping gene sequencing. Partial fragments of the translation initiation factor 2 gene (*infB*, 453 nt), the glucose-6-phosphate isomerase gene (*pgi*, 393 nt) and the recombinase A gene (*recA*, 447 nt) were amplified by PCR and sequenced as described previously (Hedegaard *et al.*, 2001; Nørskov-Lauritsen *et al.*, 2005). Amplification was carried out with 30 cycles (94 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min) using proof-reading polymerase. With two strains of *H. aphrophilus* and three strains of *H. paraphrophilus*, insufficient priming with the internal primer *infB* 1748r was observed, and the *infB* amplicons from these strains were sequenced with primer *infB* 1186f and *infB* 1254f (5′-CGGCGAAGCAGGTGGGATTAC-3′). With some strains of *Actinobacillus actinomycetemcomitans*, amplification of the *recA* gene resulted in more than one PCR product, and the desired amplicon in these strains was sequenced with the internal primers *recA* 129f (5′-CGGTTCTTTCGGTCTTGA-3′) and *recA* 568r (5′-CCAAGCAGTTGGCGTTTTT-3′).

Partial fragments [433–441 nt, corresponding to positions 45–483 in *Escherichia coli* (GenBank accession no. J01695)] of the 16S rRNA gene were amplified and sequenced using the primers 16S 8f (Angen *et al.*, 1999) and 16S 536r (Paster & Dewhirst, 1988).

Other methods. Phylogenetic analysis and construction of dendrograms were performed using MEGA version 2.1 (Kumar *et al.*, 2001). DNA–DNA hybridization was carried out at the Deutsche Sammlung von Micro-organismen und Zellkulturen GmbH (Braunschweig, Germany). Restriction fragment length polymorphism (RFLP) of 16S rRNA gene PCR products was investigated by

Table 1. Primers used for amplification and sequencing of the *nadV* gene and flanking DNA

Numbering in primer names is with reference to the *Actinobacillus actinomycetemcomitans* sequence. I, Deoxyinosine.

Primer	Sequence (5′–3′)
thrA 419r	GCTTCAAACCAIGCITTCATCAT
thrA 23r	CCSCCRAATTTIAGTACICGCAT
nadV 82f	CATTGGCTSCAATATCC
nadV 98r	GGATATTGSARCCAATG
nadV 406f	TTGCCTAGCTATATTGAAACTGC
nadV 424r	TTTCAATATAGCTAGGCAACCA
nadV 764r	TCATAGGTGGCACGTTCATTTTCT
nadV 1165f	TGGGCRATGAARGCIAGTGC
nadV 1184r	GCACTIGCYTTCATYGCCCA
yggS 39r	TTGATGAATTTGCTGTAACTG
yggS 89f	AATTATTGGCCGTTTCTA
yggS 254r	TGYAACGGSCCRATAAAATG
yggS 270r	TCAAAATATTCTGCCACCAA
yggS 636f	GATTAAATGTGGTTCTACGATGGT

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amplification using primers 16S 340f and 16S 1387r (*E. coli* numbering) and digestion with *Hha*I or *Hin*fI (Riggio & Lennon, 1997). Fragments were separated on 2 % agarose gels containing 10 μg ethidium bromide ml^{-1} and visualized under UV light.

RESULTS

The *nadV* gene in *H. aphrophilus* and *H. paraphrophilus*

The *nadV* gene was identified in *H. aphrophilus* as described in Methods, and the 1386 nt sequence of the gene from the type strain is deposited under GenBank accession number DQ223269. The gene encodes a polypeptide of 462 amino acids showing 74·5 % amino acid sequence identity with the corresponding enzyme from *Actinobacillus actinomycetemcomitans*. The *nadV* gene in *H. aphrophilus* is located between the genes for aspartokinase I (*thrA*) and a conserved hypothetical protein (*yggS*) (Fig. 1). The same gene order is observed in *Actinobacillus actinomycetemcomitans*. The ten isolates of *H. paraphrophilus* all carried a *nadV* pseudogene between *thrA* and *yggS*. In nine cases, a short (88 nt), 5′-truncated version was found (Fig. 1). With one isolate (strain 30 = CCUG 41544), a positive PCR result was observed in the initial screening using primers *nadV* 82f and *nadV* 1184r. A 3 kb fragment of the *nadV* gene locus was sequenced from this strain and has been deposited under GenBank accession number DQ223272. The NAD-dependent phenotype could be ascribed to a single nucleotide deletion in the *nadV* gene at position 282–286, where a 6 nt homopolymer was terminated after five thymidine residues resulting in a reading-frame shift and a pseudogene of 1385 nt. The deletion was verified from both sequencing directions in several PCR amplicons.

The spacer region between *thrA* and *yggS* was sequenced from five representatives of *H. segnis* using the primers *thrA* 419r and *yggS* 254r. No evidence of a *nadV* pseudogene between *thrA* and *yggS* was discernible in representatives of *H. segnis* (Fig. 1).

