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Neisseria zalophi sp. nov., isolated from oral cavity of California sea lions (*Zalophus californianus*)

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Abstract
Three independent strains of *Neisseria* sp. were isolated from the oral cavity of California sea lions (*Zalophus californianus*) that were admitted to The Marine Mammal Center facilities in California, USA. The strains were isolated from oral swabs by cultivation on Trypticase Soy agar with 5% sheep blood under aerobic conditions. The 16S rRNA gene sequence of these three strains shared 99% similarity, but demonstrated only 97–98% nucleotide similarity to the phylogenetically closest relatives such as *N. canis, N. zoodegmatis, N. animaloris*, and *N. dumasiana*. These three strains also shared 99% sequence similarity of their *rplF, rpoB*, and *gyrB* gene sequences. Based on the biochemical tests alone (i.e., without genetic analysis of housekeeping genes), it is difficult to discriminate this novel species from *N. canis*; however, it can be easily discriminated from all phylogenetically closely related species using the sequencing analysis of its housekeeping genes (e.g., *rplF, rpoB*, or *gyrB* genes). Thus, genetic testing is indispensable for accurate identification of this species in a routine laboratory practice. The species is an obligate aerobe and able to grow in Mueller–Hinton broth supplemented with 6% NaCl, but the phylogenetically closely related species (*N. canis, N. zoodegmatis, N. animaloris*, and *N. dumasiana*) were not. Based on these phenotypic and genotypic characteristics and phylogenetic data, we conclude that these new strains represent a novel species of the genus *Neisseria*, for which the name *Neisseria zalophi* sp. *nov*. is proposed. The type strain is CSL 7565^T (= ATCC BAA2455^T = DSM 102031^T).

Keywords *Neisseria zalophi* sp. nov. · *Neisseria* · California sea lions · Phylogeny

Introduction

The genus of *Neisseria* contains 28 species and subspecies (http://www.bacterio.net/neisseria.html and https://www.ncbi.nlm.nih.gov/Taxonomy) that represent commensals of humans and other animals (Knapp 1988; Liu et al. 2015) and the well-known human pathogens, *Neisseria meningitidis* and *Neisseria gonorrhoeae*, which are responsible for meningococcal disease and gonorrhea, respectively (Virji 2009), and can also establish asymptomatic infections in humans. Except for these human pathogens, all other established *Neisseria* species have been isolated from healthy or sick animals (domesticated and wildlife), are mostly commensals, and are able to colonize the oropharynx, nasopharynx, tonsillar tissues, upper respiratory, and urogenital tracts

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(Cantas et al. 2011; Hansen et al. 2015; Liu et al. 2015; Wang et al. 2016; Wroblewski et al. 2017). During our other studies focusing on the isolation and phylogenetic characterization of *Mycoplasma* spp. isolated from different animal sources (Volokhov et al. 2011, 2012b), in 2007–2008, we isolated three independent strains of *Neisseria* sp. from oral cavity swabs of wild California sea lions (*Zalophus californianus;* the family *Otariidae*) that were admitted for treatment to The Marine Mammal Center facilities in Sausalito, California, USA. The California sea lions are coastal eared seals native to western North America, and their natural habitat ranges from southeast Alaska to central Mexico, including the Gulf of California (NOAA 2015). *Neisseria* microflora of California sea lions and other *Otariidae* species (and overall other marine mammals) remain to be elucidated, with only a few publications describing isolation or detection of *Neisseria mucosa, N. flavescens*, and unknown (cultivated and uncultured) *Neisseria* spp. in these hosts (Bik et al. 2016; Hernandez-Castro et al. 2005; Smith et al. 1978).

In this study, we report the isolation of a novel *Neisseria* species, which was isolated from the oral cavity of California sea lions and characterized using a polyphasic taxonomic approach, including sequence analysis of the 16S rRNA gene and other housekeeping genes and characterization of their biochemical and chemotaxonomic features. Based on the findings, we conclude that these isolates represent a novel species of the genus *Neisseria*, and which in comparison with all previously described established *Neisseria* species demonstrated significant nucleotide sequence differences for the analyzed multiple genetic loci.

Materials and methods

Isolation and cultivation of Neisseria strain

Oral swabs were collected from live California sea lions that were admitted to the Marine Mammal Center facilities in Sausalito, California, USA, in 2007–2008. Swabs were placed in sterile cryotubes and frozen at − 80 °C until laboratory analysis. In the laboratory, the swabs were used to inoculate sheep blood agar plates (Trypticase Soy agar [TSA] with 5% sheep blood; Becton Dickinson) using the quadrant streak method. The inoculated plates were incubated at 36 ± 1 °C in ambient air supplemented with 5% CO_2. Bacterial growth on plates was visibly observed after 2–3 days. Individual colonies from the agar plates were subcultivated to generate pure bacterial cultures. After triple sub-culturing from single colonies, the isolates were maintained in Difco Columbia broth (Becton Dickinson). These strains (CSL7565^T, CSL7624, and CSL7640) and the reference strains (*N. canis* DSM 18000^T, *N. zoodegmatis* ATCC 29859^T, and *N. animaloris* ATCC 29858^T) were routinely cultured on TSA with 5% sheep blood or in the Columbia broth and saved as bacterial stocks in the Columbia broth supplemented with 15% (v/v) glycerol at − 80 °C. Hereafter, in the following text, the indication of the species name only refers to the type strain of that species.

