**Neisseria dumasiana** sp. nov. from human sputum and a dog’s mouth

Danielle Wroblewski,†‡ Jocelyn Cole,† Jana McGinnis,† Maria Perez,‡ Harriet Wilson,§ Lisa A. Mingle,† Kimberlee A. Musser†, and William J. Wolfgang†,‡,*

**Abstract**

Three independent isolates of Gram-reaction-negative cocci collected from two New York State patients and a dog’s mouth in California were subjected to a polyphasic analysis. The 16S rRNA gene sequence similarity among these isolates is 99.66 to 99.86%. The closest species with a validly published name is *Neisseria zoodegmatis* (98.7% 16S rRNA gene sequence similarity) with six additional species of the genus *Neisseria* with greater than 97% similarity. Average nucleotide identity (ANI) and genome-to-genome distance calculator (GGDC 2.0) analysis on whole genome sequence data support the three novel isolates as being from a single species that is distinct from all other closely related species of the genus *Neisseria*. Phylogenetic analysis of 16S rRNA gene sequences and ribosomal multilocus sequence typing (rMLST) indicate the novel species belongs in the genus *Neisseria*. This assignment is further supported by the predominant cellular fatty acids composition of C16:0, summed feature 3 (C15:0 iso 2-OH), and C18:ω7c, and phenotypic characters. The name *Neisseria dumasiana* sp. nov. is proposed, and the type strain is 93087T (=DSM 104677T=LMG 30012 T).

Between 2009 and 2012 the Wadsworth Center received two clinical isolates (designated 93087T and 124861) from New York State patients and one non-clinical isolate (114725 from a dog’s mouth) (Table 1). 16S rRNA gene sequences were determined as previously described [1]. Using nearly full-length 16S rRNA gene sequences (1463 nt), the three strains had a sequence similarity between 99.66 and 99.86%. Based on this finding we performed a polyphasic analysis to determine if the three isolates represented a single novel species and to further characterize that species.

A search for closely related species was performed with the 16S rRNA gene sequence from strain 93087T at the EZBioCloud website (http://www.ezbiocloud.net) against the database of type strains with validly published prokaryotic names [2]. The search returned seven species of the genus *Neisseria* with 16S rRNA gene similarities greater than 97%: *Neisseria zoodegmatis*, *N. shayeganii*, *N. animaloris*, *N. canis*, *N. dentiae*, *N. wadsworthii*, and *N. weaveri* (similarities of 98.7, 98.3, 98.1, 98.0, 97.8, 97.5 and 97.3%, respectively). For organisms with 16S rRNA gene sequence similarities greater than 97% it is recommended that an alternative assay be employed to determine inclusion or exclusion from a species [3].

Strains of closely related species of the genus *Neisseria* and the three novel isolates were cultured on chocolate agar plates (Becton Dickinson) supplemented with 10% horse blood at 37°C in a 5% CO2 atmospheric chamber.

DNA was extracted from isolates using the DNeasy Blood and Tissue kit (Qiagen). Library preparation was performed using a Nextera XT kit (Illumina) and whole-genome sequencing (WGS) was performed on a MiSeq (Illumina) as previously described [4].

Shotgun sequence reads were deposited in the short read archive at NCBI (BioProject PRJNA 359492) (Table 1). De novo assembly of the genomes was performed using SPAdes software implemented at BaseSpace (Illumina). Annotation was performed at NCBI [5]. WGS assemblies...
for *Neisseria* strains 09387ᵀ, 114725 and 124861 were deposited at GenBank/EMBL/DDBJ under the accession numbers MTAC00000000, MTAA00000000 and MTAB00000000 respectively (Table 1). WGS assemblies for the type strains of *N. canis*, *N. zoodegmatis*, *N. dentiae* and *N. animaloris* were also deposited at GenBank/EMBL/DDBJ under the accession numbers MTBL00000000, MTBM00000000, MTB00000000 and MTBN00000000, respectively. The genome sequences of strains *N. wadsworthii* 9715ᵀ, *N. shayeganii* 871¹, and *N. weaveri* NCTC 13585 used for whole-genome comparisons are available in the GenBank/EMBL/DDBJ database under the accession numbers AGAZ00000000, AGAY00000000 and LT571436 respectively.

