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Mageeibacillus indolicus gen. nov., sp. nov: A novel bacterium isolated from the female genital tract

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Abstract

Three isolates of a bacterium recovered from human endometrium using conventional culture methods were characterized biochemically and subjected to 16S rRNA gene sequencing and phylogenetic analysis. Isolates were non-motile, obligately anaerobic, non-spore forming, asaccharolytic, non-cellulolytic, indole positive, Gram positive rods. Cell wall fatty acid profiling revealed $C_{14:0}$, $C_{16:0}$, $C_{18:2\ \omega6,\ 9c}$, $C_{18:1\ \omega9c}$ and $C_{18:0}$ to be the major fatty acid composition. The DNA mol % G+C was determined to be 44.2%. 16S rRNA gene sequence analysis revealed only 91% sequence similarity with the closest cultivated bacterial isolate, *Saccharofermentans acetigenes*. Based on genotypic and phenotypic data, all three isolates are considered to be members of the same species and data suggest it represents a novel genus and species in the order *Clostridiales* with an association with *Clostridium* rRNA cluster III within the family *Ruminococcaceae*. We propose the name, *Mageeibacillus indolicus* gen. nov., sp. nov. The type strain is BAA-2120^T and CCUG 59143^T.

Keywords

bacterial vaginosis associated bacterium 3; *Mageeibacillus indolicus* gen. nov., sp. nov.; taxonomy; genital tract

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1. Introduction

Bacterial vaginosis (BV) is a common polymicrobial syndrome affecting women of reproductive age. In women with BV, the vaginal microbiome is characterized by a higher abundance and greater diversity of both facultative and anaerobic organisms and a decrease in *Lactobacillus* species. BV is associated with an increased risk of acquiring and transmitting sexually transmitted infections including human immunodeficiency virus type 1 (HIV) and herpes simplex virus type 2 [1, 2, 3]. Despite treatment, one in four women fails antibiotic therapy and the proportion of women who develop recurrent BV increases over time [4]. Failure to describe all of the etiologic agents through conventional culture-based methods has limited our current understanding of the syndrome as well as the identification of alternative treatment regimens.

Advances in molecular approaches, specifically the sequencing and phylogenetic analysis of the 16S rRNA gene has enhanced the understanding of the complex bacterial biota of the human vagina. Fredricks *et al*. reported the use of molecular methods for detecting fastidious and non-cultivable organisms highly specific for BV, including three novel species designated BV-associated bacterium 1, 2, and 3 (BVAB1, BVAB2 and BVAB3) in the order *Clostridiales* [5, 6, 7]. Although clinical criteria or the Nugent scoring method to detect altered vaginal flora were not implemented in their study, Zhou *et al*. also found novel species in the order *Clostridiales* in vaginal communities not dominated by *Lactobacillus* species [8].

In this study, we characterized three bacterial isolates (0009-5^{T}, 0004-9, 0019-D) that were indistinct from each other, and were classified as “anaerobic Gram positive rods” by initial biochemical testing. Further characterization of this group using phenotypic and genotypic approaches suggested that these isolates represent a novel genus and species that we here designate *Mageeibacillus indolicus* gen. nov., sp. nov.

2. Methods

2.1. Isolation and cultivation

The isolates were obtained from endometrial biopsy samples from women being evaluated for pelvic inflammatory disease (PID) in a protocol approved by the University of Pittsburgh Institutional Review Board. Briefly, a Pipelle® endometrial sampling device was inserted through the cervix, into the uterine cavity and the biopsy was aspirated by suction. The biopsy was initially inoculated onto Brucella agar supplemented with 5% sheep blood (bioMerieux, Durham, NC) and incubated anaerobically at 37°C for four to seven days. After the initial isolation and identification, all isolates were frozen at −80°C in litmus milk (Becton Dickinson, Franklin Lakes, NJ). Immediately prior to advanced testing, isolates were thawed, inoculated onto the same media and incubated as described above. Isolates were subcultured at least twice before testing to ensure purity.