Strain CSL7565^T was isolated from oral swab collected on December 4, 2007 from a male/yearling captured at Santa Cruz, Santa Cruz County, California, and the animal died in treatment on December 5, 2007 due to pneumonia. Strain CSL7624 was isolated from an oral swab collected on May 1, 2008 from a male/pup captured at Moss Landing Harbor, Monterey County, California, and the animal died in treatment on May 3, 2008 due to malnutrition. Strain CSL7640 was isolated from an oral swab collected on May 20, 2008 from a female/pup captured at Santa Cruz, Santa Cruz County, California, and the animal died in treatment on May 20, 2008 due to malnutrition.

Morphological and biochemical analysis

Cell morphology was observed using the conventional Gram stain procedure and motility was examined by inoculation in a semisolid Columbia broth. Gram staining was performed using the BD Gram-Staining Kit (Becton Dickinson, USA). The temperature range (18, 22, 30, 37, 40, 41, 42, and 43 °C) for growth was determined by incubating cultures for 7 days in the Columbia broth. The toleration of NaCl concentration for growth was determined in the Columbia broth and in Mueller–Hinton broth (BD) with 0–10% (w/v) NaCl (at increments of 1%), and the cells were grown at 36 ± 1 °C in ambient air supplemented with 5% CO_2. Anaerobic growth was tested for up to 2 weeks on TSA with 5% sheep blood, Wilkins–Chalgren anaerobe agar, and Columbia broth using GasPak EZ anaerobe pouch system (Becton Dickinson, USA). *Bacteroides fragilis* (ATCC 25285; BD Biosciences) was used as the indicator culture to confirm anaerobic conditions when the GasPak EZ anaerobe pouch system was used. Catalase activity was detected by the formation of bubbles in 3% (v/v) H_2O_2 solution. An oxidase activity test was performed using a commercial dropper oxidase reagent (Becton Dickinson, USA). Conventional tube tests (CTA—Cystine Tryptic agar media for carbohydrate fermentation studies from Remel), API NH and API ZYM strips (bioMérieux), and GEN III MicroPlate™—Biolog tests were used to determine physiological and biochemical characteristics of the strains. All commercial tests [API strips and Biolog plates] were conducted according to the manufacturer's recommendations. The novel *Neisseria* species strains were also tested for growth in Schaedler broth, Brucella broth, and Luria–Bertani broth for 24 h, and for growth on MacConkey agar or in MacConkey broth (both from Becton Dickinson) for 5 days and on Thayer–Martin-Modified Agar with VCNT (vancomycin, colistin, nystatin, and trimethoprim)

and on Martin–Lewis Agar with lincomycin (both from Hardy Diagnostics) for 7 days. All cultures were incubated at 36 ± 1 °C in ambient air supplemented with 5% CO_2.

Chemotaxonomic characterization

The fatty acid composition of *Neisseria zalophi* sp. nov. CSL 7565^T and *N. canis* (the selected reference species that is phenotypically closest to the isolated *Neisseria* sp. strains) was determined by gas chromatography with flame ionization detection (GC-FID). The strains were grown in Columbia broth and Luria–Bertani (LB) broth at 37.0 ± 1.0 °C for 48 h, and prepared for GC analysis as previously described (Whittaker et al. 2007). Bacterial cells were harvested in triplicate and saponified with 3.75 N NaOH in 1:1 distilled water/methanol for 5 min. at 100 °C. Free fatty acids were neutralized with 6N HCl and extracted with 1:1 petroleum ether/ethyl ether. After removal of the extraction solvent, fatty acid methyl esters (FAME) were prepared by esterification with 1% sulfuric acid in dry methanol at 80 °C and extracted with hexane. GC-FID analyses were performed with an Agilent 6890N gas chromatograph (Agilent Technologies, Palto Alto, CA, USA) equipped with a flame ionization detector, a split split-less injection port, and a SP2560 capillary column (100 m × 0.25 mm, 0.20 µm film thickness, Supelco, Bellefonte, PA). The GC oven was maintained at 160 °C, and hydrogen was used as carrier gas at 1.0 mL/min. The injection port split ratio was set to 1:100, and the injection volume was 1 µL. Identification of fatty acids was achieved as described previously (Delmonte et al. 2009), and separations were achieved by two-dimensional gas chromatography with online reduction, as previously described (Delmonte et al. 2013).

