NOTE**Phylogenetic analysis of *Fusobacterium alocis* and *Fusobacterium sulci* based on 16S rRNA gene sequences: proposal of *Filifactor alocis* (Cato, Moore and Moore) comb. nov. and *Eubacterium sulci* (Cato, Moore and Moore) comb. nov.**

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Genes encoding the 16S rRNA of *Fusobacterium alocis* ATCC 35896^T and *Fusobacterium sulci* ATCC 35585^T were sequenced. These sequences did not have any affinity with the 16S rRNA gene sequences of members of the genus *Fusobacterium*. *Fusobacterium alocis* ATCC 35896^T and *Fusobacterium sulci* ATCC 35585^T belonged to *Clostridium* cluster XI; the species most closely related to these strains were *Filifactor villosus* NCTC 11220^T and *Eubacterium infirmum* W1471, respectively. Two new combinations are proposed: *Filifactor alocis* (Cato, Moore and Moore) comb. nov. (type strain ATCC 35896^T) and *Eubacterium sulci* (Cato, Moore and Moore) comb. nov. (type strain ATCC 35585^T).

Keywords: phylogeny, *Fusobacterium alocis*, *Fusobacterium sulci*, 16S rRNA analysis

Fusobacterium alocis ATCC 35896^T (T=type strain) and *Fusobacterium sulci* ATCC 35585^T were originally isolated from gingival sulci of patients with gingivitis or periodontitis (Cato *et al.*, 1985). Later, *Fusobacterium alocis* was also found in feline soft tissue infections (Love *et al.*, 1987). Both species are obligately anaerobic, non-spore-forming rods, which are biochemically relatively inert. They have been described as Gram-negative, although no exact information on their cell wall type is available (Cato *et al.*, 1985). They produce butyrate but not isobutyrate or isovalerate, and for that reason they have been associated with the genus *Fusobacterium* (Moore *et al.*, 1984). Both species grow poorly, which makes their identification by biochemical methods difficult. The soluble cellular protein content of the two species differs from that of other fusobacteria (Cato *et al.*, 1985; Love *et al.*, 1987). Furthermore, *Fusobacterium sulci* ATCC 35585^T has been distinguished from other

fusobacteria by its cellular fatty acid composition (Moore *et al.*, 1994).

In this work, sequences of genes encoding 16S rRNA (16S rDNA) of two strains of *Fusobacterium alocis* ATCC 35896^T and two strains of *Fusobacterium sulci* ATCC 35585^T were determined. The comparative 16S rDNA sequence analysis showed that neither *Fusobacterium alocis* ATCC 35896^T nor *Fusobacterium sulci* ATCC 35585^T belongs to the genus *Fusobacterium*. Both are members of *Clostridium* cluster XI (Collins *et al.*, 1994).

Bacterial strains and culture conditions

Two strains of both *Fusobacterium alocis* ATCC 35896^T and *Fusobacterium sulci* ATCC 35585^T were studied. One of each strains was obtained from the Anaerobe Reference Laboratory of the Finnish National Public Health Institute. The second strains of *Fusobacterium alocis* ATCC 35896^T and *Fusobacterium sulci* ATCC 35585^T were ordered directly from the American Type Culture Collection (ATCC). Bacterial strains were cultured on fastidious anaerobic agar plates (LAB M) with or without 7% (w/v) bovine blood at 37 °C in a CO₂/H₂ atmosphere (Oxoid Gas generating kit BR38). Bacterial cells were collected

Abbreviations: RDP, Ribosomal Database Project; SSU rRNA, small-subunit rRNA.

The EMBL accession numbers for the 16S rRNA sequences of *Fusobacterium alocis* ATCC 35896^T and *Fusobacterium sulci* ATCC 35585^T are AJ006962 and AJ006963, respectively.

from the plate using a sterile plastic inoculation loop and were suspended in sterile PBS (0.135 M NaCl, 2.4 mM KH_2PO_4 , 6.9 mM K_2HPO_4 , pH 7.4). The cells were collected by centrifugation (12000 r.p.m. for 3 min; Hettich EBA 12R), frozen in liquid nitrogen and stored at -135°C until used.

