1.0 Microorganisms

1.1 Does your work involve the use of biological agents? ☒ YES ☐ NO
(non-pathogenic and pathogenic biological agents including but not limited to bacteria and other microorganisms, viruses, prions, parasites or pathogens of plant or animal origin)? If no, please proceed to Section 2.0

Do you use microorganisms that require a permit from the
If YES, please give the name of the species _____
What is the origin of the microorganism(s)? _____

Dr. S. Barr
Level 3 Modification

Please describe the risk (if any) of escape and how this will be mitigated:

Please attach the CFIA permit.

Please describe any CFIA permit conditions:

1.2 Please complete the table below:

Full Scientific Name of Biological Agent(s)* (Be specific)	Is it known to be a human pathogen? YES/NO	Is it known to be an animal pathogen? YES/NO	Is it known to be a zoonotic agent? YES/NO	Maximum quantity to be cultured at one time? (in Litres)	Source/ Supplier	PHAC or CFIA Containment Level
Gardnerella vaginalis	☒ Yes ☐ No	☐ Yes ☒ No	☐ Yes ☒ No		**Dr. G. Reid**	☐ 1 ☒ 2 ☐ 2+ ☐ 3
Atopobium vaginae	☒ Yes ☐ No	☐ Yes ☒ No	☐ Yes ☒ No		**Dr. G. Reid**	☒ 1 ☐ 2 ☐ 2+ ☐ 3
Prevotella bivia	☒ Yes ☐ No	☐ Yes ☒ No	☐ Yes ☒ No		**Dr. G. Reid**	☐ 1 ☒ 2 ☐ 2+ ☐ 3
Lactobacillus iners	☒ Yes ☐ No	☐ Yes ☒ No	☐ Yes ☒ No		**Dr. G. Reid**	☐ 1 ☒ 2 ☐ 2+ ☐ 3
Leptotrichia amnionii	☒ Yes ☐ No	☐ Yes ☒ No	☐ Yes ☒ No		**Dr. G. Reid**	☐ 1 ☒ 2 ☐ 2+ ☐ 3
	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No			☐ 1 ☐ 2 ☐ 2+ ☐ 3
	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No			☐ 1 ☐ 2 ☐ 2+ ☐ 3
	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No			☐ 1 ☐ 2 ☐ 2+ ☐ 3

Please attach a Material Safety Data Sheet or equivalent from the supplier if the bacterium used is not on this link:
http://www.uwo.ca/humanresources/docandform/docs/ohs/CFIA_Ecoli_list.pdf

Additional Comments: **I could not find info on the Biosafety level of Leptotrichia amnionii. It is a gram negative anaerobic bacterium and an opportunistic pathogen of the female urogenital tract. As such, will use Level 2**

Please include a ONE page research summary or teaching protocol in lay terms.
Forms with summaries more than one page will not be reviewed.

Purpose: Pilot project
We wish to perform a pilot experiment to test the influence of organisms commonly associated with bacterial vaginosis (listed above) on HIV-1 infection. We will mix replication-competent HIV-1 with each of the organisms listed above, or combinations thereof, and analyze the ability of HIV-1 to infect target cells. The level of infection will be quantified using a reporter cell line such as GHOST as described in my Level 3 BARF (BIOUWO0224). Depending on the results, we will further explore the mechanism of bacteria-induced enhancement of HIV-1 infectivity such as determining if HIV-1 adheres to the bacteria or if the bacteria induces changes in the target cell that make the cell more permissive to infection. A future modification will be submitted to outline project specifics beyond this if warranted.

Please explain how the biological agents are used in your project and how they are stored and disposed of. The BARF without this description will not be reviewed.