PCR amplification, gene sequencing, phylogenetic analysis, and determination of DNA G + C content

Genomic DNA was isolated from 1.0 mL of actively growing overnight broth culture using the DNeasy Blood and Tissue Kit (QIAgen, Chatsworth, CA) with pretreatment for Gram-negative bacteria according to the manufacturer's protocol. PCR amplification of the entire 16S rRNA gene, the 16S-23S rRNA intergenic transcribed spacer region (ITS), and, nearly, the entire 23S rRNA gene were performed using primers designed during this study (Table S1). The partial *argF* (ornithine carbamoyltransferase) and *recA* (DNA recombination/repair protein RecA) gene sequences from these strains were amplified using the previously published primers and PCR conditions (Zhou and Spratt 1992). The partial *rpoB* (DNA-directed RNA polymerase subunit beta), *rpoD (*RNA polymerase sigma factor RpoD*), polI* (DNA-directed DNA polymerase I), *ribII* (ribonuclease II), *aspS* (aspartate–tRNA ligase), *gyrB* (DNA gyrase subunit B), and porin precursor (*por*) gene sequences from these strains were amplified by PCR with the primers presented in Table S1. The selection of these genetic loci was based on our knowledge that they are part of "core genome" genes and should be present in all *Neisseria* species, as well as these genes represent more nucleotide sequence diversity in comparison with the 16S rRNA gene. The primers were designed based on nucleotide sequences of the corresponding genes of other *Neisseria* spp. (*N. canis, N. zoodegmatis, N. animaloris*, and *N. shayeganii*) available in GenBank.

The amplification mixture for all PCRs was conducted using contained 5 µL of 10 × HotStarTaq PCR buffer, 1.5 mM $MgCl_2$, 200 mM dNTP mixture, 1 mM of each primer, and 2.5 U HotStarTaq Plus DNA Polymerase (Qiagen) in a final volume of 50 µL including 3 µL of DNA template. All PCR reactions in this study were conducted under the following conditions: a polymerase activation step at 95 °C for 5 min then 40 cycles of 95 °C for 30 s, 55 °C (for all primers, except Neiss_zal_rpoBF/Neiss_zal_rpoBF for which 60 °C was used) for 60 s and 72 °C for 60 s, and a final extension at 72 °C for 10 min. PCR products were detected by electrophoresis through 1% TAE-agarose gels containing ethidium bromide concentrations followed by UV visualization. Amplicons were directly sequenced with the same primers used for PCR amplification and then with internal (walking) primers when needed.

The nucleotide sequences of the amplified genes from the novel strains were compared to the GenBank nucleotide database using the BLASTN algorithm. All DNA sequences from this study were deposited in GenBank under accession numbers KY847877-KY847916 and MG697220 (see Table S2). Nucleotide sequences were aligned with the publicly available Clustal X software (http://www.clustal.org). Inter- and intra-species similarity for each gene was analyzed using the BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Phylogenetic analysis was conducted using the MEGA 7 software (Kumar et al. 2016), and phylogenetic trees were constructed in the MEGA 7 software using neighbor-joining and minimum evolution algorithms. The robustness of the individual branches was estimated by bootstrapping with 1000 replicates.

Assessment and prediction of genome G + C content for the type strain CSL 7565^T in comparison with *N. canis, N. zoodegmatis, N. animaloris*, and *N. meningitidis* were performed by analyzing the G + C content for the 16S rRNA, 23S rRNA, *rpoB, gyrB, polI*, and *recA* gene sequences. This approach for the assessment and prediction of genome G + C content based on the comparison between G + C content of individual housekeeping genes (protein-encoding genes) and G + C contents of genomic DNA that has been determined experimentally by the HPLC (high-performance liquid chromatography) technique has been successfully demonstrated and published for multiple bacterial species (Fournier et al.

2006; Kim et al. 2015; Kuhnert and Korczak 2006; Volokhov et al. 2012a; Zavala et al. 2005). Previously published data demonstrate minimal differences (± 1–5%) between genome G + C contents based on the analysis of individual housekeeping genes and the results of G + C contents estimated using HPLC.

Results and discussion

Three independent strains (CSL7565^T, CSL7624, and CSL7640) named *Neisseria zalophi* as a result of this study were isolated from the oral cavity of California sea lions (*Zalophus californianus*). These three strains grew readily (24–48 h) on Columbia agar (or broth), Wilkins–Chalgren anaerobe agar, in Schaedler broth, Brucella broth, Luria–Bertani broth, and on TSA with 5% sheep blood incubated at 36 ± 1 °C in ambient air supplemented with 5% CO2, but no growth was detected under anaerobic conditions. Like other *Neisseria* species (except catalase-negative *N. elongata*), these strains produced catalase and oxidase, and the cells were non-motile and Gram-negative.

