Haemophilus influenzae may be untypable by the multilocus sequence typing scheme due to a complete deletion of the fucose operon

The Gram-negative, non-motile coccobacillus *Haemophilus influenzae* is a commensal of the upper respiratory tract and is involved in various infections including otitis media, epiglottitis, conjunctivitis and meningitis. *H. influenzae* is also frequently isolated from patients with chronic infections of the upper and lower respiratory tract (Aubrey & Tang, 2003; Kilian, 2005; Turk, 1984).

There are several methods for subdividing and typing of bacterial species. Serotyping divides encapsulated strains of *H. influenzae* into six serotypes (a–f) reflecting the capsular polysaccharides produced by the bacterium (Kilian, 2005; Pittman, 1931). Most isolates from invasive infections are capsulate (Aubrey & Tang, 2003; Harper & Tilse, 1991), but serotyping does not characterize unencapsulated (nontypable) strains, which comprise the majority of isolates cultured in the clinical microbiological laboratory (Harper & Tilse, 1991; Musser *et al.*, 1986). Multilocus sequence typing (MLST) is a sequence-based approach that allows direct comparison of results between laboratories, and MLST schemes have been developed for several bacterial species including *H. influenzae* (Meats *et al.*, 2003). Fragments of the housekeeping genes *adk* (adenylate kinase), *atpG* (ATP synthase F1 subunit gamma), *frdB* (fumarate reductase iron–sulfur protein), *fucK* (fuculokinase), *mdh* (malate dehydrogenase), *pgi* (glucose-6-phosphate isomerase) and *recA* (RecA protein) are sequenced and assigned allele numbers. The combination of allele numbers at the seven loci defines the MLST sequence type

The GenBank/EMBL/DDBJ accession numbers for the DNA sequences generated in this study are GQ859140–50.

A table giving the sequences of primers used in this study is available with the online version of this paper.

(ST) and more than 600 *H. influenzae* STs are presently included in the database (www.mlst.net).

Fucose is utilized as an energy and carbon source in bacteria and fuculokinase is one of four enzymes involved in the fucose pathway in *H. influenzae* (Chen *et al.*, 1987; Herman, 1971). The genes encoding fuculokinase (*fucK*) and the other three enzymes in the pathway, L-fucose permease (*fucP*), L-fucose-phosphate aldolase (*fucA*) and L-fucose isomerase (*fucI*), constitute a regulatory unit. Also part of this operon is *fucR*, an L-fucose operon activator, and *fucU* (fucose operon protein) (Chen *et al.*, 1987; Herman, 1971). The fucose operon spans the region from nt 642737 to 647375 in *H. influenzae* KW20 Rd (Fleischmann *et al.*, 1995; Peterson *et al.*, 2001) (Fig. 1). Variant strains closely related to *H. influenzae* are negative for *fucK*, and the presence of *fucK* has been used as a marker of *H. influenzae* (Nørskov-Lauritsen *et al.*, 2009; Nørskov-Lauritsen, 2009). In the present study, we have analysed the fucose operon in two isolates of *H. influenzae*, PN127 (CCUG 58365) and CCUG 30048, which failed to hybridize with a *fucK*-specific probe (Nørskov-Lauritsen *et al.*, 2009; Nørskov-Lauritsen, 2009).

Phenotypic profiles were generated with the *Neisseria*–*Haemophilus* card for the VITEK 2 (bioMérieux) and supplemented with selected biochemical tests (Kilian, 2005), and biotypes were defined as described by Kilian (2005). PCR amplification of the entire fucose operon (approx. 10 kb) was performed with the Expand 20 kb^{PLUS} PCR System (Roche). MLST analysis was performed as described by Meats *et al.* (2003). The *H. influenzae* database containing 627 STs was downloaded on 17 April 2009 from the MLST website (www.mlst.net). Also included in the phylogenetic analysis were

the type strains of *H. influenzae* (HK 389^T), *Haemophilus aegyptius* (HK 367^T) and *Haemophilus haemolyticus* (HK 386^T), seven reference strains of *H. influenzae* (HK 61, HK 1136, HK 1212, HK 2067, HK 2122, CCUG 35214 and KW20 Rd), and 31 strains previously classified as variant strains (Nørskov-Lauritsen *et al.*, 2009). *fucK* was excluded from the concatenated sequences used for cluster analysis. Phylogenetic analyses were conducted using MEGA version 4.0 (Tamura *et al.*, 2007). The population structure of *H. influenzae* was divided into phylogenetic groups I and II (Meats *et al.*, 2003; Musser *et al.*, 1988).