Crude DNA from *H. aphrophilus* was able to confer NAD-independence on *H. paraphrophilus*. DNA from *H. aphrophilus* strains 11^T, 12 and 13 was equally effective, with saturating values for transformation around 0·1 μg per plate. *H. paraphrophilus* strains 21^T, 22, 23 and 26 were competent for transformation at efficiencies of 10^{-2} to 10^{-3}. Strain 30 could be transformed to NAD-independent growth at an efficiency of approximately 10^{-5}. The remaining *H. paraphrophilus* strains appeared to be incompetent for transformation (transformation efficiencies below 10^{-8}, the detection limit of the experimental set-up).

The transformation of *H. paraphrophilus* was stable, as evaluated by the preserved ability of NAD-independent growth following 10 subcultivations on chocolate agar. A full-length *nadV* gene could be amplified and sequenced from the transformants (not shown). The functional *nadV* gene was inserted in the former locus of the pseudogene, as evaluated by the disappearance of the 946 bp band after PCR amplification of DNA from transformed cells with primers *thrA* 419r and *yggS* 39r (PCR amplicon sizes are shown in Supplementary Fig. S1 available in IJSEM Online).

Estimation of phylogeny

Fig. 2 shows minimal-evolution dendrograms based on comparison of partial sequences (393–453 nt) of the housekeeping genes *infB*, *pgi* and *recA* plus the 16S rRNA gene. A common 'actinomycetemcomitans branch', supported by bootstrap values of 61 to 98 (of 100 replications), is apparent in all four gene trees. The 40 study strains are positioned on the 'actinomycetemcomitans branches' in all trees except in the comparison of *infB* sequences (Fig. 2a), where seven representatives of *H. segnis* cluster with *H. influenzae* and *H. parainfluenzae*. This unexpected position is probably the result of a recombination event affecting an ancestor of the seven strains (Hedegaard *et al.*, 2001; Nørskov-Lauritsen *et al.*, 2005). Two other recombination events are indicated in the dendrograms, but in these cases the gene transfer is confined within the 'actinomycetemcomitans branch'. Five strains of *H. aphrophilus* (strains 11^T, 13, 14, 18 and 20) cluster with *H. segnis* in the *recA* tree, rather than with the other representatives of *H. aphrophilus* and *H. paraphrophilus* (Fig. 2c). Two strains of *H. aphrophilus* and three strains of *H. paraphrophilus* (strains 15, 19, 27, 28 and 30) constitute a separate group distinct from the major cluster of *H. aphrophilus*/*H. paraphrophilus* in the comparison of *infB* sequences (Fig. 2a).

In none of the individual gene trees was a division observed of *H. aphrophilus* and *H. paraphrophilus* according to current speciation.

The four gene fragments from each strain were concatenated and a phylogenetic analysis was performed. In the resulting

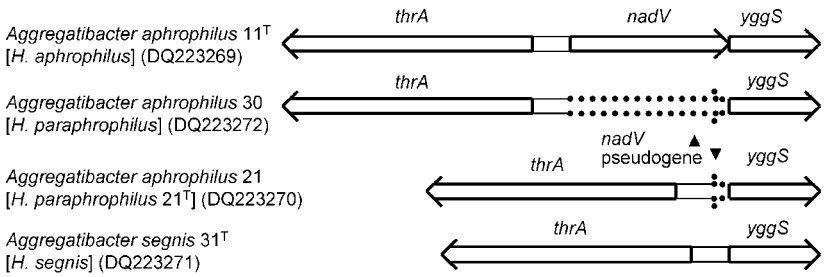

Fig. 1. Corresponding chromosomal regions from selected study strains. Nucleotide sequence accession numbers are given in parentheses. See text for details.

