Isolation and characterization of *Neisseria musculi* sp. nov., from the wild house mouse

Nathan J. Weyand,† Mancheong Ma,† Megan Phifer-Rixey,‡ Nyiawung A. Taku,† María A. Rendón,† Alyson M. Hockenberry,† Won J. Kim,† Al B. Agellon,§ Nicolas Biais,‡ Taichi A. Suzuki,‡ Lily Goodyer-Sait,§ Odile B. Harrison,§ Holly B. Bratcher,§ Michael W. Nachman,§ Martin C. J. Maiden,§ and Magdalene So†

1Department of Immunobiology and BIO5 Institute, University of Arizona, Tucson, AZ 85719, USA
2Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, AZ 85721, USA
3School of Animal and Comparative Biomedical Sciences, University of Arizona, Tucson, AZ 85721, USA
4Department of Biology, Brooklyn College of the City University of New York, Brooklyn, NY 11210, USA
5Department of Zoology, University of Oxford, Oxford OX1 3PS, UK

Members of the genus *Neisseria* have been isolated from or detected in a wide range of animals, from non-human primates and felids to a rodent, the guinea pig. By means of selective culture, biochemical testing, Gram staining and PCR screening for the *Neisseria*-specific internal transcribed spacer region of the rRNA operon, we isolated four strains of the genus *Neisseria* from the oral cavity of the wild house mouse, *Mus musculus* subsp. *domesticus*. The isolates are highly related and form a separate clade in the genus, as judged by tree analyses using either multi-locus sequence typing of ribosomal genes or core genes. One isolate, provisionally named *Neisseria musculi* sp. nov. (type strain AP2031T=DSM 101846T=CCUG 68283T=LMG 29261T), was studied further. Strain AP2031T/*N. musculi* grew well *in vitro*. It was naturally competent, taking up DNA in a DNA uptake sequence and pilT-dependent manner, and was amenable to genetic manipulation. These and other genomic attributes of *N. musculi* sp. nov. make it an ideal candidate for use in developing a mouse model for studying *Neisseria*-host interactions.

The family *Neisseriaceae*, a large family of Gram-negative bacteria, are most well known for two members that cause diseases of importance to human health, *Neisseria meningitidis* and *Neisseria gonorrhoeae*. The other members of the family are commensals of humans and other animals. *N. meningitidis* and *N. gonorrhoeae* also exhibit commensal-like behaviour in that they establish asymptomatic infections in man at high frequency (Caugant & Maiden, 2009; Read, 2014; Turner et al., 2002). This behaviour may be due to traits they inherited from their commensal forebears as they evolved a more pathogenic lifestyle (Marri et al., 2010).

At least ten commensal species of the genus *Neisseria* are known to colonize humans, and an equal number or more have been isolated from, or detected in, a wide range of animals (Bennett et al., 2014a; Liu et al., 2015). Commensal species of the genus *Neisseria* have been isolated from the oropharynx and urogenital tract of man, the oral cavity and throat of guinea pigs, the liver and faeces of ducks, the oral cavity and dental plaque of cows, the upper respiratory tract and lung of dogs, and the oral cavity of a rhesus macaque (see Table 2 in Liu et al., 2015; Barrett & Sneath, 1994; Bennett et al., 2014a; Murphy et al., 2005; Weyand et al., 2013).
Many aspects of Neisseria–host interactions can be more readily dissected using a small animal model, for example, by pairing the genetically tractable mouse, Mus musculus, with a species of the genus Neisseria that naturally colonizes the animal. We therefore sought to isolate a mouse-dwelling strain of the genus Neisseria. Through selective culturing, biochemical testing and genotyping, we obtained four isolates of the genus Neisseria, representing a novel species in the genus, from the oral cavity of wild mice (Mus musculus subsp. domesticus) trapped in two geographically distinct regions in North America. One isolate, AP2031T, was chosen for further study. AP2031T grew well in vitro. It was naturally competent; DNA transformation was greatly enhanced by the presence of the neisserial DNA uptake sequence (DUS) and by an intact pilT gene. We propose that strain AP2031T be classified as the type strain of Neisseria musculi sp. nov.

