More Easily Cultivated Than Identified: Classical Isolation with Molecular Identification of Vaginal Bacteria

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ABSTRACT

Background: Women with bacterial vaginosis (BV) have complex communities of anaerobic bacteria. There are no cultivated isolates of several bacteria identified using molecular methods and associated with BV. It is unclear if this is due to the inability to adequately propagate these bacteria, or to correctly identify them in culture.

Methods: Vaginal fluid from 15 women was plated on six different media using classical cultivation approaches. Individual isolates were identified by 16S rRNA gene sequencing, and compared with validly described species. Bacterial community profiles in vaginal samples were determined using broad-range 16S rRNA gene PCR and pyrosequencing.

Results: We isolated and identified 101 distinct bacterial strains spanning six phyla including 1) novel strains with <98% 16S rRNA sequence identity to validly described species, 2) closely related species within a genus, 3) bacteria previously isolated from body sites other than the vagina, and 4) known bacteria formerly isolated from the vagina. Pyrosequencing showed that novel strains *Peptoniphilaceae* DNF01163 and *Prevotellaceae* DNF00733 were prevalent in women with BV.

Conclusions: We isolated a diverse set of novel and clinically significant anaerobes from the human vagina using conventional approaches with systematic molecular identification. Several previously "uncultivated" bacteria are amenable to conventional cultivation.

Keywords: bacterial cultivation, human vagina, bacterial vaginosis, anaerobes, *Dialister*, *Eggerthella*, *Megasphaera*

Bacterial vaginosis (BV) is characterized by a dramatic increase in the abundance of fastidious anaerobes and facultative bacteria, with a concomitant reduction in select *Lactobacillus* species that are typically markers of health [1-5]. The microbiota in BV is heterogeneous with sub-groups of women having vaginal bacterial communities dominated by different bacteria such as *Prevotella* spp., *Leptotrichia/Sneathia* spp., BV-associated bacterium-1 (BVAB-1), or *Gardnerella vaginalis*, while in others there is no dominant bacterium [4]. Classical cultivation methods have routinely isolated numerous bacteria representing several different phyla from the human vagina in women with BV [6-10], but many bacteria detected in the vagina using molecular methods have not been successfully cultivated.

The lack of cultivated isolates that are clearly identified as BV-associated bacteria hinders our ability to explore the pathogenesis of BV. Cultivation provides critical information on bacterial phenotypes, and allows us to explore bacterial interactions among themselves, and with the human host. Cultivation also enables experimental manipulation of the isolates in the laboratory for testing hypotheses about pathogenesis and the role of virulence factors [11-15]. Isolates are important for generation of whole genome sequencing data, and interpretation of metagenomic data from vaginal samples, which inform the functional potential of individual species and their community.

Molecular surveys of the vaginal microbiota have shown that there are numerous bacteria associated with BV that are yet to be easily cultivated, such as *Eggerthella, Megasphaera*, *Dialister*, *Leptotrichia* and *Sneathia* species, and members of the Order *Clostridiales* including BVAB1 and BVAB2 [4, 16]. Cultivation studies of vaginal bacteria are typically not paired with identification of bacteria using advanced molecular methods, potentially leading to misclassification or under-representation of isolates obtained using these conventional approaches. In this study, we created a collection of over 100 different vaginal bacterial isolates obtained using classical cultivation methods coupled with systematic 16S rRNA

sequencing for isolate identification. We submitted DNA from bacteria that were under-represented in available culture collections for whole genome sequencing (WGS) by the Human Microbiome Project (HMP) Initiative to contribute to the bank of reference genomes [17].

METHODS

Sample Collection: Baseline vaginal samples were collected from 15 women attending the Public Health, Seattle and King County Sexually Transmitted Diseases Clinic (STD clinic) between April 2011 and July 2012 (Supplementary Table 1). We collected additional vaginal samples from 6 of 15 women at follow-up visits that were at least a month apart; one woman returned for one follow-up visit, four returned for two follow-ups and one woman returned for three follow-ups resulting in a total of 27 samples that were used for cultivation experiments and deep sequencing analysis (Supplementary Table 1). BV was diagnosed by Amsel criteria [18], and all 27 samples were also subjected to Gram stain interpretation using the Nugent method [19]. Of the 27 samples, 19 were collected when women had BV determined by both Amsel and Nugent criteria. Eight samples were collected when women did not have BV by Amsel criteria, however 3 of 8 were positive for BV by Nugent criteria (Supplementary Table 1). The study was approved by the Institutional Review Board at the Fred Hutchinson Cancer Research Center. All women provided written informed consent. A pelvic examination with speculum was performed for collection of samples. Polyurethane foam swabs (Epicenter Biotechnologies, Madison, WI) were brushed against the lateral vaginal wall for collection of vaginal bacteria. Swabs for cultivation experiments were transported in anaerobic Port-A-Cul™ tubes (Becton Dickinson, Franklin Lakes, NJ) from the clinic to the lab within two hours of collection, and processed in the lab within four hours of sample collection to maximize recovery of bacterial isolates. Swabs for molecular studies were stored at -80^{o}C until DNA extraction. Vaginal fluid was also collected for Gram staining, pH, saline preparation with microscopy, and potassium hydroxide preparation with microscopy.

