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ORIGINAL ARTICLE

Differences in the composition of vaginal microbial communities found in healthy Caucasian and black women

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The maintenance of a low pH in the vagina through the microbial production of lactic acid is known to be an important defense against infectious disease in reproductive age women. Previous studies have shown that this is largely accomplished through the metabolism of lactic acid bacteria, primarily species of *Lactobacillus*. Despite the importance of this defense mechanism to women's health, differences in the species composition of vaginal bacterial communities among women have not been well defined, nor is it known if and how these differences might be linked to differences in the risk of infection. In this study, we defined and compared the species composition of vaginal bacterial communities in 144 Caucasian and black women in North America. This was carried out based on the profiles of terminal restriction fragments of 16S rRNA genes, and phylogenetic analysis of 16S rRNA gene sequences of the numerically dominant microbial populations. Among all the women sampled, there were eight major kinds of vaginal communities ('supergroups') that occurred in the general populace at a frequency of at least 0.05 ($P = 0.99$). From the distribution of these supergroups among women, it was possible to draw several conclusions. First, there were striking, statistically significant differences ($P = 0.0$) in the rank abundance of community types among women in these racial groups. Second, the incidence of vaginal communities in which lactobacilli were not dominant was higher in black women (33%) as compared to Caucasian women (7%). Communities not dominated by lactobacilli had *Atopobium* and a diverse array of phylotypes from the order *Clostridiales*. Third, communities dominated by roughly equal numbers of more than one species of *Lactobacillus* were rare in black women, but common in Caucasian women. We postulate that because of these differences in composition, not all vaginal communities are equally resilient, and that differences in the vaginal microbiota of Caucasian and black women may at least partly account for known disparities in the susceptibility of women in these racial groups to bacterial vaginosis and sexually transmitted diseases.

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Introduction

The high levels of estrogen in reproductive age women cause large amounts of glycogen to be deposited in the vaginal epithelium (Paavonen, 1983), and it is subsequently metabolized primarily by bacterial populations to produce organic acids (Boskey *et al.*, 1999). The resulting low pH (4–4.5) of the vagina creates a restrictive environment that precludes the growth of many pathogenic organisms. Over the years, a large number of different species of lactic acid bacteria, primarily species of *Lactobacillus*, have been demonstrated to reside in the human vagina (Redondo-Lopez *et al.*, 1990; Antonio *et al.*, 1999) and be key players in this process. These species effectively constitute an ecological guild – a group of species that have similar requirements and play a similar role within a community – and maintaining high numbers of these populations is a hallmark of healthy conditions. Events that lead to decreased numbers of lactic acid bacteria in the vagina and the concomitant increase in the abundances of other bacteria are collectively known as bacterial vaginosis (BV; Thorsen *et al.*, 1998; Koumans and Kendrick,

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2001). During BV, there is an increase in vaginal pH (>4.5) that is often accompanied by the production of odor, and a thin, grayish white vaginal discharge (Sobel, 2000).

BV commonly occurs in women of reproductive age. It affects between 20% and 25% of the general population, and up to 50% of women attending sexual health clinics (Newton *et al.*, 1997; Morris *et al.*, 2001; Yen *et al.*, 2003; Sobel, 2005). It is the most common cause of vaginal symptoms prompting women to seek medical care (Morris *et al.*, 2001; Marrazzo, 2006), and a significant problem because it predisposes individuals to sexually transmitted diseases including human immunodeficiency virus (HIV) (Sewankambo *et al.*, 1997; Taha *et al.*, 1998; Martin *et al.*, 1999), preterm birth, pelvic inflammatory disease, endometritis and infertility (Hillier *et al.*, 1995; Sweet, 1995; Wiesenfeld *et al.*, 2002; Leitich *et al.*, 2003; Haggerty *et al.*, 2004). The causes of BV are not well understood, and specific infectious agent(s) have not been identified. However, a number of bacterial species are known to be common in women with BV, including *Gardnerella vaginalis*, *Mobiluncus* sp., *Mycoplasma hominis* and species of *Clostridiaceae* (Thorsen *et al.*, 1998; Koumans and Kendrick, 2001; Fredricks *et al.*, 2005), but their role in the pathogenesis of BV has not been established. The occurrence of BV has been associated with behaviors that upset the normal balance of bacteria in the vagina including new or multiple sex partners, douching, and the use of an intrauterine device for contraception (http://www.cdc.gov/std/bv/STDFact-Bacterial-Vaginosis). This has led some to suggest that BV is not an infectious disease at all but rather a set of symptoms that reflect an ecological disturbance that is accompanied by changes in the relative abundance of autochthonous organisms in the vagina (http://www.cdc.gov/std/bv/STDFact-Bacterial-Vaginosis). Curiously, the incidence of BV varies markedly among racial and ethnic groups (Rajamanoharan *et al.*, 1999; Royce *et al.*, 1999), ranging from 6% in Asians and 9% in whites, to 16% in Hispanics and 23% in African Americans. The reasons for differences in the incidence of BV among racial groups are unknown, but they cannot be explained by differences in socio-demographics, sexual activity, health behavior or hygiene alone (Goldenberg *et al.*, 1996; Royce *et al.*, 1999).

