Classification of Centers for Disease Control Group Eugonic Fermenter (EF)-4a and EF-4b as *Neisseria animaloris* sp. nov. and *Neisseria zoodegmatis* sp. nov., respectively

Peter Vandamme,¹ Barry Holmes,² Hervé Bercovier³ and Tom Coenye¹

Correspondence

Peter Vandamme

Peter.Vandamme@UGent.be

¹Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium

²National Collection of Type Cultures, HPA Centre for Infections, Colindale, London, UK

³Hebrew University Medical School, Ein Karem, Jerusalem, Israel

A polyphasic taxonomic study was performed on isolates classified as Centers for Disease Control Group Eugonic Fermenter (EF)-4a and EF-4b. Comparative 16S rRNA gene sequence analysis confirmed that group EF-4a and EF-4b belong to the genus *Neisseria* with *Neisseria canis* and *Neisseria dentiae* as the nearest phylogenetic neighbours. DNA–DNA hybridizations and biochemical analyses demonstrated that isolates of group EF-4a and EF-4b represent two novel species within this sublineage of the genus *Neisseria*. Based on the results of the present study, isolates of group EF-4a and EF-4b are classified as *Neisseria animaloris* sp. nov. (type strain LMG 23011^T=NCTC 12228^T) and *Neisseria zoodegmatis* sp. nov. (type strain LMG 23012^T=NCTC 12230^T), respectively.

The designation Centers for Disease Control Group Eugonic Fermenter 4 (EF-4) was first used by Tatum *et al.* (1974) for a group of Gram-negative saccharolytic organisms predominantly recovered from human wounds resulting from dog or cat bites. EF-4 isolates are considered to be commensal organisms found in the oral cavity of dogs and cats, although systemic infections in humans and animals have also been reported (Ganière *et al.*, 1995). Two biotypes can be distinguished based on the presence (EF-4a) or absence (EF-4b) of arginine dihydrolase activity (Holmes & Ahmed, 1981). In addition, both biotypes can be distinguished based on DNA G+C content (Bercovier *et al.*, 1982), cellular fatty acid content (Dees *et al.*, 1981) and whole-cell protein analysis (Holmes *et al.*, 1990). Analysis of rRNA gene sequence similarities indicated that EF-4 strains were closely related to the genus *Neisseria* (Rossau *et al.*, 1989) and this was later confirmed by a detailed numerical phenotypic taxonomic study (Barrett & Sneath, 1994). In the present study, a polyphasic taxonomic study was performed to classify both taxa formally.

EF-4 strains used in this study are listed in Table 1 and were well-chosen representatives of previous taxonomic studies

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains LMG 23011^T and LMG 23012^T are DQ006842 and DQ006843, respectively.

The fatty acid contents of *Neisseria animaloris* sp. nov., *Neisseria zoodegmatis* sp. nov. and related taxa are presented in a supplementary table in IJSEM online.

(Holmes *et al.*, 1990; Rossau *et al.*, 1989). All strains were grown aerobically on trypticase soy agar (BBL) at 37 °C unless otherwise indicated.

DNA for 16S rRNA gene sequencing was prepared by heating one or two colonies at 95 °C for 15 min in 20 µl lysis buffer containing 0.25 % (w/v) SDS and 0.05 M NaOH. Following lysis, 180 µl distilled water was added to the lysate. 16S rRNA genes were amplified using oligonucleotide primers complementary to highly conserved regions of bacterial 16S rRNA genes. The forward primer was 5'-A-GAGTTTGATCCTGGCTCAG-3' (hybridizing at positions 8–27, according to the *Escherichia coli* numbering system) and the reverse primer was 5'-AAGGAGGTGATCCAGCC-GCA-3' (hybridizing at positions 1541–1522). PCR products were purified using a NucleoFast 96 PCR clean-up kit (Macherey–Nagel). Sequencing reactions were performed by using a BigDye terminator cycle sequencing kit (Applied Biosystems) and purified with a Montage SEQ₉₆ sequencing reaction clean-up kit (Millipore). Sequencing was performed with an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). The eight sequencing primers used are listed in Coenye *et al.* (1999). Sequence assembly was performed using the AUTOASSEMBLER program (Applied Biosystems). Sequences were aligned with sequences retrieved from GenBank using CLUSTAL_X (Thompson *et al.*, 1997). Phylogenetic analyses and bootstrap analysis (1000 replicates) were subsequently performed using the BioNumerics 4.01 software package (Applied Maths). A phylogenetic tree was constructed using the neighbour-joining method (Saitou &

Table 1. List of strains studied

Strain designations beginning with CL are designations used by the National Collection of Type Cultures Identification Services Laboratory.