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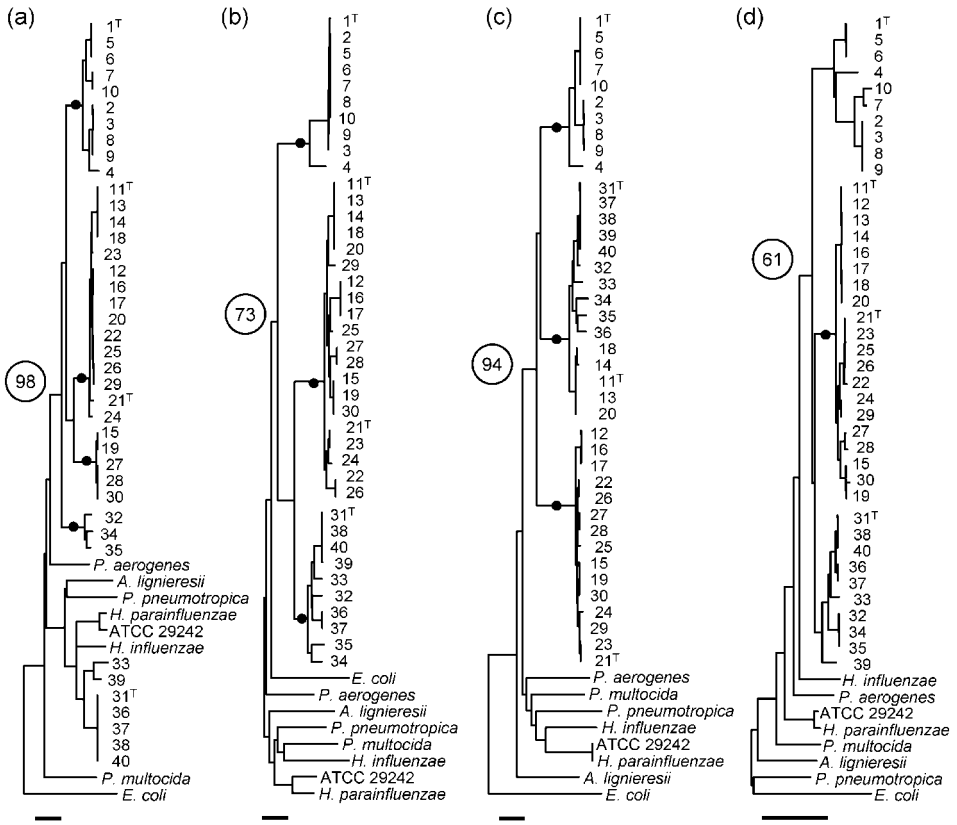

Fig. 2. Minimal-evolution dendrograms based on partial sequences of separate gene fragments. Evolutionary distances are given in terms of number of base substitutions weighted by the Kimura two-parameter model. Bars, 5 substitutions per 100 nucleotides. The percentages of bootstrap replications supporting the individual 'actinomycetemcomitans branches' are shown as encircled numbers. Solid circles (●) indicate nodes supported by ⩾99 % of bootstrap replications. (a) Translation initiation factor 2 gene, *infB* (453 nt); (b) glucose-6-phosphate isomerase gene, *pgi* (393 nt); (c) recombinase A gene, *recA* (447 nt); and (d) 16S rRNA gene (433–441 nt). Individual nucleotide sequence accession numbers are listed in Supplementary Table S1 in IJSEM Online.

tree, all 40 of the study strains were positioned on a common 'actinomycetemcomitans branch' supported by a bootstrap value of 100 (Fig. 3). A relatively large difference is observed between strain 4 (=PH791/56=HK 961=CCUG 51669) and the other representatives of *Actinobacillus actinomycetemcomitans*. This strain was isolated from an actinomycotic lesion by Per Holm at the Statens Serum Institut in Copenhagen in the 1950s. It belongs to serogroup e and carries the unusual 16S rRNA type V sequence (*Actinobacillus actinomycetemcomitans* RNA types as defined by Kaplan *et al.*, 2002). Strain 3, the other serogroup e strain, does not part from the bulk of *Actinobacillus actinomycetemcomitans* sequences.

The recombination events inferred from individual gene comparisons are discernible in the dendrogram based on the concatenated sequences, with the cluster of *H. aphrophilus*/*H. paraphrophilus* being divided into three separate subgroups and *H. segnis* into two. Yet, the ten representatives of *Actinobacillus actinomycetemcomitans*, the 20 representatives of *H. aphrophilus* and *H. paraphrophilus* and the ten representatives of *H. segnis* are located in three separate clusters, each supported by a bootstrap value of 100.

RFLP analysis of 16S rRNA

Successful discrimination between *H. aphrophilus* and *H. paraphrophilus* was reported using RFLP analysis of a 1045 bp PCR-amplified 16S rRNA gene fragment (Riggio & Lennon, 1997). We could not confirm this finding. The RFLP pattern after digestion with *Hha*I of the type strain of *H. aphrophilus* (Fig. 4, lane 1) was in accordance with the expected sizes of the restriction fragments (634, 279, 100 and 32 bp) based on the deposited nucleotide sequence of the type strain (GenBank accession no. AY362906). Seven other *H. aphrophilus* strains and four *H. paraphrophilus* strains had an RFLP pattern of this type (type 1). The *Hha*I RFLP pattern of the type strain of *H. paraphrophilus* (Fig. 4, lane 2) was in accordance with the expected sizes of the restriction fragments (734, 279 and 32 bp) based on the deposited

