INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY

TAXONOMIC DESCRIPTION

Srinivasan *et al.*, *Int. J. Syst. Evol. Microbiol.* 2021;71:004702

DOI 10.1099/ijsem.0.004702

MICROBIOLOGY SOCIETY

Megasphaera lornae sp. nov., *Megasphaera hutchinsoni* sp. nov., and *Megasphaera vaginalis* sp. nov.: novel bacteria isolated from the female genital tract

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Abstract

Six strictly anaerobic Gram-negative bacteria representing three novel species were isolated from the female reproductive tract. The proposed type strains for each species were designated UPII 199-6^T, KA00182^T and BV3C16-1^T. Phylogenetic analyses based on 16S rRNA gene sequencing indicated that the bacterial isolates were members of the genus *Megasphaera*. UPII 199-6^T and KA00182^T had 16S rRNA gene sequence identities of 99.9% with 16S rRNA clone sequences previously amplified from the human vagina designated as *Megasphaera* type 1 and *Megasphaera* type 2, members of the human vaginal microbiota associated with bacterial vaginosis, preterm birth and HIV acquisition. UPII 199-6^T exhibited sequence identities ranging from 92.9 to 93.6% with validly named *Megasphaera* isolates and KA00182^T had 16S rRNA gene sequence identities ranging from 92.6–94.2%. BV3C16-1^T was most closely related to *Megasphaera cerevisiae* with a 16S rRNA gene sequence identity of 95.4%. Cells were coccoid or diplococcoid, non-motile and did not form spores. Genital tract isolates metabolized organic acids but were asaccharolytic. The isolates also metabolized amino acids. The DNA G+C content for the genome sequences of UPII 199-6^T, KA00182^T and BV3C16-1^T were 46.4, 38.9 and 49.8mol%, respectively. Digital DNA–DNA hybridization and average nucleotide identity between the genital tract isolates and other validly named *Megasphaera* species suggest that each isolate type represents a new species. The major fatty acid methyl esters include the following: $C_{12:0}$, $C_{16:0}$, $C_{16:0}$ dimethyl acetal (DMA) and summed feature 5 ($C_{15:0}$ DMA and/or $C_{14:0}$ 3-OH) in UPII 199-6^T; $C_{16:0}$ and $C_{16:1}$*cis* 9 in KA00182^T; $C_{12:0}$; $C_{14:0}$ 3-OH; and summed feature 5 in BV3C16-1^T. The isolates produced butyrate, isobutyrate, and isovalerate but there were specific differences including production of formate and propionate. Together, these data indicate that UPII 199-6^T, KA00182^T and BV3C16-1^T represent novel species within the genus *Megasphaera*. We propose the following names: *Megasphaera lornae* sp. nov. for UPII 199-6^T representing the type strain of this species (=DSM 111201^T=ATCC TSD-205^T), *Megasphaera hutchinsoni* sp. nov. for KA00182^T representing the type strain of this species (=DSM 111202^T=ATCC TSD-206^T) and *Megasphaera vaginalis* sp. nov. for BV3C16-1^T representing the type strain of this species (=DSM 111203^T=ATCC TSD-207^T).

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Keywords: bacterial vaginosis; genital tract bacteria; human vagina; *Megasphaera*; *Negativicutes*; *Veillonellaceae*.
Abbreviations: ANI, average nucleotide identity; BM, basic medium; BV, bacterial vaginosis; dDDH, digital DNA–DNA hybridization; DMA, dimethyl acetal; FAME, fatty acid methyl ester; PID, pelvic inflammatory disease; PYG-mod, peptone–yeast–glucose modified; PYG-mod-YG, PYG-mod medium supplemented with 1% yeast extract and 1% glucose; SCFA, short chain fatty acid.
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UPII 199-6^T, KA00182^T and BV3C16-1^T have been deposited in the American Type Culture Collection (ATCC TSD-205, ATCC TSD-206 and ATCC TSD-207, respectively) and the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (DSM 111201, DSM 111202 and DSM 111203). Accession numbers for the 16S rRNA sequences for UPII 199-6^T, KA00182^T and BV3C16-1^T are MT906439, KP192298 and JN809775 respectively. Accession numbers for whole genome sequences for UPII 199-6^T, KA00182^T and BV3C16-1^T are PRJNA64689, PRJNA272074 and PRJNA89631, respectively.
Two supplementary figures are available with the online version of this article

The genus *Megasphaera* was created by Rogosa when describing the type species *Megasphaera elsdenii*, originally classified as *Peptostreptococcus elsdenii* [1] but revised when the bacterium did not exhibit a key characteristic of the genus *Peptostreptococcus*. *M. elsdenii* is Gram-negative by direct staining and by electron microscopy while *Peptostreptococcus* species are Gram-positive [1]. Eight additional *Megasphaera* species belonging to the phylum *Firmicutes*, class *Negativicutes*, order *Veillonellales* and family *Veillonellaceae* have subsequently been validly named [2–7]. *Megasphaera* species are Gram-negative, with a peculiar diderm cell wall structure typical for the *Negativicutes* [8], obligate anaerobes that are often detected in intestinal environments [9]. *Megasphaera indica* and *Megasphaera massiliensis* were isolated from human faeces [5, 10], and *Megasphaera micronuciformis* from a human liver abscess [7]. *M. elsdenii* and *Megasphaera hexanoica* were cultivated from sheep and cow rumen respectively [1, 3], while *Megasphaera stantonii* was cultured from a chicken cecum [6]. Three *Megasphaera* species have been isolated from brewery samples or spoiled beer including *Megasphaera cerevisiae*, *Megasphaera sueciensis* and *Megasphaera paucivorans* [2, 4].

Cultivation-independent molecular investigations have reported that *Megasphaera* species in the female genital tract are associated with adverse health outcomes in women. Novel *Megasphaera* sequences were first noted in the human vagina by Zhou *et al.* in a study of five women using cloning and sequencing methods [11]. Fredricks *et al.* identified two distinct *Megasphaera* sequence types in a study evaluating association of vaginal bacteria with the common dysbiotic condition, bacterial vaginosis (BV) [12] and validated these observations using targeted PCR assays in women with and without BV [13, 14]. Subsequently, several studies have reported the association of these two *Megasphaera* sequence types with BV using molecular approaches [15–26]. *Megasphaera* type 1 was shown to be useful for the molecular diagnosis of BV [13, 14, 18, 22, 27–31] and has been included as a target in commercially available nucleic acid amplification tests for the diagnosis of BV [28]. Vaginal *Megasphaera* species have also been shown to be associated with increased risk for HIV acquisition [32, 33] and among a group of mostly South African women, *Megasphaera* type 2 was associated with increased risk but not *Megasphaera* type 1 [34]. Furthermore, pregnant women with a prior history of preterm delivery and increasing levels of *Megasphaera* type 1 through mid-pregnancy were more likely to experience spontaneous preterm delivery [35]. An association between *Megasphaera* species and spontaneous preterm birth was also noted in a case-control study of mostly African American women [36]. A recent study showed that women with pelvic inflammatory disease were more likely to test positive for *Megasphaera* species among other anaerobes [37].

Our groups have previously isolated *Megasphaera* bacterial species [38–40] and have demonstrated that *Megasphaera* type 1 and type 2 bacterial isolates are susceptible to clindamycin and the nitroimidazoles used to treat BV including metronidazole, tinidazole and secnidazole [38, 39]. *Megasphaera* species have also been detected in the human oral cavity [17, 19, 41, 42], rectum [17, 19], stool [43–45] and male genitourinary microbiome [46–48]. Here, we systematically characterize three novel *Megasphaera* species from the human genital tract including isolates previously designated as *Megasphaera* type 1 and type 2 and compare them to validly published *Megasphaera* species. We propose the names *Megasphaera lornae* sp. nov. for UPII 199-6^T, *Megasphaera hutchinsoni* sp. nov. for KA00182^T and *Megasphaera vaginalis* sp. nov. for BV3C16-1^T as they represent novel species within the genus *Megasphaera*. For each of these proposed type strains, we have also characterized a second isolate including DNF00751 for *M. lornae*, UPII 135-E for *M. hutchinsoni* and HL562 for *M. vaginalis*.

