Neisseria oralis sp. nov., isolated from healthy gingival plaque and clinical samples

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A polyphasic analysis was undertaken of seven independent isolates of Gram-negative cocci collected from pathological clinical samples from New York, Louisiana, Florida and Illinois and healthy subgingival plaque from a patient in Virginia, USA. The 16S rRNA gene sequence similarity among these isolates was 99.7–100 %, and the closest species with a validly published name was *Neisseria lactamica* (96.9 % similarity to the type strain). DNA–DNA hybridization confirmed that these isolates are of the same species and are distinct from their nearest phylogenetic neighbour, *N. lactamica*. Phylogenetic analysis of 16S and 23S rRNA gene sequences indicated that the novel species belongs in the genus *Neisseria*. The predominant cellular fatty acids were $C_{16:0}$, summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH) and $C_{18:1}\omega7c$. The cellular fatty acid profile, together with other phenotypic characters, further supports the inclusion of the novel species in the genus *Neisseria*. The name *Neisseria oralis* sp. nov. (type strain 6332^T =DSM 25276^T =LMG 26725^T) is proposed.

Abbreviation: CFA, cellular fatty acid.

The GenBank/EMBL/DDBJ accession numbers for the partial 16S and 23S rRNA gene sequences of strain 6332^T are JN104029 and JN104031, respectively. Accession numbers for the partial 23S rRNA gene sequences of *N. elongata* subsp. *elongata* LMG 5124^T, *N. sicca* LMG 5290^T, *N. lactamica* LMG 26610^T, *N. macacae* LMG 26611^T and *N. animalis* LMG 26609^T are JQ042809–JQ042813, respectively. The complete genome sequence of strain F0314 is available as NCBI genome reference sequence accession ID ADEA00000000; the genome can also be viewed at the Human Oral Microbiome Database as oral taxon 014 (http://www.homd.org/taxon=014). Accession numbers for the 16S and 23S rRNA gene sequences of other strains described in the paper are listed in Table 1.

Three supplementary tables and a supplementary figure are available with the online version of this paper.

In 2005, the Wadsworth Center Bacteriology Laboratory received two independent isolates of bacteria from clinical specimens as part of their reference testing for New York State laboratories (designated 6332^T and 8261) that had identical nearly full-length 16S rRNA gene sequences. The most closely related species, based on sequence similarity, was *Neisseria lactamica* (96.9 % similarity to the type strain returned by EzTaxon; Chun *et al.*, 2007), suggesting that the isolates may represent a novel species (Tindall *et al.*, 2010). A BLAST search of GenBank revealed an additional isolate with 100 % sequence similarity, *Neisseria* sp. oral taxon 014 strain F0314 (GenBank accession no. GQ131417), which we acquired (Dewhirst *et al.*, 2010). Four additional isolates were acquired from Associated Regional and University Pathologists (ARUP) Laboratories and found to have 99.7–99.9 % 16S rRNA gene sequence similarity to the three other isolates. Suggesting a widespread prevalence for this organism, the GenBank BLAST search also identified other studies in which the same 16S rRNA gene sequence was detected (sequence similarity >99.6 %) by culture-independent methodologies. The sources of these clones were subgingival plaque (healthy and diseased), healthy skin and respiratory samples from cystic fibrosis patients, (Dewhirst *et al.*, 2010; Grice *et al.*, 2009; Paster *et al.*, 2001; Rylev *et al.*, 2011; Staudinger *et al.*, 2011; van der Gast *et al.*, 2011). In the polyphasic study described here, the seven acquired isolates were found to belong to a single novel species, for which we propose the name *Neisseria oralis* sp. nov.

The seven isolates were maintained at 37 °C in a 5 % CO_2 atmosphere on trypticase soy agar (TSA) plates (Becton Dickinson) supplemented with 5 % sheep blood. Four of the isolates were from blood cultures; one was from urine, one was from paracentesis fluid and one was from subgingival oral biofilm at a healthy site (Table 1). A representative strain, 6332^T, was chosen as the type strain.

16S rRNA gene sequences (1463–1515 nt) were determined for the isolates as described previously (Dewhirst *et al.*, 2010; Wolfgang *et al.*, 2011); see Table 1 for GenBank accession numbers. Additionally, for strain F0314, the whole-genome shotgun sequence (accession no. ADEA01000039) had been deposited previously at GenBank as part of the Human Microbiome Project (Dewhirst *et al.*, 2010; Nelson *et al.*, 2010).