Oral swabs were collected from 36 healthy wild mice (M. musculus subsp. domesticus) live-trapped in two distinct regions of North America (Table S1, available in the online Supplementary Material). A subset of these mice (TAS216–TAS220) was sampled immediately for members of the genus Neisseria, then humanely euthanized for use in another study. All other mice were brought back to the lab and housed individually or in breeding pairs in static microisolation cages, with food and water ad libitum. Cages were changed weekly or biweekly in a laminar flow hood following standard protocols to minimize cross-cage contamination. After initial breeding, all animals were housed individually or in same-sex pairs for at least 6 weeks to ensure eradication of mouse pathogens that pose a risk to animal care facilities. Serologically negative mice were made available for sampling organisms of the genus Neisseria. All collection and husbandry activities were conducted in accordance with University of Arizona IACUC policies.

Mice that had passed the quarantine period were sampled for members of the genus Neisseria. The oral cavity was swabbed using the BD BBL CultureSwab Plus Transport System (Fisher Scientific). To enrich for growth of bacteria of the genus Neisseria, swab suspensions in GCB medium base (Beckton Dickinson) were plated on GCB agar containing vancomycin (2 mg l−1) and trimethoprim (3 mg l−1), and the plates were incubated for 48 h at 37°C, 5% CO2.

To identify and speciate members of the genus Neisseria, we proceeded as follows. A portion from each colony growing on GCB vancomycin/trimethoprim agar was used for oxidase testing as described by Chesbrough (2006) using oxidase reagent (PML Microbiologicals). Oxidase+ colonies were Gram-stained, and the internal transcribed spacer (ITS) region in the rRNA operon of Gram− colonies was amplified by colony PCR, using primers specific to sequences that are highly conserved among species of the genus Neisseria (see Table S2 for primers). ITS+ colonies were streak-purified, assigned a strain number, and stored at −80°C in GCB+glycerol (20%, v/v). We identified four isolates that were oxidase+, Gram− and Neisseria ITS+. Two of these isolates, AP2031T and AP2119, were from mice trapped in Tucson, Arizona, USA (TUSA8 and TAS218, respectively), and two isolates, AP2104 and AP2105, were from mice trapped in a haystack near Parkland, Alberta, Canada (TAS116 and TAS118, respectively) (Table S1). To further characterize these four isolates, their rplF sequences were determined and compared with those of other species of the genus Neisseria. A neighbour-joining tree was drawn with rplF sequences using MEGAS (Tamura et al., 2011). All three codon positions were included. Bootstrap support values (2000 replicates) greater than 70% were added to nodes. Sequence comparisons of rplF, an approach that distinguishes closely related species in the genus Neisseria (Bennett et al., 2014b), indicated the four isolates were tightly clustered in a separate clade (Fig. 1a). Surprisingly, they were not as related to Neisseria animalis, a guinea pig isolate, as they were to Neisseria dentiae, a cow dental plaque isolate (Berger, 1960; Sneath & Barrett, 1996).

We conducted average nucleotide identity (ANI) analysis for all four mouse isolates using BioEdit 7.2.5 (Hall, 1999). Only three polymorphic sites were detected in the rplF fragment sequenced. Since the four isolates share 99.4% sequence identity, we conclude they belong to the same species, N. musculi sp. nov. This conclusion is based on the fact that rplF gene trees are more concordant with trees based on multi-locus sequence typing (MLST) of ribosomal genes (rMLST), the gold standard for identification of species of the genus Neisseria, whereas 16S rRNA gene trees lack such resolution (Bennett et al., 2014b).