While there are many reasons for concern about BV, the link between BV and an increased risk for acquisition of the HIV (Sewankambo *et al.*, 1997; Taha *et al.*, 1999) is particularly menacing. The number of women with HIV and acquired immunodeficiency syndrome (AIDS) has steadily increased worldwide and by the end of 2005, 17.5 million women were infected with HIV (http://www.niaid.nih.gov/factsheets/womenhiv.htm). More than 90% of HIV infections in adolescent and adult females result from heterosexual intercourse, where the risk of acquiring HIV is 2–4 times higher if the woman has BV (Sewankambo *et al.*, 1997; Martin *et al.*, 1999; Taha *et al.*, 1999). Myer *et al.* (2005) argue that interventions to reduce the occurrence of BV may have an impact on the spread of HIV, and that almost one-third of all new HIV infections might be prevented if all cases of BV could be cured. However, the existing treatments for BV (oral or vaginal administration of metronidazole) are plagued by high failure rates, with symptoms reoccurring in 54% of women within 3 months following antibiotic treatment (Bradshaw *et al.*, 2006). Measures that stabilize vaginal microbial communities and prophylaxis against BV may emerge if we develop an accurate understanding of the species composition and ecology of vaginal ecosystems in normal healthy women.

In this study, we tested the hypothesis that there were fundamental differences in the composition and structure of bacterial communities in the vaginas of Caucasian and black women in North America. To do this, we determined the identity and rank abundance of bacterial populations in vaginal bacterial communities of 144 healthy, reproductive age women in these racial groups. The data showed that within each racial group there were distinct differences in community composition, which allowed us to group women into categories that we referred to as 'supergroups'. There were significant differences in the rank abundance of these communities in Caucasian and black women, and we postulate that these differences in community composition and structure may account for the differential risk to BV and various vaginal infections of women in these racial groups.

Materials and methods

Subjects and clinical study design

As part of a study on the prevalence of *Staphylococcus aureus* carriage, 3012 healthy menstruating women between the ages of 13 and 40 years were enrolled from five sites in North America (Parsonnet *et al.*, 2005). The subjects were recruited by Hill Top Research Inc. in Cincinnati, OH, USA, East Brunswick, NJ, USA, St Petersburg, FL, USA, Scottsdale, AZ, USA and Winnipeg, Manitoba, Canada. The racial profile of the women corresponded with that of the 1990 USA census: 80% white, 12% black, 5% Hispanic and 3% Asian. Subjects were eligible for enrollment if they had regular menstrual cycles (21–35 days); used tampons at least occasionally; were able to read, write and understand English; did not bathe or shower within 2 h of their scheduled visit; refrained from douching, vaginal medications, suppositories, feminine sprays, genital wipes or contraceptive spermicides for 48 h before their scheduled visit; and were willing to comply with all other protocol requirements. Subjects were not eligible if they were participating in another clinical study; were pregnant, actively trying to get pregnant or suspected they were pregnant; had a gynecologi-

cal abnormality as judged by the study's medical personnel; had an infection of the genitals within the past 6 weeks; had been medically diagnosed as having diabetes, kidney failure, hepatitis, AIDS (HIV positive) or toxic shock syndrome; or were currently taking (within the last 30 days) immunosuppressive drugs, chemotherapy, systemic antimicrobial or antifungal drugs, or antimicrobials to treat a vaginal infection. Subjects completed a demographic questionnaire and classified themselves into one of the four distinct racial groups: white, black, Hispanic or Asian. The study protocol and informed consent document were reviewed and approved by Hill Top's Institutional Review Board. Informed consent from all subjects was documented before participation in the study.