At the time of isolating the first strain CSL 7565^T (in 2007) and other two strains CSL7624 and CSL7640 (in 2008), their 16S rRNA gene sequences showed significant nucleotide difference in comparison with the 16S rRNA gene sequences of known species of *Neisseria*. The similarities were as low as 95–98%, and the closest known relatives were *N. canis, N. zoodegmatis*, and *N. animaloris*, which have been recovered from the oral cavity of dogs or from human wounds resulting from dog or cat bites (Berger 1962; Dewhirst et al. 2012). Their 16S rRNA gene sequences also demonstrated 98% of nucleotide similarity to the 16S rRNA gene of the recently described species, *N. dumasiana* sp. nov. (Wroblewski et al. 2017). Despite the 97–98% of sequence similarity for the 16S rRNA genes between the strain CSL 7565^T and *N. canis, N. zoodegmatis, N. animaloris*, and *N. dumasiana*, multiple species-specific nucleotide substitutions within the 16S rRNA and 23S rRNA genes were observed for all these species. In addition, the 16S rRNA gene sequences of these isolated *Neisseria* strains showed high sequence similarity (99%) to approx. ≥ 1000 sequences of the entire 16S rRNA genes from the uncultured *Neisseria* spp. detected by metagenomic analysis in oral and stomach samples collected from healthy California sea lions (Bik et al. 2016). Evidence from that study may represent indirect evidence that *Neisseria* spp. and possibly this novel *Neisseria* sp. are commensal bacteria in healthy California sea lions. However, the pathogenicity of this new species for California sea lions, especially for younger animals, is unknown and should be investigated in the future.

Since the 16S rRNA genes of the previously published species *N. canis, N. zoodegmatis, N. animaloris*, and *N. dumasiana* demonstrated the highest nucleotide sequence similarity to the 16S rRNA gene of the type strain CSL 7565^T, additional nucleotide sequencing analysis was performed for other selected housekeeping genes (the partial *argF, recA, rpoB, rpoD, polI, ribII, aspS, gyrB*, and porin precursor) for all of these type species (Table 1). The nucleotide sequence similarities among the *rplF, gyrB, rpoB, recA, argF, rpoD, polI, ribII, aspS*, and porin precursor genes of the type strain CSL 7565^T and the closest neighboring-type strains (*N. canis, N. zoodegmatis, N. animaloris*, and *N. dumasiana*) ranged from 70 to 87% (Table 1). These nucleotide sequence differences clearly demonstrate that the strain CSL 7565^T, *N. canis, N. zoodegmatis, N. animaloris*, and *N. dumasiana* are closely related but different species of the genus of *Neisseria*. The sequence analyses of the above-mentioned housekeeping genes were found to be suitable for the unambiguous identification of CSL 7565^T, *N. canis, N. zoodegmatis, N. animaloris*, and *N. dumasiana* to the species level; however, in routine laboratory practice, the use of only one or two of these genes (e.g., *rpoB* and *gyrB*) will be sufficient for the identification of these bacteria to the species level. In addition, based on the sequence of *rpoB* gene of the strain CSL 7565^T, we designed species-specific

Table 1 Sequence similarities (%) for the *16S rRNA, rplF, gyrB, rpoB, recA, argF, rpoD, polI, ribII, aspS*, and porin precursor genes between *Neisseria zalophi* sp. nov. CSL 7565T and closely related *Neisseria*-type species

Strain	Similarity (%) with strain CSL 7565^T										
	16S rRNA	*rplF*	*gyr*B	*rpo*B	*rec*A	*argF*	*rpoD*	*polI*	*ribII*	*aspS*	*porin precursor*
Neisseria canis DSM 18000^T	97	75	77	81	84	76	73	83	73	87	68
Neisseria animaloris ATCC 29858^T	98	81	82	84	85	72	82	83	82	87	70
Neisseria zoodegmatis ATCC 29859^T	98	81	82	83	85	82	81	83	83	87	72
N. dumasiana 93087^T	98	81	84	84	86	81	81	83	83	87	78

Only the 16S rRNA, *rplF, rpoB*, and *gyrB* genes were sequenced for CSL7640 and CSL7624 strains, which demonstrated 99% nucleotide similarity of the same genes of the type strain CSL 7565^T

primers (see Table S1) that produced PCR fragment with size of 1407 bp from strains CSL 7565^T, CSL7624, and CSL7640, but did not amplify any PCR product from the closest neighboring-type strains. Based on *in silico* analysis (Christen 2008) using BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) of these designed primers against the different *Neisseria* spp. sequences available in GenBank, we can also conclude that these primers are specific only to this *Neisseria* sp. isolated from California sea lions.

For phylogenetic analysis of these *Neisseria* sp. strains in comparison with other established *Neisseria* spp., three gene sequences (the *16S rRNA, rplF*, and *rpoB*) were selected. The 16S rRNA gene is the commonly used gene for phylogenetic studies, including for *Neisseria* spp. (Harmsen et al. 2001; Mechergui et al. 2014b). Inter-species diversity among *Neisseria* spp. for the 16S rRNA gene ranges from approximately 1–5%, which can be insufficient for precise phylogenetic resolution and identification of closely related species; for example, the 16S rRNA genes of type strains of *N. macacae* and *N. sicca*, *N. meningitidis* and *N. polysaccharea*, share 99% nucleotide similarity to each other. Thus, the sequencing of additional, more diverse genes, should be used to correctly identify *Neisseria* spp. by genetic testing (Harmsen et al. 2001; Mechergui et al. 2014a). In this study, we used *rplF* and *rpoB* as additional genes for phylogenetic analysis of the novel *Neisseria* sp. in comparison with other established *Neisseria* spp. It was demonstrated previously that these genes are more diverse than the 16S rRNA gene and are useful for phylogenetic analyses and species identification (Adekambi et al. 2009; Bennett et al. 2014; Volokhov et al. 2012b). Based on the phylogenetic analysis performed on these three selected gene sequences (16S rRNA, *rplF*, and *rpoB*), the novel *Neisseria* sp. does not belong to any known *Neisseria* species (see Fig. 1, S1, S2).