Primary examination of cell morphology, Gram reaction and motility by dispersion in chopped meat broth (Becton Dickinson, prepared in-house) were examined with light microscopy at 1000X and 400X, respectively. Cell wall structure was also assessed using the

rapid potassium hydroxide (KOH) test previously performed by Halebian *et al*. [9] Colony morphology was described at 20X with a dissecting microscope. Aerotolerance was tested by subculturing onto a Brucella agar plate, supplemented with 5% sheep's blood (bioMerieux) and incubated separately in 5–7% CO_2 and an AnaeroPack® system containing a Pack-MicroAero sachet and jar (Mitsubishi Gas Company, Tokyo, Japan) at 37°C for 48 and 96 hours, respectively. Initial biochemical tests performed included catalase (3%) (Fisher Scientific, Pittsburgh, PA), spot indole (prepared in-house), urease (Becton Dickinson), oxidase (Becton Dickinson) and susceptibility to the special potency ID discs; vancomycin, 5μg and colistin, 10μg (Becton Dickinson). Lipase and lecithinase activity on Egg Yolk agar (bioMerieux), carbohydrate fermentation testing using peptone-yeast (PY) broth with the separate addition of glucose and ribose (prepared in-house), as well as PRAS esculin and gelatin hydrolysis (Anaerobe Systems, Morgan Hill, CA) were also tested.

Due to poor growth in broth, growth stimulation testing was performed and included the separate addition of 0.5% hemin (Sigma-Aldrich, St. Louis, Missouri), 0.5% arginine (Sigma-Aldrich), 0.001% sodium bicarbonate (Fisher Scientific), 0.5% Tween 80 (Beckton Dickinson), 5% horse serum (Life Technologies, Carlsbad, CA) and 1% glucose (Beckton Dickinson) to chopped meat carbohydrate (CMC) broth and PY broth. All initial biochemical testing and growth stimulation testing was performed according to Summanen *et al*. [10].

Cellulose degradation was assessed using a plate assay developed by Kasana *et al*. [11]. Briefly, inoculation on carboxymethylcellulose agar (prepared in-house) was performed and plates were incubated anaerobically at 37°C for 96 hours. Gram's iodine was then flooded onto the plate to assess for zones of hydrolysis (agar without the uptake of Gram's iodine) around the colonies suggesting cellulase production by the organism.

Optimal temperature for growth was determined by inoculating and incubating the isolates anaerobically as described above at varying temperatures. Minimum and maximum temperatures for growth were 35°C and 42°C, respectively.

Demonstration of spores was performed according to Johnson, Summanen and Finegold [12]. Briefly, a turbid suspension was made in CMC broth supplemented with 5% horse serum and incubated anaerobically at 37°C for one week followed by heat and ethanol treatments.

Additional biochemical testing was performed using API rapid ID 32A and API ZYM enzyme detection panels (bioMerieux) according to the manufacturer's instructions.

2.2. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing using the agar dilution method was performed to test susceptibility of the three strains to metronidazole, clindamycin, cefoxitin and doxycycline according to the Clinical Laboratory Standards Institute guidelines (CLSI) for anaerobic bacteria, 5th edition [13].

2.3. Fatty Acid Methyl Extraction Analysis

Fatty acid methyl extraction (FAME) was performed to determine the cell wall fatty acid composition. Because of the poor growth observed in PY broth, the standard growth medium used for FAME for comparison to their standard libraries at Microbial ID, the following recommendation by personnel at Microbial ID for processing the organisms for FAME was performed; isolates were cultured on Brucella agar with 5% sheep blood as previously described and colonies were harvested at two days growth, used to make a turbid suspension in DNAse free water and pelleted by centrifugation at 12 000 r.p.m for two minutes. The supernatant was discarded and the pellet was frozen at −80°C. The pellets were sent on dry ice overnight to Microbial ID (Newark, DE) where fatty acid analysis was performed by gas chromatography using a standard protocol, the MIDI Sherlock MIS system [14].