A sample from each woman was taken near the mid-vagina using a sterile swab. All swabs were taken between days 1 and 10 of the menstrual cycle. A saline-lubricated speculum was used to minimize contamination of the sample by the flora of the labia during entry and withdrawal of the swab. The swab was placed in a sterile cryovial and stored at -70°C until analysis. Upon collection of the vaginal sample, the attending health care practitioner noted any signs of possible genital infections (e.g., discharge, cervicitis or foul odor); none of the subjects in this study had signs of vaginal infection.

From the 3000+ swabs available, we drew random subsets so there would be 15 individuals from each of the five locations (Manitoba, Canada and Ohio, New Jersey, Arizona in the US), and an equal numbers of participants from each of three age groups: 13–18, 19–35 and 36–40 years old (Table 1). It was possible to enroll a full complement of self-declared Caucasian women ($n=75$). This was also true for black women, except for subjects from Manitoba where too few 13–18 and 35–40 years old self-declared black women enrolled in the previous study (Parsonnet *et al*., 2005). Consequently, samples from only 69 black women were included in the present study.

Terminal restriction fragment length polymorphism analysis of 16S rRNA genes

For the analysis of terminal restriction fragment length polymorphisms (T-RFLPs) of 16S rRNA genes, internal regions of 16S rRNA genes in each sample were amplified in two separate reactions using fluorescently labeled primer pairs, 8fm–926r and 49f–926r (based on *Escherichia coli* position). Primer 8fm was labeled with VIC, 49f was labeled with NED and 926r was labeled with 6-FAM (Applied Biosystems, Foster City, CA, USA). DNA amplification was performed as previously described (Zhou *et al*., 2004) except an annealing temperature of 60°C was used for primer pair 49f–926r. The profiles of terminal restriction fragments from the microbial communities in each sample were determined as follows. A mixture of the two fluorescently labeled amplicons was equally divided and separately digested with *Msp*I and *Hae*III, then the digested products were recombined. *Msp*I and *Hae*III were chosen because they have been shown empirically and through *in silico* analyses to provide the greatest resolution of populations likely to be found in vaginal samples. The resulting mixture had six fluorescently labeled terminal restriction fragments from the use of three fluorophores and two restriction enzymes and this allowed for the high resolution of microbial communities. T-RFLP profiles were determined using an ABI PRISM 3100 DNA Analyzer and GeneScan software (Applied Biosystems) as previously described (Coolen *et al*., 2005) using CST ROX 25–1000 (BioVentures Inc., Murfreesboro, TN, USA) as an internal standard.

Cluster analysis of T-RFLP data

The algorithms described by Abdo *et al*. (2006) were used for identifying the threshold for defining peaks and for the cluster analysis of T-RFLP data. First, true peaks were identified once a threshold (baseline) had been defined. Second, hierarchical clustering was carried out to identify those fragments with lengths close enough to justly group them in the same length category. Third, the Euclidean distances between T-RFLP profiles were calculated and these were hierarchically clustered based on average linkage (unweighted pair group method and arithmetic mean) and a dendrogram was constructed. Finally, three cluster criteria were employed to identify a statistically meaningful number of groups in the data.

Table 1 Number of vaginal swabs from Caucasian and black women in various geographic locations and age groups

Location	*Black*			*Caucasian*			*Total*
	13–18 years	*19–35 years*	*35–40 years*	*13–18 years*	*19–35 years*	*35–40 years*	
OH, USA	5	5	5	5	5	5	30
FL, USA	5	5	5	5	5	5	30
AZ, USA	5	4	5	5	5	5	29
NJ, USA	5	5	5	5	5	5	30
Manitoba, Canada	3	5	2	5	5	5	25
Total	23	24	22	25	25	25	144

A 'coverage sampling approach' (Abdo *et al.*, 2006) was employed to identify the fewest samples that accounted for 85% of the phylotype diversity found within a cluster. In doing so, we assume that in all profiles terminal restriction fragments with a unique size represented a single phylotype. This reduced the total number of samples that needed to be analyzed while at the same time assuring that each cluster was adequately sampled (Abdo *et al.*, 2006). The samples specified were used to construct clone libraries of amplified 16S rRNA genes for subsequent sequence analysis.