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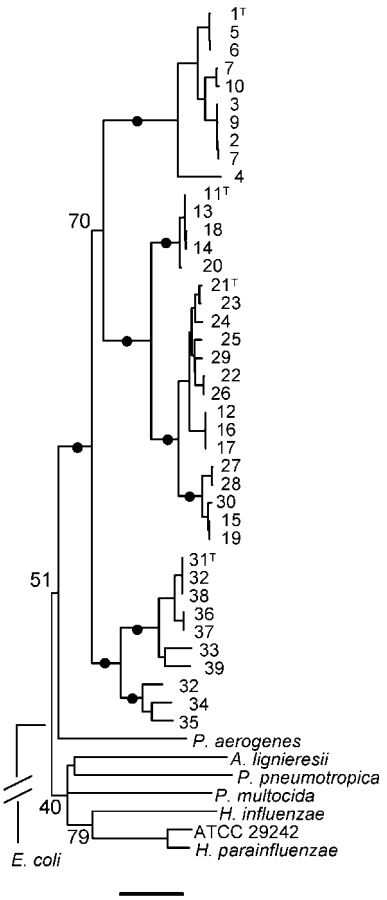

Fig. 3. Minimal-evolution tree based on concatenated sequences from four gene fragments (1726–1734 nt). Solid circles (●) indicate nodes supported by 100 % of bootstrap replications. Values to the left of nodes are percentages of bootstrap replications supporting the node. Bar, 5 substitutions per 100 nucleotides (weighted by the Kimura two-parameter model).

nucleotide sequence of the type strain (GenBank accession no. M75042). Five other *H. paraphrophilus* strains and two *H. aphrophilus* strains had an RFLP pattern of this type (type 2). The difference between the two *Hha*I RFLP patterns is caused by the presence of an additional *Hha*I recognition site (nt 442–446 in GenBank accession no. AY362906) in RFLP type 1 strains. The site is included in the 16S rRNA gene fragments sequenced for calculation of phylogeny and was verified in the sequences from all of the RFLP type 1 strains (individual nucleotide sequence accession numbers are listed in Supplementary Table S1).

Identical RFLP patterns were observed with the type strains of *H. aphrophilus* and *H. paraphrophilus* after digestion with *Hin*fI (Fig. 4, lane 4 and 5), in accordance with the expected sizes (661 and 275 bp, plus five fragments of 32 bp or less) based on the deposited nucleotide sequences. This pattern was observed with all 20 strains of *H. aphrophilus* and *H. paraphrophilus* (not shown).

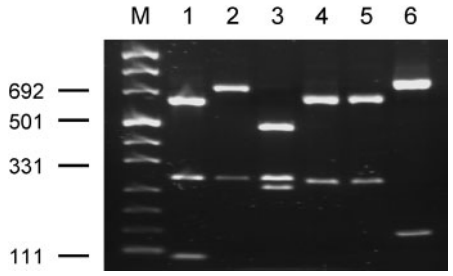

Fig. 4. RFLP analysis of a 1045 bp PCR-amplified 16S rRNA gene fragment from selected type strains digested with *Hha*I (lane 1–3) or *Hin*fI (lane 4–6). Lanes: M, size marker (in bp); 1 and 4, *Aggregatibacter aphrophilus* (*H. aphrophilus*) 11^T; 2 and 5, *Aggregatibacter aphrophilus* 21 (*H. paraphrophilus* 21^T); 3 and 6, *H. parainfluenzae* HK 409^T.

None of our strains could generate the RFLP pattern described by Riggio & Lennon (1997) as typical of *H. paraphrophilus* (*Hha*I, 481, 279 and 253 bp; *Hin*fI, 791 and 145 bp). Fragments of the described size were, however, produced by the type strain of *H. parainfluenzae* (Fig. 4, lane 3 and 6).

DNA–DNA hybridization and phenotype

In addition to dependence on growth factors, seven characters of value in the discrimination between *H. aphrophilus* and *H. paraphrophilus* are listed in the second edition of *Bergey's Manual of Systematic Bacteriology* (Kilian, 2005). These characters (fermentation of D-galactose, sorbose, melibiose, melezitose and raffinose, plus oxidase and H_2S) were tested with the 20 study strains of *H. aphrophilus* and *H. paraphrophilus*. The results are shown in Table 2, together with the characteristics reported by Kilian (2005). We could not reproduce the expected phenotype described in *Bergey's Manual* (Kilian, 2005). Fermentation of D-galactose and raffinose, reported qualities of *H. aphrophilus*, were also detected with the majority of the V factor-dependent strains, and the expected fermentation of sorbose, melibiose and melezitose by strains of *H. paraphrophilus* did not take place (Table 2). Tests for production of H_2S (discoloration of lead acetate paper after 48 h) and oxidase were difficult to interpret. Some blackening of the lead acetate paper was observed with most of the strains, and distinction between positive and negative reactions was not straightforward. A difference in the degree of blackening of the strip between strains of *H. aphrophilus* and *H. paraphrophilus* could not be discerned, however. The development of colour in the oxidase test ranged from negative to ambiguous or delayed reactions. Some strains were scored as positive in the majority of trials, but the blue colour tended to develop at the end of the allowed observation time (10 s). The outcome of the oxidase test suffered from inter- and intraobserver variability; however, a larger proportion of positive strains was

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repeatedly noted with *H. paraphrophilus* than with *H. aphrophilus*.