Unique sequence traits that are repetitive genomic elements called the DNA uptake sequence were previously identified in *Neisseria* species (Frye et al. 2013). A 12-base sequence called AG-DUS (AGGCCGTCTGAA) reported to be overrepresented repeat in the genomes of *N. subflava, N. flavescens, N. mucosa, N. bacilliformis, N. weaveri*, and *N. elongata* subsp. glycolytica (Frye et al. 2013). Our analysis of the sequenced housekeeping genes for the type strain CSL 7565^T revealed that two AG-DUS sequences exist in its DNA-directed DNA polymerase I gene (KY847911). This also supports that this novel species is a member of the genus *Neisseria*.

The phylogenetic relatedness of the type strain CSL 7565^T was compared biochemically to the type strains of *N. canis, N. zoodegmatis, N. animaloris*, and *N. dumasiana* (93087^T) (Table 2). The biochemical characteristics of CSL 7565^T, CSL7640, CSL7624, *N. canis, N. zoodegmatis*, and *N. animaloris* were determined using the standard laboratory methods (conventional tube biochemical tests) and rapid commercial systems, including API NH and API ZYM strips, which are designed and commonly used for biochemical characterization of *Neisseria* spp. (Barbe et al. 1994; D'Amato et al. 1978). The phenotypic characteristics of *N. dumasiana* (93087^T) were adopted from a recently published study (Wroblewski et al. 2017). In addition, Biolog Gen III plates (Biolog-Inc 2013) were used for for biochemical testing of the type strains (CSL 7565^T, *N. canis, N. zoodegmatis, N. animaloris*). All applied commercial tests failed to produce the identification of this novel *Neisseria* sp. to the species level, but the testing results were useful to determine the biochemical capability of these strains. The Biolog's Gen III system automatically indicated *N. zoodegmatis* to be the closest relative species to the type strain CSL 7565^T.

The Biolog GEN III MicroPlate™ results (Table S3) indicated that all species tested did not utilize glycyl-L-proline, L-arginine, L-aspartic acid, L-histidine, L-pyroglutamic acid, and L- and D-serine. CSL 7565^T and *N. canis* were positive for utilization of L-alanine and L-glutamic acid, and *N. animaloris* was positive for utilization of L-glutamic acid but not for L-alanine, and *N. zoodegmatis* was negative for utilization of both L-glutamic acid and L-alanine. CSL 7565^T was also positive for utilization of methyl pyruvate and α-ketoglutaric acid, but *N. canis, N. animaloris*, and *N. zoodegmatis* were negative or scored as borderline results. In the Biolog GEN III test, CSL 7565^T and *N. animaloris* were positive for utilization of α-D-glucose, and *N. canis* and *N. zoodegmatis* were scored as borderline results for utilization of this substrate. In contrast with this Biolog's result, all novel strains CSL 7565^T, CSL7640, and CSL7624 were negative for the utilization of D-glucose in the conventional tube test and in the API NH test, and *N. canis, N. animaloris*, and *N. zoodegmatis* were positive (see Table 2).

Based on the biochemical tests alone, it was difficult to discriminate the strains of the new species (CSL 7565^T, CSL7640, and CSL7624) from *N. canis*. The most helpful phenotypic features for the discrimination of novel *Neisseria* sp. from *N. canis* were colony color and D-glucose utilization. From our data, the GEN III MicroPlate™ Biolog testing did not provide sufficient discrimination among these species; however, it can be used as a supplementary test.

The strains CSL 7565^T, CSL7640, and CSL7624 were able to grow in Mueller–Hinton broth supplemented with 6% NaCl, in comparison with *N. canis* and *N. zoodegmatis*, which only tolerated 2% NaCl, and *N. animaloris*, which was able to tolerate 3% NaCl (see Table 2). The higher tolerance of this novel species to NaCl at concentration > 3% might explain the ability of this novel species to survive in the oral cavity of sea lions, which would be regularly exposed to sea water, known to have a salt concentration of approximately 3.5% (Oren 2008).