The genome of isolate AP2031T, from Tucson mouse TUSA8, was sequenced at the Oxford Genomic Center, Wellcome Trust Centre for Human Genetics, University of Oxford, UK. Samples were quantified using PicoGreen (Invitrogen), and their integrity was assessed using 1% E-Gel EX (Invitrogen). DNA was fragmented using the Episonic system and sized using a Tapestation D1200 system (Agilent/Lab901). Libraries were constructed with a NEBNext DNA Sample Prep Master Mix Set 1 kit (New England BioLabs), and adaptors were ligated using Illumina Adapters (Multiplexing Sample Preparation Oligonucleotide kit). Ligated libraries were size-selected using AMPure magnetic beads (Agencourt), PCR-enriched for 10 cycles as per Illumina recommendations, and purified with AMPure XP beads (Agencourt). Size distribution was determined using a Tapestation 1DK system (Agilent/Lab901), and multiplex pool concentrations were determined by PicoGreen (Invitrogen). Pooled libraries were quantified using the quantitative PCR Library Quantiﬁcation kit and MX3005PTM instrument (Agilent). Sequencing was performed using the Illumina HiSeq 2000 system to generate 100 nt paired-end reads. Resulting short-read sequences were assembled de novo using the VelvetOptimiser algorithm as part of an in-house pipeline developed at the Department of Zoology, University of Oxford. The minimum output contig size was set to 200 bp with the scaffolding option switched off; all other program settings were left at default. No read trimming was performed. Draft genome sequences (ID 29520) were deposited in the Neisseria pubmlst.org database (www.pubmlst.org/neisseria). The
Fig. 1. (a) Evolutionary relationship of mouse Neisseria isolates AP2031T (in grey box), AP2119, AP2104 and AP2105, to 50 isolates of the genus Neisseria, deduced from a neighbour-joining tree reconstructed from their rplF sequences. Bootstrap values ≥70% are noted (2000 replications). Bar, 0.04 substitutions per nucleotide position. PubMLST ID or GenBank accession numbers proceed the isolate and species notations. Taxa included for rplF comparisons were previously analysed by Bennett et al. (2014b). (b) Neighbour-net tree reconstructed from 51 concatenated ribosomal gene sequences, with AP2031T highlighted in a grey box. Bar, 0.01 substitutions per nucleotide position.
DNA G+C content of AP2031\textsuperscript{T} was 53.3 mol%, consistent with other species in the genus (Tonjum, 2005).

A detailed analysis of AP2031\textsuperscript{T} genome content will be presented in a future paper. Genomic data from AP2031\textsuperscript{T} was compared with a representative isolate dataset containing all known species of the genus \textit{Neisseria} for which whole-genome sequencing data were available (Bennett et al., 2012). MLST of 51 ribosomal genes (rMLST) (Fig. 1b) and 246 \textit{Neisseria} core genes (cgMLST) (Fig. S1) was used to generate trees as described previously, enabling relationships between isolates to be determined and speciation (Bratcher et al., 2014; Jolley et al., 2012). Sequences were extracted from the bacterial isolate genome sequence database (BIGSdb) (Jolley & Maiden, 2010), aligned using MAFFT and imported into Splitstree thereby generating neighbour-net trees. Nucleotide sequences used for rMLST and cgMLST are listed in Table S3. These analyses confirmed the \textit{rplF} findings on the evolutionary relationship of AP2031\textsuperscript{T} to other species of the genus \textit{Neisseria}.

\textit{rplF}, rMLST and cgMLST phylogenetic analysis revealed AP2031\textsuperscript{T} to be most closely related to \textit{N. dentiae}. The \textit{rplF} ANI between AP2031\textsuperscript{T} and \textit{N. dentiae} was 85.1%. Other members of the genus, as expected, had lower ANI values (see Table S4). The ANI calculator at http://enve-omics.ce.gatech.edu/ani/ was used to estimate the ANI between the AP2031\textsuperscript{T} and \textit{N. dentiae} draft genome data sets. This tool calculates ANI as described by Goris et al. (2007). Consistent with the \textit{rplF} analysis, an ANI value of 86.8% was obtained for the draft genomes of AP2031\textsuperscript{T} and \textit{N. dentiae}. Given that ANI values lower than 95% delineate the genomes of different species (Goris et al., 2007), our findings further indicate AP2031\textsuperscript{T} and the other mouse \textit{Neisseria} isolates represent a novel species in the genus \textit{Neisseria}.

