Prevalence and Abundance of Uncultivated Megasphaera-Like Bacteria in the Human Vaginal Environment

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Cultivation-independent analysis of 16S rRNA gene sequences in vaginal samples revealed two previously unrecognized, uncultivated Megasphaera-like phylotypes. Phylogenetic analysis and environmental distribution suggest that these Megasphaera types may be unique to the vaginal environment. Quantitative PCR suggests that both phylotypes are present in higher concentrations in women with bacterial vaginosis.

Bacterial vaginosis (BV) is the most common cause of vaginal irritation and is associated with serious morbidities such as adverse pregnancy outcomes (20, 21) and increased risk of human immunodeficiency virus infection (7, 17, 25, 27). No single etiologic agent has been implicated as the cause of BV, and the syndrome has been referred to as a polymicrobial disorder (9, 22). Cultivation-independent analyses of 16S rRNA gene sequences show that previously unrecognized species are prevalent in the vaginal flora (4, 10, 11, 13, 14, 28, 30). Among these are uncultivated species most closely related to Megasphaera (11, 14, 28, 30). The clinical significance of vaginal Megasphaera is unknown. To investigate the association of Megasphaera with various grades of vaginal flora, we developed quantitative real-time PCR (qRT-PCR) assays targeting two Megasphaera-like 16S phylotypes, termed Megasphaera types 1 and 2, and evaluated their prevalence and relative abundance in patients whose vaginal flora had been clinically and microscopically defined as normal, intermediate, or BV.

DNA extracted (High Pure PCR template preparation kit; Roche Molecular Diagnostics, Penzberg, Germany) from 41 vaginal swab specimens used originally for detection of Mycoplasma genitalium to determine the etiology of mucopurulent cervicitis was studied here. The LSU Health Sciences Center Institutional Review Board protocol describing the study population and sampling is available upon request. The vaginal flora of each patient was characterized clinically using Amsel’s criterion (1) and Nugent vaginal Gram stain scores (18, 29). Normal and BV flora were defined by Nugent scores of ≤3 and ≥7, respectively. Plasmids were purified using a QIAprep spin miniprep kit (Qiagen, Valencia, CA). Genomic and plasmid DNA were quantified using a TBS-380 fluorometer (Turner Biosystems, Sunnyvale, CA) and Quant-iT PicoGreen double-strand-DNA reagent (Invitrogen, Carlsbad, CA). Plasmoid copies were calculated with the DNA copy number calculator at the URI Genomics and Sequencing Center web site (24). PCR primers targeting Megasphaera type 1 and type 2 were designed using Primrose (2). The primer sequences for type 1 are 5′GACGGATGCCAACAGTATCCGTCCG3′ and 5′AAATTCGACAGTTTTCCGTCCTCCCTC′; the primers for type 2 are 5′GGCGAAAGTGTTAAATAGCCATC3′ and 5′ACTCAAGTCITCCAGTTCCGGTC3′. Cross-reactivity between Megasphaera type 1 and 2 assays was tested using plasmids cloned from our vaginal PCR survey (14), and results were negative. Total bacterial 16S rRNA gene concentration was measured in vaginal DNA specimens as previously described (3) using the primer sequences 5′CTTACGGGAGGCAGACG3′ and 5′ATTACC CGCGCTGCTGGC′. In our analyses, the sensitivity of this assay was limited to 104 templates per qRT-PCR. All assays were performed on an iCycler (Bio-Rad, Hercules, CA) using iQ-SYBR green PCR supermix (Bio-Rad) with 10 ng of vaginal template DNA and a 0.5 μM final concentration of each primer. Temperature cycling for all assays was 95°C for 2.5 min, followed by 40 cycles at 95°C for 30 s, 64°C for 30 s, and 72°C for 30 s; annealing was 65°C for the total bacterial assay. Cloned Megasphaera type 1 and 2 16S rRNA genes from vaginal clone libraries (10) were used to generate 10-fold serial dilutions for standard curves. Threshold cycles from standard curves were used to calculate the number of Megasphaera type 1 and 2 16S rRNA genes in vaginal DNA specimens (8). All qRT-PCR products were visualized in ethidium bromide-stained agarose gels and sequenced to confirm specificity (Davis Sequencing, Inc., Davis, CA).