16S rRNA gene sequencing

Bacterial cells were suspended in 500 μl sterile lysis buffer [10 mM Tris/HCl (pH 7.5), 1 % (w/v) Triton X-100, 0.5 mM EDTA]. Cells were incubated for 1 h at 37°C with 100 μg lysozyme and 10 units mutanolysin (Sigma) and after that for 1 h at 56°C with 50 μg proteinase K (Boehringer Mannheim). The DNA was extracted twice with phenol/chloroform-isoamyl alcohol and precipitated with sodium acetate and ethanol. Finally, the DNA was dissolved in 100 μl water. The total PCR volume was 50 μl , containing 5 μl sample, 2 units Dynazyme DNA polymerase (Finnzymes Oy), 10 μmol each dNTP, 10 pmol each PCR primer (Jalava *et al.*, 1995; Weisburg *et al.*, 1991) and 5 μl $10\times$ PCR buffer (Finnzymes). The PCR steps (94°C for 30 s, 55°C for 30 s, 72°C for 1 min) were repeated 38 times using a GenAmp PCR System 2400 (Perkin-Elmer). The amplified product (40 μl) was separated and purified by 1.5 % (w/v) SeaPlaque agarose gel electrophoresis (FMC BioProducts). DNA was separated from agarose using β -agarase I (New England Biolabs) according to manufacturer's instructions with minor modifications. Cyclic sequencing procedures were carried out using the Thermo Sequenase Dye Terminator Cycle Sequencing Pre-mix kit (Amersham Life Science). Sequencing products were resolved using 6 % (w/v) polyacrylamide gel and a 373A Stretch DNA Sequencer (Applied Biosystems). Sequencing primers have been described previously by Jalava *et al.* (1995).

Sequence comparisons

Partial 16S rDNA sequences of the two *Fusobacterium alocis* ATCC 35896^T strains were compared to each other using the GAP program of the sequence software package of the Genetics Computer Group (GCG). The sequences were 99.9 % identical (1472 bases compared). There was only one unresolved position separating these two sequences. The two partial 16S rDNA sequences of *Fusobacterium sulci* ATCC 35585^T were also almost completely identical: 99.9 % identity over 1378 bases compared including one unresolved position. The 16S rDNA sequences of *Fusobacterium alocis* ATCC 35896^T and *Fusobacterium sulci* ATCC 35585^T were further compared together to the prokaryotic small subunit (SSU) rRNA sequence database of the Ribosomal Database Project II (RDP) using the distance matrix program version 1.1 (Maidak *et al.*, 1997). Twelve best-matching sequences which were 80 % or more complete were included in the analysis. In the similarity calculation, 1405 alignment positions and full ambiguity code matching but not gaps

(insertions or deletions) were included in the match/mismatch count. Among the SSU rRNA sequences of the RDP, the best match for the 16S rDNA sequence of *Fusobacterium alocis* ATCC 35896^T was the 16S rRNA sequence of *Filifactor villosus* NCTC 11220^T (Collins *et al.*, 1994); the similarity was 92.6 %. The next best match was 85.6 % to two sequences, one of *Clostridium bifermantans* NCIMB 19716^T and the other of *Clostridium ghonii* NCIMB 10636^T. The best match between 16S rDNA of *Fusobacterium sulci* ATCC 35585^T and SSU rRNA sequences in the RDP was 96.2 % to two sequences of *Eubacterium* sp. strain W1471. These two sequences can be found in the EMBL sequence database under accession numbers Z36273 and U13039. The accession number U13039 corresponds to 16S rDNA sequence of *Eubacterium infirmum* W1471 described by Cheeseman *et al.* (1996). The next best match was 91.3 % to the sequence of '*Clostridium aminobutyricum*' DSM 2634 ('*C. aminobutyricum*' is not a valid name).

The 16S rDNA sequences determined in this study were compared to 16S rDNA sequences of the other species in the genus *Fusobacterium* using the GAP program. Similarities were between 74.0 and 76.9 %. When the *Fusobacterium alocis* ATCC 35896^T sequence was compared to that of *Fusobacterium alocis* ATCC 35896^T (accession number X55406) published by Lawson *et al.* (1991), the similarity was 76.7 %. Since we sequenced the 16S rDNA of two *Fusobacterium alocis* ATCC 35896^T strains and the sequences obtained were almost identical, we conclude that our finding cannot be due to contamination or a laboratory error, and that Lawson *et al.* (1991) have erroneously sequenced some other *Fusobacterium* species instead of *Fusobacterium alocis* ATCC 35896^T.

Phylogenetic analysis and proposal of new combinations

The 16S rRNA sequences of *Fusobacterium alocis* ATCC 35896^T and *Fusobacterium sulci* ATCC 35585^T were further analysed by reconstructing phylogenetic trees. Firstly, the sequences were aligned with the best matching sequences obtained with the distance matrix program. These sequences were copied from the RDP and the alignment was done manually using the ARB sequence editor (Grimm *et al.*, 1998). Five short (< 20 bases) ambiguous areas were removed from the alignment. The final alignment consisted of 1259 bases and included positions between 102 and 1391 (*Escherichia coli* numbering obtained from Gutell, 1994). When calculated from the edited alignment, the similarity between *Fusobacterium alocis* ATCC 35896^T and *Filifactor villosus* NCTC 11220^T 16S rRNA sequences was 92.9 % and that between *Fusobacterium sulci* ATCC 35585^T and *E. infirmum* W1471 was 95.2 %.