During passage on agar plates, \textit{N. musculi} sp. nov. (type strain AP2031\textsuperscript{T}) gave rise to colonies with smooth and rough phenotypes (Fig. S2). Images of colony morphology were acquired using a Cannon SLR digital camera attached to a Wild M7 zoom microscope (Heerbrugg, Switzerland) mounted on a Bausch and Lomb stage. All colonies of AP2031\textsuperscript{T} were circular and convex, and varied in opacity. Smooth colonies had shiny, unwrinkled surfaces, while rough colonies had bumpy, undulating surfaces. These phenotypes were interchangeable: smooth colony variants would give rise to rough colony variants and vice versa (data not shown). This process is reminiscent of colony phase variation observed for \textit{N. gonorrhoeae}, which is caused by variable expression of surface components (Bhat et al., 1991; Long et al., 1998; Swanson & Barrera, 1983; Swanson et al., 1971; Walstad et al., 1977). The mechanism underlying AP2031\textsuperscript{T} colony phase variation is under investigation.

Smooth and rough variants formed biofilms when cultured on glass, and scanning electron microscopy revealed their distinct morphologies. Scanning electron microscopy was performed on AP2031\textsuperscript{T} cells as described by Higashi et al. (2011). Biofilms formed by the rough variant were taller and contained multiple layers of cells (Fig. 2). Rough variant cells are best described as diplococcobacilli: short rods often found in pairs. The rods were approximately 0.4 µm in width and 0.5–0.8 µm in length (Fig. 2a, c, e, g). The
**Fig. 2.** Scanning electron microscopy images of cells of strain AP2031\(^T\) shown as side (a–d) and aerial (e–i) views. Images of rough (a, c, e, g) and smooth (b, d, f, h, i) colony variants after 3 h of growth on glass slides are shown. Boxed sections of images are shown at higher magnification in adjoining panels (c, d, e, f, i). White arrows in panel (i) indicate indentations along sheaths. Acquisition voltage, distance (mm) and scale bar are indicated.
shape of smooth variant cells was difficult to discern. Many appeared to be encased in sheaths of varying length with occasional indentations along their sides (Fig. 2b, d, f, h, i and S3). Several commensal species of the genus Neisseria are rod-shaped (e.g. Neisseria elongata, Neisseria weaveri, Neisseria shayeganii, Neisseria bacilliformis, Neisseria zoodegmati, Neisseria animaloris) (Ganiere et al., 1995; Liu et al., 2015; Veyrier et al., 2015), but there are no reports, to our knowledge, of sheath-like structures associated with cells of members of the genus Neisseria. The surfaces of smooth and rough variants were decorated with fibres, with rough variant fibres being shorter and less abundant. The nature of these fibres remains to be determined.

All species of the genus Neisseria studied to date are competent for DNA transformation. Playing important roles in this process are the Type IV pilus (Tfp) and the DUS, a 10 nt sequence, 5′-GCCGTCTGAA-3′, that is highly enriched in neisserial genomes. Tfp-mediated DNA transformation involves, among other things, the binding of the DUS to Tfp-associated protein ComP, and PiT-driven retraction of the Tfp (Cehovin et al., 2013; Hamilton & Dillard, 2006; Wolfgang et al., 1998). Two DUS variants, distinguished by two bases immediately preceding the 10-mer, are abundant in members of the genus Neisseria. The surfaces of smooth and rough variants were decorated with fibres, with rough variant fibres being shorter and less abundant. The nature of these fibres remains to be determined.

To determine the number of copies of DUS in AP2031 genome, and to extract the DUS and surrounding sequences into a fasta file for constructing the WebLogo, Perl regular expression pattern matching in combination with the fuzznuc program from EMBOSS was used (Crooks et al., 2004; Rice et al., 2000). There are 3893 copies of the 10-mer DUS in AP2031T, of which 2614 are agDUS and 119 are atDUS (Fig. S4). AP2031T has a complete set of genes for the biogenesis of the Tfp including pilT, which encodes the motor protein for the Tfp fibre (Table S5). The abundance of the neisserial DUS and the presence of Tfp genes strongly suggest that AP2031T is naturally competent for DNA transformation.