2.4. Electron Microscopy

For transmission electron microscopy, cells were fixed in 2.5% gluteraldehyde and negative staining was performed on copper grids with a formver membrane. Micrographs were recorded on a Jeol JEM-1011 electron microscope operating at 80kV. For scanning electron microscopy, cells were fixed in 2.5% gluteraldehyde and 1% osmium tetroxide (OsO_4). Specimens were dehydrated in ethanol, coated with 3.5 nm of gold/palladium and analyzed with a JEM-6335F field emission SEM at 3kV.

2.5. DNA mol% G+C content determination

0009-5^T was submitted to the J. Craig Venter Institute for whole genomic sequencing as part of the Human Microbiome Project. The DNA mol% G+C content was determined by analyzing the complete genomic sequence using Molecular Evolutionary Genetics Analysis (MEGA4) software [15].

2.6. DNA isolation, 16S rRNA gene sequencing and phylogenetic analysis

Whole genomic DNA was extracted as previously described in Antonio *et al*. [16]. DNA from the three isolates was PCR-amplified using broad-range primers 27F (5´-AGAGTTTGATCMTGGCTCAG-3´) (Frank *et al.*) [17] and 1541R (5´-AAGGAGGTGATCCAGCCGCA-3´) (Löeffler *et al*.) [18] to amplify nearly complete 16S rRNA genes. Each 50 μl PCR reaction contained 1X Pfu Buffer, 0.8 mM deoxynucleotide triphosphate mixture, 1.5 U PfuTurbo Hot Start DNA Polymerase (Stratagene-Agilent, Santa Clara, CA), 0.2 μM forward and reverse primers. The PCR conditions included a pre-melt at 95 °C for 10 min and then 40 cycles of 95°C for 30 seconds (melt), 55°C for 30 seconds (annealing) and 72°C for 30 seconds (extension), followed by a final extension step at 72 °C for 7 min. The PCR products were visualized after electrophoresis using 1.5% agarose gels and staining with Gel-Red (Biotium, Hayward, CA). The PCR products were sequenced (Big Dye Version 3, Life Technologies) using a panel of broad-range 16S rRNA gene-directed sequencing primers to ensure that every base was read at least 2 times. The sequencing primers included 27F, 1541R, 338F (5´-ACTCCTRCGGGAGGCAGCAG-3´), 1060F (5´-TGTCGWCAGCTCGTGYYGTGA-3´), 806R (5´-GGACTACCAGGGTATCTAAT-3´), 338R (5´-CTGCTGCCTCCCGYAGGAGT-3´) and

1407F (5´-TGYACWCACCGCCCGTC-3´) (Loy *et al*.) [19]. Raw sequence data were edited and assembled using Sequencher (Gene Codes, Ann Arbor, MI). A multiple sequence alignment was created from the consensus sequence of the novel isolates and type strains of close relatives obtained from NCBI GenBank and Ez Taxon databases [20]. Evolutionary analyses were conducted in MEGA6 [21]. A phylogenetic tree was constructed with 26 sequences using the Neighbor-Joining method to infer evolutionary history [22]. Evolutionary distances were computed using the Jukes-Cantor method [23] and bootstrap analysis (1000 replicates) was performed to determine the reliability of the inferred tree [24]. All positions containing gaps and missing data were eliminated and a total of 1203 positions were included in the final dataset.

2.7. GenBank Accession Numbers

The partial 16S rRNA gene sequences from the isolates were submitted to GenBank and the Accession numbers for bacterial isolates 0004-9, 0009-5^T, and 0019-D are GQ900631, GQ900632 and GQ900633, respectively. The whole genomic sequence for 0009-5^T was submitted to GenBank and the Accession number is CP001850.

3. Results and Discussion

All three isolates were non-motile, vancomycin sensitive, colistin resistant, obligately anaerobic rods that stained Gram negative with blunt or tapered ends and were asaccharolytic, non-cellulolytic, catalase negative, indole positive, urease negative, oxidase negative and were negative for esculin and gelatin hydrolysis, and lecithinase and lipase activity. A negative rapid KOH test (absence of stringing reaction with the addition of bacteria to a 3% KOH solution) validated disk susceptibility results and confirmed the bacteria as a Gram positive rod. There was no demonstration of spores following heat and ethanol treatments. The optimal temperature for growth ranged from 35–42°C. The DNA mol% G+C content was determined to be 44.2%. Table 1 shows phenotypic and genotypic data which are useful for differentiating *Mageeibacillus indolicus* from other taxa in the *Clostridium* rRNA cluster III and related organisms.