To identify the type strains most closely related to 6332^T for inclusion in our polyphasic analysis, the BLAST (Altschul *et al.*, 1997) and megaBLAST (Zhang *et al.*, 2000) search programs were used with the 16S rRNA gene sequence against the database of type strains of prokaryotic species with validly published names (Chun *et al.*, 2007). The 50 sequences with the highest scores were then selected for the calculation of pairwise sequence similarity using the global alignment algorithm implemented at the EzTaxon server (http://www.eztaxon.org/; Chun *et al.*, 2007). Five of the most similar type strains by sequence similarity (96.1–96.9 %) were acquired for this study, *Neisseria lactamica* LMG 26610^T, *N. animalis* LMG 26609^T, *N. macacae* LMG 26611^T, *N. elongata* subsp. *elongata* LMG 5124^T and *N. sicca* LMG 5290^T.

Previous studies indicate that, for organisms with more than 97 % 16S rRNA gene sequence similarity, alternative methods must be employed, such as DNA–DNA hybridization, to determine inclusion with or exclusion from closely related species (Tindall *et al.*, 2010). Although none of the most closely related organisms crossed this threshold, *N. lactamica* LMG 26610^T, with a similarity of 96.9 %, was the closest, and so was selected for DNA–DNA hybridization analysis. DNA was purified as described by Logan *et al.* (2000) and hybridization was performed using the microplate method described by Ezaki *et al.* (1989), with modifications described by Willems *et al.* (2001). Means of two reciprocal hybridization values were determined for all pairwise combinations of strains 6332^T, 8261 and 11-26358 and *N. lactamica* LMG 26610^T (Table S1, available in IJSEM Online). Based on a 70 % cut-off value for defining species (Wayne

Table 1. Strains used in this study

Strain	Isolation date	Source	GenBank accession no.	
			16S rRNA gene	23S rRNA gene
6332^T (=LMG 26725^T =DSM 25276^T)	July 2005	Blood, 55-year-old male; Dutchess County, NY, USA	JN104029	JN104031
8261 (=LMG 26726 =DSM 25277)	October 2005	Blood, 56-year-old female; Saratoga County, NY, USA	JN104030	JN104032
F0314 (=11-24509)	June 1982	Subgingival oral biofilm, 23-year-old female; Blacksburg, VA, USA	GQ131417	JN986585
11-26358	NK	Urine, 56-year-old; FL, USA	JN986581	JN986586
11-26359	NK	Blood, >89-year-old; OH, USA	JN986582	JN986587
11-26360	NK	Blood, 19-month-old; LA, USA	JN986583	JN986588
11-26361	NK	Paracentesis fluid, 80-year-old; IL, USA	JN986584	JN986589

NK, Not known.

et al., 1987), strains 6332^T, 8261 and 11-26358 belong to the same species, and that species is distinct from *N. lactamica*.

For cellular fatty acid (CFA) analysis, the seven strains of *N. oralis* sp. nov. and the five most closely related type strains were cultured aerobically for 48 h on trypticase soy broth agar (TSBA) at 37 °C and harvested in late-exponential growth phase. Fatty acid methyl esters were prepared according to the manufacturer's instructions (Sherlock Microbial Identification Systems; MIDI, Inc.) and analysed on an Agilent Technologies 6890N gas chromatograph. For the type strain 6332^T, the predominant CFAs were $C_{16:0}$, summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH) and $C_{18:1}\omega7c$. These were the same as the predominant CFAs of the most closely related type strains (Table 2). The variation seen among these strains in Table 2 was similar to that seen among the seven strains of *N. oralis* (Table S2). Hence, the CFA composition is concordant with assigning the novel species to the genus *Neisseria*, but is not useful in distinguishing among the species within the genus.

Phylogenetic analysis was performed using nearly full-length 16S rRNA gene sequences from the seven strains of *N. oralis* and the top 50 BLAST hits returned for type strain 6332^T from the EzTaxon database (Chun *et al.*, 2007). These sequences were aligned using the CLUSTAL W utility in MEGA 4.0.2 (Tamura *et al.*, 2007) and exported to PhyML 3.0 (Guindon & Gascuel, 2003). A rooted maximum-likelihood tree was recreated using the general time reversible model, six substitution rate categories and five random starting trees (Guindon & Gascuel, 2003). Tree reliability was evaluated using the approximate likelihood ratio test (aLRT) (Anisimova & Gascuel, 2006) (Fig. 1). The seven novel strains reside in a well-supported clade that harbours *N. lactamica* as its closest relative as well as *N. elongata*, two species of *Kingella* and the one species of *Eikenella*. The other most closely related species (based on sequence distance) reside in other, distinct clades (Fig. 1).