The novel *Megasphaera* species characterized here were isolated from either an endometrial biopsy or a vaginal fluid sample obtained from women participating in two separate research studies. The research study at the University of Pittsburgh evaluated women for pelvic inflammatory disease (PID); endometrial biopsy samples were collected in a protocol approved by the University of Pittsburgh Review Board (IRB approval number: PRO 010010112) [39, 49]. The research study at the Fred Hutchinson Cancer Research Center sought to isolate novel anaerobes from the human vagina [40] and was approved by the institutional review board at the Fred Hutchinson Cancer Research Center (IRB approval number: IR 7363). All participants provided written informed consent. UPII 199-6^T was isolated from an endometrial biopsy tissue sample from a woman being evaluated for PID. An endometrial sampling device (Pipelle) was inserted through the cervix into the uterine cavity and a biopsy was aspirated by suction. The biopsy was inoculated onto Brucella agar supplemented with 5% laked sheep blood, hemin and Vitamin K (Brucella blood agar) (bioMérieux or Hardy Diagnostics) and incubated anaerobically at 37 °C for 4–7 days. An anaerobic atmosphere for all experiments was created using a trimix with 90% N_2, 5% H_2 and 5% CO_2 (Airgas). KA00182^T was isolated on Brucella blood agar from a vaginal fluid sample which was diluted, plated and incubated anaerobically at 37 °C for 5–7 days. BV3C16-1^T was isolated from a vaginal sample obtained from a woman with BV through enrichment in a liquid basic medium (BM) and incubated at 37 °C for 2 to 4 days, followed by isolation on trypticase yeast extract blood agar plates with anaerobic incubation at 37 °C for at least 2 weeks. BM comprised Solution A and a reducing agent solution which had the following components per litre of media: Solution A contained 1 g yeast extract, 1 g casamino acids, 1.04 g KH_2PO_4 and 1.11 g K_2HPO_4; reducing agent solution contained 0.4 g NH_4Cl, 0.1 g $MgCl_2{\cdot}6H_2O$, 0.05 g $CaCl_2{\cdot}2H_2O$, 1 ml trace elements SL10, 0.0025 g resazurin, 0.05 g $FeCl_2{\cdot}4H_2O$ and 0.5 g L-cysteine HCl. The two solutions were prepared separately to avoid precipitation and chemical interactions during autoclaving. After preliminary identification using 16S rRNA gene sequencing, isolates were frozen at −80 °C in litmus milk (Becton Dickinson) or glycerol stocks (10% v/v). Prior to use of the bacterial isolates in experiments, scrapings from frozen stocks were plated on Brucella blood agar and incubated anaerobically for 48–72 h

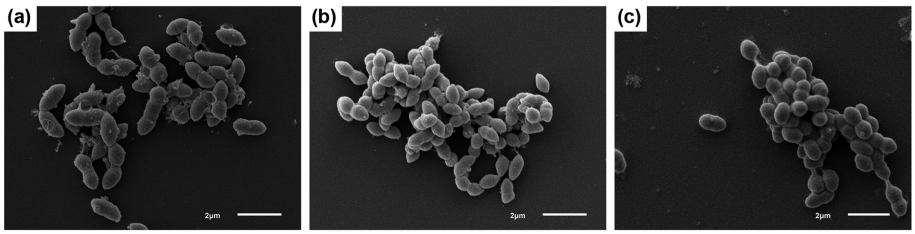

Fig. 1. Scanning electron micrograph of cells of (a) UPII 199-6^T, (b) KA00182^T, and (c) BV3C16-1^T. Cells were cultured for 2 days in PYG-mod-YG. Bar, 2 µm.

at 37 °C. Individual colonies were sub-cultured at least twice and examined by Gram stain or 16S rRNA gene sequencing to ensure purity.

Validly named *Megasphaera* species were used as reference strains for comparisons with the genital tract strains. *M. cerevisiae* DSM 20462^T [2], *M. elsdenii* DSM 20460^T [1], *M. indica* DSM 25563^T [5], *M. massiliensis* DSM 26228^T [10], *M. micronuciformis* DSM 17226^T [7], *M. paucivorans* DSM 16981^T and *M. sueciensis* DSM 17042^T [4] were obtained from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (DSMZ). Strains were maintained anaerobically on Brucella blood agar or peptone–yeast–glucose modified (PYG-mod) medium [50] supplemented with 1% yeast extract and 1% glucose (PYG-mod-YG) at 37 °C. Bacterial characteristics for *M. stantonii* AJH 120^T [6] and *M. hexanoica* MH^T [3] were obtained from the original manuscripts.

Optimal temperature was tested by growing the isolates on Brucella blood agar and incubating them at different temperatures. Optimal pH was tested by growing the isolates in PYG-mod-YG broth adjusted to varying pH with 2 M HCl or 1 N NaOH solutions. OD_{600} measurements were conducted in an Epoch 2 microplate reader (BioTek). UPII 199-6^T, KA00182^T and BV3C16-1^T were strict anaerobes. UPII 199-6^T exhibited growth between 35–37 °C (optimal, 37 °C) and pH 5.0–6.5 (optimal, 6.0–6.5). KA00182^T grew between 30–42 °C (optimal, 35–37 °C) and pH 5.5–7.5 (optimal, 5.5). BV3C16-1^T had a growth range between 30–37 °C (optimal, 35–37 °C) and pH 5.0–7.5 (optimal, 7.0). Motility and spore formation were assessed in duplicate using previously described methods [51, 52]. For the motility assay, *Paeniclostridium sordellii* DSM 2141 was used as a positive control and *Gardnerella vaginalis* ATCC 14018 was used as a negative control. *P. sordellii* and *Escherichia coli* DNF00564 were used as positive and negative controls, respectively, for the spore formation assay. All three *Megasphaera* isolate types from the genital tract were non-motile and non-spore-forming.

UPII 199-6^T, KA00182^T and BV3C16-1^T were grown anaerobically at 37 °C for 2 days on Brucella blood agar to assess colony and cellular morphologies. Colonies of strain UPII 199-6^T were convex, entire, glossy and off-white in colour with a diameter of 0.5–0.7 mm. Colonies of strain KA00182^T were convex, entire, glossy and off-white in colour with a diameter of 0.4–0.7 mm. Colonies of strain BV3C16-1^T were small, convex, and translucent with a diameter of 0.3–0.4 mm. All isolates stained as Gram-negative cocci or short coccobacilli. Scanning electron microscopy (SEM; Fig. 1) and transmission electron microscopy (TEM; Fig. 2) were performed to further evaluate cellular morphology. Bacterial cultures grown in PYG-mod-YG medium were centrifuged to form a pellet and fixed in 0.5 strength Karnovsky's fixative (2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.3) overnight at 4 °C for both SEM and TEM. For SEM, duplicates of each sample (50 µl) were applied in a pool on poly-L-lysine coated coverslips for 30 min, rinsed with 0.1 M sodium cacodylate buffer and treated with 1% osmium tetroxide for 1 h. The coverslips were rinsed with cacodylate