BACTERIA WORK:
All bacteria will be cultured in our Level 2 laboratory (DSB 3006B) or the shared Level 2 room (DSB 3004E) and stored at 4C or -80C in DSB3006B. Bacteria will be contained within stoppered vessels or flasks. Bacteria will be transported to the Level 3 in an approved leak-proof transport vessel. Bacteria will be mixed with HIV-1 in the Level 3 facility. All bacteria coming in contact with HIV-1 will be fixed before leaving the Level 3 facility in a final concentration of 2% formaldehyde for 30 mins. All equipment coming in contact with the bacteria will be bleached and washed with soapy water and/or autoclaved and incinerated.

VIRAL WORK:
As outlined in my BARF (BIOUWO0224).

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Bacteria

ATCC® Number: 14018™ Order this Item **Price:** $40.00

Preceptrol® Culture

Organism: *Gardnerella vaginalis* (Gardner and Dukes) Greenwood and Pickett deposited as *Haemophilus vaginalis* Gardner and Dukes

Designations: 594 [NCTC 10287]

Isolation: vaginal secretions

Depositor: CD Dukes

Biosafety Level: 2

Shipped: freeze-dried

Growth Conditions: ATCC medium70: Casman's medium base with 5% rabbit blood
Alternate medium 1685: NYC III medium
Temperature: 37.0°C

Permits/Forms: In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.

Cross References: Nucleotide (GenBank) : AX110990 Sequence 1723 from Patent WO0123604.
Nucleotide (GenBank) : M58744 Gardnerella vaginalis 16S ribosomal RNA.

Type Strain: yes [5878] [36887] [53555]

Comments: taxonomy [8198]

References:

5878: Int. Bull. Bacteriol. Nomencl. Taxon. 12: 76, 1962.
7131: Gardner HL, Dukes CD. Hemophilus vaginalis vaginitis. Ann. N.Y. Acad. Sci. 83: 280-289, 1959. PubMed: 13826525
8198: Criswell BS, et al. Haemophilus vaginalis 594, a gram-negative organism?. Can. J. Microbiol. 17: 865-869, 1971. PubMed: 4999344
9999: Gardner HL, Dukes CD. Haemophilus vaginalis vaginitis: a newly defined specific infection previously classified non-specific vaginitis. Am. J. Obstet. Gynecol. 69: 962-976, 1955. PubMed: 14361525
10891: Gardner HL, et al. The prevalence of vaginitis; a study in incidence. Am. J. Obstet. Gynecol. 73: 1080-1085, 1957. PubMed: 13411080
36887: Skerman VB, et al. Approved lists of bacterial names. Int. J. Syst. Bacteriol. 30: 225-420, 1980.
53555: Greenwood JR, Pickett MJ. Transfer of Haemophilus vaginalis Gardner and Dukes to a new genus, Gardnerella: G. vaginalis (Gardner and Dukes) comb. nov.. Int. J. Syst. Bacteriol. 30: 170-178, 1980.

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Bacteria

ATCC® Number: 55195™ Order this Item

Price: $255.00

Organism:	*Lactobacillus iners* deposited as Unidentified coryneform
Designations:	AB107
Isolation:	patient with bacterial vaginosis
Depositor:	North Carolina A&T State University
Biosafety Level:	2
Shipped:	freeze-dried
Growth Conditions:	ATCC medium1685: NYC III medium **Temperature:** 35.0°C Duration: 5% CO2
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.
Comments:	Formerly known as Gardnerella vaginalis. However, 16s sequencing indentifies this organism as Lactobacillus iners.
Applications:	produces FcrV protein protein V [11616] produces protein V, which binds with all four subclasses of human IgG [11616]
References:	11616: Allen JW. Protein V:A IGG binding factor. US Patent 5,128,451 dated Jul 7 1992

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Bacteria

ATCC® Number: BAA-55™ Order this Item

Price: $255.00

Organism: *Atopobium vaginae* Rodriguez Jovita et al.