Strain designation	Source
<i>Neisseria animaloris</i> sp. nov. (EF-4a)	
LMG 23011 ^T (=NCTC 12228 ^T =CL 191/78 ^T =ATCC 29858 ^T =CDC D8251 ^T)	Thumb wound, Wisconsin, USA
LMG 23009 (=NCTC 12226=CL 480/77)	Dog-bite wound, London, UK
LMG 23010 (=NCTC 12227=CL 643/80=CIP 268.80)	Abscess following dog-bite, Montreuil, France
<i>Neisseria zoodegmatis</i> sp. nov. (EF-4b)	
LMG 23012 ^T (=NCTC 12230 ^T =CL 194/78 ^T =ATCC 29859 ^T =CDC D5986 ^T)	Dog-bite wound, Hawaii, USA
LMG 23013 (=NCTC 12229=CL 641/80=CIP 784.80)	Dog-bite wound, Paris, France
LMG 23014 (=NCTC 12231=CL 724/80=CDC C669)	Vagina, Canada

Nei, 1987); bases that could not unequivocally identified were discarded for analyses. Almost complete 16S rRNA gene sequences were determined for two EF-4 strains, LMG 23011^T and LMG 23012^T. Partial sequences of about 450 bases were generated for strains LMG 23009, LMG 23010, LMG 23013 and LMG 23014. The 16S rRNA gene sequence of LMG 23011^T shared the highest similarity values with *Neisseria canis* LMG 8383^T (97.9%), group EF-4b strain LMG 23012^T (97.7%) and *Neisseria dentiae* LMG 23015^T (97.1%). The 16S rRNA gene sequence of LMG 23012^T was most similar to those of *N. canis* LMG 8383^T (98.1%) and *N. dentiae* LMG 23015^T (97.9%). 16S rRNA gene sequence similarities of strains LMG 23011^T and LMG 23012^T with *Uruburuella suis* CCUG 47806^T and other *Neisseria* species were below 97%. The 447-base fragment of the 16S rRNA gene sequence of strain LMG 23010 was 100% similar to the corresponding fragment of strain LMG 23011^T; the 447-base fragment of the 16S rRNA gene sequence of strain LMG 23009 differed by two bases from the corresponding fragment of the other two strains (data not shown). Finally, the 452-base fragments of the 16S rRNA gene sequence of strains LMG 23013 and LMG 23014 were 100% similar to the corresponding fragment of strain LMG 23012^T (data not shown).

In order to determine the degree of relatedness between representative group EF-4a and EF-4b strains, DNA–DNA hybridization experiments were performed between strains LMG 23011^T, LMG 23012^T, *N. dentiae* LMG 23015^T and *N. canis* LMG 8383^T. High molecular mass DNA was prepared as described by Pitcher *et al.* (1989) and DNA–DNA hybridizations were performed with photobiotin-labelled probes in microplate wells as described by Ezaki *et al.* (1989) using an HTS7000 Bio Assay Reader (Perkin-Elmer) for fluorescence measurements. The hybridization temperature was 39 °C. Reciprocal experiments were performed for every pair of strains. The hybridization value between strains LMG 23011^T and LMG 23012^T was 42%, which is in agreement with the previously reported value of 31% (Rossau *et al.*, 1989). Hybridization values between these EF-4a and EF-4b reference strains and *N. canis* LMG 8383^T (36 and 50%, respectively) and *N. dentiae* LMG 23015^T (31 and 43%,

respectively) were also low to intermediate. These DNA–DNA hybridization results demonstrate that EF-4a and EF-4b isolates represent two distinct and novel species within this bacterial lineage.

All strains were biochemically characterized in a range of 66 conventional biochemical tests by methods described previously (Holmes *et al.*, 1986). Certain strains were additionally tested for nitrite reduction through to gas by the method described by Holmes *et al.* (1975). The species descriptions as presented below are based on data obtained for the isolates presented in Table 1 and for an additional 19 strains of group EF-4a and 12 strains of group EF-4b studied by Holmes *et al.* (1990). Differential characteristics between isolates from group EF-4a and EF-4b and their closest phylogenetic neighbours are shown in Table 2.