No hemolysis was observed on sheep blood agar plates after incubation for 5 days for either CSL 7565^T, CSL7640,

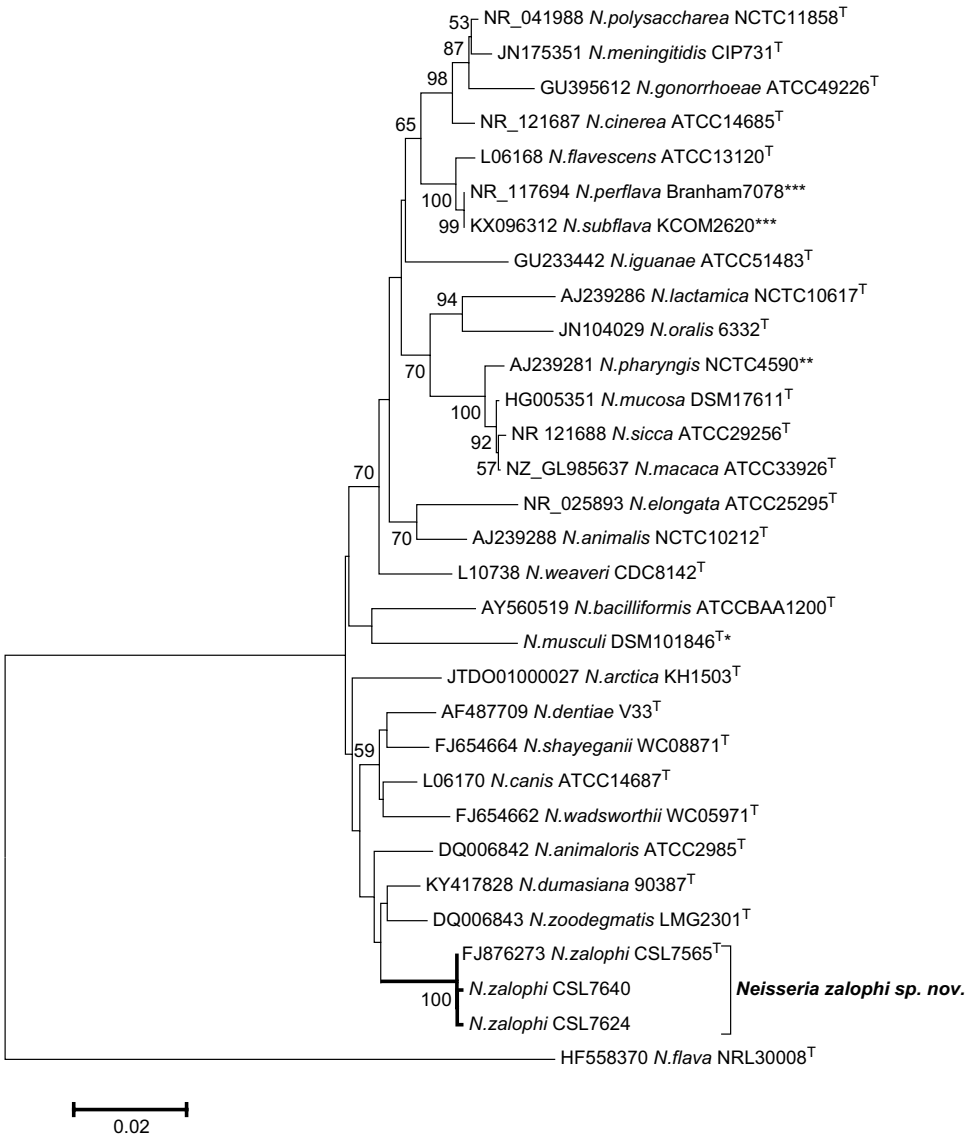

Fig. 1 Dendrogram showing the phylogenetic relationships of *Neisseria zalophi* sp. nov. with other *Neisseria* spp. based on nucleotide sequence data for the 16S rRNA gene (1400 nt in the length). The trees were constructed by the minimum evolution method in MEGA 7. The bootstrap values presented at corresponding branches were evaluated from 1000 replications, and bootstrap values lower than 50% are not included on the tree. GenBank accession numbers are indicated for each strain used in creating the dendrograms. The letter "T" at the end of strain identification numbers indicates type strains. *The 16S rRNA gene sequence of *Neisseria musculi* sp. nov. was provided by Dr. Nathan J. Weyand (Weyand et al. 2016). **The previously described 16S rRNA gene sequence of *N. pharyngis* NCTC4590 (Smith et al. 1999) is included in this phylogenetic analysis; however, there is no type strain designated for this species. ***For type strains of *N. perflava* LMG 5284^T and *N. subflava* LMG 5313^T, the only short sequences (457 nt) of the 16S rRNA gene are available (GenBank accession numbers AJ247246 and AJ247249, respectively); therefore, for this phylogenetic analysis, we used the full-length 16S rRNA gene sequences of *N. perflava* Branham 7078 and *N. subflava* KCOM2620, which demonstrated complete identity to the partial 16S rRNA gene sequences of their type strains. *N. flava* is used as an outgroup sequence in this tree due to significant difference of its 16S rRNA gene to other *Neisseria* spp.