DNA hybridization data were generated by measurement of the renaturation rate of hybrid DNA mixtures. The DNA–DNA relatedness between the type strains of *H. aphrophilus* and *H. paraphrophilus* was 77 ± 4 % (mean $\pm$ range of duplicate measurements), which is above the threshold value of 70 % that has been suggested for the definition of bacterial species (Wayne *et al.*, 1987). The DNA relatedness of *Actinobacillus actinomycetemcomitans* with selected type strains of the *Pasteurellaceae* is listed in Supplementary Table S2 available in IJSEM Online.

DISCUSSION

Khairat (1940) based his original description of *H. aphrophilus* on a single strain serially isolated from a case of infective endocarditis. He described a dependence on X factor at the initial isolation, but stated that the characteristic was absent during anaerobic growth and was lost upon subculture. King & Tatum (1962) did not observe X factor dependence with their 34 strains, and this led Boyce *et al.* (1969) to reinvestigate four of the original Khairat isolates. All four isolates were capable of growth in the absence of added haemin, but a larger number of colonies could grow from small inocula if haemin was added to the medium. The results were taken to indicate the presence of X factor-independent variants, and, with the variants being the minority, the species should be regarded as dependent on X factor (Boyce *et al.*, 1969).

We suggest the opposite conclusion. The described dependence on haemin in growth experiments is only relative, not absolute, as is the case with the 'truly' dependent species *H. influenzae*, *Haemophilus haemolyticus* and *Haemophilus haemoglobinophilus*. Moreover, the stimulation of growth by haemin was described as transient (Khairat, 1940) or inconsistent (Boyce *et al.*, 1969). The biochemical synthesis of haem is well characterized, and the original strain of Khairat possesses all the enzymes of the haem biosynthetic pathway (White & Granick, 1963). When requirement for X factor is defined as a lack of ability to synthesize porphyrins from δ-aminolaevulinic acid, *H. aphrophilus* is not dependent on X factor (Table 2). We therefore propose *H. aphrophilus* to be classified as an X factor-independent species.

In addition to growth factor requirements, a number of characters of value in the discrimination between *H. aphrophilus* and *H. paraphrophilus* are listed by Kilian (2005) in *Bergey's Manual*. We could not reproduce this pattern of characteristics with the present study material (Table 2). Similar results were reported by Tanner *et al.* (1982), who found *H. paraphrophilus* to be unable to ferment melibiose and melezitose and to be negative in tests for oxidase and H_2S. Moreover, they found the ability to ferment galactose and raffinose to be variably associated with both *H. aphrophilus* and *H. paraphrophilus* (Tanner *et al.*, 1982). The data on which the description in *Bergey's Manual* is based was compiled from several studies using a variety of phenotypic tests, sometimes unspecified. We are unable to explain the discrepancies, but, with the possible exception of the oxidase test, we conclude that these characters are not related to the V factor-dependent phenotype.

By multilocus enzyme electrophoresis, strains of *H. aphrophilus* were closely related, whereas strains assigned to *H. paraphrophilus* included distantly related lineages (Caugant *et al.*, 1990). The difference was caused by three atypical strains, ATCC 29242, HK 296 and HK 321. When

Table 2. Differential characteristics of *H. aphrophilus* and *H. paraphrophilus*

Characters quoted from *Bergey's Manual* are as compiled by Kilian (2005); w, weak reaction. Data from this study show the number of strains that tested positive; d, weak to delayed reaction (see text for details).

Character	*Bergey's Manual*		This study	
	H. aphrophilus	*H. paraphrophilus*	*H. aphrophilus* ($n=10$)	*H. paraphrophilus* ($n=10$)
Porphyrin (X)*	w	+	10	10
NAD (V)†	−	+	0	10
Oxidase production	−	+	d	d
H_2S production	−	+	d	d
Fermentation of:				
D-Galactose	+	−	10	9
Sorbose	−	+	0	0
Melibiose	−	+	0	0
Melezitose	−	+	0	0
Raffinose	+	−	10	7

*Synthesis of porphyrins from δ-aminolaevulinic acid.
†Requirement for V factor.

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these strains were excluded from evaluation of the data, *H. aphrophilus* and *H. paraphrophilus* coalesced into one genetically homogeneous group (Caugant *et al.*, 1990). It is now clear that the aberrant strains were mislabelled. ATCC 29242 belongs to the species *H. parainfluenzae* (see below). Strains HK 296 and HK 321 were only tentatively assigned to *H. paraphrophilus*, although they differed from other strains of this species in several properties (Kilian, 1976; Tanner *et al.*, 1982). They are included in the present study (strains 33 and 34, respectively) and cluster with strains of *H. segnis* by multilocus sequence phylogeny (Fig. 3). The phenotype of these strains was reinvestigated and found to comply with *H. segnis* (not shown).