Direct Plating for Isolation of Bacteria: The format for plating has been described in detail [7]. Briefly, vaginal swabs were vortexed in 1.5 mL of reduced Hank's solution [20, 21], and the resulting vaginal fluid was serially diluted in reduced saline. Aliquots (100 µL) from a range of dilutions were plated on Brucella Blood Agar with hemin and vitamin K (Hardy Diagnostics, Santa Maria, CA), Rogosa Agar selective for lactobacilli [22], Human Blood Tween (HBT) bilayer selective for *G. vaginalis,* and Brucella Laked Blood Agar with kanamycin, hemin, and vitamin K selective for *Prevotella* spp. (both from Becton Dickinson, Franklin Lakes, NJ), and incubated under anaerobic conditions at 37°C for 5 to 7 days. Another set of plates including HBT bilayer, Columbia Blood Agar with 5% sheep blood and Chocolate Agar (both from Hardy Diagnostics, Santa Maria, CA) were incubated for up to 72 hours at 37°C in 5-10% CO_2. Bacterial isolates from the plates were selected based on morphologic characteristics of the bacterial colonies using stereomicroscopy, Gram staining, and acridine orange staining followed by epifluorescence microscopy.

Identification and Phylogeny of Cultivated Isolates: DNA from bacterial pellets was extracted using the Biostic® Bacteremia DNA Isolation Kit (MoBio Laboratories Inc, Carlsbad, CA) and eluted in 75-150 µL buffer. Bacterial DNA was subjected to broad-range PCR using primers targeting a conserved sequence of the 16S rRNA gene [23, 24]. Amplification was confirmed by gel electrophoresis, amplicons were cleaned with the DNA Clean & Concentrator™-5 Kit (Zymo Research, Irvine, CA) and submitted for Sanger sequencing (Shared Resources, Fred Hutch). Sequence alignments were performed using BioEdit v.7.1.11 [25]. The 16S rRNA sequences were compared with sequences in GenBank [26] and EzTaxon [27] for taxonomic identification of each cultivated isolate. Phylogenetic trees were constructed using MEGA version 6 [28]. Select strains were submitted to BEI Resources, and 16S rRNA sequences have been submitted to the NCBI GenBank database (see Supplementary Table 1 for accession numbers).

Molecular Profiling of the Vaginal Microbiota: DNA from vaginal swabs was extracted using the Biostic® Bacteremia DNA Isolation Kit, eluted in 150 µL buffer. Sham swabs without human contact were processed as controls to assess contamination from reaction buffers or sample collection swabs. We performed community 16S rRNA gene PCR with pyrosequencing using 454 Life Sciences Titanium technology (Roche, Branford, CT) targeting the V3-V4 region of the 16S rRNA gene [4, 5] to determine the bacterial community in each vaginal swab sample. Sequence reads were classified using a phylogenetic placement tool *pplacer* [29] and a curated reference set of vaginal bacteria [4]. The prevalence of each bacterial isolate was determined by comparing isolate sequences to pyrosequencing reads using vsearch v1.9.10 (https://github.com/torognes/vsearch) with an alignment threshold of 99% identity. Pyrosequencing reads have been submitted to the NCBI Short Read Archive (SRP071678, SRX950298-SRX950303, SRX950306, SRX950317, SRX950348, SRX950350).

Preparation of DNA for Whole Genome Sequencing: Genomic DNA (gDNA) from bacterial pellets was extracted using the ZR Fungal/Bacterial DNA MidiPrep Kit (Zymo Research, Irvine, CA) and eluted in 150 µL buffer. A NanoDrop 1000 was used to determine gDNA concentrations, and gDNA quality was assessed using A260/280 and A260/230 ratios. Prior to submission for whole genome sequencing, isolate gDNA was sequence confirmed by broad-range 16S rRNA gene PCR and Sanger sequencing. DNA from 31 strains (Supplementary Table 1) was submitted to one of two HMP sequencing centers, Human Genomic Medicine, J. Craig Venter Institute (JCVI), Rockville, MD, or The Genome Center, Washington University School of Medicine, St. Louis, MO. A sub-set of these isolates were deposited in Biodefense and Emerging Infections Research Resources Repository (BEI Resources).