Cellular fatty acid analysis was carried out with a loopful of well-grown cells after an incubation period of 48 h. Fatty acid methyl esters were prepared, separated and identified using the Microbial Identification System (Microbial ID) as

Table 2. Phenotypic characteristics useful for differentiation of *Neisseria animaloris* sp. nov. and *Neisseria zoodegmatis* sp. nov. from related taxa

Taxa: 1, *N. animaloris* sp. nov. (22 strains); 2, *N. zoodegmatis* sp. nov. (15 strains); 3, *N. canis* LMG 8383^T; 4, *N. dentiae* LMG 23015^T; 5, *U. suis* (five strains; data from Vela *et al.*, 2005). All data are from this study unless otherwise indicated. Results for nitrite reduction to gas were determined from only three strains of *N. animaloris* (LMG 23011^T, LMG 23009 and LMG 23010) and three strains of *N. zoodegmatis* (LMG 23012^T, LMG 23013 and LMG 23014). NA, Not available.

Characteristic	1	2	3	4	5
Arginine dihydrolase activity	+	–	–	–	–
Facultatively anaerobic	+	+	–	+	+
Growth on MacConkey agar	+	+	+	+	–
Nitrite reduction	+	+	–	+	+
Nitrite reduction to gas	+	–	–	+	NA

described previously (Vandamme *et al.*, 1992). *N. canis*, *N. dentiae* and strains from group EF-4a and EF-4b could all be distinguished through quantitative differences in their cellular fatty acid profiles (see Supplementary Table S1 in IJSEM Online). In particular, the relative distribution of 16:1 ω 7c, 16:0 and 16:0 2-OH was useful to distinguish strains from group EF-4a and EF-4b.

The allocation of the two novel species to a specific genus is not straightforward given the polyphyletic nature of the genus *Neisseria*. Clearly, *N. canis* and *N. dentiae* are the nearest phylogenetic neighbours of both taxa. In a neighbour-joining phylogenetic tree, rather than clustering with the other *Neisseria* species, they form a distinct lineage together with *U. suis*. The sequence similarity levels of the 16S rRNA genes of isolates from group EF-4a and EF-4b, *N. canis* and *N. dentiae* with *U. suis* range from 96.8 (EF-4a) to 95.5 % (EF-4b). However, neither the EF-4a–*U. suis* lineage, nor the lineage composed of isolates from group EF-4a and EF-4b, *N. canis*, *N. dentiae* and *U. suis*, is supported by a high bootstrap value (Fig. 1). In general, these organisms are rather unreactive and there is little to distinguish members of the genera *Neisseria* and *Uruburuella* in the characters they exhibit. Although the capacity to grow in facultatively anaerobic conditions was reported to distinguish *Uruburuella* strains from members of the genus *Neisseria* (Vela *et al.*, 2005) (which are generally considered strictly aerobic), we found the type strain of *N. dentiae* to

be facultatively anaerobic, just like group EF-4a and EF-4b strains. *N. canis*, however, is strictly aerobic. There are therefore no clear phenotypic grounds to distinguish the genus *Uruburuella* from the genus *Neisseria* (even if all of the members of the lineage composed of group EF-4a and EF-4b, *N. canis*, *N. dentiae* and *U. suis* were transferred into the genus *Uruburuella*). Therefore, pending a more general reassessment of the taxonomy of the genus *Neisseria*, we find it most appropriate to allocate strains from group EF-4a and EF-4b to the genus *Neisseria*.

Description of *Neisseria animaloris* sp. nov.

Neisseria animaloris (a.ni.mal.or'is. L. n. *animal* an animal; L. n. *os* the mouth; N.L. gen. n. *animaloris* of an animal's mouth).

Colonies are circular, convex, entire, opaque, shiny, smooth and haemolytic. All 22 strains studied by Holmes *et al.* (1990) are positive for acid production (in peptone water medium) from glucose, arginine dihydrolase production, catalase production, cytochrome oxidase production, growth at 37 °C, growth at room temperature (18–22 °C), growth on MacConkey agar and nitrate reduction. Most strains are positive for (exceptions in parentheses) fermentation in the Hugh and Leifson O–F test (negative: LMG 23009, CL 579/78; oxidative reaction: CL 608/78) and gelatinase production (plate method; CL 820/79). All strains are