H. parainfluenzae may be difficult to discriminate from *H. paraphrophilus* without extended phenotypic examination. Confusion of these two species has occurred with several reference strains from the international culture collections. ATCC 7901 was deposited by Margaret Pittman as *H. parainfluenzae* (strain 429) and is a quality control strain for bioMérieux Vitek and IDS products. It has been shown to be a mislabelled strain of *H. paraphrophilus* (Potts *et al.*, 1986), as confirmed by our results (strain 29). ATCC 29242 (=NCTC 10558) is one of the original three strains of *H. paraphrophilus* (strain Haywood) deposited by K. Zinnemann. The strain is insignificantly related to reference strains of both *H. aphrophilus* and *H. paraphrophilus* by overall DNA–DNA similarity (Tanner *et al.*, 1982) and clusters with the type strain of *H. parainfluenzae* by 16S rRNA gene sequence (Dewhirst *et al.*, 1992). The latter identification is confirmed by the housekeeping gene comparisons of this study (Fig. 2a–c). By phenotype, ATCC 29242 complies with *H. parainfluenzae* biotype I (negative in lactose fermentation and in the production of indole and urease and positive in ornithine decarboxylase).

Strains of *H. parainfluenzae* mistaken as *H. paraphrophilus* may also have been decisive in the reported discrimination between *H. aphrophilus* and *H. paraphrophilus* by RFLP analysis. The *Hha*I and *Hin*fI RFLP patterns reported for five strains of *H. paraphrophilus* by Riggio & Lennon (1997) could be generated with the type strain of *H. parainfluenzae* (Fig. 4), but not with any of our 20 strains of *H. aphrophilus* and *H. paraphrophilus*. Ribotyping based on *Eco*RI-digested DNA has previously failed to discriminate *H. aphrophilus* from *H. paraphrophilus* (Sedlacek *et al.*, 1993). Separation into discrete entities has however been reported using multivariate analysis of enzyme data (API ZYM; Myhrvold *et al.*, 1992) and of carbohydrate data from lipopolysaccharides (Brondz & Olsen, 1990).

H. aphrophilus and *H. paraphrophilus* do not merit separate species rank when evaluated by DNA–DNA hybridization, the currently accepted reference methodology. They are discriminated by a single pivotal character, the dependence on V factor of *H. paraphrophilus*. Our results show that this character can arise from a single nucleotide deletion in the *nadV* gene and that the V factor-dependent phenotype is naturally reversible in strains competent for transformation. Thus, the division into two species of *H. aphrophilus* and *H. paraphrophilus* is not justified, and we propose the two taxa to be united. According to the *Bacteriological Code*, the specific epithet *aphrophilus* has priority over *paraphrophilus*.

It is widely accepted that *H. aphrophilus* is misclassified as a member of the genus *Haemophilus* and that *Actinobacillus actinomycetemcomitans*/*Haemophilus actinomycetemcomitans* is misclassified in both of the genera *Haemophilus* and *Actinobacillus*. In a previous study, we showed that eight representatives of the 'actinomycetemcomitans branch' form a monophyletic group by multilocus sequence phylogeny (Nørskov-Lauritsen *et al.*, 2005). In the present work, we extended the investigation to a larger collection of strains. A single strain (strain 4) parted from the other representatives of *Actinobacillus actinomycetemcomitans* at the four examined gene loci and may represent a distinct subgroup of this species. A separate position (ET division VI) has previously been demonstrated for this strain by multilocus enzyme electrophoresis (Poulsen *et al.*, 1994). Seventeen other strains cluster unexpectedly in only one of four gene loci, and these 17 gene fragments are most likely recruited from other species by recombination. If the division of *H. aphrophilus* into two subclusters by *infB* comparison is caused by recombination, it involves a hitherto unrecognized and possibly extinct species on the 'actinomycetemcomitans branch'.

The multilocus sequence approach provides a buffer against the distorting effects of recombination at a single gene locus. Despite the *infB* sequences recruited outside the 'actinomycetemcomitans branch' by seven representatives of *H. segnis* (Fig. 2a), a phylogenetic analysis of concatenated sequences locates all 40 study strains on a common branch supported by a bootstrap value of 100. The interrelationship of the study strains is closer than the clustering of three strains of *H. influenzae* and *H. parainfluenzae*, which is supported by a bootstrap value of 79 (Fig. 3). The species on the 'actinomycetemcomitans branch' also form a monophyletic group (RNA cluster 13) by comparison of near-full-length 16S rRNA sequences from 114 members of the family *Pasteurellaceae* (Olsen *et al.*, 2005). Representatives of the related RNA clusters 14 (*P. aerogenes*), 15 (*P. pneumotropica*) and 16 (*H. influenzae*) are included in the multilocus sequence phylogenetic analysis and appear unrelated to the strains of the 'actinomycetemcomitans branch' at the generic level (Fig. 3). The 'actinomycetemcomitans branch' is separate from other evolutionary lineages in the family *Pasteurellaceae*, and the generation of a new genus merits consideration.