Following five days of anaerobic incubation at 37°C on Brucella agar supplemented with 5% sheep’s blood (bioMerieux), colony morphology revealed a one millimeter, entire, flat fried egg with a transparent pink edge and slight iridescent cream center. Longer incubation resulted in a slight spreading of colonies and larger colony.

Growth was not supported in PY or CMC broth alone. Increased growth was not observed in either broth when supplemented as described above, however the initial inoculum was maintained in PY supplemented with glucose, arginine, sodium bicarbonate, hemin and horse serum. Growth was also maintained in CMC supplemented with horse serum.

The same positive reactions to twelve substrates were observed for all three strains following API ID 32A and API ZYM testing and include the following; indole, arginine arylamidase, phenylalanine arylamidase, leucine arylamidase, tyrosine arylamidase, glycine arylamidase, α-fucosidase, histidine arylamidase, serine arylamidase, valine arylamidase, acid phosphatase, and napthol-AS-BI-phosphohydrolase suggesting amino acids are utilized

as a primary source of carbon. Enzymatic activity was observed for alanine arylamidase for two of the three strains tested (0019-D and 0004-9). Reactions to substrates were rated as positive regardless if color change was weak or robust.

Electron micrograph images (available as supplementary data, S1 and S2) taken from the strains grown on Brucella agar revealed rods approximately 1.25 μm in length and 588 nm in diameter occurring in chains or in small aggregates. S1 shows a Gram positive cell wall with a relatively thin layer of peptidoglycan and may explain why the organism stains Gram negative.

Predominant fatty acids (> 5%) observed among all strains were of the unbranched saturated and unsaturated forms; $C_{14:0}$ (4.83–11.09%), $C_{16:0}$ (30.13–39.31%), $C_{18:2\ \omega6,\ 9c}$ (7.62–10.18%), $C_{18:1\ \omega9c}$ (25.52–32.75%) and $C_{18:0}$ (5.69–10.89%). Minor fatty acids present consisted of unbranched and branched iso- and anteiso- forms; $C_{12:0}$ (1.18–1.95%), $C_{15:0}$ (1.85–3.05%), iso-$C_{15:0}$ (0–0.41%), anteiso-$C_{15:0}$ (0–0.66%), $C_{16:0\ \text{aldehyde}}$ (0–0.58%), $C_{16:1\ \omega7c}$ (1.23–1.56%), $C_{16:0\ \text{DMA}}$ (0–0.93%), anteiso-$C_{17:0}$ (0.49–0.61%), $C_{17:0}$ (1.56–2.56%). The Sherlock MIS software used to analyze fatty acid composition profiles did not recognize the pattern generated for comparison to known cultivated and tested bacteria, further supporting the novelty of this bacterium.

Sexually Transmitted Diseases Treatment guidelines recommend treatment of BV with metronidazole or clindamycin and PID with cefoxitin in combination with doxycycline with or without metronidazole [33]. These antibiotics were chosen for susceptibility testing because these novel strains were found to be highly associated with BV and these microorganisms were recovered from the upper genital tract of women being evaluated for pelvic inflammatory disease. Breakpoints of resistance for metronidazole, clindamycin and cefoxitin are ≥ 32 μg ml^{-1}, ≥ 8 μg ml^{-1}, and ≥64 μg ml^{-1}, respectively. CLIS guidelines do not list breakpoints for doxycycline for anaerobes, so susceptibility could not be determined. The range of minimal inhibitory concentrations observed for the three isolates for doxycycline however was (4–16 μg ml^{-1}). Although only three isolates were tested, no resistance to metronidazole, clindamycin or cefoxitin was observed among the strains and the ranges of minimal inhibitory concentrations for those drugs were (0.25–0.5 μg ml^{-1}), (0.125 μg ml^{-1}) and (4–8 μg ml^{-1}), respectively.