Designations: CCUG 38953 [CIP 106431, DSM 15829]

Isolation: vaginal flora from a healthy woman, Goteborg, Sweden, 1998 [49736]

Depositor: CCUG

History: ATCC <<--CCUG<<--I. Mattsby

Biosafety Level: 1

Shipped: freeze-dried

Growth Conditions: ATCC medium1377: Haemophilus ducreyi medium
Temperature: 37.0°C
Atmosphere: Anaerobic

Permits/Forms: In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.

Cross References: Nucleotide (GenBank) : ACGK01000000 Atopobium vaginae DSM 15829, whole genome shotgun sequencing project
Nucleotide (GenBank) : Y17195 16S rRNA sequence

Type Strain: yes(type strain)

Comments: This strain has been sequenced as a reference genome for the NIH Human Microbiome Project.

References: 49736: Rodriguez Jovita M, et al. Characterization of a novel Atopobium isolate from the human vagina: description of Atopobium vaginae sp. nov.. Int. J. Syst. Bacteriol. 49: 1573-1576, 1999. PubMed: 10555338

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Bacteria

ATCC® Number: 29303™ Order this Item **Price:** $205.00

Organism: *Prevotella bivia* (Holdeman and Johnson) Shah and Collins deposited as *Bacteroides bivius* Holdeman and Johnson

Designations: VPI 6822 [653C, NCTC 11156]

Isolation: Endometrium

Depositor: LV Holdeman

History: ATCC <<--LV Holdeman<<--D. Blazevic 653C

Biosafety Level: 2

Shipped: freeze-dried

Growth Conditions: ATCC medium593: Chopped meat medium
Temperature: 37.0°C
Duration: anaerobic

Permits/Forms: In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.

Cross References: Nucleotide (GenBank) : L16475 Prevotella bivia ATCC 29303 16S ribosomal RNA gene, complete sequence.

Type Strain: yes [8428] [8904] [36887]

Applications: media testing [92430] [92428]

References:

8428: Holdeman LV, Johnson JL. Bacteroides disiens sp. nov. and Bacteroides bivius sp. nov. from human clinical infections. Int. J. Syst. Bacteriol. 27: 337-345, 1977.
8904: Shah HN, Collins DM. Prevotella, a new genus to include Bacteroides melaninogenicus and related species formerly classified in the genus Bacteroides. Int. J. Syst. Bacteriol. 40: 205-208, 1990. PubMed: 2223612
36887: Skerman VB, et al. Approved lists of bacterial names. Int. J. Syst. Bacteriol. 30: 225-420, 1980.
92428: Medical microbiology -- Culture media -- Part 2: Ready-to-use blood culture systems. Berlin, Germany:Deutsches Institut fur Normung;DIN DIN 58942-2: 2004, 2004
92430: Medical microbiology--Culture media -- Part 4: Transport systems for specimens containing bacteria. Berlin, Germany:Deutsches Institut fur Normung;DIN DIN 58942-4: 2003, 2003

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Leptotrichia amnionii sp. nov., a Novel Bacterium Isolated from the Amniotic Fluid of a Woman after Intrauterine Fetal Demise

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Clinical Research Center, Marshfield Medical Research and Education Foundation,[1] Department of Obstetrics and Gynecology, Marshfield Clinic,[2] and Marshfield Laboratories,[3] Marshfield, Wisconsin 54449, and Department of Molecular, Cellular, and Developmental Biology, The University of Colorado, Boulder, Colorado 80309[4]

Received 18 March 2002/Returned for modification 21 June 2002/Accepted 30 June 2002

A novel bacterium was isolated and characterized from the amniotic fluid of a woman who experienced intrauterine fetal demise in the second trimester of pregnancy. The bacterium was a slow-growing, gram-negative anaerobic coccobacillus belonging to the genus *Leptotrichia*. Unlike *Leptotrichia sanguinegens*, the isolate did not grow in chopped-meat glucose broth or on sheep blood agar upon subculturing. The isolate was characterized by sequencing and analyzing its 16S rRNA gene. The 1,493-bp 16S ribosomal DNA sequence had only 96% homology with *L. sanguinegens*. Several phylogenetic analyses indicated that *L. amnionii* is a distinct species and most closely related to *L. sanguiegens*.