A dendrogram illustrating the relationship between vaginal Megasphaera phylotypes and Megasphaera spp. from other environments was created using 28 nearly full-length (~1,390-nt) Megasphaera sequences from studies of gut, oral, vaginal, and other environments. Some shorter Megasphaera type 1 and 2 (~900-nt) sequences were added to the tree to document the origin of all members of the two vaginal phylotypes. Sequences were obtained from the Ribosomal Database Project (15) and from GenBank. Sequences were aligned using ClustalW (5). Alignments were checked and adjusted using Jalview (6). A Dialister pneumosintes sequence was used as an outgroup (16). The tree was constructed using the neighbor-joining method (19) with the Jukes-Cantor model implemented in the PAUP software (26). Bootstrap analysis (100 replicates) was performed to test the significance of the nodes.

Statistical calculations were performed using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, California) and the Student’s t test (23). Statistical significance was defined as P ≤ 0.05. To determine the etiology of mucopurulent vaginal swab specimens used originally for detection of Mycoplasma genitalium, we developed quantitative real-time PCR (qRT-PCR) assays targeting two Megasphaera-like 16S rRNA genes from vagi- nal PCR survey (14), and results were negative. Total bacterial 16S rRNA gene concentration was measured in vaginal DNA specimens as previously described (3) using the primer sequences 5′CTTACGGGAGGCAGACG3′ and 5′ATTACC CGCGCTGCTGGC′. In our analyses, the sensitivity of this assay was limited to 104 templates per qRT-PCR. All assays were performed on an iCycler (Bio-Rad, Hercules, CA) using iQ-SYBR green PCR supermix (Bio-Rad) with 10 ng of vaginal template DNA and a 0.5 μM final concentration of each primer. Temperature cycling for all assays was 95°C for 2.5 min, followed by 40 cycles at 95°C for 30 s, 64°C for 30 s, and 72°C for 30 s; annealing was 65°C for the total bacterial assay. Cloned Megasphaera type 1 and 2 16S rRNA genes from vaginal clone libraries (10) were used to generate 10-fold serial dilutions for standard curves. Threshold cycles from standard curves were used to calculate the number of Megasphaera type 1 and 2 16S rRNA genes in vaginal DNA specimens (8). All qRT-PCR products were visualized in ethidium bromide-stained agarose gels and sequenced to confirm specificity (Davis Sequencing, Inc., Davis, CA).

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Differences in the qRT-PCR values obtained for Megasphaera types 1 and 2 across patient groups defined by Nugent scores were evaluated by Student’s t test analysis. The significance of the association between patient groups (BV and normal) and presence of Megasphaera types 1 and 2 was measured by Fisher’s exact test. Statistical significance was set at an \( p < 0.05 \), and all tests were two-sided.

The dendrogram shows that Megasphaera type 1 and type 2 sequences form two well-supported clades (Fig. 1). No sequences in these clades originate from oral, gut, rumen, or other environments, suggesting that Megasphaera type 1 and 2 organisms may be uniquely adapted to the genitourinary environment. Megasphaera sequences within the type 1 clade share a high degree of similarity (98 to 99%) with each other, as do those within the type 2 clade (98 to 99%). Sequences within the type 1 and 2 clades share 95 to 96% similarity with those of their nearest relatives. This suggests that phylotypes 1 and 2 likely represent new species (23).

Measurements of Megasphaera type 1 and type 2 and total bacterial 16S rRNA gene concentrations in vaginal specimens of 41 patients are shown in Fig. 2. Vaginal specimens are arranged according to increasing Nugent score. Megasphaera type 1 was more prevalent than Megasphaera type 2, as it was detected in 38 (76%) versus 22 (52%) patients, respectively (\( p < 0.0004 \)) (Fig. 2A and B). Megasphaera type 1 concentrations were dramatically higher (5 orders of magnitude) in all patients with the highest possible indication of BV (Nugent = 10, Amsel = 4) than in patients with the highest possible indication of normal flora (Nugent = 0, Amsel = 0) (\( p = 0.0114 \)). Meanwhile, total bacterial concentrations between these extreme patient groups differed by 2 orders of magnitude (\( p < 0.0001 \)). Megasphaera type 1 appears to have a stronger association with BV (\( p = 0.0072 \)) than type 2 (\( p = 0.0366 \)). The quantitative analyses in this study correlate with previous, presumably less quantitative, broad-range molecular studies in which PCR amplification of 16S rRNA genes and clone library analyses showed that Megasphaera type 1 clones were more prevalent and abundant in vaginal specimens of BV patients.
than *Megasphaera* type 2 (11). However, it should be noted that even though *Megasphaera* type 1 seems to be strongly associated with BV, it was detected in a number of women with clinically normal Nugent scores, both in this study and in PCR-based analyses of normal patients by others (12). Thus, measurements of species abundance may provide a more informative view of the vaginal ecology.

**Nucleotide sequence accession numbers.** GenBank accession numbers EF120358 and EF120359 were assigned to the sequences determined in this study.

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