H. influenzae strains PN127, CCUG 30048 and KW20 Rd were subjected to PCR with primers flanking the fucose operon. The forward primer HI_0608.1002f was positioned in locus HI_0608 and the reverse primer HI_0616.1708r in locus HI_0616 (*hepA*) (Fig. 1; primer sequences are given in Supplementary Table S1 available in JMM Online). Visualization of the PCR products by gel electrophoresis revealed a product of approximately 10 kb for *H. influenzae* KW20 Rd, as expected. In contrast, both PN127 and CCUG 30048 gave rise to amplicons of only approximately 2 kb. Sequencing of the fucose operon region in PN127 and CCUG 30048 showed that nucleotides 641308–648266 (numbers referring to KW20 Rd) were missing. This deleted region includes all the genes in the fucose operon plus 140 and 143 nt of the flanking intergenic regions upstream and downstream of the operon, respectively. Instead, a 54 nucleotide region is present at position 641309, between *folD* and *hepA*, in these two strains (Fig. 1; GenBank accession nos GQ859140 and GQ859141). The 54 nucleotide region appears to contain no putative open reading frames and to have no homologous sequences in the NCBI database.

Fig. 1. Graphical representation of the fucose operon sites in selected *H. influenzae* genomes. (a) *H. influenzae* KW20 Rd (GenBank accession no. NC_000907). The fucose operon (white) contains the loci HI_0610–HI_0615. Numbers refer to positions in *H. influenzae* KW20 Rd. (b) PN127 and CCUG 30048. The entire fucose operon is missing (GenBank accession nos GQ859140 and GQ859141, respectively).

We also performed PCR targeting the individual genes of the fucose operon, *fucP*, *fucA*, *fucU*, *fucK*, *fucI* and *fucR*

(primer sequences are given in Supplementary Table S1). Products of the expected size were obtained with all *H.*

influenzae reference strains, but not with CCUG 30048 and PN127. Thus, the fucose operon was not translocated to other parts

Fig. 2. Neighbour-joining tree of 627 STs from the *H. influenzae* MLST database, 7 *H. influenzae* reference strains (HK 61, HK 1136, HK 1212, HK 2067, HK 2122, CCUG 35214 and KW20 Rd), type strains of *H. influenzae* (HK 389^T), *H. aegyptius* (HK 367^T) and *H. haemolyticus* (HK 386^T), 31 variant strains closely related to *H. influenzae*, and PN127 and CCUG 30048 from this study. Phylogenetic group I is indicated in blue, phylogenetic group II in yellow and variant cluster in red. Strains with equivocal allocation to group II are shown in grey.

of the genome by genomic rearrangement in PN127 and CCUG 30048. PCR with primers HI_0608.1002f and HI_0616.1708r on the *H. influenzae* reference strains in all cases produced products of approximately 10 kb, indicating that the six *fuc* genes are located sequentially, similar to the arrangement in *H. influenzae* KW20 Rd.

An essential question is the identity and allocation to species of the two investigated strains. CCUG 30048 was isolated from the nasopharynx of a child and is consistent by phenotype with *H. influenzae* biotype IV, except for the detectable emission of hydrogen sulphide (Nørskov-Lauritsen *et al.*, 2009). By 16S rRNA gene sequence comparison (GenBank accession no. EU909661), it is related to *H. influenzae* serotype e strains, which constitute a distant lineage within the borders of the species (Nørskov-Lauritsen *et al.*, 2009). Phylogenetic analysis based on concatenated housekeeping gene fragments revealed an intermediary position related to both phylogenetic group II strains (including serotype e and serotype f strains; yellow in Fig. 2) and variant strains excluded from the species ('non-haemolytic *Haemophilus haemolyticus*'; red in Fig. 2). Nine STs from the MLST database clustered with CCUG 30048, but none of these were from reference strains with validated allocation to a phylogenetic group or species. However, three adjacent STs (ST 461–3) represent invasive isolates cultured from blood, compatible with a classification within the pathogenic species *H. influenzae* rather than other non-influenzae *Haemophilus* commensal species. Strain PN127 was isolated in pure culture from sputum of a patient hospitalized for pneumonia and is consistent by phenotype with *H. influenzae* biotype III. The 16S rRNA gene sequence (GenBank accession no. FJ939591) is identical to that of the full genome sequenced strain Rd, and multilocus analysis allocated PN127 to phylogenetic group I (Fig. 2). The strain

clearly belongs to the core of the species as evidenced by both clinical occurrence and phenotypic and genetic characterization.