Comparing our novel isolate sequences with sequences in GenBank using the NCBI BLAST tool revealed that the 16S rRNA gene sequences were most closely related (≥ 98%) to the sequences from uncultivated bacterial clones detected by PCR and designated as BVAB3 [5], a vaginal clone (AY995273) in the study by Zhou *et al*., [8] and skin clones (GQ043025, GQ038494) reported by Grice *et al*., [34]. The novel isolate sequences were also distinct from sequence types obtained from clones previously described as BVAB1 (sequence similarity, 82%) and BVAB2 (sequence similarity, 89%) from the human vagina [5].

The closest bacterial isolate *Saccharofermentans acetigenes* described by Chen *et al*. [25] had a 91% sequence similarity. Phylogenetic analysis revealed that the novel bacterium formed deep branches with *S. acetigenes* and *Fastidiosipila sanguinis* described by Falsen *et*

al. [26], both of which are associated with *Clostridium* rRNA cluster III (as defined by Collins *et al*.) [35] and are now included in the family *Ruminococcaceae* [36]. These associations are supported by high bootstrap values as shown in Figure 1. The high level of sequence divergence suggests that strain 0009-5^T could represent a novel species of a new genus in this cluster.

This study resulted in identification of our novel isolate as the fastidious uncultivated clone previously designated as BVAB3 [5]. Numerous studies have been conducted regarding BV-associated bacteria, specifically BVAB3, and its role in the etiology of BV and resulting sequelae remain unclear. While it has been associated with BV in some studies [5, 6], others have reported that this organism was linked to BV in univariate but not multivariate models [37]. It remains to be determined whether BVAB3 is critical to the development of BV in some women. In addition to the vagina and endometrium, this organism has also been detected from anal swabs of women having BV, suggesting that extragenital reservoirs for this organism can occur [38]. However, this organism was never recovered from the oral cavity and was only in 4% of anal swab samples [38]. Foxman *et al*. evaluated the role of several BV-associated bacteria with preterm birth and reported that pregnant women having this organism had a decreased risk of preterm delivery [39]. BV has been linked with increased shedding of HIV [1]. Among HIV infected women, those having BVAB3 were more likely to shed HIV even when on effective antiretroviral therapy [40]. Further research will be required to elucidate the role of this organism in BV and reproductive tract sequelae.

Description of *Mageeibacillus* gen. nov.

Mageeibacillus: Ma.gee.i.ba.cil'lus. N.L. n. Mageea, named after Magee; L. masc. n. bacillus, a rod; N.L. masc. n. Mageeibacillus, a rod isolated at Magee-Womens Research Institute, and where biochemical identification was performed. Cells are Gram positive, catalase negative, oxidase negative, non-motile, asaccharolytic, obligately anaerobic, non-spore forming rods. Cellulose is not degraded. Optimal temperature range for growth is 35–42°C. Predominant fatty acids observed are unbranched saturated and unsaturated forms. The type species is *Mageeibacillus indolicus*.

Description of *Mageeibacillus indolicus* sp. nov.

Mageeibacillus indolicus (in.dol'icus. N.L. masc. adj. indolicus for pertaining to the ability of the organism to produce indole).

Colonies are entire, 2mm, flat fried egg with a transparent pink edge and slight iridescent cream center at eight days growth on Brucella agar supplemented with 5% sheep blood incubated anaerobically at 37°C. Cells are Gram positive but stain Gram negative, are vancomycin sensitive, colistin resistant, non-motile, catalase, oxidase, urease, lipase and lecithinase negative, non-cellulolytic and are indole positive. Rods are approximately 1.25 μm in length and 588 nm in diameter occurring in chains or in small aggregates when grown as previously mentioned. Optimal temperature for growth is 37°C. Strains are asaccharolytic but enzymatic activities are present for arginine arylamidase, phenylalanine arylamidase, leucine arylamidase, tyrosine arylamidase, glycine arylamidase, α-fucosidase, histidine arylamidase, serine arylamidase, valine arylamidase, acid phosphatase and napthol-AS-BI-

phosphohydrolase. Susceptibility testing to metronidazole, clindamycin and cefoxitin was performed with no resistance observed, however only three isolates were tested. Predominant fatty acids observed were $C_{14:0}$, $C_{16:0}$, $C_{18:2\ \omega6,\ 9c}$, $C_{18:1\ \omega9c}$ and $C_{18:0}$. The DNA base composition is 44.2 mol% G+C.