Molecular-based diagnostic and identification methods for fastidious or uncultivable bacteria have resulted in the recognition of many new pathogenic microorganisms (3, 16). One of the most successful methods is PCR amplification and sequencing of the bacterial 16S rRNA gene. This method has been successfully applied to environmental as well as clinical samples (6, 15). The large rRNA sequence databases at GenBank and at Ribosomal Database Project II allow for a quick comparison of 16S ribosomal DNA (rDNA) sequences and accurate identification of bacteria that are difficult to identify on the basis of phenotypic properties alone (10). The use of this method has greatly expanded the list of indigenous microbial flora of humans and has helped in recognizing the numerous opportunistic pathogens that cause infections related to severe physiological stress and immunosuppression due to chemotherapy.

Leptotrichia species are slow-growing, gram-negative anaerobic flora of the oral cavity and genital tract (5). Colonization by *Leptotrichia* species has been reported in over 40% of children less than a year old (19). *Leptotrichia buccalis*, which is considered indigenous oral flora, has been associated with endocarditis in patients with Down's syndrome (2) and bacteremia in neutropenic children and adults (14, 22). They seem to colonize permucosal implants of edentulous patients (12) and, not surprisingly, are often considered contaminants if isolated from clinical specimens.

Leptotrichia sanguinegens has recently been proposed as an agent of postpartum and neonatal bacteremia (4). It has not been identified from a healthy individual. We describe an isolate that is related to the species *L. sanguinegens*, but is different in its genotypic properties and nutritional requirements (4). For this isolate, we proposed the name "*L. amnionii* sp. nov." (from "amnion," the extraembryonic membrane enveloping the embryo in utero and containing the amniotic fluid), to signify its source of isolation.

CASE REPORT

A 27-year-old previously healthy, multiparous female in the second trimester of pregnancy presented to the emergency room with severe headache, neck and back pain, and a temperature of 102°F. The abdominal examination demonstrated no guarding or rebound. A purulent vaginal discharge was noted, but the physical examination was otherwise normal. Fetal heart tones were present. The patient was hospitalized. Initial laboratory values demonstrated a leukocyte count of 7,800 with the differential showing 1 metamyelocyte, 10 band forms, 85 segmented neutrophils, and 3 lymphocytes. The hemoglobin level was 11.5 g/dl, and the C-reactive protein level was 11.4 mg/dl. A urinalysis was unremarkable, showing no evidence of infection. A wet preparation of the vaginal discharge demonstrated no abnormal organisms or evidence of significant vaginal infection. A PCR test for *Chlamydia trachomatis* was negative. Cerebral spinal fluid evaluation was normal. The symptoms gradually resolved, and the patient was discharged. Six days later, the patient was seen in the outpatient clinic. No fetal heart tones could be heard, and an ultrasound confirmed an intrauterine fetal demise. The patient was admitted for uterine evacuation by labor induction. Prior to induction, an amniocentesis was performed. The amniotic fluid was turbid and brown in color and had a distinct foul smell. A gram stain of the amniotic fluid demonstrated gram-negative coccobacilli. A slow-growing, gram-negative anaerobic coccobacillus was recovered. Scant growth of *Bacteroides fragilis* and *Propionibacterium acnes* was observed in cultures of the placenta. The mother was given amoxicillin-clavulanic acid and had an uneventful recovery.

