

## *Neisseria bacilliformis* sp. nov. Isolated from Human Infections

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Most *Neisseria* species are gram-negative cocci or diplococci; currently, *N. elongata* is the only species of human origin with a bacillary morphology. Here, we report isolation and characterization of eight strains of another bacillary *Neisseria* species from human infections. The organisms caused or contributed to either oral cavity-related or respiratory tract infections, and two strains were isolated from blood cultures. The 16S rRNA gene sequences of these organisms, being homogenous or nearly so (99.4 to 100% identity), matched at <96% known *Neisseria* species and formed a distinct group within the genus. Analysis of the cellular fatty acids showed broad similarity with a few *Neisseria* species. The organisms were gram negative and measured 0.6  $\mu\text{m}$  by 1.3 to 3.0  $\mu\text{m}$ . They grew well on chocolate agar and on sheep blood agar but did not grow on modified Thayer-Martin agar. They were positive for oxidase and negative for indole production. There was no acid production from dextrose, lactose, maltose, or sucrose. The tests for catalase reaction, nitrate reduction, and tributinin varied with the strains. These results suggest that these organisms represent a novel species within the genus *Neisseria*, for which the name *Neisseria bacilliformis* sp. nov. is proposed. The type strain is MDA2833 = ATCC BAA-1200<sup>T</sup> = CCUG50858<sup>T</sup>. Distinction between *N. bacilliformis* and *N. elongata* can be made confidently by 16S rRNA gene sequencing or cellular fatty acid profiling but may be difficult by morphology or routine biochemical tests.

The genus *Neisseria* includes a group of closely related gram-negative bacteria that are primarily commensal inhabitants of the mucus membranes of mammals. There are currently 12 *Neisseria* species of human origin, with *N. meningitidis* and *N. gonorrhoeae* being important pathogens and the others being opportunistic (13). Based on phylogenetic studies of house-keeping genes, *Neisseria* species have been classified into five groups, represented by *N. meningitidis*, *N. flavescens*, *N. cinerea*, *N. pharyngis*, and *N. elongata* (22).

Of the known *Neisseria* species, *N. elongata* (of human origin) and *N. weaveri* (of dog origin and occasionally isolated from human wounds from dog bites) are the only species with a bacillary morphology; all others are cocci or diplococci (2, 5, 11, 13). It has been suggested, therefore, that the diplococcal morphology should not be the sole criterion for the identification of *Neisseria* (5). Because of the rare occurrence of these bacillary *Neisseria* spp., however, some clinical microbiology laboratories are unfamiliar with this morphology; when encountered, these asaccharolytic bacilli may be confused with *Pasteurella* spp. or *Moraxella* spp.

In this study, we report the isolation and characterization of eight strains of another bacillary *Neisseria* species from various human infections and diverse geographic locations. Significant differences from other *Neisseria* spp. in phylogeny and phenotypic features were found, leading us to propose for these strains the designation *Neisseria bacilliformis* sp. nov.

### MATERIALS AND METHODS

**Bacterial strains and cultures.** Eight strains were analyzed. They were from the microbiology laboratory of The University of Texas M. D. Anderson Cancer Center (MDACC) in Houston, Texas, a 500-bed comprehensive care cancer hospital (three MDA strains isolated from September 2002 to January 2004); the Hackensack University Medical Center (HUMC), a 700-bed tertiary care hospital in New Jersey (strain HUMC1166); and the Culture Collection of the University of Goteborg (CCUG) in Sweden (four strains). Approximately 2,100 wound cultures and 30,000 blood cultures were performed yearly at MDACC. All subcultures were plated on blood agar and chocolate agar (BBL Becton Dickinson Microbiology Systems, Cockeysville, MD) and incubated aerobically at 35°C with 5% CO<sub>2</sub>.