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of *Neisseria animaloris* sp. nov. (EF-4a) and *Neisseria zoodegmatidis* sp. nov. (EF-4b). Bar, 1 % sequence dissimilarity.

negative for acid production (in peptone water medium) from adonitol, arabinose, cellobiose, dulcitol, glycerol, inositol, lactose, maltose, mannitol, raffinose, rhamnose, salicin, sorbitol, starch, sucrose, trehalose and xylose. All strains are negative for acetoin production (Voges–Proskauer test; incubation at 37 °C for 2 days and incubation at room temperature for 5 days), casein digestion, gluconate oxidation, β -galactosidase production (ONPG test), gas production from glucose in peptone water medium, H₂S production (by lead acetate paper and triple-sugar iron agar methods), indole production, KCN tolerance, lysine decarboxylase production, malonate utilization, methyl red test at room temperature (incubation for 5 days), motility (hanging drop preparation at both 37 °C and room temperature), ornithine decarboxylase production, phenylalanine deamination, pigment production, production of extracellular DNase, reduction of 0.4 % (w/v) selenite, urease production and utilization of citrate (Simmons' medium). Most strains are negative for gelatinase production (stab method; CIP 655.75, CIP 310.77, CIP 181.80), methyl red test at 37 °C (incubation for 2 days; CL 820/79) and utilization of citrate (Christensen's medium; LMG 23011^T). DNA G + C content is around 50 mol%; the DNA G + C content of the type strain is 49.3 mol% (Rossau *et al.*, 1989). Characteristics for the type strain are the same as described above for the species except for the utilization of citrate in Christensen's medium, which is positive in the type strain.

The type strain, LMG 23011^T (=NCTC 12228^T=CL 191/78^T=ATCC 29858^T=CDC D8251^T), was isolated from a thumb wound in Wisconsin, USA. Strains LMG 23009 (=NCTC 12226) and LMG 23010 (=NCTC 12227) are reference strains.

Description of *Neisseria zoodegmatis* sp. nov.

Neisseria zoodegmatis (zoo.deg'ma.tis. Gr. n. *zoon* an animal; Gr. n. *degma* a bite; N.L. gen. n. *zoodegmatis* of an animal's bite).

Colonies are circular, convex, entire, opaque, shiny, smooth and haemolytic. All 15 strains studied by Holmes *et al.* (1990) are positive for catalase production, cytochrome oxidase production, growth at 37 °C, growth at room temperature (18–22 °C) and growth on MacConkey agar. Most strains are positive for (exceptions in parentheses) acid production (in peptone water medium) from glucose (C5663, CL 149/79), fermentation in the Hugh and Lefson O-F test (negative: LMG 23014, A8412, B2939, D3138), gelatinase production (plate method; LMG 23012^T, B2939, C5663, D7300, E764, E928, CL 149/79), nitrate reduction (B2939) and utilization of citrate (Christensen's medium; C3248, C5663, D3138, D7300, E928, CL 392/80). All strains are negative for acid production (in peptone water medium) from adonitol, arabinose, cellobiose, dulcitol, glycerol, inositol, lactose, maltose, mannitol, raffinose, rhamnose, salicin, sorbitol, starch, sucrose, trehalose and xylose. All strains are negative for acetoin production (Voges–Proskauer test;

incubation at 37 °C for 2 days and incubation at room temperature for 5 days), arginine dihydrolase production, casein digestion, gluconate oxidation, β -galactosidase production (ONPG test), gas production from glucose in peptone water medium, gelatinase production (stab method), H₂S production (by lead acetate paper and triple-sugar iron agar methods), indole production, KCN tolerance, lysine decarboxylase production, malonate utilization, methyl red test (both incubation at 37 °C for 2 days and incubation at room temperature for 5 days), motility (hanging drop preparation at 37 °C and room temperature), ornithine decarboxylase production, phenylalanine deamination, pigment production, production of extracellular DNase, reduction of 0.4 % (w/v) selenite, urease production and utilization of citrate (Simmons' medium). The DNA G + C content of *N. zoodegmatis* strains is around 50 mol% (Rossau *et al.*, 1989); the DNA G + C content of the type strain is 50.5 mol% (Rossau *et al.*, 1989).

The type strain, LMG 23012^T (=NCTC 12230^T=CL 194/78^T=ATCC 29859^T=CDC D5986^T), was isolated from a dog-bite wound in Hawaii, USA. Strains LMG 23013 (=NCTC 12229) and LMG 23014 (=NCTC 12231) are reference strains.