To seek further support for the novel species designation, 23S rRNA gene sequences were determined for the seven new isolates as well as the five most closely related type strains. Sequencing was performed as described previously (Wolfgang *et al.*, 2011). Sequence similarity for the seven novel strains ranged from 99.4 to 100 %. A maximum-likelihood tree was reconstructed from sequences generated in this study as well as sequences from closely related species for which data were available at GenBank (Fig. S1) (note that the 23S rRNA gene sequence was not available for the type strain of some species; e.g. the sequence of strain MS11 was used for *Neisseria gonorrhoeae*). The seven isolates of *N. oralis* formed a clade that is distinct from all other *Neisseria* species, further supporting the novel species designation.

As in other studies, our phylogenetic analysis using the 16S rRNA gene sequence reveals that the genus *Neisseria* is polyphyletic (Vandamme *et al.*, 2006; Wolfgang *et al.*, 2011). We found that *N. oralis* resides in a well-supported clade that harbours two additional species of *Neisseria*, two species of *Kingella* and *Eikenella corrodens* (Fig. 1). This clade is distinct from the clade that harbours the type species of the genus, *N. gonorrhoeae*. As such, it is necessary to identify additional characters that support or reject the inclusion of the novel species in closely related genera. Characteristics that support inclusion of our isolates in the genus *Neisseria* are the formation of Gram-negative-staining cocci, the presence of catalase and oxidase activities, the absence of motility and the CFA profile (Tønjum, 2005; Weyant *et al.*, 1984). Characteristics that support exclusion from closely related genera are the absence of catalase activity in the genera *Bergeriella*, *Eikenella* and *Kingella*, the formation of long filaments and gliding motility in the genera *Alysiella*, *Simonsiella* and *Conchiformibium*, which additionally reside in distinct clades (Tønjum, 2005), and the fact that the single species of the genus *Uruburuella* falls in a separate clade and has additional characters that distinguish it from our isolates (Vela *et al.*, 2005).

Characteristics that aid in distinguishing all seven strains of *N. oralis* from the closely related type strains examined in this study are the ability to reduce nitrate, the absence of acid phosphatase activity and acid production from

Table 2. CFA compositions of *Neisseria* type strains

Strains: 1, 6332^T; 2, *N. elongata* subsp. *elongata* LMG 5124^T; 3, *N. sicca* LMG 5290^T; 4, *N. animalis* LMG 26609^T; 5, *N. lactamica* LMG 26610^T; 6, *N. macacae* LMG 26611^T. Values are percentages of total fatty acids, and were determined in this study. Fatty acid methyl esters for which no values were greater than 1 % were omitted. The three most prevalent fatty acids for each strain are in bold. ND, Not detected.

Fatty acid	1	2	3	4	5	6
Saturated						
$C_{12:0}$	7.8	5.8	8.9	6.9	9.6	5.2
$C_{14:0}$	3.5	3.6	2.1	3.7	5.7	3.4
$C_{16:0}$	**33.7**	**33.7**	**19.4**	**34.7**	**34.3**	**31.4**
$C_{17:0}$	ND	1.1	ND	ND	ND	ND
$C_{18:0}$	0.4	0.7	0.3	1.0	0.4	0.3
Unsaturated						
$C_{16:1}\omega5c$	0.2	ND	1.2	0.5	0.7	1.1
$C_{17:1}\omega6c$	ND	0.3	0.2	1.3	ND	ND
$C_{18:1}\omega7c$	**17.4**	**21.4**	**20.8**	**19.0**	**11.2**	**14.6**
Hydroxy						
$C_{12:0}$ 3-OH	5.3	4.2	6.1	0.3	6.1	3.3
$C_{16:0}$ 2-OH	ND	ND	1.1	ND	ND	1.9
Summed features*						
Summed feature 2	3.3	2.9	4.0	7.9	3.6	2.6
Summed feature 3	**27.5**	**25.4**	**33.8**	**24.2**	**27.6**	**35.3**

*Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI System. Summed feature 2 contained one or more of $C_{12:0}$ ALDE?, $C_{14:0}$ 3-OH and iso-$C_{16:1}$ I. Summed feature 3 contained $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH.