MATERIALS AND METHODS

Microbiology. The amniotic fluid and the placenta tissue specimens were cultured on blood and chocolate agar under both aerobic and anaerobic conditions at 37°C. The template DNA for 16S rDNA PCR was prepared from a few colonies that were isolated on the prereduced blood agar incubated anaerobically. The DNA was extracted with a Qiagen DNA extraction kit (Qiagen, Inc.,

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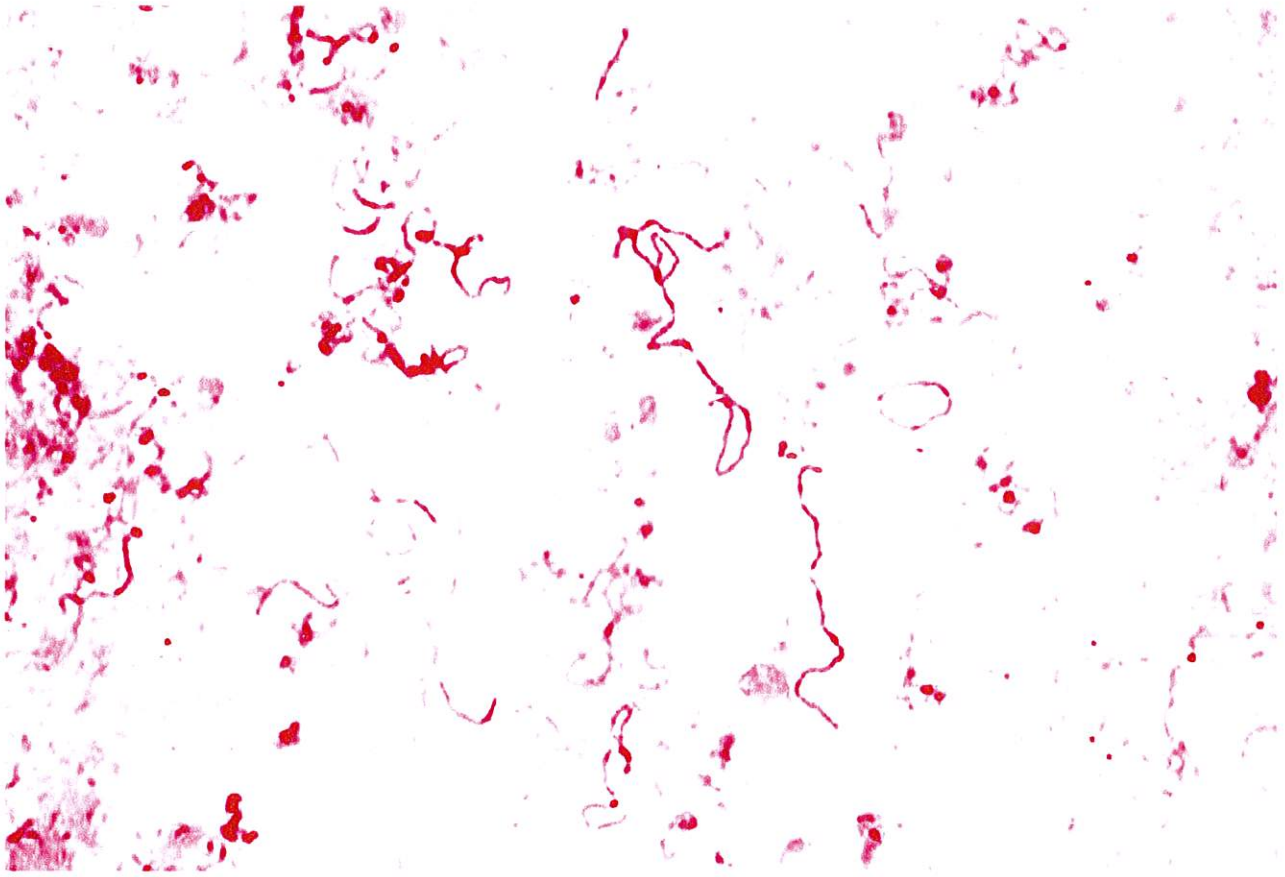

FIG. 1. Gram stain of amniotic fluid colonies demonstrating gram-negative pleomorphic bacilli.

Valencia, Calif.). Broad-range prokaryotic PCR primers (23) and nested sequencing primers (17, 23) were used to amplify and sequence the 16S gene rRNA. The methodology has been described previously (17).