CSL7624, or *N. canis*. *N. dumasiana* was also reported to be a non-hemolytic species (Wroblewski et al. 2017). Two other species, *N. zoodegmatis* and *N. animaloris*, were reported to be hemolytic (Vandamme et al. 2006). No growth of either CSL 7565^T, CSL7640, CSL7624, and *N. canis* was observed on MacConkey agar or in MacConkey broth after 5 day incubation. *N. dumasiana* was previously reported as species that does not grow on MacConkey agar (Wroblewski et al. 2017); however, both *N. zoodegmatis* and *N. animaloris* are able to grow on MacConkey agar (Vandamme et al. 2006). No growth of the new species (CSL 7565^T, CSL7640, and CSL7624) was observed for 7 days

Table 2 Result of biochemical testing for *Neisseria zalophi* sp. nov., in comparison with the phylogenetically closely related *Neisseria* species

Tests	*Neisseria zalophi* sp. nov.			*Neisseria canis*	*Neisseria animaloris*	*Neisseria zoodegmatis*	*Neisseria dumasiana*	Testing systems
	ATCC BAA-2455^T	CSL7640	CSL7624	DSM 18000^T	ATCC 29858^T	ATCC 29859^T	93087^T	
Colony color	Gray	Gray	Gray	Yellow	Gray	Yellow	Gray	(24–48 h) On the Columbia agar, Wilkins–Chalgren anaerobe agar and on Trypticase Soy agar (TSA) with 5% sheep blood
Urea	−	−	−	−	−	−	−	Conventional tube test (Remel)
Nitrate	+	+	+	+	+	+	+	
Oxidase	+	+	+	+	+	+	+	
Catalase	+	+	+	+	+	+	+	
Hemolysis on sheep blood agar	−	−	−	−	+	+	−	(48–72 h) On Trypticase Soy agar (TSA) with 5% sheep blood
Indole	−	−	−	−	−	−	−	Conventional tube test (Remel)
Voges-Proskauer	−	−	−	−	−	−	ND	
D-Glucose	−	−	−	(+)	+	+	+ (−)[a]	
D-Fructose	−	−	−	−	−	−	−	
D-Maltose	−	−	−	−	−	−	−	
Sucrose	−	−	−	−	−	−	−	
Growth at room temperature (18–22 °C)	+	+	+	+	+	+	+	
Growth in presence of NaCl	6%	6%	6%	2%	3%	2%	2%	In Mueller–Hinton broth (BD)
Growth on MacConkey agar	−	−	−	−	+	+	−	Conventional test
Anaerobic growth	−	−	−	−	(+)	−	+	
Citrate utilization on Simmons citrate agar	−	−	−	−	+	−	−	
Penicillinase	−	−	−	−	−	−	−	API NH strip test
D-Glucose	−	−	−	+	+	+	+	
D-Fructose	−	−	−	−	−	−	−	
D-Maltose	−	−	−	−	−	−	−	
Sucrose	−	−	−	−	−	−	−	
Ornithine decarboxylase	−	−	−	−	−	−	−	
Urease	−	−	−	−	−	−	−	
Lipase	−	−	−	−	−	−	−	
Alkaline phosphatase	−	−	−	−	−	−	−	
Beta galactosidase	−	−	−	−	−	−	−	
Proline arylamidase	+	+	+	+	+	+	+	
Gamma glutamyl transferase	−	−	−	−	−	−	−	
Indole	−	−	−	−	−	−	−	

Table 2 (continued)

Tests	*Neisseria zalophi* sp. nov.			*Neisseria canis*	*Neisseria animaloris*	*Neisseria zoodegmatis*	*Neisseria dumasiana*	Testing systems
	ATCC BAA-2455^T	CSL7640	CSL7624	DSM 18000^T	ATCC 29858^T	ATCC 29859^T	93087^T	
Alkaline phosphatase	−	−	−	−	+	+	ND	API ZYM strip test
Esterase (C4)	+	+	+	+	+	+	ND	
Esterase lipase (C8)	+	+	+	+	−	−	ND	
Lipase (C14)	−	−	−	−	−	−	ND	
Leucine acrylamldase	+	+	+	+	+	+	ND	
Valine acrylamldase	+	+	+	+	+	+	ND	
Cystine acrylamldase	+	+	+	+	−	+	ND	
Trypsin	−	−	−	−	−	−	ND	
α-Chymotrypsin	−	−	−	−	−	−	ND	
Acid phosphatase	+	+	+	+	+	+	ND	
Naphthol-AS-BI-phosphohydrolase	+	+	+	+	+	+	ND	
α-Galactosidase	−	−	−	−	−	−	ND	
β-Galactosidase	−	−	−	−	−	−	ND	
β-Glucuronidase	−	−	−	−	−	−	ND	
α-Glucosidase	−	−	−	−	−	−	ND	
β-Glucosidase	−	−	−	−	−	−	ND	
N-acetyl-β-glucosaminidase	−	−	−	−	−	−	ND	
α-Mannosidase	−	−	−	−	−	−	ND	
α-Fucosidase	−	−	−	−	−	−	ND	