The evolutionary distance matrix of the aligned sequences was calculated using the two-parameter model for multiple substitutions (Kimura, 1980). The tree was constructed from the distance matrix by the

Fig. 1. Neighbour-joining tree of the 16S rRNA gene sequences of *Fusobacterium alocis* ATCC 35896^T and *Fusobacterium sulci* ATCC 35585^T and related species of the *Clostridium* cluster XI. *Clostridium litoreale* DSM 5388^T was used as an outgroup. The numbers above branches are bootstrap percentages from 1000 resampled datasets. Bar, 0.013 difference per nucleotide.

neighbour-joining method (Saitou & Nei, 1987). The reliability of the tree topology was estimated by the bootstrap method (Felsenstein, 1985). These analyses were done using the PHYLIP program package (Felsenstein, 1993). The neighbour-joining tree with bootstrap percentages drawn by NJPLOT (Thompson *et al.*, 1994) is shown in Fig. 1. The alignment was further

analysed using the DNAPENNY program (Felsenstein, 1985). Phylogenetic trees were produced according to the parsimony principle using a branch-and-bound tree search algorithm. The analysis yielded four minimum length trees (trees not shown). Trees obtained according to the distance and the parsimony principle were manually compared and the following obser-

vations were made. (1) *Eubacterium* species, '*C. aminobutyricum*' and *Fusobacterium sulci* ATCC 35585^T clustered together in all trees. This '*Eubacterium* cluster' was well supported in the bootstrap analysis (bootstrap value of 100%). (2) In all trees, *Fusobacterium sulci* ATCC 35585^T clustered with two *Eubacterium* strains (*Eubacterium* sp. W1471 and *E. infirmum* W1471). This cluster also had a high bootstrap value (97.8%). (3) *Filifactor villosus* NCTC 11220^T and *Fusobacterium alocis* ATCC 35896^T formed a relatively deep branch in all trees and this cluster had a high bootstrap value (100%).

The database comparison and the phylogenetic analyses showed that *Fusobacterium alocis* ATCC 35896^T and *Fusobacterium sulci* ATCC 35585^T belonged to *Clostridium* cluster XI. This cluster, proposed by Collins *et al.* (1994), is taxonomically heterogeneous and contains probably several different genera. The closest relative of *Fusobacterium alocis* ATCC 35896^T is *Filifactor villosus* NCTC 11220^T (Collins *et al.*, 1994; Love *et al.*, 1979), which forms a deep branch in cluster XI. Both species are biochemically relatively inert, although some biochemical properties like the production of isobutyrate and isovalerate and utilization of pyruvate, separate *Filifactor villosus* NCTC 11220^T from *Fusobacterium alocis* ATCC 35896^T. Colonial and cellular morphology and the capability of *Filifactor villosus* NCTC 11220^T to form spores also differentiate these two species (Love *et al.*, 1979; Cato *et al.*, 1985). Our phylogenetic analysis included several other species from *Clostridium* cluster XI. In all the trees, *Fusobacterium alocis* ATCC 35896^T clustered together with *Filifactor villosus* NCTC 11220^T and they formed a well-separated (high bootstrap value) long branch. We conclude that despite some biochemical and morphological differences, there are no good reasons to exclude *Fusobacterium alocis* ATCC 35896^T from the genus *Filifactor*.

Fusobacterium sulci ATCC 35585^T is related to asaccharolytic oral *Eubacterium* species, the closest relative being *E. infirmum* W1471 (Cheeseman *et al.*, 1996). None of these *Eubacterium* species was included in the analysis done by Collins *et al.* (1994). In our analysis, these *Eubacterium* species clustered with '*C. aminobutyricum*', which forms a deep branch in *Clostridium* cluster XI, indicating that these asaccharolytic *Eubacterium* species also belong to *Clostridium* cluster XI. The 16S rRNA gene similarity of *Fusobacterium sulci* ATCC 35585^T and *E. infirmum* W1471 is rather high (95.2%), although less than 97%, which suggests that these species belong to the same genus but represent different species (Stackebrandt & Goebel, 1994). Both species produce butyrate and acetate but in general they are biochemically inactive. They differ from each other by cell morphology and size. *E. infirmum* W1471 cells are 0.5 × 1.0–2.0 µm in size and they occur singly (Cheeseman *et al.*, 1996). *Fusobacterium sulci* ATCC 35585^T cells are longer (1.0–7.0 µm) and they also occur in pairs and short chains (Cato *et al.*, 1985). The DNA G+C content

(measured by the thermal denaturation method) of *E. infirmum* W1471 is 38 mol% and that of *Fusobacterium sulci* ATCC 35585^T is 34 mol%. These morphological features as well as the different DNA base compositions support the idea that *E. infirmum* W1471 and *Fusobacterium sulci* ATCC 35585^T are related, but separate species.