**Sequencing of the 16S rRNA gene and phylogenetic analysis.** Amplification of the 16S rRNA gene by PCR and subsequent sequencing of the amplicon were performed as described previously (9). Briefly, extracted genomic DNA was amplified by a set of highly conserved bacterial primers: 5' GCGTGCTAAC ACATGCAAGTC 3' and 5' AGGAGGTGATCCAACCGCA 3' (positions 42 to 1539 of the sequence listed under GenBank accession no. J01859 [*Escherichia coli*]). The 1,490-bp amplicon was sequenced by using these and a few sets of internal primers and the dye terminator method in an ABI automated sequencer (Applied Biosystems, Foster City, CA). Sequence homology searches were performed through queries of GenBank by BLAST matches or through alignment of two sequences (1). Phylogenetic analysis was performed by using the Drawtree program of PHYLIP ([www.molgen.mpg.de](http://www.molgen.mpg.de)) and ClustalW multiple alignments ([www.ebi.ac.uk/clustalw](http://www.ebi.ac.uk/clustalw)) (6).

**Phenotypic characterization.** Biochemical tests were performed in conventional tube media (BBL Becton Dickinson Microbiology Systems, Cockeysville, MD) and miniaturized API 20NE (bioMérieux, Marcy l'Etoile, France). Routine control organisms (*N. meningitidis* and *N. lactamica*) were included in the tests. Cellular fatty acids were analyzed in a commercial laboratory by gas-liquid chromatography and Sherlock Version 4.5 software and library RCLN505.00 (Microbial ID, Inc., Newark, DE). Antibiotic susceptibility tests were performed using Etest (Biodisk, Solna, Sweden) on Mueller-Hinton agar with a 48-h incubation, and results were interpreted according to the breakpoints set for *Pseudomonas aeruginosa* and non-*Enterobacteriaceae* by the Clinical and Laboratory Standards Institute (formerly National Committee for Clinical Laboratory Standards) (16). Three CCUG strains required blood Mueller-Hinton agar and 5% CO<sub>2</sub> incubation for 24 h for the test.

**Nucleotide sequence accession numbers and strain depositions.** The 16S rRNA gene sequences of MDA2833, MDA1552, and CCUG38158 were depos-

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TABLE 1. Sources and clinical significance of four strains of a bacillary *Neisseria* sp.

Feature(s)	Strain			
	MDA2833	MDA0990	MDA1552	HUMC1166
Isolation source, yr, location	Submandibular wound, 2003, Houston, Tex.	Sputum, 2002, Houston, Tex.	Sputa (4 days apart), 2004, Houston, Tex.	Lung abscess, 2004; New Jersey
Patient age (yr), sex	53, male	66, female	69, female	62, male
No. of colonies	Few	Many	Many	Many
Coisolate(s)	Non-group A $\beta$ -streptococcus, <i>Capnocytophaga</i> sp., <i>Veillonella</i> sp., and normal flora	Few <i>Klebsiella pneumoniae</i> isolates, few <i>Candida albicans</i> isolates, and normal flora	Few yeast	<i>Enterococcus faecium</i>
Significance	Contributory to wound infection	Contributory to possible bronchitis	Probable cause of bronchitis	Cocause of abscess
Underlying condition(s)	Right mandibulectomy for carcinoma	Brain lymphoma; bacteremia treated with gentamicin and nafcillin	Lung cancer lobectomy; pneumonia in other lung treated with vancomycin	Lymphoma
Treatment	Surgical drainage; amoxicillin/clavulanate, clindamycin	Gentamicin and nafcillin	Aztreonam, ciprofloxacin, and vancomycin	Surgical resection; ceftriaxone and piperacillin/tazobactam
Outcome	Recovered	Recovered	Recovered	Recovered

ited in the GenBank database as AY560519, DQ117531, and DQ117530, respectively. Strains MDA2833, MDA0990, MDA1552, and HUMC1166 were deposited at CCUG as CCUG50858 through CCUG50861. MDA2833 was also deposited at ATCC as BAA-1200.