Phylogenetic analysis of 16S rRNA gene sequences

Primers FD1f and RD1r (Weisburg *et al.*, 1991) without fluorescent labels were used to generate nearly full-length 16S rRNA amplicons of 16S rRNA genes in vaginal samples. These mixtures of amplicons were used for library construction as described previously (Zhou *et al.*, 2004). We were unable to construct a clone library from one singleton (B1-10) because the yield of genomic DNA was too low.

Approximately 100 clones of each sample were randomly chosen from each library and the cloned DNA fragments were partially sequenced using an ABI 3730 PRISM DNA Analyzer. High-quality sequences with less than 3% uncalled bases and more than 500 bp long were analyzed by using BLAST (Altschul *et al.*, 1997) to identify similar sequences from among the *Eubacterial* type strains found in the Ribosomal Database Project (RDP; Cole *et al.*, 2003) that were at least 1200 nt long. The most closely related RDP sequences and each sequence from a library were clustered based on genetic similarity using a neighbor joining method (Saitou and Nei, 1987). The resulting clusters were used to define operational taxonomic units (OTUs) in which phylogenetically related clones with $\geqslant$90% sequence similarity to a reference strain were presumed to be members of the same genus, and those whose sequences were $\geqslant$97% similar were considered to be members of the same species. Clones with $\leqslant$90% sequence similarity to any sequence in the RDP were classified as novel organisms. Clones representing each OTU were chosen for detailed phylogenetic analysis by bidirectional sequencing of the entire 16S rRNA gene fragment. These sequences were edited and assembled, then phylogenetic trees were constructed as previously described (Zhou *et al.*, 2004) to depict the phylogenetic relationships that exist among members of the communities. The gene sequences reported in this paper have been deposited in GenBank and were assigned accession numbers AY995236–AY995274.

Statistical modeling and analysis

To evaluate differences in the distribution of microbial community supergroups among Caucasian and black women, we used a method proposed by Schütte *et al.* (in preparation). We constructed a contingency table (Table 2) with columns of individuals with similar vaginal microbial communities and rows of ethnicity. The counts within each row, associated with each ethnicity, were assumed to be independent and multinomial-distributed. This probabilistic model was then compared to a reduced multinomial model that assumed no ethnic-based differences in the distribution of community supergroups. Comparison between the full model that accounts for ethnicity to the reduced model was carried out using a likelihood ratio test and the bootstrap (Efron and Tibshirani, 1997; Schütte *et al.*, in preparation). Based on the above modeling assumptions, the likelihood function under the full model that accounts for ethnicity was as follows:

$$L(x|N,P_f) = \prod_{i\in\{W,B\}} \binom{n_i}{x_{iI}x_{iII}\ldots x_{iVII}} \prod_{j\in\{I,II,\ldots,VIII\}} p_{ij}^{x_{ij}} \quad (1)$$

where x was a matrix $\{x_{ij}\}$ that represented the counts of individuals having a given microbial community supergroup j associated with the ith ethnicity (i.e., that occupied cell ij of the matrix); $N=\{n_W, n_B\}$ that represented the total row counts of white and black women, respectively; and P_f was a matrix of parameters, $\{p_{ij}\}$ representing the probabilities of having a microbial community supergroup j associated with the ith ethnicity. As stated above this model assumes that ethnicity impacted the vaginal community structure. There were 16 parameters associated with this model (one parameter per each cell). Under the multinomial model, we estimated only seven parameters per row as the last parameter could be deduced from the others. The maximum likelihood estimates of these parameters were merely the proportions of the cell counts x_{ij} to the total sample size of the corresponding row n_i, (x_{ij}/n_i).