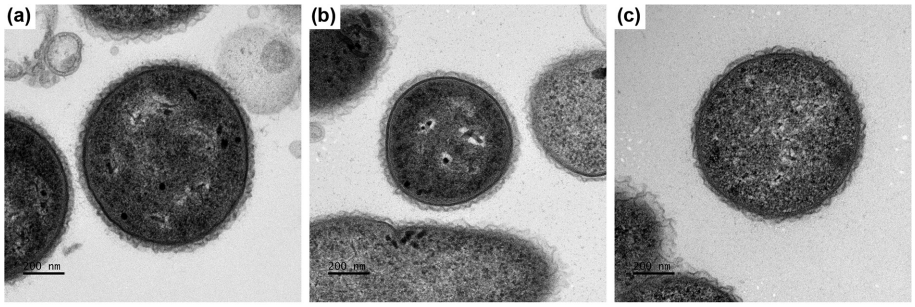

Fig. 2. Transmission electron microscope image of cells of (a) UPII 199-6^T, (b) KA00182^T, and (c) BV3C16-1^T. Cells were cultured for 2 days in PYG-mod-YG. Bar, 200 nm.

buffer, dehydrated through a graded series of alcohols, infiltrated with hexamethyldisilazane and allowed to air dry. Coverslips were mounted on stubs and sputter coated with gold/palladium. Samples were imaged on a JEOL 6610 LV SEM at 5kV. For TEM, after fixation, cells were pelleted, rinsed with 0.1 M cacodylate buffer, treated with 1% osmium tetroxide for 1 h, rinsed with cacodylate buffer and dehydrated through a graded series of alcohols and propylene oxide. Pellets were embedded in Eponate12 resin (Ted Pella). Sections (70 nm) were cut using a Leica EM UC7 ultramicrotome, contrasted with uranyl acetate and lead citrate, and imaged on a JEOL JEM 1400 transmission microscope at 120kV. Digital images were acquired with a Gatan Ultrascan 1000XP digital camera system. UPII 199–6^T cells had rounded ends, were often arranged in pairs and 0.9–1.5 µm long and 0.9–1.1 µm wide (Table 1, Figs 1 and 2). KA00182^T cells were oval with tapered ends and were arranged in singlets, pairs and chains along their tapered ends. KA00182^T cells were 0.5–0.7 µm long and 0.5–0.7 µm wide (Table 1, Figs 1 and 2). BV3C16-1^T cells were larger with rounded ends, often occurring in pairs with cells being 1.0–1.4 µm long and 0.7–1.1 µm wide (Table 1, Figs 1 and 2).

The cellular fatty acid composition of UPII 199-6^T, KA00182^T and BV3C16-1^T were compared with the reference *Megasphaera* strains except *M. hexanoica* and *M. stantonii*. All bacterial strains were grown on Brucella blood agar and colonies were harvested at 2 days growth, resuspended in DNAse free water and pelleted by centrifugation at 12000 r.p.m. for 2 min. Cell pellets were frozen at −80 °C. Fatty acid analysis was conducted using gas chromatography with the Sherlock Fatty Acid Analysis System by Microbial ID. The most abundant fatty acids of the three novel *Megasphaera* species from the genital tract were: UPII 199-6^T, $C_{12:0}$, $C_{16:0}$, $C_{16:0}$ dimethyl acetal (DMA) and summed feature 5 ($C_{15:0}$ DMA and/or $C_{14:0}$ 3-OH); KA00182^T, $C_{16:0}$ and $C_{16:1}$*cis* 9; BV3C16-1^T, $C_{12:0}$, $C_{14:0}$ 3-OH and summed feature 5 (Table 2). Similar patterns were noted in the second isolate of each proposed type strain (Table 2). While the three novel species shared some similarities with other *Megasphaera* type strains, proportions were different, and differences were also noted in other cellular fatty acids thereby distinguishing them from other validly named *Megasphaera* type strains.

Initially, simple biochemical tests were performed for presumptive identification to the genus level [9, 53, 54] and included catalase (Fisher Scientific), spot indole (prepared in-house), oxidase (Becton Dickinson), nitrate reduction (Anaerobe Systems) and H_2S production (Hardy Diagnostics). Biochemical characterization of the genital tract isolates and the reference strains were conducted in duplicate using the Biolog Anaerobe Identification Test Panel to evaluate metabolism of sugars and amino acids [54, 55] according to the manufacturer's instructions. We also used the Pre-Reduced Anaerobically Sterilized (PRAS) medium to assess sugar fermentation [56] and gas production. The genital tract *Megasphaera* isolates that were tested did not produce gas, H_2S, or indole and were negative for catalase, oxidase and nitrate reductase (Table 1). Like *M. paucivorans* and *M. sueciensis,* the genital tract *Megasphaera* species did not metabolize sugars including glucose, fructose, sucrose and lactose. Moreover, none of the *Megasphaera* species tested metabolized gelatin. They were positive in the Biolog tests for organic acids such as lactate, pyruvate and α-ketovalerate. KA00182^T, UPII 135-E and HL562 metabolized succinate but UPII 199-6^T, DNF00751 and BV3C16-1^T yielded weak results; none of the other *Megasphaera* type strains with available results metabolized succinate. Specific differences between the genital tract *Megasphaera* species were noted in amino acid metabolism. Of the amino acids tested, UPII 199-6^T only metabolized serine, which was also positive for all other *Megasphaera* strains tested. In contrast, BV3C16-1^T and HL562 metabolized all five amino acids tested including alanine, alanyl-L-glutamine, glutamine, glutamate and serine while KA00182^T tested positive for alanyl-L-glutamine, glutamate and serine.

To characterize the metabolic end products, all isolates were grown in PYG-mod-YG at 37 °C in triplicate for 24–48 h. Cell supernatants were used for detection of short chain fatty acids and organic acids using ^{1}H-NMR spectroscopy. NMR analyses of bacterial supernatants were made using a Bruker AVANCE III 800 MHz spectrometer equipped with a cryoprobe or a room temperature probe suitable for ^{1}H inverse detection with Z-gradients at 298 K. 500 µl media was mixed with 100 µl phosphate buffer prepared in deuterated water (0.1 M; pH=7.4) containing 50 µM TSP (3-(trimethylsilyl) propionic-2,2,3,3-d_4 acid sodium salt). The solution was placed in a 5 mm NMR tube and one-dimensional ^{1}H NMR spectra were obtained using a one pulse sequence that included residual water signal suppression by pre-saturation during the relaxation delay. For each sample, 32 k data points were acquired using a spectral width of 9615 Hz and a relaxation delay of 10 s. The data were processed using a spectral size of 32 k points and by multiplying with an exponential window function with a line broadening of 0.5 Hz. The resulting spectra were phase and baseline corrected and referenced with respect to the internal TSP signal. Metabolite peaks in the spectra were then assigned using chemical shift databases [57–59] and the peak assignments were confirmed based on the spectra of standard compounds obtained under identical conditions. The peak areas were obtained by integration with reference to the internal reference signal from TSP. Using these peak areas, along with the known concentration of TSP and the number of protons each peak represented in the molecule, short chain fatty acid metabolites concentrations in the supernatants were determined. Bruker Topspin version 3.0 and 3.1 software packages were used for NMR data acquisition and processing, respectively. NMR experiments were performed at the Northwest Metabolomics Research Center at University of Washington. Similar to other validly named *Megasphaera* type strains, UPII 199-6^T, KA00182^T and BV3C16-1^T produced butyrate, fumarate, isovalerate, 2-methylbutyrate and 2-aminobutyrate (Table 1, Fig. S1, available in the online version of this article). The genital tract isolates did not produce *n*-caproate, or *n*-valerate. Moreover, none of the

Table 1. Comparison of the characteristics of genital tract *Megasphaera* species with validly named species of the genus *Megasphaera*