RESULTS

**Sources and significance.** The sources and clinical significance of the four strains from MDACC and HUMC are shown in Table 1. These organisms caused or contributed to respiratory tract-related infections. All patients suffered from underlying cancer, with two having related surgery. These organisms were not identifiable previously through routine biochemical tests. The four strains from CCUG had incomplete histories (Table 2): two were isolated from human blood and two from the head and neck region (possible infections). These organisms were previously identified, with low confidence, as *N. elongata*, through biochemical tests and/or analysis of cellular fatty acids. From both tables, the diverse geographic origins of the eight strains are evident.

**Analysis of 16S rRNA gene sequences.** The 16S rRNA genes of the MDACC and HUMC strains were sequenced to near full length (1,490 bp). The sequences of the CCUG strains were determined to 1,490 bp for CCUG45926 and CCUG38158 and to 1,050 bp for CCUG38963 and CCUG50611.

Identical sequences were found for five strains: MDA2833,

MDA0990, HUMC1166, CCUG38963, and CCUG50611. Queries through GenBank BLAST showed that the organisms most closely matched *N. meningitidis* (GenBank accession no. AL162758 and many others) at 95.7% (1,410 of 1,473 bp). The matches with all other established *Neisseria* species, including the bacillary organisms *N. elongata* and *N. weaveri*, were at or less than 95%. These results suggest that these organisms are likely identical and belong to the genus *Neisseria*. With significant phylogenetic distance from known *Neisseria* species, a novel species is probable.

The 16S rRNA gene sequences of MDA2833 matched at 99.4% (1,471 of 1,480 bp) that of MDA1552, at 99.5% (1,472 of 1,480 bp) that of CCUG45926, and at 99.5% (1,472 of 1,480 bp) that of CCUG38158. The sequences of MDA1552 and CCUG45926 were essentially identical (one nucleotide difference), and they matched at 99.5% (1,485 of 1,493 bp) that of CCUG38158. Therefore, MDA1552, CCUG45926, and CCUG38158 are likely variants of the MDA2833 group organisms.

A phylogenetic tree depicting the relationships among MDA2833, its variants, and other representative *Neisseria* species is shown in Fig. 1. This analysis suggests that MDA2833 and its variants form a distinct group within the genus and that they are likely closer to *N. weaveri*. Sequence comparisons with other member genera in the family *Neisseriaceae* were also made;

TABLE 2. Sources of four CCUG strains of a bacillary *Neisseria* sp. with insufficient clinical data

Feature(s)	Strain			
	CCUG38963	CCUG50611	CCUG45926	CCUG38158
Isolation source, yr, location	Human ear, 1998, Sweden	Human blood, 2005, Sweden	Oral fistula, 2001, Sweden	Human blood, 1997, Sweden
Patient age (yr), sex	43, unknown	83, male	Unknown	Unknown
Previous ID <sup>a</sup>	<i>N. elongata</i> subsp. <i>nitroreducens</i>	<i>N. elongata</i> subsp. <i>nitroreducens</i>	<i>N. elongata</i> subsp. <i>elongata</i>	<i>N. weaveri</i> or <i>N. elongata</i>

<sup>a</sup> ID, identification by biochemical tests and cellular fatty acid analyses with low confidence.