The type stain is BAA-2120^T and CCUG 59143^T, isolated from the human genital tract.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Three bacteria were isolated from the endometrium with conventional culture methods.
- Isolates are anaerobic, asporulous, asaccharolytic, indole positive, positive rods.
- Sequencing revealed only 91% similarity to cultivated organisms in GenBank.
- All data suggest a novel genus and species within the Clostridium rRNA cluster III.

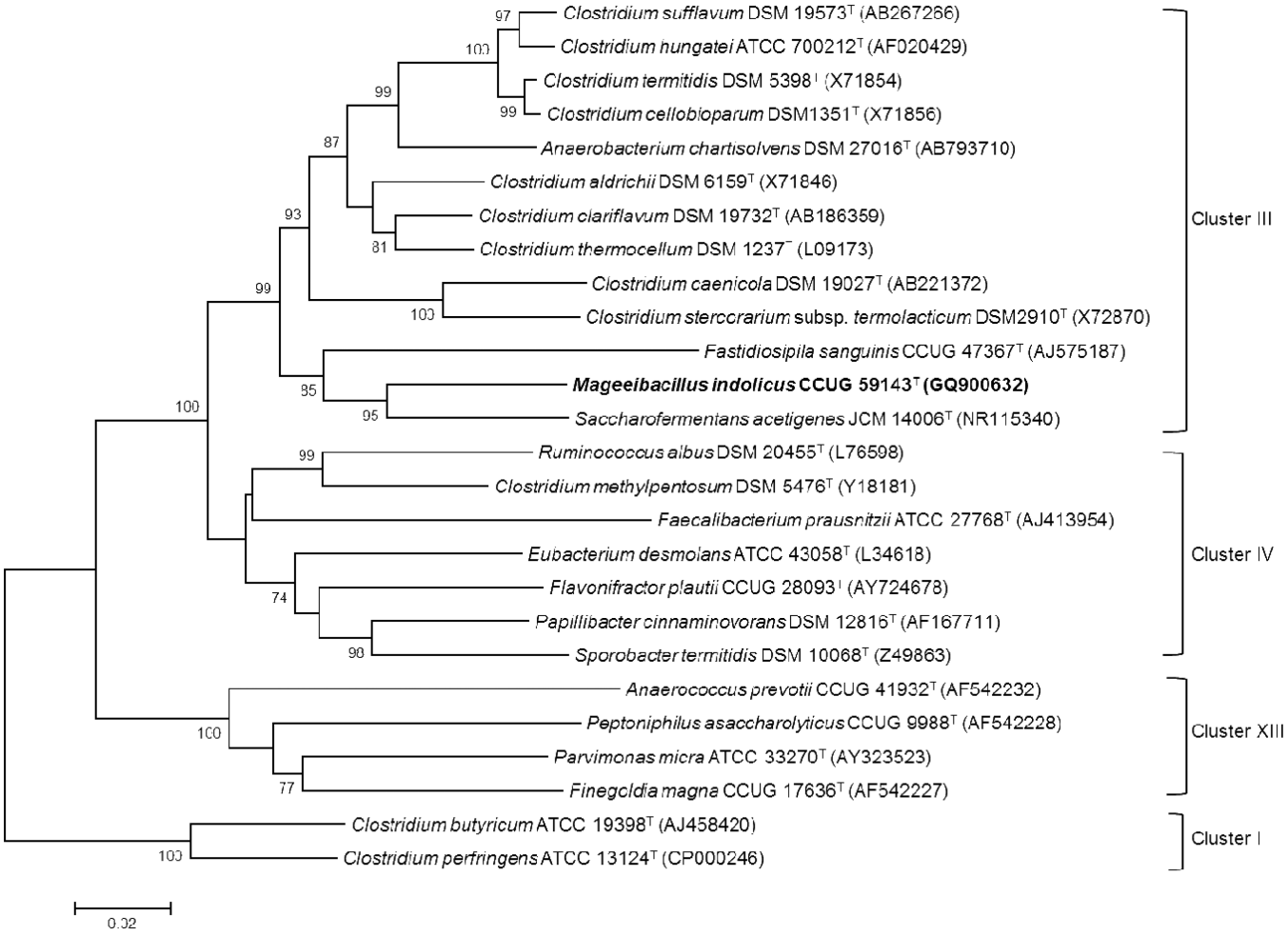

Figure 1.