RESULTS

Bacterial Isolates. We isolated and identified 101 different bacterial strains spanning six phyla from a screen of 704 isolates obtained from 27 vaginal samples (Supplementary Table 1). As expected, the vast

majority of isolates were from the *Firmicutes* (n=43) and *Actinobacteria* (n=36). We also isolated bacteria belonging to the *Bacteroidetes* (n=14), *Proteobacteria* (n=6), *Fusobacteria (Fusobacterium nucleatum)*, and *Tenericutes (Mycoplasma hominis)*. Bacterial isolates were categorized into four groups described below in bold. Select strains are depicted in the phylogenetic tree (Figure 1), and colony morphology and Gram stains of one isolate from each of the four groups below are displayed in Figure 2.

Novel Strains: Of the 101 strains, 11 (10.9%) had <98% sequence identity to validly described species (Supplementary Table 1, Figure 1) including *Dialister* sp. Type 2 DNF00626 (95.7% sequence identity) and *Eggerthella* sp. Type 1 DNF00809 (90.3% sequence identity). Both are fastidious bacteria of clinical significance to BV [4, 30]. All novel isolates belonged to the phyla *Actinobacteria*, *Firmicutes* or *Bacteroidetes*.

Closely Related Species within a Genus: We obtained three or more phylogenetically distinct species from some genera including *Prevotella* (n=9), *Peptoniphilus* (n=3), and *Lactobacillus* (n=7) (Supplementary Table 1). Among the *Prevotella* species, we obtained both *Prevotella amnii* DNF00058 and *Prevotella timonensis* DNF01181 (Figure 1), designated as *Prevotella* genogroup 1 and *Prevotella* genogroup 2 in earlier molecular studies of the vaginal microbiota [16, 30]. Among the lactobacilli, we isolated *L. crispatus*, *L. jensenii*, *L. coleohominis*, and *L. vaginalis* mostly from women without BV, and *L. iners* and *L. gasseri* from women with and without BV. *L. iners* isolates were obtained only using media containing blood such as HBT, Columbia Blood Agar or Brucella Blood Agar, but not from Rogosa Agar, which is consistent with previous observations [31].

Identical Species Found in Other Body Sites, but not The Vagina: Additional searches of databases were conducted for bacteria that are not typically noted in molecular screens of the vaginal microbiota, and with >99% sequence identity with validly described species, in order to determine if they had been previously isolated from the vagina. Searches were conducted in NCBI PubMed

(http://www.ncbi.nlm.nih.gov/pubmed) and GenBank (http://www.ncbi.nlm.nih.gov/genbank/) databases with the search string "name of bacterium, vagina." Bacteria were scored as "not previously isolated from the vagina" if there were no publications reporting these bacteria as vaginal isolates, or no sequence data was available from cultured vaginal isolates. Seventeen bacteria (16.8%) not previously isolated from the vagina were identified in the study (Supplementary Table 1). Examples include *Atopobium deltae* DNF00019 previously isolated from human blood [32], and *Fenollaria massiliensis* DNF00604 formerly isolated from human osteoarticular samples [33] (Supplementary Table 1, Figure 1).

Previously Cultivated Members of the Vaginal Microbiota: Nineteen strains obtained (18.8%) have been previously isolated from the vagina including *Dialister micraerophilus*, *Veillonella* spp., *Anaerococcus* spp., and *Peptostreptococcus anaerobius* (Supplementary Table 1, Figure 1). Several isolates of *Atopobium vaginae* and *G. vaginalis* were cultivated; *A. vaginae* was isolated from HBT and Brucella Blood agar plates that were incubated under anaerobic conditions, while *G. vaginalis* was isolated from all plates tested except Rogosa agar under either aerobic or anaerobic conditions (data not shown).

Comparative Analysis of 16S rRNA Sequences Obtained from Isolates vs. those Found by Community PCR with High-Throughput Sequencing: The prevalence of each bacterial isolate was determined by comparing isolate sequences to pyrosequencing reads from the set of 25 samples. Two samples had fewer than 500 sequence reads and were excluded from this analysis. The 16S rRNA sequences of 60 isolates (59.4%) were detected in the vaginal community (Supplementary Table 2). Seven of the eleven sequences (63.6%) from novel bacteria were present, and *Dialister* sp. Type 2 DNF00626, *Eggerthella* sp. Type 1 DNF00809, *Peptoniphilaceae* DNF01163 and *Megasphaera* sp. Type 1 DNF00751 sequences were detected in over 50% of samples. *Prevotellaceae* DNF00733 (designated *Prevotella* genogroup 7 in molecular screens) was detected in 40% of samples. Among the genera with

multiple species isolated, we noted that the number of species detected by deep sequencing was consistently lower than the number of species isolated. For example, our molecular survey did not detect certain bacteria cultivated in this study such as *Lactobacillus vaginalis* DNF00112, several *Actinomyces*, and *Bifidobacterium* spp., or any *Corynebacterium* or *Propionibacterium* sequences. Likewise, only four of eighteen sequences (22.2%) from isolates classified as previously isolated from other body sites were detected by deep sequencing including *Atopobium deltae* DNF00019, *Facklamia hominis* DNF00119, *Fenollaria massiliensis* DNF00604, and *Gemella haemolysans* DNF01167. In contrast, most isolates (73.4%) classified as previously isolated from the vagina were detected by their 16S rRNA sequences in the community.