Strains: 1, *M. lornae* UPII 199-6^T; 2, *M. lornae* DNF00751; 3, *M. hutchinsoni* KA00182^T; 4, *M. hutchinsoni* UPII 135-E; 5, *M. vaginalis* BV3C16-1^T; 6, *M. vaginalis* HL562; 7, *M. cerevisiae* DSM 20462; 8, *M. elsdenii* DSM 20460; 9, *M. hexanoica* MH; 10, *M. indica* DSM 25563; 11, *M. massiliensis* DSM 26228; 12, *M. micronuciformis* DSM 17226; 13, *M. paucivorans* DSM 16981; 14, *M. stantonii* AJH120; 15, *M. sueciensis* DSM 17042. Substrate utilization was evaluated using the Biolog anaerobe identification panel. +, Positive; –, negative; w, weak; ng, no growth; ND, not determined; R, resistant; S, susceptible. For tinidazole and secnidazole, the lowest concentrations (μg ml^{-1}) at which we noted a clearance zone are shown. Production of SCFAs was measured after growth in PYG-mod-YG at 37 °C for 24–72 h. ;

Characteristic	1	2	3	4	5	6	7	8	9*	10	11	12	13	14*	15
Cell size (μm)†	0.9–1.5	–	0.5–0.7	–	1.0–1.4	–	1.4	1.2–1.9	0.8–1.2	1.2–2.3	0.87	0.4–0.6	1.2–1.9	1.1–1.8	1.0–1.4
Growth at 45 °C†	–	ND	–	ND	–	ND	–	–	–	–	+	ND	–	+	–
Utilization of:															
D-Glucose‡	–	–	–	–	–	–	+/–	–/+	–	+	+	–	–	–	–
D-Fructose‡	ng	ND	–	ng	–	–	+	+	+	+	+	–	–	+	–
Lactose‡	–	ND	–	ng	–	–	–	–	ND	–	–	–	–	ND	–
Sucrose	–	–	–	–	–	–	–	–	ND	–	+	–	–	ND	–
Dextrin	–	–	–	–	–	–	–	–	ND	+	+	–	–	ND	–
D-Mannitol	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–
D,L-Lactate	+	+	+	+	+	+	+	+	W	+	+	+	+	–	+
Pyruvate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Succinate	W	W	+	+	W	+	–	–	ND	–	–	–	–	ND	–
α-Ketovalerate	+	+	+	+	+	+	+	+	ND	+	+	+	+	ND	+
L-Alanine	–	–	–	–	+	+	–	+	ND	+	+	+	+	ND	–
L-Alanyl-L-Glutamine	–	–	+	–	+	+	+	–	ND	+	+	+	+	ND	–
L-Glutamine	–	–	–	–	+	+	+	–	ND	–	+	+	+	ND	–
L-Glutamate	–	+	+	+	+	+	–	+	ND	–	+	–	+	ND	–
L-Serine	+	+	+	+	+	+	+	+	ND	+	+	+	+	ND	+
Production of:															
Gas	ND	–	–	ND	–	–	+	+	+	+	+	–	–	ND	–
H_2S	–	–	–	–	–	–	–	–	+	–	+	–	–	ND	–
Indole	–	–	–	–	–	–	–	–	–	–	–	–	–	ND	–
Presence of:															
Oxidase	–	–	–	–	–	–	–	–	–	–	–	–	–	ND	–
Catalase	–	–	–	–	–	–	–	–	–	–	–	–	–	ND	–

Continued

Table 1. Continued

Characteristic	1	2	3	4	5	6	7	8	9*	10	11	12	13	14*	15
Nitrate reduction	–	–	–	–	–	–	–	–	–	–	–	–	–	ND	–
Susceptibility to:															
Bile (1 mg)	S	S	S	S	S	S	S	S	S	S	S	S	S	ND	S
Colistin (10 µg)	R	R	S	R	R	R	S	S	S	S	S	S	R	R	R
Vancomycin (5 µg)	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R
Kanamycin (1000 µg)	S	S	S	S	S	S	S	S	ND	S	S	S	S	ND	S
Clindamycin	S	S	S	S	S	S	S	S	ND	S	S	S	S	ND	ND
Metronidazole	S	S	S	S	S	S	S	S	ND	S	S	S	S	ND	ND
Tinidazole	ND	0.5	0.125	ND	0.25	0.125	0.25	1	ND	0.5	1	0.5	0.25	ND	ND
Secnidazole	ND	0.5	0.25	ND	0.25	0.125	0.25	0.5	ND	0.25	0.5	0.5	0.25	ND	ND
Short chain fatty acids:															
2-Aminobutyric acid	W	–	+	W	+	+	+	+	ND	+	+	+	+	ND	+
2-Methylbutyric acid	+	–	+	+	+	+	+	+	ND	W	W	+	W	ND	+
Acetic acid	W	+	–	–	–	–	–	+	+	W	–	–	W	+	+
Butyric acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Formic acid	W	+	W	W	+	–	W	+	ND	+	–	–	W	+	W
Fumaric acid	+	+	+	+	+	+	+	+	ND	+	+	+	+	ND	+
Isobutyric acid	+	–	+	+	+	+	+	+	+	W	W	+	W	ND	+
Isovaleric acid	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactic acid	W	W	W	W	+	+	–	–	ND	W	W	W	–	–	–
n-caproic acid	–	–	–	–	–	–	–	+	+	+	+	–	–	ND	–
n-valeric acid	–	–	–	–	–	–	–	–	+	–	–	–	+	ND	+
Propionic acid	+	+	–	+	+	+	–	+	+	–	–	+	+	+	+
Succinic acid	–	–	–	–	–	–	+	+	ND	+	+	–	–	ND	–

*Characteristics for 9, *M. hexanoica* MH are from Jeon *et al*. [3], and for 14, *M. stantonii* AJH120 are from Maki and Looft [6].
†Data for cell size and growth at 45 °C was determined for UPII 199-6^T, KA00182^T and BV3C16-1^T. Cell size and growth at 45 °C for the other species were obtained from the original publications [1–7, 9].
‡D-Glucose, D-fructose and lactose fermentation was also assessed using the Pre-Reduced Anaerobically Sterilized (PRAS) medium. +, Positive by both methods, –, negative by both methods; +/–, positive by Biolog and negative by PRAS; –/+, negative by Biolog and positive by PRAS; ng, no growth; ND, not determined.

Table 2. Fatty acid methyl ester (FAME) analysis of genital tract *Megasphaera* species and comparison with validly named species of the genus *Megasphaera*

Strains: 1, UPII 199-6^T; 2, DNF00751; 3, KA00182^T; 4, UPII 135-E; 5, BV3C16-1^T; 6, HL562; 7, *M. cerevisiae* DSM 20462; 8, *M. elsdenii* DSM 20460; 9, *M. hexanoica* MH; 10, *M. indica* DSM 25563; 11, *M. massiliensis* DSM 26228; 12, *M. micronuciformis* DSM 17226; 13, *M. paucivorans* DSM 16981; 14, *M. stantonii* AJH120; 15, *M. sueciensis* DSM 17042. Values are percentages of total fatty acids. Fatty acids less than 1% of the total in all of the strains are not listed. –, Not detected. Major components are marked in bold text.