Neighbor-joining phylogenetic dendrogram showing the phylogenetic position of *Mageeibacillus indolicus* CCUG 59143T among closely related species using 16S rRNA sequences. The tree was rooted with *C. butyricum* ATCC 19398T and *C. perfringes* ATCC 13124T. Numbers at branch points represent bootstrap support (%) based on an analysis of 1000 resampled datasets. GenBank accession numbers of the 16S rRNA gene sequences are provided in parentheses. Bar, 2% sequence divergence.

Table 1

Characteristics for differentiating related organisms within *Clostridium* rRNA cluster III and cluster IV [a, b]

	Mageeibacillus indolicus	**Saccharofermentans acetigenes**	**Fastidiosipila sangunis**	**Clostridium caenicola**	**Clostridium clariflavum**	**Clostridium sufflavum**	**Flavonifractor plautii**	**Ruminococcus albus**
% similarity		91%	88%	88%	90%	89%	86%	84%
Characteristics								
Cluster	III	III	III	III	III	III	IV	IV
Cellular Morphology	rod	coccobacillary	cocci	rod	rod	rod	rod	cocci
Spores	neg	pos	neg	pos	pos	pos	neg	neg
Gram stain	neg	pos	pos	neg	variable	neg	variable	pos
Motility	neg	neg	neg	pos	NO	pos	pos	neg
Flagella	neg	neg	neg	PB	P	P	P	neg
Cellulose degradation	neg	neg	neg	pos	pos	pos	neg	neg
Growth at 60°C	neg	neg	neg	pos	pos	neg	neg	neg
Mol % G+C	44.2	55.6	33	51.1	36.9	40.7	58–61.6	42.6–45.8
Predominant Fatty Acids (>5%)	$C_{14:0}$, $C_{16:0}$, $C_{18:2\omega6,9c}$, $C_{18:1\omega9c}$, $C_{18:0}$	iso-$C_{15:0}$, anteiso-$C_{15:0}$, iso-$C_{14:0}$, 3-OH	$C_{14:0}$, $C_{16:0}$, $C_{18:2\omega6,9c}$, $C_{18:1\omega9c}$, $C_{18:0}$	iso-$C_{15:0}$, anteiso-$C_{15:0}$, $C_{16:0}$, iso-$C_{17:0}$, anteiso-$C_{17:0}$	$C_{16:0}$, iso-$C_{16:0}$, $C_{16:0}$ DMA, iso-$C_{17:0}$	iso-$C_{15:0}$, iso-$C_{14:0}$, $C_{16:0}$ DMA,	Unknown compound between $C_{12:0}$ and $C_{13:0}$, $C_{14:0}$, Unknown compound between anteiso-$C_{15:0}$ and $C_{15:0}$, $C_{16:0}$, $C_{17:1\omega8c}$, $C_{18:0}$	iso-$C_{14:0}$, Unknown compoud between iso-$C_{14:0}$ and $C_{14:0}$, $C_{14:0}$, anteiso-$C_{15:0}$, $C_{15:0}$, iso-$C_{16:0}$, $C_{16:0}$

[a] Characteristics are described as (neg) negative, (pos) positive, (ND) not determined, (NO) not observed, (PB) polar bundle, (P) peritrichous.

[b] Data from: Chen *et al.* [25], Falsen *et al.* [26], Culture Collection University of Goteborg, [27] Shiratori *et al.* [28], Nishiyama *et al.* [29], Carlier *et al.* [30] Ezaki [31], Ifkovits and Ragheb [32]