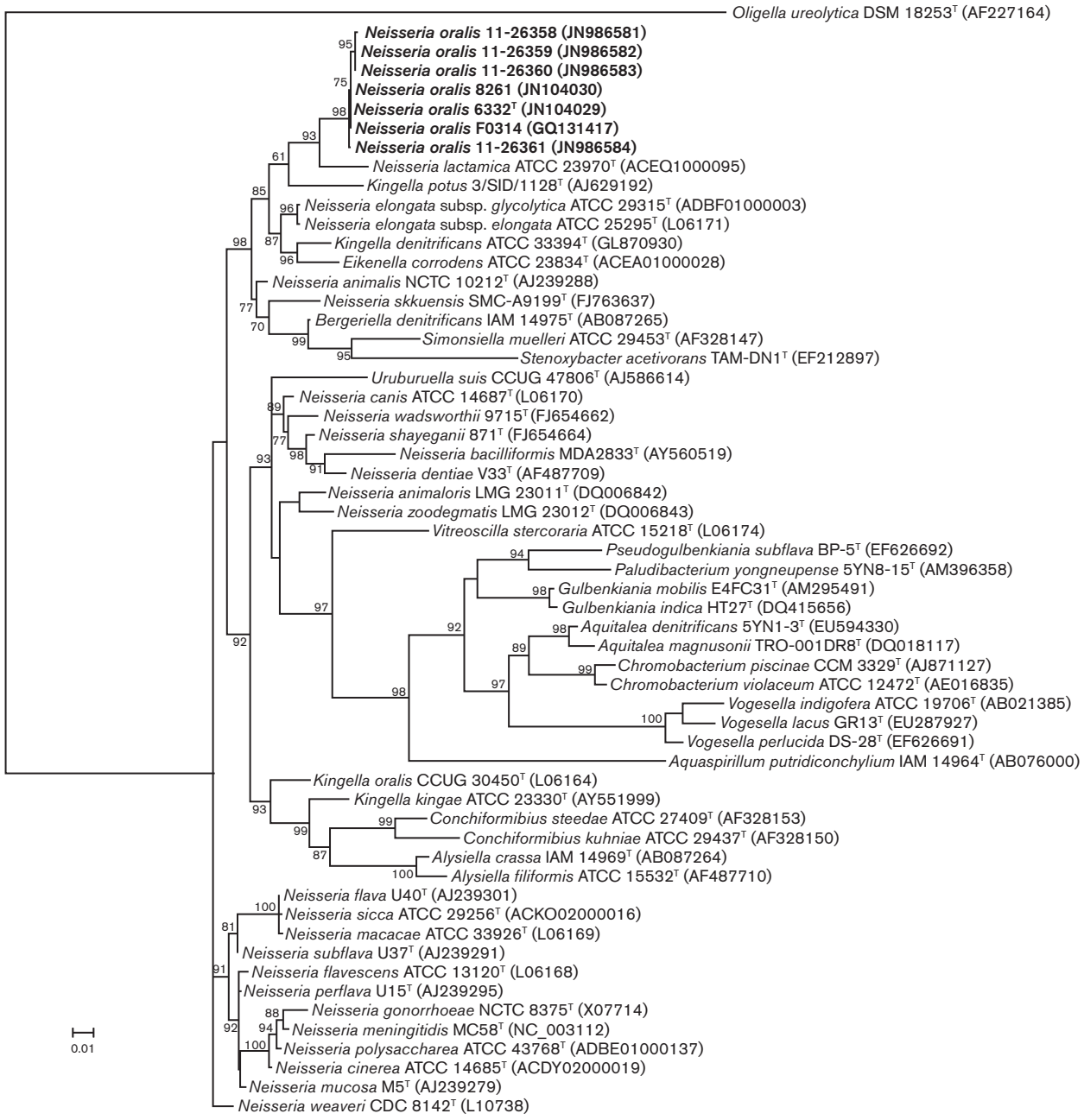

Fig. 1. Maximum-likelihood phylogenetic tree reconstructed from 16S rRNA gene sequences of strains of *N. oralis* sp. nov. and the 50 closest taxa returned from a BLAST search of the EzTaxon database. The tree was rooted with *Oligella ureolytica* DSM 18253^T. Branch probability is indicated at nodes and was determined by the aLRT; probabilities below 50 % are not shown. Bar, 0.01 expected changes per site.

D-glucose, maltose and sucrose (Tables 3 and S3) [for methods, see Forbes *et al.* (1998) and Kohlerschmidt *et al.* (2009)]. Other species, not analysed in this study, that may also be excluded on the basis of the absence of nitrate reduction are *N. gonorrhoeae*, *N. cinerea*, *N. dentiae*, *N. flavescens*, *N. meningitidis*, *N. subflava*, *N. weaveri* and *N. polysaccharea* (Janda & Gaydos, 2007; Tønjum, 2005). For the remaining taxa that reduce nitrate, with the exception of *Neisseria mucosa* and *N. elongata* subsp. *nitroreducens*, the absence of acid production from glucose, maltose and/or sucrose is diagnostic. For *N. mucosa* and *N. elongata* subsp. *nitroreducens*, the presence of large mucoid colonies or rod-shaped cells, respectively, are diagnostic.