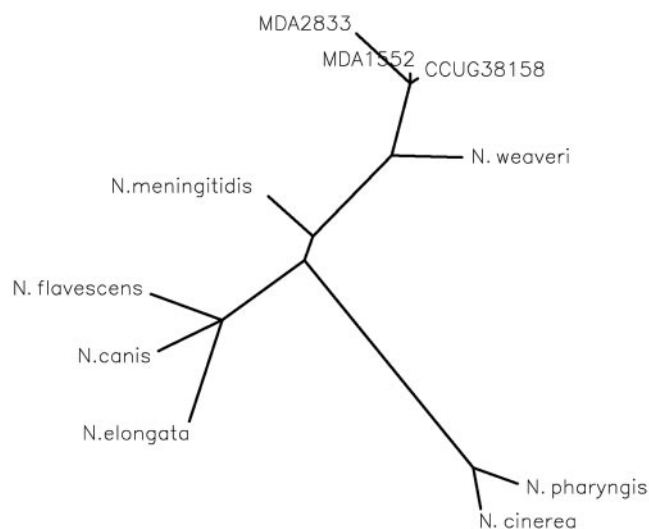


FIG. 1. Phylogenetic tree of MDA2833 and its variants in relation to representative *Neisseria* species. Strain designations and GenBank accession numbers are as follows: *N. canis* ATCC 14687<sup>T</sup>, L06170; *N. cinerea* LNP1646, AJ239287; *N. flavescens* ATCC 13120<sup>T</sup>, L06168; *N. elongata* subsp. *elongata* ATCC 25295<sup>T</sup>, L06171; *N. meningitidis* M7825, AF382301; *N. pharyngis* NCTC4590, AJ239281; and *N. weaveri* ATCC 51223<sup>T</sup>, L10738.

MDA2833 matched *Eikenella corrodens* (AY286546 and others) and *Kingella* spp. (L06164 and others) at levels of 94% to 95%.

**Fatty acid analysis.** The cellular fatty acids of the three MDA strains were analyzed, showing broad similarities with but substantial differences from a few known *Neisseria* species,

such as *N. weaveri*, *N. elongata*, *N. flava*, *N. polysaccharea*, and *N. flavescens* (Table 3). These results again suggest possible species status for these organisms. The fatty acid peaks for the three strains were very similar, particularly for the major peaks of 16:0, 16:1 ω7c/16:1 ω6c, and 18:1 ω7c.

**Culture and phenotypic characteristics.** Incubated aerobically at 35°C with 5% CO<sub>2</sub>, all strains grew well on sheep blood agar and chocolate agar plates. The colonies were round, smooth, glistening, and light gray and measured 0.5 to 1 mm at 24 h. Picked up with a cotton swab, the organisms were light yellow in color. No hemolysis was observed within 72 h. The organisms also grew on Trypticase soy agar, with the colony size being approximately half of that on chocolate agar. No growth was observed on modified Thayer-Martin agar or with incubation at ambient air and temperature. The organisms were gram negative and bacillary. A Gram stain and an electron photomicrograph of MDA2833 are shown in Fig. 2; the organism measures 0.6 μm by 1.3 to 3.0 μm.

The biochemical reactions of the eight strains are shown in Table 4. The organisms were all positive for oxidase and negative for indole production. None produced acids from dextrose, lactose, maltose, or sucrose or showed much reaction on miniaturized API 20NE tests. For the five strains within the MDA2833 group, all reduced nitrate to nitrite and were negative for catalase, and four were also positive for tributinin. For the three variant strains, all were negative for nitrate reduction and tributinin, and MDA1552 and CCUG45926 (identical 16S rRNA genes) were positive for catalase.

The strains were tested against a panel of antibiotics. They were all susceptible to amikacin (MIC, 3 to 16 μg/ml), imi-

TABLE 3. Cellular fatty acid analysis of three strains of a bacillary *Neisseria* sp., with comparison to *N. elongata* subsp. *elongata*