Genomic relatedness was determined using average nucleotide identity (ANI, implemented at http://www.ezbiocloud.net) and genome-to-genome distance calculator (GGDC) 2.0 (implemented at http://ggdc.dsmz.de/distcalc2.php) [2, 6]. ANI and GGDC values below 96 % and 70 %, respectively, are appropriate for species delineation [7, 8]. The ANI distance between the three novel strains was 96.7 to 97.7 %, whereas the distance to the seven most closely related species with validly published names was 74.9 to 89.0 % (Table S1, available in the online Supplementary Material). The GGDC values among the three novel strains ranged from 77.5 to 80 %, whereas the distance to the seven closely related species was 25.2 to 40.4 % (Table S2). Taken together these results indicate that the three novel isolates represent a single novel species that is distinct from the seven most closely related species of the genus *Neisseria* with validly published names.

Phylogenetic analysis was performed using nearly full-length 16S rRNA gene sequences for all species with validly published names with a sequence similarity of >92 % to strain 93087ᵀ returned from the EzBioCloud database. Sequences were aligned using ClustalW implemented in MEGA 7.0 software package [9]. To remove poorly aligned positions and divergent regions, GBlocks 0.91b was implemented at Phylogeny (http://phylogeny.lirmm.fr/phylo.cgi/index.cgi), using default settings [10]. An unrooted maximum-likelihood tree was produced with MEGA 7.0 using the Tamura–Nei model, with 100 bootstrap replications to establish branch reliability (Fig. 1). The three novel isolates are most closely related to several species of the genus *Neisseria*, but branch support is generally low and does not necessarily support inclusion of the novel species in the genus *Neisseria*. Several other organisms in the genus *Neisseria* are also poorly distinguished by this method [11].

To improve phylogenetic resolution, the ribosomal multilocus sequence typing (rMLST) scheme of Jolley and Maiden [12, 13] was implemented at the *Neisseria* Multilocus Sequence Typing website (http://pubmlst.org/neisseria/). The genomic sequences for the three novel isolates were uploaded to http://pubmlst.org/neisseria/ where the rMLST alleles were extracted, concatenated and aligned to rMLST sequences from 57 species of the genus *Neisseria* and other closely related genera. GBlocks 0.91b was implemented as described in the previous paragraph. An unrooted maximum-likelihood tree was created using the online version of PhyML 3.0 (http://atgc.lirmm.fr/phyml) [14]. Branch reliability was evaluated using the approximate likelihood ratio test (aLRT) [15] (Fig. 2). The three novel isolates reside in a strongly supported clade harbouring only species of the genus *Neisseria*. This clade includes four of the most closely related species (*N. zoodegmatis*, *N. animaloris*, *N. dentiae* and *N. weaveri*) supporting placement of the novel species in the genus *Neisseria*. Interestingly, the other three most closely related species (*N. wadsworthii*, *N. canis* and *N. shayeganii*) reside in a clade that includes organisms from the genera *Eikenella*, *Simonsiella* and *Kingella*. As previously described, these results support the need for a more comprehensive assessment of phylogenetic relationships in the family *Neisseriaceae* by whole-genome sequencing [11].

Cellular fatty acids (CFA) analysis was performed on the three novel strains and the seven most closely related strains. The isolates 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 as described by the Sherlock Microbial Identification Systems (MIDI) and identified on an Agilent Technologies 6890N gas chromatograph. For the type strain 93087ᵀ, the predominant CFA were C₁₀:₀, summed feature 3 (C₁₆:₁ω₇c/15:₀ iso 2-0H), and C₁₈:₁ω₇c. Except for *N. shayeganii*, the predominant CFA for the most closely

<table>
<thead>
<tr>
<th>Strain ID</th>
<th>Source</th>
<th>Date isolated</th>
<th>NCBI accession number for</th>
<th>rMLST* accession</th>
</tr>
</thead>
<tbody>
<tr>
<td>93087ᵀ</td>
<td>Sputum, 79 year-old female; Saratoga County, NY, USA</td>
<td>6/25/09</td>
<td>KY417828 MTAC00000000 SAMN06192246</td>
<td>40199</td>
</tr>
<tr>
<td>114725</td>
<td>Domestic dog mouth, Placer County, CA, USA</td>
<td>9/1/10</td>
<td>KY417829 MTAA00000000 SAMN06192247</td>
<td>40200</td>
</tr>
<tr>
<td>124861</td>
<td>Sputum, 76 year-old male; Broome County, NY, USA</td>
<td>2/10/12</td>
<td>KY417830 MTAB00000000 SAMN06192248</td>
<td>40201</td>
</tr>
</tbody>
</table>

*Accession for *Neisseria* PubMLST database [13].
related species were the same (Table 2). The unusual CFA profile for \textit{N. shayeganii} was observed and discussed previously [1]. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS data were obtained for strain 93087, as previously described [4]. Strain 93087 has prominent peaks...
Fig. 2. Maximum-likelihood phylogenetic tree using an rMLST scheme alignment [12] for the three strains of *Neisseria dumasiana* sp. nov. and 57 additional taxa. Branch probabilities >50% are shown at the nodes and are determined by the approximate likelihood ratio test (aLRT) [15]. Bar, expected changes per site.
at m/z 7080.052, 7695.846 and 8312.158 that were not present in the spectra of the most closely related type strain *N. zoodegmati* *s* DSM 21643$^T$ (Fig. S1).