Phylogenetic analysis. The rDNA sequence of the *L. amnionii* sp. nov. was aligned with a database of archaeal, bacterial, and eucaryal SSU rRNA sequences (ca. 10,000 sequences in total) by using the ARB software package (18). Both BLAST analysis and the parsimony insertion tool of ARB tentatively placed the *L. amnionii* sequence within the bacterial division of *Fusobacteria*. Consequently, a subset of the ARB alignment, which included the *Leptotrichia* species of the division *Fusobacteria* (including the species of the genus *Leptotrichia*), as well as members of other, outlying bacterial divisions, was selected for phylogenetic analysis. Both full-length data sets and sequence alignments minimized by the use of the Lane mask (8) were analyzed. The sequences of *Methanococcus jannaschii* and *Sulfolobus acidocaldarius* were selected as out-groups for phylogenetic analysis. The dendrogram presented in Fig. 2 was constructed by evolutionary distance analysis (neighbor joining with Olsen correction) with the ARB package (18). The robustness of this tree was assessed by bootstrap resampling (>100 replicates) of evolutionary distance trees by using weighted least-squares mean analysis with Kimura two-parameter or maximum-likelihood correction of evolutionary distances (PAUP* version 4.0b2) (20). Parsimony and maximum-likelihood analyses (ARB or PAUP*) provided results that were substantially similar to those of the evolutionary distance algorithm.

Nucleotide sequence accession number. The 16S rRNA sequence of the *L. amnionii* sp. nov. was deposited in GenBank and given accession no. AY078425.

RESULTS AND DISCUSSION

Numerous gram-negative coccobacilli were observed in the amniotic fluid along with numerous neutrophils. Anaerobic culture of the amniotic fluid on blood and chocolate agar resulted in very small gray colonies, <1 mm in diameter, following 72 h of incubation. Gram stain of the colonies revealed gram-negative coccobacilli, including some filamentous forms (Fig. 1). There was no growth on blood agar incubated under aerobic conditions, nor was there anaerobic growth on kanamycin and vancomycin or Mueller-Hinton agars upon subculturing. Viral cultures were negative. Since the bacterium resembled *L. sanguinegens*, it was inoculated into chopped meat glucose (CMG) broth and incubated under aerobic conditions (4). This medium did not sustain growth, as evidenced by the lack of turbidity of the medium. The organism was extremely fastidious and did not survive beyond the third subculture. There was insufficient growth to perform biochemical or fatty acid analysis.

The isolate was identified and characterized by PCR amplification of the 16S rRNA gene by using broad-range eubacterial primers FD1 and RD1 (17, 23). The PCR product was directly sequenced as described previously (17). A 1,493-nucleotide consensus sequence was created and edited with DNAsis software (Hitachi Corporation) and compared with the sequences deposited in the GenBank database. The submitted sequence had only 96% homology to *L. sanguinegens* (GenBank accession no. L37789).

Phylogenetic analysis. Based on its unique source of isolation, inability to grow on known special media, such as CMG