Fatty acid	% of indicated fatty acid in strain			
	MDA2833	MDA0990	MDA1552	<i>N. elongata</i> subsp. <i>elongata</i> <sup>a</sup>
11:0	0.42			
12:0	6.97	5.09	7.65	5.96
12:0 3OH	5.45	4.00	6.45	4.17
Unknown	0.44		0.41	
14:0	2.43	2.74	1.56	4.73
15:0	0.76	0.29	0.58	
12:0 aldehyde	3.57	3.25	5.07	1.57
16:1 ω7c/16:1 ω6c	<b>20.77</b>	<b>23.22</b>	<b>16.35</b>	<b>27.50</b>
16:0	<b>26.40</b>	<b>29.68</b>	<b>30.31</b>	<b>36.92</b>
17:1 ω8c	0.29	0.19	0.00	
17:0	0.38	0.20	0.45	
17:1 iso I/ante iso B				3.25
16:0 3OH	0.64	0.72	0.73	
18:0 ante/18:2 ω6,9c	4.28	4.02	1.98	
18:1 ω9c	3.97	3.53	1.66	2.43
18:1 ω7c	<b>20.73</b>	<b>21.41</b>	<b>24.50</b>	<b>11.07</b>
18:0	2.25	1.45	2.30	1.78
20:4 ω6,9,12,15c	0.26	0.23		
Total	100.01	100.02	100.00	99.38
Similarity index (out of 1.000), organism	0.678, <i>N. flavescens</i> 0.652, <i>N. polysaccharea</i> 0.638, <i>N. weaveri</i>	0.808, <i>N. weaveri</i> 0.649, <i>N. polysaccharea</i> 0.528, <i>N. flava</i>	0.558, <i>N. elongata</i> 0.522, <i>N. weaveri</i>	Not applicable
Interpretation	<i>Neisseria</i> sp.	<i>N. weaveri</i>	<i>Neisseria</i> sp.	<i>N. elongata</i>

<sup>a</sup> Data are averages of multiple runs in the MIDI database. (courtesy of Microbial ID, Inc., Newark, DE). Figures in bold represent major peaks.

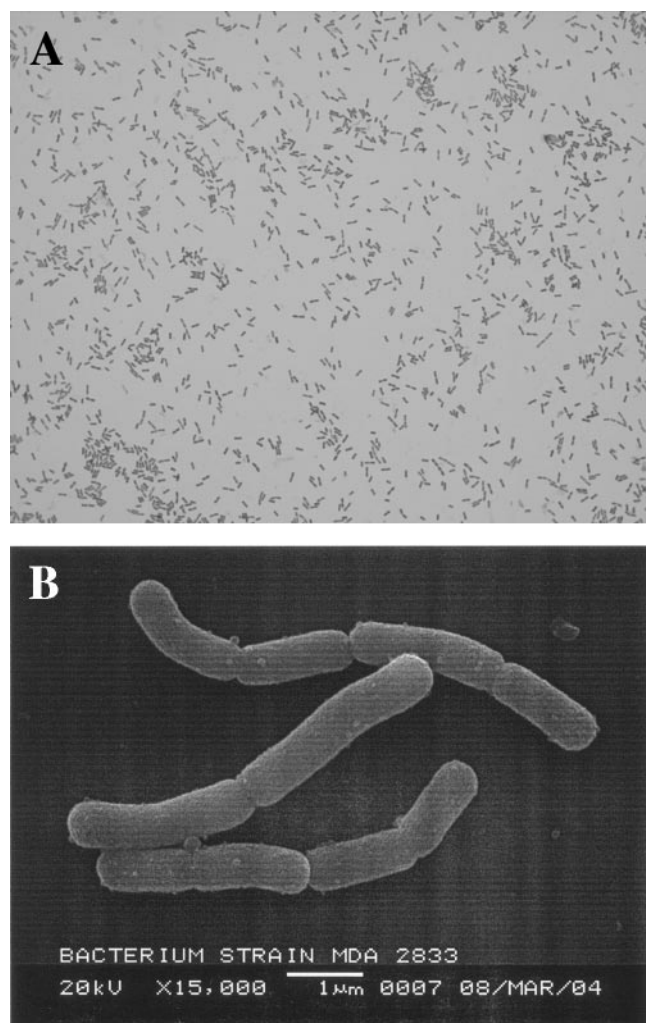


FIG. 2. Gram staining (magnification,  $\times 1,000$ ) (top) and electron microscopy (bar, 1  $\mu\text{m}$ ) (bottom) of MDA2833, a bacillary *Neisseria* sp.

penem (MIC, 0.032 to 0.09  $\mu\text{g/ml}$ ), ticarcillin/clavulanate (MIC, 0.016 to 0.25  $\mu\text{g/ml}$ ), and trimethoprim-sulfamethoxazole (MIC, 0.002 to 0.5  $\mu\text{g/ml}$ ) and were either fully or intermediately susceptible to cefepime (MIC, 3 to 12  $\mu\text{g/ml}$ ) and ciprofloxacin (MIC, 0.034 to 2  $\mu\text{g/ml}$ ).