To identify bacteria that were both cultivated and numerically abundant in this study (>0.1% of all reads), we compared bacterial sequence reads with isolates obtained (Table 1). We isolated 23 out of 36 abundant bacteria (63.9%) using conventional plating methods. The analysis also highlighted notable exceptions including *Leptotrichia amnionii* and BVAB1 (Table 1).

DISCUSSION

Meta-omics technologies can provide great insight into the composition and function of bacterial communities, but do not fully substitute for the information gleaned from observing the properties of isolated microbial cells. Metagenomic and metatranscriptomic methods are useful for obtaining important clues regarding the genomic potential and gene expression profiles of bacteria in a community [34, 35], but so far they have not provided a complete picture of microbial properties and interactions. Many microbes remain resistant to cultivation in the lab [36, 37], and this is a serious impediment to better understanding of microbial interactions and pathogenesis. Here, we sought to expand the bank of cultivated bacterial isolates from the human vagina based on the assumption that many bacteria will grow in the lab using conventional cultivation methods, but are poorly identified

using conventional techniques. We predicted that using molecular identification tools would help close this gap. We used plating on conventional media to isolate vaginal bacteria, and paired this with molecular identification of isolates using 16S rRNA gene sequencing. A set of 101 different strains belonging to six phyla were obtained in the study illustrating that a wide range of bacteria can be cultivated from the vagina using classical approaches. We also examined the composition of the vaginal microbiota in the same set of samples using broad-range PCR and pyrosequencing. We determined prevalence of the bacterial isolate sequences in these samples, and identified bacteria that were not isolated using conventional cultivation in this study. A sub-set of 31 isolates has been submitted for whole genome sequencing based on the guidelines set by the HMP for the selection of reference genomes (http://hmpdacc.org/reference_genomes/reference_genomes.php). Briefly, bacterial isolates for WGS were selected for at least one of the following criteria: novelty or uniqueness of species, established clinical significance, abundance in the vagina, identical species found in a different body site or the opportunity to explore pan-genomes.

By using systematic 16S rRNA-based identification, we obtained 11 novel bacteria whose 16S rRNA sequences were <98% identical to validly described species. Of the 11 isolates, three bacteria are highly associated with BV (*Dialister* sp. Type 2, *Eggerthella* sp. Type 1, and *Megasphaera* sp. Type 1) [4, 16, 30]. Although not dominant members of the vaginal bacterial community, these strains are prevalent in women with BV. *Eggerthella* sp. Type 1 16S rRNA sequence was detected in 85.5% of women with BV compared with 9.5% of women without BV; *Dialister* sp. Type 2 in 72.6% of women with BV versus 4.8% without and *Megasphaera* sp. Type 1 in 67.5% of women with BV and 12.4% of women without BV [4]. Interestingly, in an analysis that examined association of bacterial taxa with individual Amsel clinical characteristics, *Dialister* sp. Type 2 and *Megasphaera* sp. Type 1 were correlated with clue cells, while *Eggerthella* sp. Type 1 was one of only two bacteria that were associated with all four clinical criteria [4]. In another study that examined vaginal bacteria in 264 women from Seattle, *Megasphaera* sp. Type 1,

combined with BVAB2 was found to be highly sensitive and specific for the diagnosis of BV [30]. This is the first report of cultivated isolates of *Dialister* sp. Type 2 and *Eggerthella* sp. Type 1 and their DNA has been submitted for WGS by HMP to generate reference genomes. The Hillier lab at Magee Women's Research Institute, Pittsburgh, has previously cultivated *Megasphaera* sp. Type 1 (strain 28L) whose genome has been sequenced (ADGP00000000) (http://hmpdacc.org/catalog/).

We cultivated several bacterial isolates that were minority members of the bacterial community but were highly prevalent. Minority status does not necessarily imply that these isolates are insignificant in the pathogenesis of BV. Low abundance organisms in the human body can function as pathogens under particular conditions. For example, *Porphyromonas gingivalis*, a low abundance but prevalent bacterium in the oral niche, can disrupt host-microbe homeostasis leading to inflammatory periodontal disease [38]. Having cultivated isolates of minority vaginal bacteria provides opportunities for future hypothesis development and *in vitro* experimentation. Potentially important novel isolates include *Peptoniphilaceae* DNF01163 and *Prevotellaceae* DNF00733, which were present in 68% and 40% of women in this study, but at low abundance.