Based on the 16S and 23S rRNA gene sequence comparisons, DNA–DNA hybridization, CFA composition and

Table 3. Characteristics that distinguish *N. oralis* sp. nov. from closely related species

Strains/species: 1, *N. oralis* sp. nov. 6332^T; 2, *N. elongata* subsp. *elongata* LMG 5124^T; 3, *N. sicca* LMG 5290^T; 4, *N. animalis* LMG 26609^T; 5, *N. lactamica* LMG 26610^T; 6, *N. macacae* LMG 26611^T; 7, *N. gonorrhoeae* [data from Janda & Gaydos (2007) and the bioMérieux package insert]. Data were obtained in this study unless indicated otherwise. ND, No data available. Characteristics listed for 6332^T were the same for all seven strains of *N. oralis* sp. nov. (see Table S3).

Characteristic	1	2	3	4	5	6	7
Acid production (peptone water) from:							
D-Glucose	+	−	−	−	+	+	+
Maltose	+	+	+	−	+	+	−
Nitrate reduction	+	−	−	−	−	−	−
Catalase reaction	+	+	+	+	+	−	+
Acid production (API NH) from:							
D-Glucose	+	−	+	−	+	−	+
Maltose	+	−	−	−	−	−	−
Sucrose	+	−	−	+	−	−	−
Enzyme activity (API NH)							
Proline 4-methoxy-β-naphthylamide	+	+	+	−	+	+	+
γ-Glutamyl 4-methoxy-β-naphthylamide	+	−	−	−	−	+	−
Enzyme activity (API ZYM)							
Esterase (C4)	−	+	−	+	+	+	ND
Acid phosphatase	−	+	+	+	+	+	ND

phenotypic and phylogenetic analyses, we propose that the seven novel strains (Table 1) represent a single novel species, *Neisseria oralis* sp. nov.

Description of *Neisseria oralis* sp. nov.

Neisseria oralis (o.ra′lis. N.L. fem. adj. *oralis* of the mouth, the source of the first isolate).

Colonies are small, circular, entire, raised, moist, yellow, weakly α-haemolytic (except 11-26358, which is β-haemolytic) and 1–1.5 mm in diameter after 48 h of growth at 37 °C in 5% CO_2. Facultative anaerobe. Growth is observed at 28 and 42 °C, with no growth at 10 °C. No growth is observed on MacConkey agar after 5 days. Cells are Gram-negative, 0.5 μm in diameter, may be present in chains and are non-motile. Produces catalase and cytochrome oxidase and reduces nitrate to nitrite. Negative for acid production using Hugh & Leifson O-F base with 1% D-mannitol and D-xylose and displays interstrain variability (see Table S3 for details) for D-glucose (6/7 positive; type strain positive), maltose (6/7 positive; type strain positive), lactose (2/7 positive; type strain negative) and sucrose (5/7 positive; type strain positive). Positive for acid production in peptone water base with 1% D-glucose and maltose and negative for acid production from adonitol, L-arabinose, dulcitol, inositol, D-mannitol, raffinose, D-rhamnose, D-salicin, D-sorbitol and D-xylose; displays interstrain variability for acid production from lactose (3/7 positive; type strain negative) and sucrose (6/7 positive; type strain positive). Negative for utilization of Simmons' citrate, hydrolysis of aesculin, urea and gelatin, indole production, decarboxylation of arginine, lysine and ornithine using Moeller's decarboxylase medium and production of H_2S in triple-sugar iron agar [for methods, see Forbes *et al.* (1998) and Kohlerschmidt *et al.* (2009)]. Using the API NH system (bioMérieux), positive for acidification of D-glucose, maltose and sucrose and for the presence of proline arylamidase and γ-glutamyl transferase activities and negative for penicillinase, lipase, alkaline phosphatase and indole production; displays interstrain variability for acidification of D-fructose (6/7 positive; type strain negative), ornithine decarboxylase (1/7 positive; type strain negative), urease (2/7 positive; type strain negative) and β-galactosidase (2/7 positive; type strain negative). Using the API ZYM system (bioMérieux), displays interstrain variability for leucine arylamidase (3/7 positive; type strain positive) and β-galactosidase (2/7 positive; type strain negative) and is negative for all other activities tested in the kit. The DNA G+C content of the type strain is 52.6 mol%. The predominant CFAs are $C_{16:0}$, summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH) and $C_{18:1}\omega7c$.

The type strain, 6332^T (=LMG 26725^T =DSM 25276^T), was isolated from whole blood of a 55-year-old male in New York, USA. Details of other strains are given in Table 1.

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