Fatty acid	1	2	3	4	5	6	7	8	9*	10	11	12	13	14*	15
$C_{9:0}$	–	–	–	–	–	–	–	–	1.6	–	–	–	–		–
$C_{10:0}$	3.1	2.8	2.8	3.3	0.3	0.3	7.0	–	1.9	0.5	0.7	0.9	–	0.3	–
$C_{11:0}$	0.8	0.3	–	0.9	–	0.1	0.9	–	3.0	–	1.1	–	–	–	–
$C_{12:0}$	**13.5**	**12.7**	9.0	**10.1**	**13.2**	**13.1**	9.2	**13.7**	**16.6**	**16.1**	**12.8**	**13.5**	**15.8**	**18.9**	**16.5**
$C_{13:0}$	–	–	–	–	–	–	–	–	3.2	–	0.9	–	–	–	0.8
$C_{14:0}$	–	1.0	1.1	0.7	4.0	2.4	5.4	5.3	8.1	2.4	5.3	4.1	3.3	0.9	4
$C_{16:0}$ aldehyde	6.1	3.0	1.6	2.1	1.8	–	2.4	2.2	–	0.8	1.1	3.7	2.0	1.1	0.8
$C_{15:0}$	–	–	–	–	1.1	–	0.9	0.7	6.3	–	3.3	–	1.1	–	0.9
$C_{16:0}$	**21**	**20.4**	**13.1**	**10.9**	9.9	8.0	7.9	**12.8**	**12.7**	3.8	7.8	7.8	8.8	8.8	3.2
$C_{17:0}$	–		–	0.6	0.5	0.4	–	0.5	5.4	–	1.5	–	0.7	–	–
$C_{18:0}$	3.2	1.6	2.0	3.4	1.2	1.3	–	1.5	9.4	–	1.3	2.0	1.5	2.3	–
$C_{11:0}$ DMA	–	0.4	0.3		–	0.1	–	–	–	–	–	–	–	–	–
$C_{14:0}$ DMA	–		–	1.0	–		1.4	–	–	–	2.2	–	1.4	–	2.6
$C_{16:0}$ DMA	**11.4**	8.3	4.3	4.3	4.9	4.7	4.5	5.6	0.3	1.4	4.1	4.8	3.9	3.3	1.4
$C_{16:1}$ *cis* 7 DMA	–	–	0.6	–	7.8	**11.6**	–	–	–	2.0	0.3	–	–	–	–
$C_{16:1}$ *cis* 9 DMA	–	3.0	8.8	–	–	–	4.8	9.8	–	7.1	7.6	7.5	7.3	7.1	6.2
$C_{19:0}$ cyc 9,10 DMA	–	–	0.4	–	0.5	–	3.9	0.5	–	–	1.7	–	–	0.5	1.4
$C_{17:0}$ DMA	–	–	–	–	–	–	–	–	2.3	–	1.0	–	–		–
$C_{18:0}$ DMA	–	–	–	–	–	–	–	0.5	–	–	0.6	–	–	0.4	–
$C_{18:1}$ DMA	–	–	–	–	–	–	–	–	–	–	0.7	–	–		0.9
$C_{18:1}$ *cis* 7 DMA	–	–	–	–	–	3.6	–	–	–	1.2	–	–	–		–
$C_{18:1}$ *cis* 9 DMA	–	–	0.9	0.5	1.4	–	1.1	4.5	–	3.7	4.9	4.7	2.9	4.8	8.5
$C_{16:1}$ *cis* 7	–	–	2.6	–	–	**10.2**	–	1.7	4.2	**10.4**	1.4	–		1.3	–
$C_{16:1}$ *cis* 9	3.0	6.3	**13.4**	9.5	10	1.1	7.6	8.9	–	7	6.4	4.5	10.2	7.1	9.6
$C_{17:1}$ *cis* 6	–	**12.3**	1.8	2.8	5.1	–	7.0	1.3	–	9.2	1.8	0.7	4.1	0.7	2.3
$C_{17:1}$ *cis* 11	–	–	–	–	–	2.2	–	–	–	–	–	–	–	–	–
$C_{18:2}$ *cis* 9,12	–	–	–	–	–	–	–	–	3.7	–	–	–	–		–
$C_{18:1}$ *cis* 6	–	–	–	–	–	–	2.5	–	–	1.1	1	–	–	–	–
$C_{18:1}$ *cis* 7	–	–	0.8	–	–	–	–	–	–	1.1	–	1	–	1.3	–
$C_{18:1}$ *cis* 9	8.5	1.3	7.1	3.7	4.0	9.9	2.5	6.7	8.2	3.7	4.6	13.8	6.6	**15.9**	10
C_{19} cyc 9,10	3.7	3.6	6.9	5.1	4.4	3.4	5.5	1.5	–	3.6	3.2	–	4.3	2.0	1.3
$C_{11:0}$ iso	0.6	0.3	1.2	3.0	0.4	0.3	0.6	–	–	–	–	0.4	–	–	–
$C_{13:0}$ iso	–	–	–	0.5	2.9	1.9	–	–	–	–	0.2	1.1	–	–	0.7
$C_{13:0}$ iso 3-OH	0.9	0.4	–	3.7	1.2	0.9	–	–	–	–	–	0.5	0.7	–	–
$C_{15:0}$ iso	–	–	–	–	1.1	0.5	–	–	–	–	–	0.5	–	–	–

Continued

Table 2. Continued

Fatty acid	1	2	3	4	5	6	7	8	9*	10	11	12	13	14*	15
$C_{15:0}$ iso 3-OH	–	–	–	1.0	1.1	0.7	–	–	–	–	–	–	–	–	–
$C_{17:0}$ iso	0.6	0.3	0.9	1.8	1.5	1.0	–	–	–	–	–	0.7	–	–	–
$C_{12:0}$ 3-OH	–	6.8	4.6	–	–	1.6	3.0	2.2	–	3.1	1.4	1.8	2.0	1.8	2.8
$C_{13:0}$ 3-OH	–		1.4	–	1.5	–	–	–	–	–	–	–	–	–	–
$C_{14:0}$ 3-OH	–	**12.2**	9.0	–	**13.8**	**13.0**	**17.3**	**12.7**	–	7.8	**14.3**	**17.2**	**14.8**	**16.2**	**13.9**
$C_{17:2}$ at 16760	–	–	–	–	–	1.0	–	–	–	–	–	–	–	1.5	–
Unknown at 14.762	–	1.7	–	–	–	–	–	–	–	–	–	–	–	3.8	–
Unknown at 17.223	–	–	–	–	0.4	–	–	–	1.5	–	1.1	–	–	–	0.8
Summed feature 2†	7.2	6.8	4.6	5.7	1.5	1.6	3	2.2	–	3.2	1.4	1.8	2	–	2.8
Summed feature 4†	0.8	1.7	5.4	4.2	4.8	3.5	3.8	5.8	1.1	5.7	3.6	5.7	5.5	–	4.9
Summed feature 5†	**14.2**	**12.2**	9	**13.8**	**13.8**	**13.0**	**17.3**	**12.7**	6.6	7.8	**14.3**	**17.2**	**14.8**	**16.2**	**13.9**
Summed feature 6†	0.9	3.0	8.8	5.4	7.8	**11.6**	4.8	9.8	2.4	7.1	7.6	7.5	7.3	–	6.2
Summed feature 7†	–	–	–	–	0.9	1.0	0.8	1.9	1.4	2.2	1.7	2.7	2.1	–	5.4
Summed feature 10†	–	–	0.8	0.5	–	0.4	–	–	–	1.1	–	1	–	–	–

*Characteristics for 9, *M. hexanoica* MH are from Jeon *et al*. [3], and for 14, *M. stantonii* AJH120 are from Maki and Looft [6]. All other data were generated in this study.
†Summed features consist of one or more fatty acids that could not be separated by the MIDI system. Summed feature 2 consists of $C_{13:1}$ *cis* 12, $C_{14:0}$ ALDE, and/or $C_{11:1}$ 2-OH; summed feature 4 consists of an unknown at 14.762 and/or $C_{15:2}$; summed feature 5 consists of $C_{15:0}$ DMA and/or $C_{14:0}$ 3-OH; summed feature 6 consists of $C_{15:0}$ anteiso 3-OH and/or $C_{16:1}$ *cis* 9 DMA; aummed feature 7 consists of $C_{17:2}$ and $C_{17:1}$ *cis* 8; aummed feature 10 consists of $C_{18:1}$ *cis* 7 and/or an unknown at 17.834.

genital tract *Megasphaera* species produced succinate (Fig. S1), which is notably higher in concentration in vaginal fluid from women with BV [60]. BV3C16-1^T produced lactate while the other genital tract isolates were weak producers.