Similarly, little is known about the role of rare members of the bacterial community in the human microbiome that are neither prevalent, nor abundant. Cultivation strategies have the potential to be more sensitive than deep sequencing methods in detecting these rare members if favorable growth conditions are provided. Theoretically, cultivating a bacterium requires just one bacterial cell to divide and form a colony under optimal growth conditions. Examples of rare bacteria isolated in this study include novel bacteria such as *Corynebacterium* sp. DNF00584 and *Olsenella* sp. DNF959, and many members of *Actinomyces*, *Bifidobacterium* and *Corynebacterium* genus. It is conceivable that some rare bacteria isolated in the vagina are not long-term colonizers of the vaginal microbiota, but are transient members of the community. For example, the *Actinomyces* are present in oral bacterial communities

[39], *Bifidobacterium* spp. are gut colonizers [40], and *Corynebacterium* spp. are key members of the skin microbiota [41], and these bacteria may have been inoculated into the vagina as a result of sexual or other practices. It is also plausible that duplicate species may have differing metabolic capabilities depending on the body site they are present in, and studies examining such questions are emerging as more isolates and genomes are available for comparison [42]. With this in mind, the HMP has recommended that duplicate species from different body sites be selected for WGS to help unravel the role of similar bacterial species in different body sites (http://hmpdacc.org/reference_genomes/reference_genomes.php); several bacteria from our study have been submitted for WGS (Supplementary Table 1).

Human microbial communities frequently have multiple species within a bacterial genus, but there is limited understanding of the function of closely related species in each ecological niche. Several species of *Prevotella* and *Lactobacillus* were isolated in this study. There is emerging evidence that not all *Lactobacillus* species are functionally equivalent in the vaginal niche. For example, *L. crispatus* and *L. jensenii* have often been associated with health in many cultivation and molecular studies. Recent molecular investigations have shown that *L. iners* is present in high abundance in women with and without BV [2, 4]. We have previously noted that *L. iners* often dramatically increases in concentrations following antibiotic treatment for BV [43, 44], and this bacterium has metabolic profiles that are intermediate between the lactobacilli often associated with health, *L. crispatus* and *L. jensenii*, and the BV-associated bacteria [5]. These observations highlight that closely related members of a bacterial community can have different metabolic capabilities in the same ecological niche. Similarly, several *Prevotella* species found in the vagina are associated with BV, but the role of each species is poorly understood. *Prevotella amnii* and *Prevotella timonensis* are both associated with BV [4, 30, 45], and have been isolated in this study. *P. buccalis*, *P. disiens* and *P. bivia* sequences were correlated with a positive amine odor, while *P. bivia* sequences were associated with elevated pH [4]. Having multiple isolates of

these closely related *Prevotella* species will enable us to explore pangenomes, the full complement of genes in a phylogenetic clade, and facilitate investigations about the genetic variation and functional redundancy of closely related species.

An important question that we addressed was what fraction of bacterial species identified in the vagina using PCR methods are ultimately cultivated using the conventional plating method. We successfully cultivated a large fraction of the bacteria known to be present in the vagina, though several species remained recalcitrant to cultivation. Prominent bacteria that we did not isolate include *Leptotrichia/Sneathia* spp., *Megasphaera* sp. Type 2, BVAB1, BVAB2, and *Mageeibacillus indolicus* (BVAB3) among others. Recently, *M. indolicus* was isolated on Brucella Blood Agar from an endometrial biopsy and has the ability to produce indole, hence the name [46]. In another study, *Sneathia amnii* was isolated on Chocolate agar plates from a vaginal swab [47]. A genome of *Megasphaera* sp. Type 2 isolated by the Hillier lab is available from the HMP collection of reference genomes (www.hmpdacc.org/catalog). Nonetheless, there are still uncultivated species from the human vagina, and novel approaches may be required to routinely culture these crucial members of the vaginal microbiota.

In conclusion, classical cultivation paired with methodical 16S rRNA sequencing for identification yielded a diverse and varied set of bacterial isolates from the human vagina, including two key members of the BV microbiota, *Dialister* sp. Type 2 and *Eggerthella* sp. Type 1, that were known only by their molecular signatures. Identification of isolates using 16S rRNA sequencing and phylogeny was valuable and robust in differentiating between bacterial strains. However, the approach was labor intensive, and neither high-throughput nor real time. Bacteria isolated from this study are being subjected to whole genome sequencing, and used to study microbial interactions *in vitro* with the goal of further illuminating the pathogenesis of BV.