For the reduced model, we assumed that the proportion of individuals belonging to a supergroup was the same for both ethnicities. Accordingly, the likelihood of the reduced model could be written as follows:

$$L(x|N,P_r) = \prod_{i\in\{W,B\}} \binom{n_i}{x_{iI}x_{iII}\ldots x_{iVIII}} \times \prod_{j\in\{I,II,\ldots,VIII\}} p_j^{\sum_{i\in\{W,B\}} x_{ij}} \quad (2)$$

where x and N were as described above, and P_r was a vector of parameters $\{p_j\}$, regardless of ethnicity. There were eight parameters associated with this model (one per column), only seven of which need to be estimated, and the maximum likelihood estimate for each of these parameters was

$$\hat{p}_i = \sum_{i\in\{W,B\}} x_{ij} \Big/ \sum_{i\in\{W,B\}} n_i$$

Table 2 Species composition of vaginal communities in healthy Caucasian and black women

Phylotype[a]	*Supergroup*[b] *(% clones)*												
	I			*II*		*III*			*IV*	*V*	*VI*	*VII*	*VIII*
	G1	*S4*	*S7*	*G2*	*G5*	*G3*	*G10*	*G12*	*G4*	*G6*	*G7*	*G9*	*G11*
Lactobacillus iners	86.1	93.3	96.8	0.6	0.0	7.0	2.4	0.0	52.1	1.0	0.0	3.6	0.0
L. crispatus	0.6	1.1	3.2	93.3	86.8	0.2	0.0	0.0	23.8	19.0	50.5	0.0	0.0
L. jensenii	0.5	0.0	0.0	1.5	10.0	0.0	0.0	0.0	6.3	0.3	49.5	0.0	0.0
L. gasseri	0.5	0.0	0.0	0.0	2.4	6.4	0.0	0.0	4.5	77.4	0.0	0.0	0.0
L. vaginas	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0
L. coleohominis	0.2	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
L. salivarius[c]	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Actinobaculum sp.[c]	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Aerococcus sp.	1.1	2.3	0.0	0.0	0.0	0.5	2.4	1.1	0.3	0.0	0.0	0.0	0.0
Anaerobranca sp.	0.1	0.0	0.0	0.0	0.0	0.1	9.8	0.0	0.0	0.0	0.0	0.0	0.0
Anaerococcus sp.[c]	0.2	0.0	0.0	0.0	0.0	3.9	0.0	1.1	0.0	0.0	0.0	0.0	0.0
Atopobium vaginae	1.5	0.0	0.0	0.0	0.0	16.9	4.9	27.8	0.0	0.3	0.0	0.0	3.4
Clostridium sp.	0.2	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Dialister sp.	0.0	0.0	0.0	0.0	0.0	2.3	4.9	3.3	0.0	0.0	0.0	0.0	0.0
Eggerthella hongkongensis	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Enterococcus faecalis	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Finegoldia magna	0.0	1.1	0.0	0.0	0.0	0.0	0.0	2.2	0.0	0.0	0.0	0.0	0.0
Gardnerella vaginalis	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.4	0.0	0.0	0.0
Gemella palasticanis	0.3	0.0	0.0	0.0	0.0	1.1	0.0	7.8	0.0	0.0	0.0	4.8	0.0
Lachnospiraceae sp.	0.4	0.0	0.0	0.0	0.0	2.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Leptotrichia sp.	0.0	0.0	0.0	0.0	0.0	2.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Megasphaera sp.	1.1	2.2	0.0	0.0	0.0	5.5	9.8	12.2	0.0	0.0	0.0	0.0	10.3
Micromonas sp.	0.4	0.0	0.0	0.0	0.0	7.0	0.0	6.7	0.0	0.0	0.0	0.0	4.5
Mobiluncus mulieris[c]	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.3
Mycoplasma sp.[c]	0.0	0.0	0.0	0.0	0.0	0.0	2.4	0.0	0.0	0.0	0.0	0.0	0.0
Peptococcus niger[c]	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Peptoniphilus sp.	0.3	0.0	0.0	0.0	0.0	1.5	9.8	1.1	0.0	0.0	0.0	0.0	3.4
Peptostreptococcus sp.[c]	0.0	0.0	0.0	0.0	0.0	5.0	29.3	0.0	0.0	0.0	0.0	0.0	0.0
Prevotella sp.	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Pseudomonas sp.	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0
Staphylococcus sp.	0.0	0.0	0.0	2.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Streptococcus sp.	0.8	0.0	0.0	1.8	0.0	0.0	0.0	0.0	11.8	0.3	0.0	91.6	0.0
Veillonella sp.	0.2	0.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Uncultured bacterium[c,d]	3.1	0.0	0.0	0.0	0.0	1.9	0.0	0.0	0.0	0.0	0.0	0.0	72.4
Novel[e]	2.3	0.0	0.0	0.1	0.6	32.5	24.3	36.7	1.0	0.8	0.0	0.0	3.7
Total number of women (per group)	50	1	1	24	9	20	2	2	10	8	4	2	2
Number of caucasian women	24	1	1	16	7	5			9	5	4	1	0
Number of black women	26			8	2	15	2	2	1	3	0	1	2
Number of women (per supergroup)	52			33		24			10	8	4	2	2