Antimicrobial susceptibility testing was performed using the agar dilution method on Brucella blood agar as per the Clinical and Laboratory Standards Institute (CLSI) guidelines [61]. The US Centers for Disease Control Sexually Transmitted Diseases Treatment guidelines recommend metronidazole, tinidazole or clindamycin for treatment for BV [62], hence these antibiotics (Sigma Aldrich) were selected for testing. We also tested secnidazole (Symbiomix), a single-dose antibiotic that has been recently approved for the treatment of BV [38, 63–65]. Concentrations tested ranged from 0.03 to 128 μg ml^{-1}. The lowest antibiotic concentration that yielded marked reduction or no growth was recorded as the minimum inhibitory concentration (MIC). The CLSI defined breakpoints to clindamycin (≤2 μg ml^{-1} sensitive, ≥8 μg ml^{-1} resistant) and metronidazole (≤8 μg ml^{-1} sensitive, ≥32 μg ml^{-1} resistant) were used for interpretation of MIC results [61]. There are no CLSI defined breakpoints for tinidazole or secnidazole. Other compounds that we examined for activity against *Megasphaera* species included bile (1 mg), colistin (10 μg), vancomycin (5 μg) and kanamycin (1000 μg). Testing for bile sensitivity was conducted using bile discs with 20% bile (Becton Dickinson) [54] that were prepared in-house. Brucella plates were inoculated using the quadrant method and the bile disc was placed in the first quadrant; any zone of inhibition around the bile disc was considered sensitive. The same approach was used for discs containing colistin, vancomycin or kanamycin. If a bacterium exhibited a zone of clearance ≥10 mm, it was marked as sensitive to colistin, vancomycin or kanamycin while if the clearance zone was <10 mm, the bacterium was considered resistant [54]. Genital tract isolates and validly named *Megasphaera* species were sensitive to bile and kanamycin (Table 1). All genital tract isolates were resistant to colistin while KA00182^T was sensitive. All *Megasphaera* species tested were sensitive to antibiotics typically used to treat BV including clindamycin and metronidazole. The lowest concentrations of secnidazole and tinidazole resulted in clearance zones suggesting sensitivity to these antibiotics. Similar to other Gram-negative bacteria, all *Megasphaera* isolates tested with the exception of DNF00751 were resistant to vancomycin.

Genomic DNA was extracted using the ZR Fungal/Bacterial DNA MidiPrep Kit (Zymo Research) according to the

Table 3. Comparison of the genome characteristics of genital tract *Megasphaera* isolates with validly named species of the genus *Megasphaera*.

Strains: 1, UPII 199-6^T; 2, DNF00751; 3, KA00182^T; 4, UPII 135-E; 5, BV3C16-1^T; 6, HL562; 7, *M. cerevisiae* DSM 20462; 8, *M. elsdenii* DSM 20460; 9, *M. hexanoica* MH; 10, *M. indica* DSM 25563; 11, *M. massiliensis* DSM 26228; 12, *M. micronuciformis* DSM 17226; 13, *M. paucivorans* DSM 16981; 14, *M. stantonii* AJH120; 15, *M. sueciensis* DSM 17042. ND, No data.

Megasphaera strains	BioProject accession	Genome length (Mb)	No. of protein coding genes	No. of tRNA genes	No. of stable RNA genes	DNA G+C content (mol%)*
1	PRJNA64689	1.64	1577	49	52	46.4
2	PRJNA257377	1.73	1819	45	47	45.9
3	PRJNA272074	1.57	1514	45	48	38.9
4	PRJNA64691	1.65	1517	50	53	38.9
5	PRJNA89631	2.21	2262	49	52	49.8
6	ND	ND	ND	ND	ND	ND
7	PRJEB19539	3.15	3354	52	54	44.8
8	PRJNA437124	2.48	2329	66	80	52.8
9	PRJNA287738	2.88	2858	53	60	49
10	ND	ND	ND	ND	ND	54.9
11	PRJEB645	2.66	2422	56	61	50.2
12	ND	ND	ND	ND	ND	46.4
13	PRJEB16204	2.91	2915	51	53	40.2
14	PRJNA471687	2.65	2590	57	67	52.6
15	ND	ND	ND	ND	ND	43.1

*DNA G+C content (mol%) for *M. indica*, *M. micronuciformis*, and *M. sueciensis* were obtained from the original references [4, 5, 7] as genomes were not available. Genome information is also not available for *M. vaginalis* HL562.

manufacturer's instructions for whole genome shotgun sequencing and submitted to one of two Human Microbiome Project (HMP) sequencing centres. UPII 199-6^T and BV3C16-1^T were sequenced at the J. Craig Venter Institute, Rockville, Maryland while KA00182^T was sequenced at the Genome Institute at Washington University School of Medicine, St. Louis, Missouri. The whole genome sequences of the genital tract isolates have been available to the scientific community since they were first deposited by the HMP (Accessions in Table 3). The 16S rRNA gene sequences for each isolate were obtained from the WGS projects or by sequencing the 16S rRNA gene (accessions in Fig. 3). All vaginal isolates characterized in this study each had one copy of the 16S rRNA gene. Validly published *Megasphaera* species harbour one (*M. cerevisiae*) to seven gene copies (*M. elsdenii*) of the 16S rRNA gene. UPII 199-6^T had a sequence identity of 96.4% to KA00182^T and 93.7% to BV3C16-1^T, while KA00182^T had a sequence identity of 92.8% with BV3C16-1^T. Evaluation of the UPII 199-6^T 16S rRNA sequence in the EZ Taxon database of validly named type strains [66] showed that this isolate had a sequence identity of 93.6% to *M. stantonii* and ranged in identity from 92.9–93.6% to validly named *Megasphaera* species. KA00182^T had the closest sequence identity to *M. micronuciformis* (94.2%), while BV3C16-1^T had the closest identity to *M. cerevisiae* (95.4%). The 16S rRNA gene from KA00182^T ranged in sequence identity from 92.6–94.2% among all validly named *Megasphaera* isolates, while the range for BV3C16-1^T was between 93.0 and 95.4% suggesting that all three isolates are novel species within the *Megasphaera* genus. NCBI BLAST searches (https://blast.ncbi.nlm.nih.gov/Blast.cgi) [67] of the 16S rRNA sequences from UPII 199-6^T, KA00182^T and BV3C16-1^T resulted in matches to uncultivated bacterial clones from the human vagina and skin. Importantly, UPII 199-6^T had a sequence identity of 99.9% with the uncultured clone 127-Q 35 (AY738672) designated as *Megasphaera* type 1. KA00182^T (AY738697) had a sequence identity of 99.9% with the uncultured clone 123-Q 3 designated as *Megasphaera* type 2. We have previously demonstrated these clones to be associated with BV [12]. A multiple sequence alignment of the 16S rRNA gene from the genital tract isolates along with validly named members of the family *Veillonellaceae* was created using the ClustalW algorithm and the evolutionary relationships were inferred by using the maximum- likelihood method based on the Tamura–Nei model [68] in MEGA X [69] (Fig. 3). All three isolates fall within the *Megasphaera* clade of validly named species. The closest neighbour of UPII 199-6^T was KA00182^T and both isolates were phylogenetically distinct from *M. elsdenii*, *M. indica* and *M. massiliensis*, the most closely related species. BV3C16-1^T was most closely related to *M. cerevisiae*. Similar results were obtained when evolutionary relationships were inferred using the neighbour-joining method (Fig. S2).