Notes

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Table 1. Abundant Bacteria Isolated by Conventional Cultivation

*Bacteria Detected by Broad-range PCR with Pyrosequencing	Isolates Obtained
Anaerococcus prevotii	Y
Atopobium vaginae	Y
Dialister micraerophilus	Y
Dialister sp. Type 2	Y
Eggerthella sp. Type 1	Y
Finegoldia magna	Y
Fusobacterium nucleatum	Y
Gardnerella vaginalis	Y
Lactobacillus crispatus	Y
Lactobacillus gasseri	Y
Lactobacillus iners	Y
Lactobacillus jensenii	Y
Megasphaera sp. Type 1	Y
Mycoplasma hominis	Y
Peptoniphilus harei	Y
Prevotella amnii	Y
Prevotella bivia	Y
Prevotella disiens	Y
Prevotella melaninogenica	Y
Prevotella timonensis	Y
Streptococcus agalactiae	Y
Streptococcus anginosus	Y
Streptococcus mitis/oralis	Y
Aerococcus christensenii	N
BV-associated bacterium 1 (BVAB1)	N
BV-associated bacterium 2 (BVAB2)	N
Gemella asaccharolytica	N
Leptotrichia amnionii	N
Mageeibacillus indolicus	N
***Megasphaera* sp. Type 2**	N
Parvimonas micra	N
Porphyromonas asaccharolytica	N
***Prevotella* genogroup 3**	N
***Prevotella* genogroup 4**	N
Sneathia sanguinegens	N
Streptococcus gallolyticus	N

*Bacteria >0.1% abundance across 25 samples in the study. Bacteria in bold were not isolated in this study.

Figure Legends

Figure 1: Diversity of bacterial isolates obtained with classical cultivation methods. Minimum evolution phylogenetic dendrogram showing relationships of representative 16S rRNA gene sequences of vaginal bacterial isolates among closely related validly described species. Numbers at branch points depict bootstrap support based on analysis of 1000 replicates. GenBank Accession numbers of the 16S rRNA sequences are provided in parentheses. Strains are color coded based on categories developed in this study. Orange: Bacteria <98% sequence identity to validly published species; Blue: Closely related species within a genus; Purple: Bacteria previously isolated from other human body sites, but not the vagina; Green: Known bacteria previously isolated from the vagina. Bar, 5% sequence divergence.