[a]The classification of clones was carried out by comparing their 16S rRNA gene sequences to those of known organisms. The genus and species names were used if the sequence similarity to a type species was >97%; the genus name only was used if the sequence similarity was <97% but >90%; and a clone was designated as novel if the sequence similarity to known organisms was <90%.
[b]Mean relative abundances of populations in clone libraries analyzed. 'G' indicates cluster of >1 sample, 'S' designates a single sample ('singleton').
[c]Not found in Caucasian women.
[d]The uncultured bacterium is a member of the order *Clostridiales*, but was unrelated to any organism described previously.
[e]'Novel' includes various phylotypes within the phylum *Firmicutes*.

The likelihood ratio test statistic utilized in comparing the two models presented in equation (1) and equation (2) was:

$$-2\ln(\Lambda) = -2\{\ln[L(x|N, \hat{P}_r)] - \ln[L(x|N, \hat{P}_f)]\} \quad (3)$$

where x and N were as described above, and $\hat{P}_f$ and $\hat{P}_r$ were a matrix of the maximum likelihood estimates of the parameters of the full model and a vector of the maximum likelihood estimates of the parameters of the reduced model, respectively. This likelihood ratio test statistic evaluated the evidence supporting rejection of the reduced model (Equation 2) in favor of the full model (Equation 1), highlighting the importance of ethnicity (if any) in explaining the variation in the count data. To establish the strength of evidence based on this test

statistic, we calculated a *P*-value using the bootstrap (Efron and Tibshirani, 1997).

Should the reduced model be rejected in favor of the full model we could conclude that there was an effect of ethnicity, and we could proceed to identify those groups that differed most and hence contributed most to the rejection of the reduced model. To accomplish this, we used a standard proportion, pair-wise comparison procedure (Moore and McCabe, 1998).

Results

Classification of vaginal microbial communities

Differences between vaginal microbial communities in Caucasian and black women from North America were identified by analyzing profiles of T-RFLPs of 16S rRNA genes. The data were subjected to cluster analysis to identify similar communities, and the number of clusters (kinds of communities) was independently assessed using three different statistical algorithms as described by Abdo *et al*. (2006). Among the 144 women sampled, there were 12 kinds of bacterial communities with two or more women, and eight that were represented by a single individual ('singletons'; Figure 1). The community types that included two or more women account for all those that occur in the populations sampled at a frequency of 0.05 ($P=0.99$). Thus, we have accounted for those vaginal communities that are commonly found in Caucasian and black women.

To identify the numerically abundant bacterial populations in each kind of community, 16S rRNA gene libraries were constructed from samples that represented 85% of the diversity in each cluster, and from all 'singletons'. The composition of each sample was determined by phylogenetic analysis of partial 16S rRNA gene sequences (Table 2). In total, 57 clone libraries were analyzed, and approximately 6000 clones ($\sim$90 from each library) were sequenced. Assuming the bacterial numbers in the original samples were on the order of 10^8 cells per ml of vaginal secretion, then comparatively rare populations present at less than $\sim 10^6$/ml would not have been sampled and are not reflected in data from the analysis of clone libraries. From these data, it was apparent that the composition of communities of singletons S4 and S7 closely resembled those in group 1 so these were combined to form supergroup I. Likewise, the composition of communities in other groups that were similar to one another were combined to make supergroups II and III (Table 2). The composition of the community in sample W27 differed from others in group 4 by having a high proportion of *Veillonella* and was therefore removed from the group.