To test the competence of AP2031T, we determined the transformation frequency of the smooth variant in the presence of genomic DNA from AP2098, a naturally occurring streptomycin-resistant (SmR) variant. AP2098 was isolated by plating the original AP2031T strain on GCB plates containing streptomycin (100 mg L−1). Genomic DNA from AP2098 was used for transformation assays using previously described procedures (Weyand et al., 2013). Transformation mixtures were plated on GCB agar with and without streptomycin (100 mg L−1). Transformation frequency was expressed as the number of SmR c.f.u./total c.f.u. per µg of DNA. For some negative controls, DNA was incubated for 20 min with 2 units of DNAse I. The frequency was 1.7 × 10−4 (SEM±4.8 × 10−6) in the presence of DNA, and >3 logs lower in its absence or when the DNA was predigested with DNAse (Table 1).

To determine the role of the DUS in AP2031T transformation, cells were incubated with synthetic DNA amplified by PCR and encoding rpsL from AP2098 (SmR), with or without the 10-mer DUS 5′-GCCGTCTGAA-3′ (see Table S2 for primers). DNA (1 µg) was used for transformation assays, and transformation frequencies were calculated as described above. The transformation frequency was nearly 3 logs higher when the transforming DNA contained the DUS (Table 1).

Finally, we determined the role of pilT in AP2031T competence. Primers NP242F and NP242R (Table S2) containing flanking sequences for the AP2031T pilT gene were used to amplify a kanamycin cassette from plasmid pNBNeiKan (synthesized by Genescript) for use in constructing gene deletions (Datsenko & Wanner, 2000). A DUS and an Escherichia coli

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<th>Table 1. Transformation frequency of mouse Neisseria strains</th>
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gDNA: genomic DNA; rpsL: PCR amplicon of rpsL locus with SmR mutation; RifR, rifampicin-resistant.
*Transformation frequencies are expressed as the number of SmR c.f.u./total c.f.u. Values are the mean from three independent experiments±SEM.
uction frequency below the limit of detection.
AP2031 from mouse be classified as representatives of Neisseria (Bennett et al., 2012; Helaine et al., 2005; Kurre et al., 2012). This analysis is confirmed by trees of the Tucson isolate, AP2031 (Sneath & Barrett, 1998). AP2031 from the wild house mouse Neisseria musculi sp. nov. from the wild house mouse

By means of selective culture, oxidase testing, Gram staining and PCR testing for the Neisseria ITS, we isolated four strains of the genus Neisseria from the oral cavity of healthy wild mice (M. musculus subsp. domesticus) trapped in Tucson, Arizona, USA, and Parkland, Alberta Province, Canada. rplF sequence comparisons indicate they are very closely related to each other and form a distinct clade in the genus Neisseria. Their closest phylogenetic neighbour is N. dentiae, a bovine isolate (Fig. 1) (Sneath & Barrett, 1996). This analysis is confirmed by trees of the Tucson mouse isolate, AP2031T, based on MLST (Figs 1b and S1). We propose that the strains of the genus Neisseria isolated from mouse be classified as representatives of N. musculi sp. nov. (type strain AP2031T). The rMLST approach unambiguously identifies members of the genus Neisseria (Bennett et al., 2012). Our rMLST and core gene analyses indicate AP2031T/N. musculi is a member of the genus Neisseria. This assignment is supported by the DUS sequence analysis of AP2031T/N. musculi (Fig. S4).

AP2031T/N. musculi sp. nov. is naturally competent; DNA transformation is greatly enhanced by the DUS and pilT, which encodes the Tfp motor protein (Table S5). Tfp is a hallmark of the genus Neisseria. Tfp consists of a fibre, composed mainly of pilin subunits, that extends from the cell into the extracellular milieu, and machinery for its assembly and many biological activities (Carbonnelle et al., 2006; Giltner et al., 2012). In addition to DNA transformation (Cehovin et al., 2013; Wolfgang et al., 1998), Tfp plays important roles in other aspects of biology of the genus Neisseria, including bacterial attachment, motility, microcolony formation and signalling to the host cell (Eriksson et al., 2012; Helaine et al., 2005; Higashi et al., 2007; Howie et al., 2005; Kurre et al., 2012; Lee et al., 2005; Merz et al., 1996, 1999, 2000; Nassif et al., 1994; Pujol et al., 1999; Winther-Larsen et al., 2001; Wolfgang et al., 1998). That AP2031T/N. musculi harbour the genes necessary for the biosynthesis, assembly and anchoring of the Tfp fibre; for the retraction and mechanotransductive properties of the fibre; and for DNA transformation (Table S5) strongly suggest that it expresses Tfp that functions similarly to those of other species in the genus.