Stability of names is one of the principles of nomenclature. The species investigated in this study are involved in serious infections of man and are regularly encountered in clinical microbiological laboratories. The findings must be communicated to physicians, and the bacterial names should ideally evoke associations with particular clinical syndromes. A renaming may cause difficulties for health-care workers, and misunderstandings could be dangerous.

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However, the same line of arguments can be raised in favour of a change. An unnatural classification is an obstacle to learning and to the advancement of clinical and scientific knowledge. An association of these species with *Actinobacillus* and *Haemophilus* is not helpful in the clinical setting. The current classification is clearly unsatisfactory and, upon acceptance, a new generic name may help in the identification of certain pathological processes.

The conspicuous growth in broth as small granules adhering to the walls of the test tube was noted in the original descriptions of '*Bacterium actinomycetem comitans*' (Klinger, 1912) and *Haemophilus aphrophilus* (Khairat, 1940). Accordingly, we propose *Aggregatibacter* gen. nov. as the generic name for the group.

Description of *Aggregatibacter* gen. nov.

Aggregatibacter (Ag.gre.ga.ti.bac′ter. L. v. *aggregare* to come together, aggregate, N.L. masc. n. *bacter* bacterial rod, N.L. masc. n. *Aggregatibacter* rod-shaped bacterium that aggregates with others).

A member of the family *Pasteurellaceae*. Gram-negative, non-motile, facultatively anaerobic rods or coccobacilli. Growth is mesophilic. Several species of the genus are capnophilic and primary isolation may require the presence of 5–10 % CO_2. There is no dependence on X factor and the requirement for V factor is variable. Granular growth in broth is common. Colonies on sheep- and horse-blood agar are greyish white and non-haemolytic. Acid is produced from glucose, fructose and maltose, whereas arabinose, cellobiose, melibiose, melezitose, salicin and sorbitol are not attacked. The fermentation of galactose, lactose, mannitol, mannose, raffinose, sorbose, sucrose, trehalose and xylose is variable and may aid in identification to the species level. Nitrate is reduced. Alkaline phosphatase is produced, but strains are negative in tests for indole, urease, ornithine and lysine decarboxylases and arginine dihydrolase. Oxidase reaction is negative or weak; catalase is variably present. IgA1 protease is not expressed. The species of the genus are intimately associated with man; they are part of the human oral flora and are occasionally recovered from other body sites, including blood and brain, as causes of endocarditis and abscesses. The type species is *Aggregatibacter actinomycetemcomitans*, originally described as '*Bacterium actinomycetem comitans*' by Klinger (1912).

Description of *Aggregatibacter actinomycetemcomitans* (Klinger 1912) comb. nov.

Aggregatibacter actinomycetemcomitans [ac.ti.no.my.ce.tem.co′mi.tans. Gr. n. *actis* a ray; N.L. n. *myces* from Gr. n. *mukes* a fungus; N.L. n. *actinomyces* ray fungus; L. part. adj. *comitans* accompanying; N.L. part. adj. *actinomycetemcomitans* (sic) accompanying an actinomycete].

Basonym: *Actinobacillus actinomycetemcomitans* (Klinger 1912) Topley and Wilson 1929.

Other synonym: *Haemophilus actinomycetemcomitans* (Klinger 1912) Potts *et al.* 1985.

Small rods which may appear as cocci in broth or actinomycotic lesions. They may occur singly, in pairs or in small clumps. Grows poorly in ambient air, but well in 5 % CO_2. Colonies on chocolate agar are small, with a diameter of $\leqslant 0{\cdot}5$ mm after 24 h, but may exceed 1–2 mm after 48 h. On primary isolation, the colonies are rough-textured and adherent and have an internal, opaque pattern described as star-like or like 'crossed cigars'. The rough phenotype is related to fimbriation and to the production of hexoseamine-containing exopolysaccharide. Cells from rough colonies grow in broth as granular, autoaggregated cells that adhere to the glass and leave a clear broth. Successive rounds of subculturing *in vitro* on solid media can result in transformation into a smooth, non-adherent colony type that exhibits planktonic growth in broth and reduced ability to colonize the mouth of experimental animals. X and V factors are not required. Acid is produced from glucose, fructose, maltose and mannose, whereas arabinose, cellobiose, galactose, lactose, melibiose, melezitose, trehalose, raffinose, salicin, sorbitol and sucrose are not fermented. Variable fermentation is observed with mannitol and xylose. Catalase-positive; ONPG is not hydrolysed. Secretes a leukotoxin of the highly conserved repeat toxin (RTX) family. Six serotypes a–f have been described. Key tests for discrimination between *Aggregatibacter actinomycetemcomitans* and V factor-independent strains of *Aggregatibacter aphrophilus* are catalase and ONPG, plus fermentation of lactose, sucrose and trehalose.

Indigenous to man, with primary habitat on dental surfaces. A particular clone of serotype b with enhanced leukotoxic activity is predominantly associated with cases of localized aggressive periodontitis. *Aggregatibacter actinomycetemcomitans* has regularly been isolated together with *Actinomyces* species from human actinomycosis. It is sometimes found in other pathological processes such as endocarditis, brain abscess and urinary tract infections. The G+C content of the DNA is 42·7 mol% (T_m).