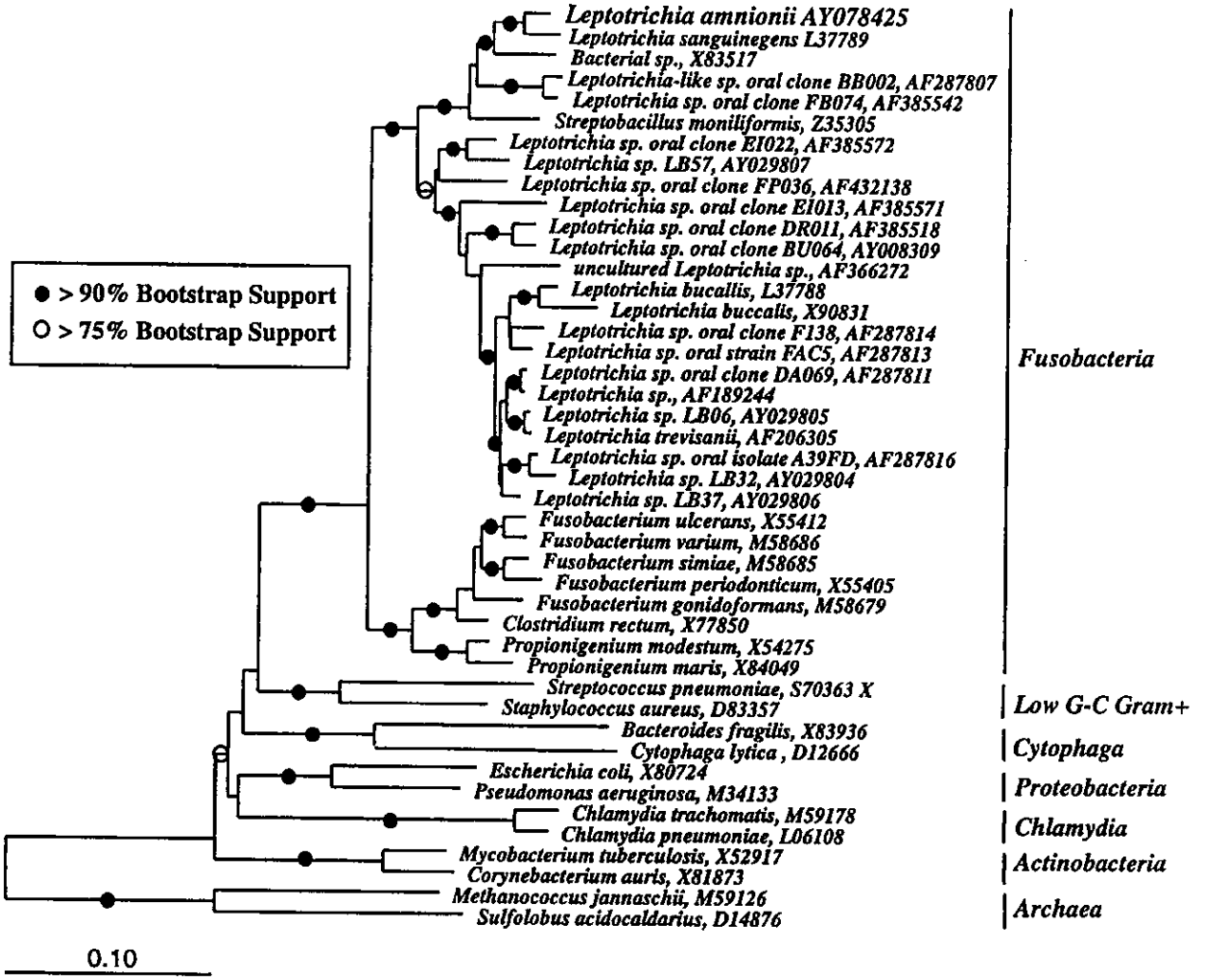

FIG. 2. Evolutionary distance dendrogram of selected leptotrichial and fusobacterial 16S rRNA sequences, including that of the *Leptotrichia*-like sp. isolate. Two archaeal species, *Methanococcus jannaschii* (M59126) and *Sulfolobus acidocaldarius* (D14876), were chosen as out-groups for phylogenetic analysis. Sequences are identified by species name and GenBank accession number. Branch points supported by >90% bootstrap values are indicated by solid circles. Open circles represent branch points with bootstrap values in the range 75 to 89%. Branch points without circles were not resolved (bootstrap values in the range <75%) as specific groups by this analysis. The bar at the bottom indicates the number of nucleotide changes per site.

broth, and unique 16S rDNA sequence, it was evident that this bacterium was related to, but different from, *L. sanguinegens* (4). The phylogenetic relationship of *L. amnionii* with other species of the bacterial division *Fusobacteria*, including species of the genus *Leptotrichia*, was inferred by evolutionary distance, parsimony, and maximum-likelihood analyses. Figure 2 shows a representative evolutionary distance dendrogram. Parsimony and maximum-likelihood analyses gave qualitatively similar results. Bootstrap resampling of data provided strong support for a specific association of *L. amnionii* with other members of the genus *Leptotrichia*. *L. sanguinegens* was identified as the closest neighbor of *L. amnionii* (bootstrap values of 99 and 100% for distance and parsimony analyses, respectively).