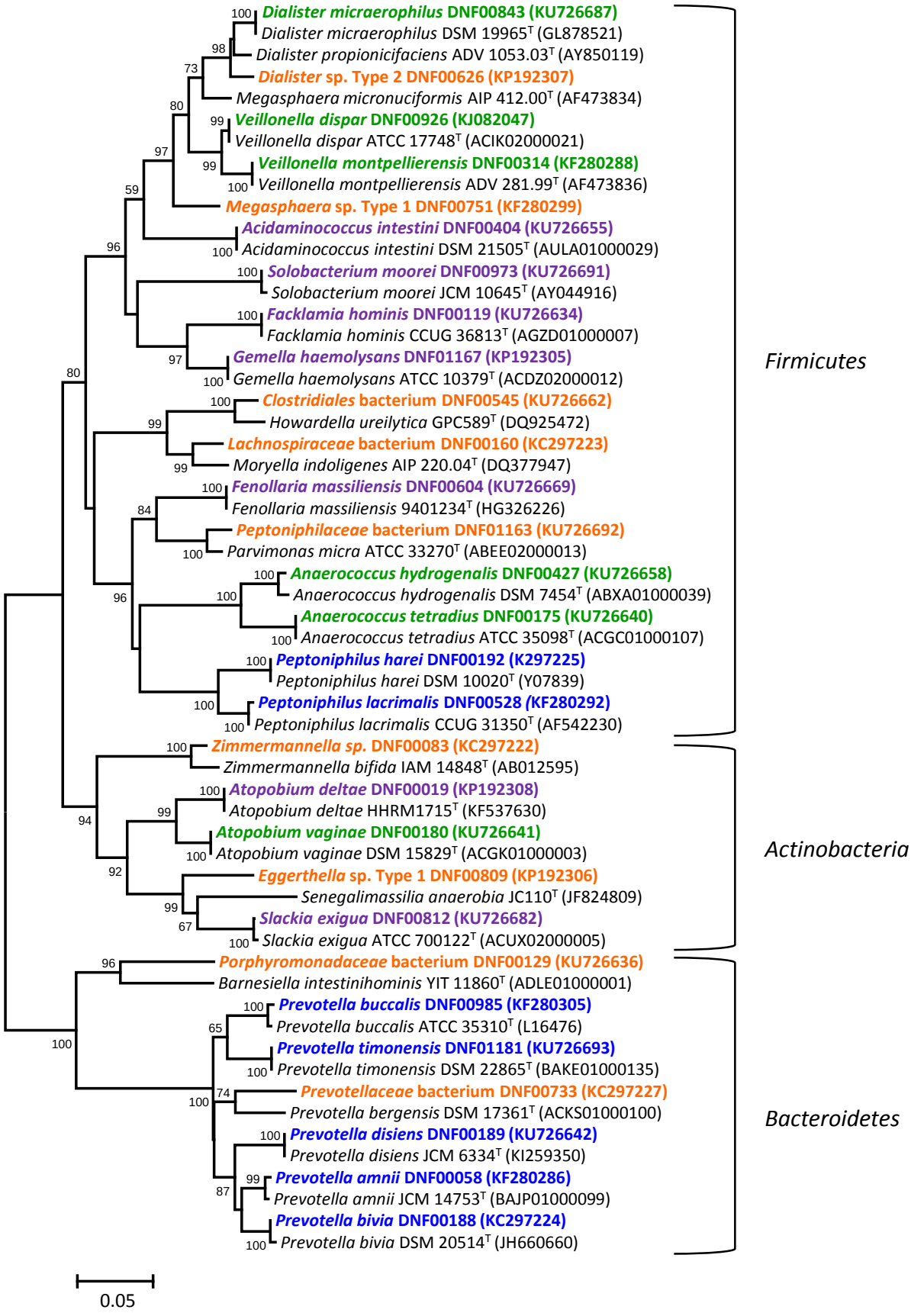
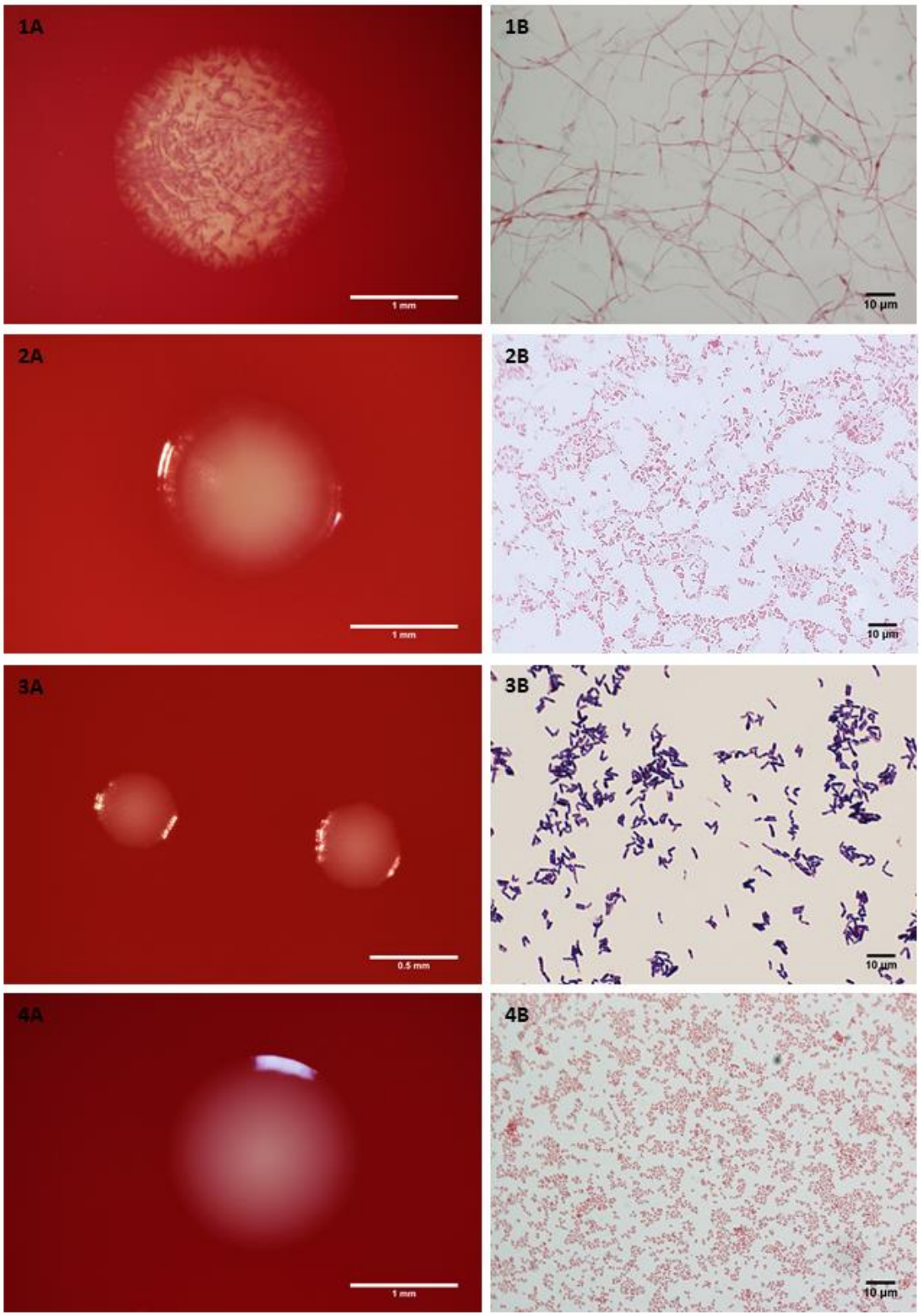