ND no data

−, negative; +, positive; (+), weakly positive

[a]The species is reported as D-glucose-positive; however, it displays inter-strain variability for D-glucose (one of the three strains is positive; type strain is negative) (Wroblewski et al. 2017)

on Thayer–Martin-modified agar, with VCNT (vancomycin, colistin, nystatin, and trimethoprim) and on Martin–Lewis agar with lincomycin, the media commonly used for isolation and cultivation of the human pathogens *N. meningitidis* and *N. gonorrhoeae*. Neither CSL 7565^T, CSL7640, CSL7624, *N. canis*, nor *N. zoodegmatis* grew under anaerobic conditions. *N. animaloris* grew weakly at anaerobic conditions, but the colonies were much smaller than when grown aerobically. *N. dumasiana* was previously reported as a facultative anaerobe and was cultivated in anaerobic atmosphere on the chocolate agar plates (Wroblewski et al. 2017). All strains grew at room temperature (18–22 °C) in the Columbia broth. The strains CSL 7565^T, CSL7640, CSL7624, and *N. canis* grew at up to 41 °C in the Columbia broth within 24 h, but no growth was observed for them at 42 and 44 °C even after 96 h incubation.

The fatty acid composition for two selected type strains, CSL 7565^T and *N. canis*, was determined by gas chromatography with flame ionization detection (GC-FID). Results were expressed as a percentage of the total fatty acids (see Table S4). The major fatty acids for these two species were $C_{16:0,}$ $C_{16:1\text{-}c9}$, and $C_{18:1\text{-}c9}$. The main differences in the percentages of fatty acids between two species or for the same species grown in two different broths were observed for $C_{12:0,}$ $C_{14:0,}$ $C_{15:0,}$ $C_{16:1\text{-}t7}$, $C_{18:1\text{-}c9}$, $C_{17:0,}$ $C_{17:1\text{-}c9}$, $C_{17:1\text{-}c11}$, $C_{18:1\text{-}c7}$, $C_{18:1\text{-}c9}$, and $C_{24:1}$. For the species tested, the growth media influenced the relative proportions of certain of the fatty acids, e.g.: $C_{12:0,}$ $C_{14:0,}$ $C_{15:0,}$ $C_{17:0,}$ $C_{17:1\text{-}c9}$, $C_{17:1\text{-}c11}$, $C_{18:1\text{-}c7}$, and $C_{24:1}$ (see Table S4). A similar effect of growth media has been previously observed for other bacterial species (Bezbaruah et al. 1988; Moss and Lewis 1967); therefore, comparative FAME analysis of *Neisseria* species should be performed using the exact same medium and culturing conditions.

Assessment and prediction of genome G + C content for the type strain CSL 7565^T in comparison with *N. canis, N.*

zoodegmatis, N. animaloris, and *N. meningitidis* were performed by the analysis of G+C content for their 16S rRNA, 23S rRNA, *rpoB, gyrB, polI*, and *recA* gene sequences (Table S5). The predicted genomic G+C content for the type strain CSL 7565^T is 50.7 ± 3.7 mol%.

Therefore, on the basis of the phenotypic, genotypic, chemotaxonomic characteristics, and phylogenetic data, these three *Neisseria* sp. strains represent a novel species of the genus *Neisseria*, for which the name *Neisseria zalophi* sp. nov. is proposed.

Description of *Neisseria zalophi* sp. nov

Neisseria zalophi (zalophi L. gen. n. zalophus, of *Zalophus californianus*, the species isolated from *Zalophus californianus*).

The cells of this species are Gram-negative, non-spore forming, and non-motile coccobacilli (approx. 0.5–0.8 µm in length) and appeared as short chains with a few diplococcobacilli forms also present. Catalase and oxidase positive. Good growth occurs on Columbia agar, Wilkins–Chalgren anaerobe agar and on Trypticase Soy agar (TSA) with 5% sheep blood. No growth of this species was observed on the MacConkey agar, Thayer–Martin-Modified Agar with VCNT (vancomycin, colistin, nystatin, trimethoprim), or Martin–Lewis Agar with lincomycin. Colonies on Columbia agar plates were about 1–2 mm in diameter, circular, entire, opaque, gray colored, convex, and smooth. Obligate aerobe, non-hemolytic species. Positive in tests for nitrate, oxidase, catalase, proline arylamidase, esterase (C4), leucine acrylamldase, valine acrylamldase, acid phosphatase, and naphthol-AS-BI-phosphohydrolase. Negative for utilization of D-glucose, D-maltose, sucrose, D-fructose, and negative for production of urease, indole, gelatinase, lipase, alkaline phosphatase, beta galactosidase, and gamma glutamyl transferase. The species is able to grow in Mueller–Hinton broth supplemented with 6% NaCl. The type strain has estimated DNA G+C content of 50.7 ± 3.7 mol%. The type strain is CSL 7565^T (= ATCC BAA2455^T = DSM 102031^T). This strain was isolated from the oral cavity of California sea lion (*Zalophus californianus*) that were admitted to the Marine Mammal Center in California, USA. The respective DPD Taxon Number is TA00368.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to declare for this research.

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