Clinical significance. Three species of *Leptotrichia*, *L. buccalis*, *L. trevisanii*, and *L. sanguinegens* (also called *Sneathia sanguinegens*) (1), have been associated with human infections (Table 1). Fifty-nine percent of the patients were immunosuppressed due to malignancy. Four cases of *L. sanguinegens* bacteremia were associated with pregnancy, and two neonates were infected (4).

L. buccalis has been well characterized and is part of the normal oral flora. It has been isolated from babies <1 year of age, and 40% of babies seem to be carriers (19). *L. trevisanii* is a recently identified bacterium that was recovered from a patient with myeloid leukemia. *L. sanguinegens* has been proposed as an agent of postpartum and neonatal bacteremia. At this time, we suspect that *L. amnionii* is indigenous to the urogenital tract and is an opportunist in the appropriate clinical situations. Like other *Leptotrichia*-related clinical cases, this bacterium was isolated from a clinical condition that is physiologically somewhat analogous to having the same stress and immunosuppression as an underlying malignancy. Hanff et al. described the presence of a strong odor from two neonatal

TABLE 1. Comparison of clinical features of *Leptotrichia* species isolated until 2001

Organisms	Source	Patient age (yr)	Sex[a]	Clinical condition	Reference
L. buccalis	Blood	15	M	Lymphocytic lymphoma	22
L. buccalis	Blood	7	F	Acute leukemia	22
L. buccalis	Blood	19	M	Acute leukemia	22
L. buccalis	Blood	73	F	Ovarian carcinoma	22
L. buccalis	Blood	46	M	Lymphocytic leukemia, cavitary pneumonia	11
L. buccalis	Blood	67	F	Lymphatic leukemia	7
L. buccalis	Blood	14	M	Osteogenic sarcoma	14
L. buccalis	Blood	9	F	Medullary aplasia	14
L. buccalis (4 cases)	Blood	43 ± 3	NK[b]	Bone marrow transplantation	9
L. sanguinegens	Blood	100	F	Pneumonia	4
L. sanguinegens (4 cases)	Blood	NK		Pregnancy/postpartum fever	4
L. sanguinegens	Blood	<1 (35 wk)	F	Suspected sepsis	4
L. sanguinegens	Blood	<1 (41.5 wk)	M	Suspected sepsis	4
Leptotrichia sp.	Blood	50	M	Myelogenous leukemia	13
L. trevisanii	Blood	46	M	Myelogenous leukemia	21
L. amnionii	Amniotic fluid	27	F	Pregnancy/intrauterine fetal demise	This report

[a] M, male; F, female.
[b] NK, not known.

cases of infection possibly due to *L. sanguinegens* (4), as was detected from the amniotic fluid in our case. It appears that *L. amnionii* is not a blood-loving microbe like *L. sanguinegens,* because it failed to grow on blood agar. Additional clinical isolates will help establish its true ecological niche and pathogenic potential.

Description of *Leptotrichia amnionii* sp. nov. The name "*L. amnionii*" (am′ n̄.on.ē.ī. L. gen. n., *amnionii*) is derived from the word "amnion." The organism is characterized by pleomorphic coccobacillus, long, nonmotile, fusiform cells. Some cells are joined end to end in a filamentous form. *L. amnionii* grows anaerobically on blood agar after 3 days of incubation and is closely related to *L. sanguinegens* based on its 16S rDNA sequences.

ACKNOWLEDGMENTS

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