Dialister micraerophilus DNF00843 (KU726687)
Dialister micraerophilus DSM 19965T (GL878521)
Dialister propionicifaciens ADV 1053.03T (AY850119)
Dialister sp. Type 2 DNF00626 (KP192307)
Megasphaera micronuciformis AIP 412.00T (AF473834)
Veillonella dispar DNF00926 (KJ082047)
Veillonella dispar ATCC 17748T (ACIK02000021)
Veillonella montpellierensis DNF00314 (KF280288)
Veillonella montpellierensis ADV 281.99T (AF473836)
Megasphaera sp. Type 1 DNF00751 (KF280299)
Acidaminococcus intestini DNF00404 (KU726655)
Acidaminococcus intestini DSM 21505T (AULA01000029)
Solobacterium moorei DNF00973 (KU726691)
Solobacterium moorei JCM 10645T (AY044916)
Facklamia hominis DNF00119 (KU726634)
Facklamia hominis CCUG 36813T (AGZD01000007)
Gemella haemolysans DNF01167 (KP192305)
Gemella haemolysans ATCC 10379T (ACDZ02000012)
Clostridiales bacterium DNF00545 (KU726662)
Howardella ureilytica GPC589T (DQ925472)
Lachnospiraceae bacterium DNF00160 (KC297223)
Moryella indoligenes AIP 220.04T (DQ377947)
Fenollaria massiliensis DNF00604 (KU726669)
Fenollaria massiliensis 9401234T (HG326226)
Peptoniphilaceae bacterium DNF01163 (KU726692)
Parvimonas micra ATCC 33270T (ABEE02000013)
Anaerococcus hydrogenalis DNF00427 (KU726658)
Anaerococcus hydrogenalis DSM 7454T (ABXA01000039)
Anaerococcus tetradius DNF00175 (KU726640)
Anaerococcus tetradius ATCC 35098T (ACGC01000107)
Peptoniphilus harei DNF00192 (K297225)
Peptoniphilus harei DSM 10020T (Y07839)
Peptoniphilus lacrimalis DNF00528 (KF280292)
Peptoniphilus lacrimalis CCUG 31350T (AF542230)
Zimmermannella sp. DNF00083 (KC297222)
Zimmermannella bifida IAM 14848T (AB012595)
Atopobium deltae DNF00019 (KP192308)
Atopobium deltae HHRM1715T (KF537630)
Atopobium vaginae DNF00180 (KU726641)
Atopobium vaginae DSM 15829T (ACGK01000003)
Eggerthella sp. Type 1 DNF00809 (KP192306)
Senegalimassilia anaerobia JC110T (JF824809)
Slackia exigua DNF00812 (KU726682)
Slackia exigua ATCC 700122T (ACUX02000005)
Porphyromonadaceae bacterium DNF00129 (KU726636)
Barnesiella intestinihominis YIT 11860T (ADLE01000001)
Prevotella buccalis DNF00985 (KF280305)
Prevotella buccalis ATCC 35310T (L16476)
Prevotella timonensis DNF01181 (KU726693)
Prevotella timonensis DSM 22865T (BAKE01000135)
Prevotellaceae bacterium DNF00733 (KC297227)
Prevotella bergensis DSM 17361T (ACKS01000100)
Prevotella disiens DNF00189 (KU726642)
Prevotella disiens JCM 6334T (KI259350)
Prevotella amnii DNF00058 (KF280286)
Prevotella amnii JCM 14753T (BAJP01000099)
Prevotella bivia DNF00188 (KC297224)
Prevotella bivia DSM 20514T (JH660660)
Firmicutes
Actinobacteria
Bacteroidetes
0.05

Figure 2: Colony morphology (A) and Gram stain of vaginal bacteria (B). Representative isolates from the four groups described in the study are shown here. 1. *Lachnospiraceae* bacterium DNF00160 2. *Prevotella massiliensis* DNF00663 3. *Corynebacterium kroppenstedtii* DNF00591 4. *Prevotella lacrimalis* DNF00528.


1A
1 mm
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