Pilins in Tfp of pathogenic members of the genus Neisseria are decorated with mono-, di- or tri-saccharides, and/or phosphorylated residues (Aas et al., 2006; Forest et al., 1999; Hartley et al., 2011; Naessan et al., 2008). These post-translational modifications are thought to contribute to Tfp bundling, neisserial interactions with human cells and protection of the bacteria from immune defenses (Chamot-Rooke et al., 2011; Jennings et al., 2011; Marceau et al., 1998). AP2031T/N. musculi sp. nov. encodes enzymes for the biosynthesis and covalent linkage of the basal monosaccharide to pilin (pglB, pglB2, pglC and pglD), but lacks the genes that add additional sugars to the monosaccharide (pglA and pglE). This suggests its pilin may be decorated with monosaccharides. It should be noted that pilins in N. elongata Tfp are not glycosylated even though the commensal harbors pgl genes (Anonsen et al., 2015). Whether this lack of glycosylation is due to control of gene expression is unclear. Finally, AP2031T/N. musculi lacks the genes (pptA and pptB) that phosphorylate pilin (Anonsen et al., 2012; Naessan et al., 2008).

In summary, we have isolated a novel species in the genus Neisseria from the oral cavity of wild mice. We determined the ability of one of these isolates, type strain AP2031T, to take up neisserial DNA, and sequenced its genome. AP2031T had the following characteristics that support its inclusion in the genus Neisseria. Firstly, it stained Gram-negative. Secondly, it had oxidase activity. Thirdly, genus-specific primers successfully amplified the 16S ITS region. Fourthly, rplF and MLST phylogenetic analysis determined that AP2031T belongs in the genus and is most closely related to N. dentiae, an established species of the genus Neisseria. Fifthly, AP2031T genome sequencing established that this isolate has over 3000 copies of the best characterized Neisseria-specific repetitive element, the DUS.

AP2031T/N. musculi sp. nov. grows readily in the lab and is amenable to genetic manipulation. These qualities of AP2031T, coupled with its genetic relatedness to other species of the genus Neisseria, make the isolate an excellent candidate for use in developing a mouse model for probing Neisseria-host interactions. Such a model will circumvent roadblocks imposed by host tropism, an issue that makes studying human-dwelling members of the genus Neisseria problematic. It will allow assessment of the role of the Type IV pilus in colonization by members of the genus Neisseria, and dissection of immune responses mounted by the host (the mouse) to the commensal species of the genus Neisseria.

Description of Neisseria musculi sp. nov.

Neisseria musculi (musculi L. gen. n. musculi of a mouse).

Cells are diplococcobacilli (approx. 0.5 to 0.8 µm in length) Gram-stain-negative and oxidase-positive. Good growth

http://jfs.microbiologyresearch.org
occurs on chocolate agar, TSA with 5% sheep blood and GCB agar. Colonies are small, circular, convex and vary between colony morphologies with margins that are entire (smooth colonies) or undulate (rough colonies). Colonies vary in opacity and are 0.5 to 1 mm in diameter after 48 h of growth in 5% CO₂ at 37°C on GCB agar. The DUS, GCCGTCTGAA, enriched in the type strain's genome confirms membership in the genus.

Type strain is AP2031ᵀ (≡DSM 101846ᵀ=CCUG 68283ᵀ=LMG 29261ᵀ) and was isolated from an oral swab of Mus musculus subsp. domesticus in Tucson AZ, USA. The type strain has a DNA G+C content of 53.3 mol%.

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References