## DISCUSSION

The above studies on phylogeny, cellular fatty acids, and phenotypic features suggest that the eight bacterial strains represent a novel species within the genus *Neisseria*, and we propose the name *Neisseria bacilliformis* sp. nov.

**Description of *Neisseria bacilliformis* sp. nov.** *Neisseria bacilliformis* (ba.cil.li.for'mis. N.L. n. *bacillus*, small rod; L. adj. suffix *formis*, shaped like; N.L. adj. *bacilliformis*, shaped like a small rod) is a gram-negative bacillus measuring 0.6  $\mu\text{m}$  by 1.3 to 3.0  $\mu\text{m}$ . The organism grows well on chocolate agar and sheep blood agar with colony size measuring 0.5 to 1 mm at 24 h. The colonies are round, smooth, glistening, and light gray in color. No growth occurs on modified Thayer-Martin agar, an ideal medium for *N. gonorrhoeae*. Biochemically, it is asaccharolytic and negative for indole production but positive for oxidase. Reactions in catalase, nitrate reduction, and tributinin tests vary according to the strain. The cellular fatty acids contain major peaks of C16:0, C16:1  $\omega 7c/16:1 \omega 6c$ , and C18:1  $\omega 7c$ . The organism is susceptible to many antibiotics tested. The type strain is MDA2833 = ATCC BAA-1200<sup>T</sup> = CCUG50858<sup>T</sup>, and the 16S rRNA gene is GenBank accession no. AY560519, which renders the single most reliable identification. Available medical information suggests that *N. bacilliformis* causes opportunistic human infections that are related to the oral cavity and respiratory tract. Blood invasion may also occur (and is likely in the presence of predisposing risk factors).

Until the present study, *N. elongata*, described first in 1970 (5), has been the sole bacillary *Neisseria* species isolated from humans. It has three subspecies currently: *N. elongata* subsp. *elongata*, *N. elongata* subsp. *glycolytica*, and *N. elongata* subsp. *nitroreducens*. All subspecies have been reported to cause hu-

TABLE 4. Positive biochemical reactions for eight strains of a bacillary *Neisseria* sp., with comparison to *N. elongata* subsp. *elongata*

Feature	No. of positive reactions/total for <sup>a</sup> :			
	MDA2833 group (n = 5)	MDA1552 group (n = 2)	CCUG38158 (n = 1)	<i>N. elongata</i> subsp. <i>elongata</i> <sup>b</sup>
Acid from:				
Dextrose	0/5	0/2	0/1	Negative
Lactose	0/5	0/2	0/1	Negative
Maltose	0/5	0/2	0/1	Negative
Sucrose	0/5	0/2	0/1	Negative
Oxidase	5 <sup>c</sup> /5	2/2	1/1	Positive
Catalase	0/5	2/2	0/1	Negative
Spot indole	0/5	0/2	0/1	Negative
Tributinin	4/5	0/2	0/1	Negative
Nitrate to nitrite	5/5	0/2	0/1	Negative
API 20NE code, identification	1000004 at 24–48 h, low discrimination	0000004 at 24–48 h, <i>Moraxella</i> spp.	0000004 at 24–48 h, <i>Moraxella</i> spp.	Not tested

<sup>a</sup> MDA2833 group included MDA2833, MDA0990, HUMC1166, CCUG38963, and CCUG50611; MDA1552 group included MDA1552 and CCUG45926.

<sup>b</sup> Data are from references 5, 7, and 13.