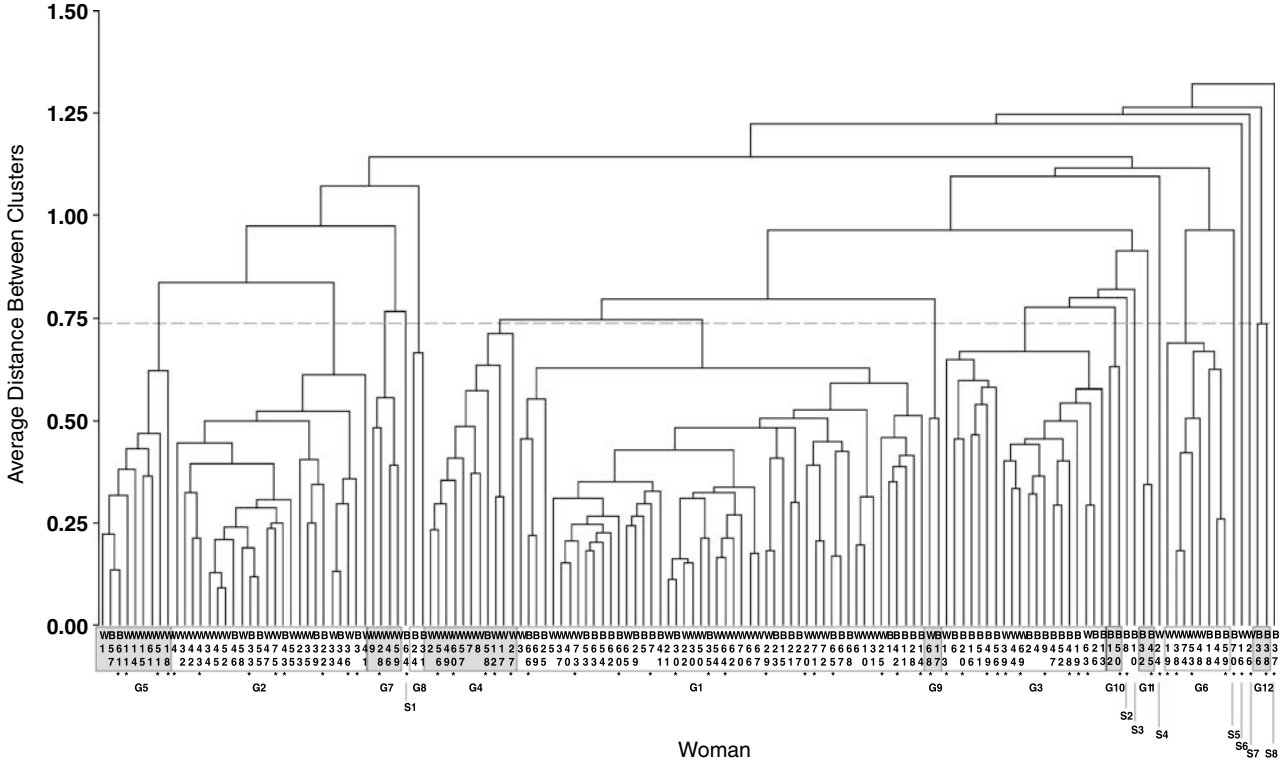

Figure 1 Clustering of vaginal microbial communities in Caucasian and black women based on data from T-RFLP analysis of 16S rRNA genes. Groups (clusters) with $\geqslant 2$ women designated with a 'G' followed by a number. Clusters consisting of a single sample (singletons) are designated with an 'S' followed by a number. The samples from each group that were used to construct 16S rRNA gene clone libraries are indicated by asterisks.

After condensing the community types into supergroups, we found there were eight supergroups with two or more women, and these accounted for 94% of the women sampled.