Acknowledgements

T.C. and P.V. are indebted to the Fund for Scientific Research – Flanders (Belgium) for a position as a postdoctoral fellow and research grants, respectively. We thank T. O. MacAdoo for advice on the etymology of names. We are extremely grateful to R. E. Weaver for the supply of cultures which made this study possible and B.H., in particular, would like to thank R. E. Weaver for years of fruitful collaboration. We thank P. Borman and E. Samyn for technical assistance.

References

- Barrett, S. J. & Sneath, P. H. (1994). A numerical phenotypic taxonomic study of the genus *Neisseria*. *Microbiology* **140**, 2867–2891.
- Bercovier, H., Escande, F., Chatelain, R. & Weaver, R. E. (1982). Group EF-4 bacteria: a newly recognised pathogen. *Adv Pathol* **1**, 27–30.
- Coenye, T., Falsen, E., Vancanneyt, M., Hoste, B., Govan, J. R. W., Kersters, K. & Vandamme, P. (1999). Classification of *Alcaligenes faecalis*-like isolates from the environment and human clinical samples as *Ralstonia gilardii* sp. nov. *Int J Syst Bacteriol* **49**, 405–413.
- Dees, S. B., Powell, J., Moss, C. W., Hollis, D. G. & Weaver, R. E. (1981). Cellular fatty acid composition of organisms frequently associated with human infections from dog bites: *Pasteurella multocida* and groups of EF-4, IIj, M-5, and DF-2. *J Clin Microbiol* **14**, 612–616.
- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.
- Ganière, J. P., Escande, F., André-Fontaine, G., Larrat, M. & Filloneau, C. (1995). Characterisation of group EF-4 bacteria from the oral cavity of dogs. *Vet Microbiol* **44**, 1–9.
- Holmes, B. & Ahmed, M. S. (1981). Group EF-4: a *Pasteurella*-like organism. In *Haemophilus, Pasteurella and Actinobacillus*, pp. 161–174.

Edited by M. Kilian, W. Frederiksen & E. L. Biberstein. London: Academic Press.

Holmes, B., Lapage, S. P. & Malnick, H. (1975). Strains of *Pseudomonas putrefaciens* from clinical material. *J Clin Pathol* **28**, 149–155.

Holmes, B., Dawson, C. A. & Pinning, C. A. (1986). A revised probability matrix for the identification of Gram-negative, aerobic, rod-shaped, fermentative bacteria. *J Gen Microbiol* **132**, 3113–3135.

Holmes, B., Costas, M. & Wood, C. A. (1990). Numerical analysis of electrophoretic patterns of group EF-4 bacteria, predominantly from dog-bite wounds of humans. *J Appl Bacteriol* **68**, 81–91.

Pitcher, D. G., Saunders, N. A. & Owen, R. J. (1989). Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Lett Appl Microbiol* **8**, 151–156.

Rossau, R., Vandenbussche, G., Thielemans, S., Segers, P., Grosch, H., Göthe, E., Mannheim, W. & De Ley, J. (1989). Ribosomal ribonucleic acid cistron similarities and deoxyribonucleic acid homologies of *Neisseria*, *Kingella*, *Eikenella*, *Simonsiella*, *Alysiella*, and Centers for Disease Control Groups EF-4 and M-5 in the emended family *Neisseriaceae*. *Int J Syst Bacteriol* **39**, 185–198.

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

Tatum, H. W., Ewing, W. H. & Weaver, R. E. (1974). Miscellaneous Gram-negative bacteria. In *Manual of Clinical Microbiology*, pp. 270–294. Edited by E. H. Lennette, E. H. Spaulding & J. P. Truant. Washington, DC: American Society for Microbiology.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.

Vandamme, P., Vancanneyt, M., Pot, B. & 10 other authors (1992). Polyphasic taxonomic study of the emended genus *Arcobacter* with *Arcobacter butzleri* comb. nov. and *Arcobacter skirrowii* sp. nov., an aerotolerant bacterium isolated from veterinary specimens. *Int J Syst Bacteriol* **42**, 344–356.

Vela, A. I., Collins, M. D., Lawson, P. A., García, N., Domínguez, L. & Fernández-Garayzábal, J. F. (2005). *Uruburuella suis* gen. nov., sp. nov., isolated from clinical specimens of pigs. *Int J Syst Evol Microbiol* **55**, 643–647.