<sup>c</sup> Weakly positive for strain HUMC1166.



man diseases (10, 12), with endocarditis being the severest: at least 14 cases for *N. elongata* subspecies *nitroreducens* (7, 8) and 2 cases each for *N. elongata* subspecies *elongata* and *N. elongata* subspecies *glycolytica*, respectively (3, 4, 8, 17) have been reported. In the present study, we found that the morphology and biochemical features of *N. bacilliformis* are similar to those of various subspecies of *N. elongata*, as described in the literature (13). In fatty acid analysis (Tables 2 and 3), all four CCUG strains and MDA1552 also resembled *N. elongata* most closely. Furthermore, the isolation frequencies for *N. bacilliformis* and *N. elongata* are similar, according to preliminary data from MDACC: three strains of *N. bacilliformis* and two strains of *N. elongata* (from sputum and blood and identified by 16S rRNA gene sequencing) during the 17-month study period (X.Y.H., unpublished data). These data, therefore, lead us to believe that some of the previous *N. elongata* cases might have been caused by *N. bacilliformis*. The morphology and asaccharolytic nature of *N. bacilliformis* may also lead to confusion with the identification of *Pasteurella* spp. and *Moraxella* spp., both of which are also commensals in the upper airway. At present, the single most reliable way to identify *N. bacilliformis* is through 16S rRNA gene sequencing. Once the fatty acid profiles have been incorporated into databases, fatty acid analyses should also provide reasonably confident identification.

The opportunistic nature of the infections caused by *N. bacilliformis* and *N. elongata* raises the question of how important clinically it is to differentiate them. Future studies, upon accumulation of more cases and clinical strains, may help to answer this question. Nonetheless, recognition of *N. bacilliformis* certainly expands the diversity of the genus and its associated morphological features and may also have meaning for *Neisseria* systematics. The evolution, gene diversification, and phylogeny of the mucosa-dwelling *Neisseria* species, especially pathogenic *N. meningitidis* and *N. gonorrhoeae*, have been subjects of considerable investigation (22, 24). The genomes of *N. meningitidis* and *N. gonorrhoeae* have been sequenced (19, 23; GenBank accession no. AE004969), which has made genome-wide comparative studies feasible and rather revealing (14, 21). Our data (Fig. 1), albeit preliminary, show that *N. bacilliformis* strains represent a distinct group within the genus, which may lead to further phylogenetic studies.

In addition to the cases from our study, a case of endocarditis caused by another probable *N. bacilliformis* strain has been reported recently in France (15). The bacillus, identified by 16S rRNA gene sequencing analysis, matches at 99% an oral *Neisseria* sp. clone, AK105 (AY005029) (20), that has 99.2% (1,443/1,454) identity with the *N. bacilliformis* type strain and 99.6% (1,456 of 1,462 bp) identity with the variant MDA1552. Unfortunately, the lack of accessible sequences of the French strain precluded a direct comparison with *N. bacilliformis*. The phenotypic features of the French strain also match *N. bacilliformis*, most closely the variants MDA1552 and CCUG45926, due to positive catalase reactions. Like our strains and most commensal *Neisseria* spp., the organism also shows susceptibility to many antibiotics tested. The patient, a 38-year-old man, developed endocarditis on top of a history of wearing a permanent cardiac pacemaker for years; dental pain with associated shivers, myalgia, and dizziness a few weeks earlier; and tenosynovitis of the left stenoacromioclavicular joint for

days. The infection was cured by antibiotic therapy and surgical treatment.

For another patient with a long-term history of bronchiectasis, lower respiratory tract infection caused by a bacillary *Neisseria* sp. has been reported (18). This organism (strain RLUH) is positive in oxidase and catalase reactions, and its 16S rRNA gene sequences (AF429995) match most closely those of *N. canis* (strain ATCC 14687<sup>T</sup>, L06170) (97.8%, 943 of 964 bp), *N. weaveri* (97.5%, 940 of 964 bp), *N. bacilliformis* (96.7%, 932 of 964 bp), and *N. elongata* subsp. *elongata* (95.3%, 919 of 964 bp). These matches thus suggest the possibility of a novel species. Overall, our data and the literature suggest that bacillary *Neisseria* spp. are likely diverse as well, although they are infrequently encountered in clinical microbiology laboratories.

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