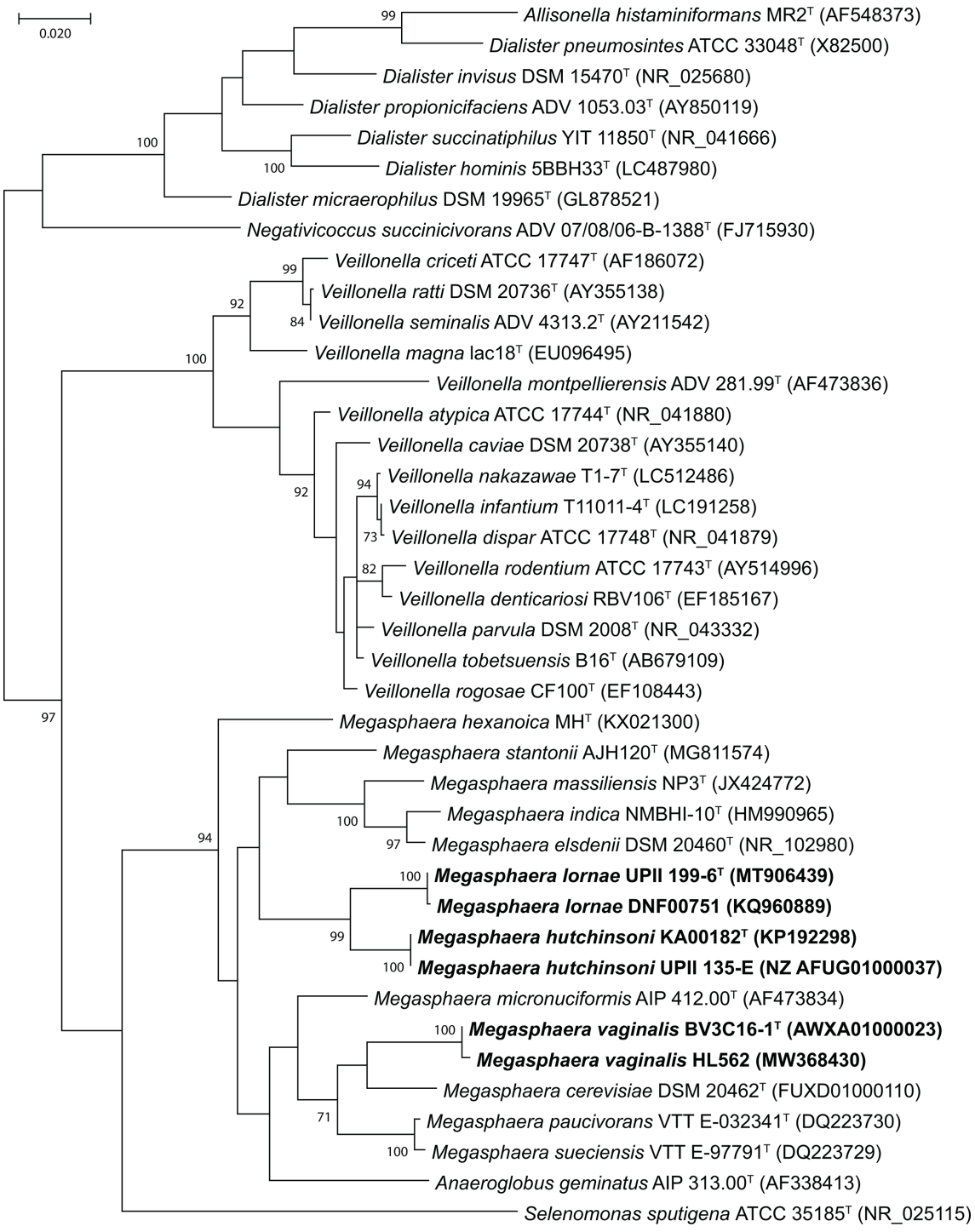

Fig. 3. Molecular phylogenetic analysis by the maximum-likelihood method based on 16S rRNA gene sequences showing the phylogenetic positions of *M. lornae*, *M. hutchinsoni* and *M. vaginalis* with closely related members of the family *Veillonellaceae*. The genital tract isolates clustered within the *Megasphaera* clade. Bootstrap values (based on 1000 replications) greater than or equal to 70% are shown as percentages at each node. Bar, 0.02 substitutions per nucleotide position. *Selenomonas sputigena* ATCC 35185 (NR_025115) from the family *Selenomonadaceae* was added as an outgroup.

(a)

	1	2	3	4	5	7	8	9	11	13	14
1		90.7	22.2	22.5	18.7	19.3	23.4	21	19.5	18.8	20.3
2	90.7		23.2	23.9	18.6	19	23.9	21.3	19.5	18.8	20.7
3	22.2	23.2		89.5	20.4	18.9	24.9	21.3	19.1	17.7	22.2
4	22.5	23.9	89.5		20.4	18.5	24.5	25.8	19.3	17.7	22.2
5	18.7	18.6	20.4	20.4		18.6	21	18.9	21.5	17.9	19.7

(b)

	1	2	3	4	5	7	8	9	11	13	14
1		99.1	82.9	83.7	76.1	76.4	76.9	76.8	76.0	74.9	77.0
2	99.1		83.8	84.9	76.3	76.2	76.3	76.5	76.3	75.1	77.1
3	82.9	83.8		99.0	77.6	77.2	78.3	76.6	77.0	75.1	77.2
4	83.7	84.9	99.0		79.0	76.8	79.0	83.4	77.7	74.9	77.6
5	76.1	76.3	77.6	79.0		75.5	76.2	76.4	79.2	74.7	76.7

Fig. 4. Genome-based species delineation using the (a) genome BLAST distance phylogeny approach. Values less than 70% are indicative of a different species. Strains: 1, UPII 199-6^T; 2, DNF00751; 3, KA00182^T; 4, UPII 135-E; 5, BV3C16-1^T; 7, *M. cerevisiae* DSM 20462; 8, *M. elsdenii* DSM 20460; 9, *M. hexanoica* MH; 11, *M. massiliensis* DSM 26228; 13, *M. paucivorans* DSM 16981; 14, *M. stantonii* AJH120; 15, *M. sueciensis* DSM 17042. *M. vaginalis* HL562, *M. indica*, *M. micronuciformis* and *M. sueciensis* did not have genomes available for this analysis. UPII 199-6^T and DNF00751 belong to the same species with a DDH value of 90.7% (*M. lornae*). KA00182^T and UPII 135-E belong to the same species with a DDH value of 89.5% (*M. hutchinsoni*). (b) Average nucleotide identity analyses. Identities of <95% indicate a novel species. The genital tract isolates (1–5) are sufficiently different from validly named *Megasphaera* species (7–15) to warrant designation as novel species. UPII 199-6^T and DNF00751 are the same species (99.1% ANI) while KA00182^T and UPII 135-E are the same species (99% ANI).

Information regarding genome lengths, predicted protein-coding genes and RNA genes were extracted from the bacterial Bioinformatics Database and Analysis Resource Center, PATRIC (Table 3) [70]. The DNA G+C content was calculated using the online service at http://ggdc.dsmz.de [71] and were different in UPII 199-6^T, KA00182^T and BV3C16-1^T from other validly named *Megasphaera* species whose genomes were available (Table 3). Genome data is not available for the type strains of *M. indica, M. micronuciformis* and *M. sueciensis* and DNA G+C content data was obtained from the original papers [4, 5, 7]. The DNA G+C content for UPII 199-6^T was similar to the DNA G+C content reported for *M. micronuciformis* DSM17226 (46.4 mol%) which was determined using a wet lab approach, HPLC [7], hence the values are not directly comparable. The striking differences between UPII 199-6^T and *M. micronuciformis* DSM17226 using several lines of evidence including phylogenetic analyses, biochemical characterization, FAME analyses and metabolite end product testing show that they are different species. Digital DNA–DNA hybridization (dDDH) was used to evaluate the relatedness of UPII 199-6^T, KA00182^T and BV3C16-1^T to each other and to the reference genomes available for validly named isolates (Fig. 4a). Genome-based species delineation was conducted using the genome BLAST distance phylogeny approach (GBDP) version 2.1 using the online web tool with the recommended settings for Formula 2 independent of genome length and robust against incomplete draft genomes (http://ggdc.dsmz.de) [71, 72]. DDH values of <70% of UPII 199-6^T, KA00182^T and BV3C16-1^T, in comparison with *M. cerevisiae*, *M. elsdenii*, *M. hexanoica*, *M. massiliensis*, *M. paucivorans* and *M. stantonii*, warrant designation as novel species [71]. These results were further supported with average nucleotide identity (ANI) analyses using a web-based ANI tool (http://enve-omics.ce.gatech.edu/aai) [73]. None of the genital tract isolates characterized here had an identity >95% on comparison with validly named *Megasphaera* species which is the suggested cut-off for a novel species (Fig. 4b).