Description of *Filifactor alocis* (Cato, Moore and Moore) comb. nov.

The description of *Filifactor alocis* comb. nov. is identical to that proposed for *Fusobacterium alocis* Cato, Moore and Moore (Cato *et al.*, 1985). The type strain is ATCC 35896^T.

Description of *Eubacterium sulci* (Cato, Moore and Moore) comb. nov.

The description of *Eubacterium sulci* comb. nov. is identical to that proposed for *Fusobacterium sulci* Cato, Moore and Moore (Cato *et al.*, 1985). The type strain is ATCC 35585^T.

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References

- Cato, E. P., Moore, L. V. H. & Moore, W. E. C. (1985). *Fusobacterium alocis* sp. nov. and *Fusobacterium sulci* sp. nov. from the human gingival sulcus. *Int J Syst Bacteriol* **35**, 475–477.
- Cheeseman, S. L., Hiom, S. J., Weightman, A. J. & Wade, W. G. (1996). Phylogeny of oral asaccharolytic *Eubacterium* species determined by 16S ribosomal DNA sequence comparison and proposal of *Eubacterium infirmum* sp. nov. and *Eubacterium tardum* sp. nov. *Int J Syst Bacteriol* **46**, 957–959.
- Collins, M. D., Lawson, P. A., Willems, A., Cordoba, J. J., Fernandez-Garayzabal, J., Garcia, P., Cai, J., Hippe, H. & Farrow, J. A. E. (1994). The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. *Int J Syst Bacteriol* **44**, 812–826.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Felsenstein, J. (1993). PHYLIP (Phylogeny Inference Package) version 3.5p. Department of Genetics, University of Washington, Seattle, WA, USA.
- Grimm, D., Merkert, H., Ludwig, W., Schleifer, K.-H., Hacker, J. & Brand, B. (1998). Specific detection of *Legionella pneumophila*: construction of a new 16S rRNA-targeted oligonucleotide probe. *Appl Environ Microbiol* **64**, 2686–2690.
- Gutell, R. R. (1994). Collection of small subunit (16S- and 16S-like) ribosomal RNA structures: 1994. *Nucleic Acids Res* **22**, 3502–3507.
- Jalava, J., Kotilainen, P., Nikkari, S., Skurnik, M., Vanttinen, E., Lehtonen, O., Eerola, E. & Toivanen, P. (1995). Use of the

polymerase chain reaction and DNA sequencing for detection of *Bartonella quintana* in the aortic valve of a patient with culture-negative infective endocarditis. *Clin Infect Dis* **21**, 891–896.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **2**, 150–174.

Lawson, P. A., Gharbia, S. E., Shah, H. N., Clark, D. R. & Collins, M. D. (1991). Intrageneric relationships of members of the genus *Fusobacterium* as determined by the reverse transcriptase sequencing of small-subunit rRNA. *Int J Syst Bacteriol* **41**, 347–354.

Love, D. N., Jones, R. F. & Bailey, M. (1979). *Clostridium villosum* sp. nov. from subcutaneous abscesses in cats. *Int J Syst Bacteriol* **29**, 241–244.

Love, D. N., Cato, E. P., Johnson, J. L., Jones, R. F. & Bailey, M. (1987). Deoxyribonucleic acid hybridization among strains of fusobacteria isolated from soft tissue infections of cats: comparison with human and animal type strains from oral and other sites. *Int J Syst Bacteriol* **37**, 23–26.

Maidak, B. L., Olsen, G. J., Larsen, L., Overbeek, R., McCaughey, M. J. & Woese, C. R. (1997). The RDP (Ribosomal Database Project). *Nucleic Acids Res* **25**, 109–110.

Moore, W. E. C., Holdeman, L. V. & Kelley, R. W. (1984). Genus II. *Fusobacterium* Knorr 1992, 4^{AB}. In *Bergey's Manual of Systematic Bacteriology*, pp. 631–637. Edited by N. R. Krieg & J. G. Holt. London: Williams & Wilkins.

Moore, L. V. H., Bourne, D. M. & Moore, W. E. C. (1994). Comparative distribution and taxonomic value of cellular fatty acids in thirty-three genera of anaerobic Gram-negative bacilli. *Int J Syst Bacteriol* **44**, 338–347.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**, 697–703.