Biochemical and phenotypic characteristics that distinguish the three novel strains from the seven most closely related species examined in this study are the mass spectra, the ability to reduce nitrate, the absence of decarboxylation of arginine, grey colonies, acid production from *O-F* sugars, and sucrose, large mucoid colonies or rod-shaped cells are present or absent. For species of the genus *Neisseria* not included in this study, the three novel strains can be distinguished from *Neisseria gonorrhoeae*, *N. cinerea*, *N. flavescens*, *N. meningitidis*, *N. subflava* and *N. polysacchara* by the ability to reduce nitrate to nitrite [18, 19]. For species of the genus *Neisseria* that reduce nitrate, the presence or absence of acid production from glucose, maltose and sucrose, large mucoid colonies or rod-shaped cells are diagnostic.

The ANI and GGDC indicate the three novel strains represent a single species that is distinct from all closely related species. Furthermore, the rMLST phylogenetic analysis, CFA composition, and biochemical characters show these

### Table 2. Fatty acid methyl esters content (%) for the three strains of the novel species and the type strains of the seven most closely related species

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8*</th>
<th>9*</th>
<th>10*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid production from O-F sugars</td>
<td>Sucrose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acid production from peptone waters</td>
<td>Glucose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>Malate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Decarboxylation of arginine</td>
<td>SAC (sucrose utilization)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>API NH activities</td>
<td>PROA (proline isomerase activity)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Colony pigmentation</td>
<td>G</td>
<td>G</td>
<td>Y/G</td>
<td>Y</td>
<td>Y</td>
<td>G</td>
<td>G</td>
<td>Y/G</td>
<td>G</td>
<td>G</td>
</tr>
</tbody>
</table>

*Data from Wolfgang et al. [20].
†Summed features represent groups of two or more fatty acids that could not be separated using the MIDI system. Summed feature 2 is composed of C$_{16:0}$ 3-OH/C$_{16:1}$ iso 2-OH.
isolates reside in the genus Neisseria. Based on these findings, we conclude that the three isolates represent a single novel species of the genus Neisseria, for which the name Neisseria dumasiana sp. nov. is proposed.

**DESCRIPTION OF NEISSERIA DUMASIANA SP. NOV.**

*Neisseria dumasiana* (du.mas.i.a’na. N.L. fem. adj. dumasiana of Dumas, to honour Nellie Dumas for over 40 years of service to advancing the practice of clinical and public health microbiology in New York State).

After 48 h of growth at 37 °C in 5% CO₂, colonies are 1.9 to 2.8 mm, grey, moist, circular, convex, entire, non-haemolytic and are facultative anaerobes. Growth is observed from 22 to 42 °C with optimal growth at 37 °C and no growth at 4 °C. Growth occurs in the presence of 2% NaCl but not with 5% or higher NaCl in brain heart infusion broth. Growth occurs in a pH range of 6 to 9.5 with optimum growth at pH 7.2. No growth occurs on MacConkey agar after 10 days of observation. Cells are Gram-negative, cocoid to coccobacilli, 0.8 µm in diameter, may be present in pairs, and are non-motile. Cells produce catalase and cytochrome oxidase, and they reduce nitrate to nitrite. Cells are negative for acid production using Hugh and Leifson Oxidative-Fermentation base (O-F) with 1% lactose, D-mannitol, maltose, sucrose and D-xylene, and display inter-strain variability for D-glucose (one of three strains is positive; type strain is negative). Cells are positive for acid production in peptone water base with 1% D-glucose; negative for acid production from lactose, maltose, D-mannitol, sucrose and D-xylene. Cells are negative for utilization of D-fructose, maltose and D-glucose, penicillinase, lipase, alkaline phosphatase, α-glutamyl-transferase activity, ornithine decarboxylase, urease and β-galactosidase. The predominant cellular fatty acids are C₁₆:0, summed feature 3 (C₁₆:1ω7c/C₁₅:0 iso 2-OH) and C₁₈:1ω7c.

The type strain, 93087T (=DSM 104677T=LMG 30012T) was isolated from sputum of a 79-year-old female in New York State, USA. The DNA G+C content of the type strain is 50.5 mol%.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**References**


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