In summary, several lines of evidence including 16S rRNA gene-based phylogeny, growth and biochemical features, cellular fatty acid composition, short-chain fatty acids produced, determination of DNA G+C content, dDDH and ANI analyses demonstrate that the six isolates characterized in this study represent three novel species within the genus *Megasphaera*. The proposed type strains are designated *M. lornae* sp. nov. UPII 199-6^T, *M. hutchinsoni* sp. nov. KA00182^T and *M. vaginalis* sp. nov. BV3C16-1^T.

DESCRIPTION OF *MEGASPHAERA LORNAE* SP. NOV.

Megasphaera lornae (lor′ nae. N.L. fem. n. *lornae* of Lorna, named in honour of Lorna Rabe, a microbiologist who isolated and contributed to the characterization of *Megasphaera* species from the female genital tract).

Strictly anaerobic, Gram-negative, non-spore forming, non-motile, coccoid-shaped micro-organism that grows as single cells, in pairs or occasional chains. Cells are 0.9–1.5 µm. Colonies are visible on Brucella blood agar after 2 days of growth and appear as convex, entire, glossy and off-white in colour and a diameter of 0.5–0.7 mm. Growth of the bacterium occurs between 35–37 °C with an optimal temperature of 37 °C in PYG-mod-YG broth. The major SCFAs produced are butyric acid and propionic acid when grown in PYG-mod-YG broth for 48 h. The bacterium is sensitive to metronidazole and clindamycin, antibiotics typically used to treat BV. Bile and kanamycin inhibit growth, while colistin and vancomycin have no impact on growth. The bacterium does not metabolize sugars including glucose, fructose, lactose, sucrose and dextrin, but metabolizes organic acids including lactate, pyruvate, α-ketoglutarate and succinate. This bacterium can metabolize serine, but not alanine, alanyl-L-glutamine, glutamine or glutamate.

The type strain is UPII 199-6^{T} (=DSM 111201^{T},=ATCC TSD-205^{T}), which was isolated from an endometrial biopsy sample from a woman being evaluated for PID. The DNA G+C content of the type strain is 46.4mol%. GenBank accession numbers for the 16S rRNA gene sequence and whole genome sequence are MT906439 and PRJNA64689, respectively.

DESCRIPTION OF *MEGASPHAERA HUTCHINSONI* SP. NOV.

Megasphaera hutchinsoni (hutch. in. so′ ni. N.L. fem. adj. *hutchinsoni*, named in honour of the Fred Hutchinson Cancer Research Center where this cultivation study was conducted).

Strictly anaerobic, Gram-negative, non-spore forming, non-motile, coccoid-shaped micro-organism that grows as singlets, pairs and chains along their tapered ends. Cells are 0.5–0.7 µm. Colonies are visible on Brucella blood agar after 2 days of growth and appear as convex, entire, glossy and off-white in colour with a diameter of 0.4–0.7 mm. Growth of the bacterium occurs between 30–42 °C with an optimal temperature of 35–37 °C in PYG-mod-YG broth. The major SCFAs produced are 2-methylbutyric acid, butyric acid, isobutyric acid, fumaric acid and isovaleric acid when grown in PYG-mod-YG broth for 24 h. The bacterium is sensitive to metronidazole, clindamycin, tinidazole and secnidazole, antibiotics used to treat BV. Bile, colistin and kanamycin inhibit growth, while vancomycin has no impact on growth. The bacterium does not metabolize sugars including glucose, fructose, lactose, sucrose and dextrin, but metabolizes organic acids including lactate, pyruvate, α-ketoglutarate and succinate. The bacterium metabolizes amino acids including alanyl-L-glutamine, glutamate and serine, but not alanine or glutamine.

The type strain is KA00182^{T} (=DSM 111202^{T}=ATCC TSD-206^{T}), which was isolated from human vaginal fluid obtained from a woman with BV. The DNA G+C content of the type strain is 38.9mol%. GenBank accession numbers for the 16S rRNA gene sequence and whole genome sequence are KP192298 and PRJNA272074, respectively.

DESCRIPTION OF *MEGASPHAERA VAGINALIS* SP. NOV.

Megasphaera vaginalis sp. nov. (va.gi.na′ lis. N.L. fem. adj. *vaginalis*, pertaining to the vagina).

Strictly anaerobic, Gram-negative, non-spore-forming, non-motile, coccoid-shaped micro-organism that grows as single cells with rounded ends, often occurring in pairs. Cells are 1.0 to 1.4 µm. Colonies are visible on Brucella blood agar after 2 days of growth and appear as small, convex and translucent with a diameter of 0.3–0.4 mm. Growth of the bacterium occurs between 30–37 °C with an optimal temperature of 35–37 °C in PYG-mod-YG broth. The major SCFAs produced are 2-aminobutyric acid, 2-methylbutyric acid, butyric acid, isobutyric acid, isovaleric acid, and propionic acid when grown on PYG-mod-YG broth for 48 h. The bacterium is sensitive to metronidazole, clindamycin, tinidazole and secnidazole, antibiotics used to treat BV. Bile and kanamycin inhibit growth, while colistin and vancomycin have no impact on growth. The bacterium does not metabolize sugars including glucose, fructose, lactose, sucrose and dextrin, but metabolizes organic acids including lactate, pyruvate, α-ketoglutarate and succinate. The bacterium metabolizes several amino acids including alanine, alanyl-L-glutamine, glutamine, glutamate and serine.

The type strain is BV3C16-1^{T} (=DSM 111203^{T}=ATCC TSD-207^{T}), which was isolated from human vaginal fluid obtained from a woman with BV. The DNA G+C content of the type strain is 49.8mol%. GenBank accession numbers for the 16S rRNA gene sequence and whole genome sequence are JN809775 and PRJNA89631, respectively.

Funding information
The work was supported by the National Institutes of Health (grant R01 HG005816 to D. N. F.) as part of the Human Microbiome Project initiative focused on technology development and NIH grant U19AI120249 to S. L. H.

Acknowledgements
We appreciate Sean Proll's help with figures and tables. Steve MacFarlane and Bobbie Schneider provided support with preparation of samples for electron microscopy.

Conflicts of interest
D. N. F. and T. F., have received a royalty from B. D., around molecular diagnosis of B. V. S. S., has received speaking honoraria from Lupin Inc. S. L. H., has served as a consultant to Hologic related to the development of diagnostic tests for bacterial vaginosis and her institution has received research funding from Cepheid and Becton-Dickinson.

Ethical statement
The research study at the University of Pittsburgh evaluated women for pelvic inflammatory disease; endometrial tissue samples were collected in a protocol approved by the University of Pittsburgh Review Board (IRB approval number: PRO 010010112). The research study at the Fred Hutchinson Cancer Research Center was designed for the isolation of novel anaerobes from the human vagina and was approved by the institutional review board at the Fred Hutch (IRB approval number: IR 7363). All participants provided informed consent.

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