Our results show that occasional isolates of *H. influenzae* lack the entire fucose operon including *fucK*. The MLST scheme developed for the unambiguous characterization of *H. influenzae* (Meats *et al.*, 2003) is unable to assign a sequence type to such strains. Furthermore, detection of *fucK* may not be suitable for specific identification of *H. influenzae* (Abdeldaim *et al.*, 2009; Nørskov-Lauritsen *et al.*, 2009).

Winnie Ridderberg, Mette G. Fenger and Niels Nørskov-Lauritsen

Department of Clinical Microbiology,
Aarhus University Hospital Skejby,
DK-8200 Aarhus N, Denmark

Correspondence:

Niels Nørskov-Lauritsen
(nielnoer@rm.dk)

Abdeldaim, G. M. K., Strålin, K., Kirsebom, L. A., Olcén, P., Blomberg, J. & Herrmann, B. (2009). Detection of *Haemophilus influenzae* in respiratory secretions from pneumonia patients by quantitative real-time polymerase chain reaction. *Diagn Microbiol Infect Dis* **64**, 366–373.

Aubrey, R. & Tang, C. (2003). The pathogenesis of disease due to Type B *Haemophilus influenzae*. *Methods Mol Med* **71**, 29–50.

Chen, Y.-M., Zhu, Y. & Lin, E. C. C. (1987). The organization of the *fuc* regulon specifying L-fucose dissimilation in *Escherichia coli* K12 as determined by gene cloning. *Mol Gen Genet* **210**, 331–337.

Fleischmann, R. D., Adams, M. D., White, O., Clayton, R. A., Kirkness, E. F., Kerlavage, A. R., Bult, C. J., Tomb, J. F., Dougherty, B. A. & other authors (1995). Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science* **269**, 496–512.

Harper, J. J. & Tilse, M. H. (1991). Biotypes of *Haemophilus influenzae* that are associated with noninvasive infections. *J Clin Microbiol* **29**, 2539–2542.

Herman, R. H. (1971). Mannose metabolism. II. *Am J Clin Nutr* **24**, 556–561.

Kilian, M. (2005). Genus III. *Haemophilus*. Winslow Broadhurst, Buchanan, Krumwiede, Rogers and Smith 1917, 561, pp. 883–904. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 2, part B. Edited by D. J. Brenner, N. R. Krieg & J. T. Staley. New York: Springer.

Meats, E., Feil, E. J., Stringer, S., Cody, A. J., Goldstein, R., Kroll, J. S., Popvic, T. & Spratt, B. G. (2003). Characterization of encapsulated and nonencapsulated *Haemophilus influenzae* and determination of phylogenetic relationships by multilocus sequence typing. *J Clin Microbiol* **41**, 1623–1636.

Musser, J. M., Barenkamp, S. J., Granoff, D. M. & Selander, R. K. (1986). Genetic relationships of serologically nontypeable and serotype b strains of *Haemophilus influenzae*. *Infect Immun* **52**, 183–191.

Musser, J. M., Kroll, J. S., Moxon, E. R. & Selander, R. K. (1988). Evolutionary genetics of the encapsulated strains of *Haemophilus influenzae*. *Proc Natl Acad Sci U S A* **85**, 7758–7762.

Nørskov-Lauritsen, N. (2009). Detection of cryptic genospecies misidentified as *Haemophilus influenzae* in routine clinical samples by assessment of marker genes *fucK*, *hap*, and *sodC*. *J Clin Microbiol* **47**, 2590–2592.

Nørskov-Lauritsen, N., Overballe, M. D. & Kilian, M. (2009). Delineation of the species *Haemophilus influenzae* by phenotype, multilocus sequence phylogeny, and detection of marker genes. *J Bacteriol* **191**, 822–831.

Peterson, J. D., Umayam, L. A., Dickinson, T. M., Hickey, E. K. & White, O. (2001). The comprehensive microbial resource. *Nucleic Acids Res* **29**, 123–125.

Pittman, M. (1931). Variation and type specificity in the bacterial species *Haemophilus influenzae*. *J Exp Med* **53**, 471–492.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.

Turk, D. C. (1984). The pathogenicity of *Haemophilus influenzae*. *J Med Microbiol* **18**, 1–16.