The type strain is NCTC 9710^T = ATCC 33384^T = CCUG 13227^T = CIP 52.106^T = DSM 8324^T. The GenBank accession number of the 16S rRNA gene sequence of the type strain is M75039.

Description of *Aggregatibacter aphrophilus* (Khairat 1940) comb. nov.

Aggregatibacter aphrophilus (aph.ro′phi.lus. Gr. n. *aphros* foam; Gr. adj. *philos* loving; N.L. adj. *aphrophilus* foam loving).

Basonym: *Haemophilus aphrophilus* Khairat 1940.

Short, regular rods, $0{\cdot}5 \times 1{\cdot}5$–$1{\cdot}7$ μm, with occasional filamentous forms. Colonies on chocolate agar incubated in air supplemented with 5–10 % extra CO_2 are high-convex, granular, yellowish and opaque and reach a diameter of 1·0–1·5 mm within 24 h. When plates are

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incubated without extra CO_2, the growth is characteristically stunted, with very small colonies interspersed with a few larger colonies. Growth in broth is granular, with heavy sediment on the bottom of the tube and adhering colonies on the walls, which are difficult to remove. The presence of haemin may enhance growth, but porphyrins are synthesized from δ-aminolaevulinic acid and X factor is not required. Some isolates are capable of synthesizing NMN from nicotinamide and 5-phosphoribosyl pyrophosphate (formerly *Haemophilus aphrophilus*), while others lack a functional nicotinamide phosphoribosyltransferase and are dependent on V factor (formerly *Haemophilus paraphrophilus*). Acid is produced from glucose, fructose, lactose, maltose, mannose, sucrose and trehalose, whereas arabinose, cellobiose, mannitol, melibiose, melezitose, salicin, sorbose, sorbitol and xylose are not fermented. Variable fermentation is observed with galactose and raffinose. H_2O_2 is not decomposed; ONPG is hydrolysed. Key tests for discrimination between V factor-dependent isolates of *Aggregatibacter aphrophilus* and strains of *H. parainfluenzae* biotype V (negative for indole, urease and ornithine decarboxylase) are fermentation of lactose and trehalose.

Aggregatibacter aphrophilus is a member of the normal flora of the human oral cavity and pharynx. May cause brain abscess and infective endocarditis and has been isolated from various other body sites including peritoneum, pleura, wound and bone. The G+C content of the DNA is 42 mol% (T_m).

The type strain is NCTC 5906^T = ATCC 33389^T = CCUG 3715^T = CIP 70.73^T. The GenBank accession number of the 16S rRNA gene sequence of the type strain is AY362906.

Description of *Aggregatibacter segnis* (Kilian 1977) comb. nov.

Aggregatibacter segnis (seg′nis. L. masc. adj. *segnis* slow, sluggish).

Basonym: *Haemophilus segnis* Kilian 1977.

Small, pleomorphic rods, often showing a predominance of irregular, filamentous forms. Colonies on chocolate agar are smooth or granular, convex, greyish white and opaque, and may take 48 h to reach a diameter of 0·5 mm. Growth in broth and fermentation media is slow, and reactions are negative or weakly positive. V but not X factor is required; CO_2 enhances growth for some strains. Small amounts of acid result from the fermentation of glucose, fructose, galactose, sucrose and maltose. Fermentation of sucrose is usually stronger than fermentation of glucose. Arabinose, cellobiose, lactose, mannitol, mannose, melibiose, melezitose, raffinose, salicin, sorbose, sorbitol, trehalose and xylose are not fermented. Catalase and β-galactosidase (hydrolysis of ONPG) are variably present. Only quantitative differences in the amount of acid produced from carbohydrates may discriminate *Aggregatibacter segnis* phenotypically from strains of *H. parainfluenzae* biotype V (negative for indole, urease and ornithine decarboxylase).

Aggregatibacter segnis is a regular member of the human oral flora, particularly in dental plaque, and can be isolated from the pharynx. It has occasionally been isolated from human infections including infective endocarditis. The G+C content of the DNA is 43–44 mol% (T_m).

The type strain is $HK316^T$ (= ATCC 33393^T = CCUG 10787^T = CCUG 12838^T = CIP 103292^T = NCTC 10977^T). The GenBank accession number of the 16S rRNA gene sequence of the type strain is M75043.

ACKNOWLEDGEMENTS

The authors wish to thank Professor Poul Jørgensen, Institute of Molecular and Structural Biology, Aarhus University, for helpful suggestions concerning inverse PCR, Professor Dr Hans G. Trüper, Rheinische Friedrich-Wilhelms-Universität, Bonn, for etymological advice, and Dr Brita G. Bruun, Department of Clinical Microbiology, Hillerød Hospital, for critical reading of the manuscript.

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