Composition of vaginal communities

Most vaginal communities (five of eight supergroups) were dominated by organisms that were phylogenetically related to *L. iners, L. crispatus*, *L. jensenii* and *L. gasseri* (Table 2). These accounted for 80% of the women sampled. Overall, *L. iners* was the most common species of *Lactobacillus* in women of both races; it was recovered in 66% of the women sampled (95/144), and was the most abundant species in communities of supergroup I, which included 36% (52/144) of the women sampled. Combinations of two or three species of lactobacilli, whose abundances were roughly equal, dominated vaginal communities in supergroups IV, V and VI, and these accounted for 15% of the women sampled. In contrast, communities of supergroup III had low numbers of lactobacilli, and exhibited greater species evenness including high numbers of clones most closely related to *Atopobium* and genera of the order *Clostridiales* including *Megasphaera, Dialister, Anaerococcus*, *Finegoldia, Peptostreptococcus* and *Eubacterium* (Figure 2). In addition, 20–30% of the clones from these communities were from miscellaneous novel clades in the phylum *Firmicutes*. Communities in supergroup VII were dominated by populations most closely related to *Streptococcus* sp., while those of supergroup VIII had high numbers of clones from a single clade of the family *Lachnospiraceae* that were unrelated to any named bacterium, but closely related (>97% 16S rRNA gene sequence similarity) to an uncultured bacterium (Figure 2).

Differences between racial groups

The kinds of microbial communities found in the vaginas of black women differed significantly from those of Caucasian women (Table 2; Figure 3). The incidence of vaginal communities dominated by various species of *Lactobacillus* (supergroups I, II, IV, V and VI) was less in black women (68%) than in Caucasian women (91%). Conversely, the incidence of communities with a high proportion of phylotypes related to strictly anaerobic bacteria was considerably higher in black women (32%, supergroups III, VII and VIII) than in Caucasian women (8%, supergroups III and VII). Particularly striking was the fact that supergroup III, which includes communities dominated by *Atopobium* sp. and other strict anaerobes, was four times more common in black women than in Caucasian women. Second, communities dominated by roughly equal numbers of more than one species of *Lactobacillus* (supergroups IV, V and VI) were found in 25% of Caucasian women but only 6% of black women.

Visual inspection of the rank-abundance distributions (Figure 3) showed that three of the eight supergroups (II, IV and VI) were more common among Caucasian women, while two supergroups (III and VIII) were more common among black women (Figure 3). To determine whether these differences were statistically significant, we used a method proposed by Schütte *et al*. (in preparation). The data were summarized in a contingency table wherein individuals were classified based on two factors: community structure and ethnicity (Table 3). Our contention was that if the relative abundances of the microbial communities differed between the two ethnic groups there would be at least one supergroup that differed between them. To test this for all the microbial community supergroups, we assumed that the counts of individuals belonging to each supergroup, within each ethnicity, follow a multinomial distribution that was independent from that of the other ethnicity. Comparison of the full multinomial model that accounted for the effect of ethnicity (Equation 1) to the reduced model that did not (Equation 2) on the basis of likelihood ratios resulted in rejecting the reduced model in favor of the full model. The resulting bootstrap *P*-value was zero (corresponding to a log-likelihood ratio test statistic equal to 29.26). Accordingly, we concluded that there was a highly significant effect of ethnicity on the frequency of community types in women of the two ethnic groups. Pair-wise comparisons were conducted to identify which of the supergroups accounted for most of the difference attributed to ethnicity (Table 4). The results showed that supergroup III contributed most to the difference between the two ethnicities ($P=0.0001$), while supergroup IV was also important ($P=0.0051$). Supergroups II, VI and VIII with *P*-values of 0.0162, 0.0051 and 0.0749, respectively, had moderate importance in explaining the observed differences in the frequency of community types in women of the two ethnic groups. It is worth noting that the proportion of women in supergroups I, V and VII showed no significant differences between the two ethnic groups.

Diversity of numerically abundant Lactobacillus

The phylogenetic relationships of the *Lactobacillus* strains were determined by comparing the 16S rRNA gene sequences from this study to those of reference strains sequenced previously. Most lactobacilli found in the vaginal communities were phylogenetically related to *L. iners*, *L. crispatus*, *L. jensenii* and *L. gasseri* (Figure 4), and were likely to be homofermentative. In contrast, *L. vaginalis* and *L. colehominis* were phylogenetically distinct and related to heterofermentative species (Pavlova *et al.*, 2002). The sequence heterogeneity among clones of *L. crispatus* and *L. jensenii* was greater than that of *L. iners* and *L. gasseri*, suggesting there are evolutionarily divergent subpopulations of *L. crispatus*

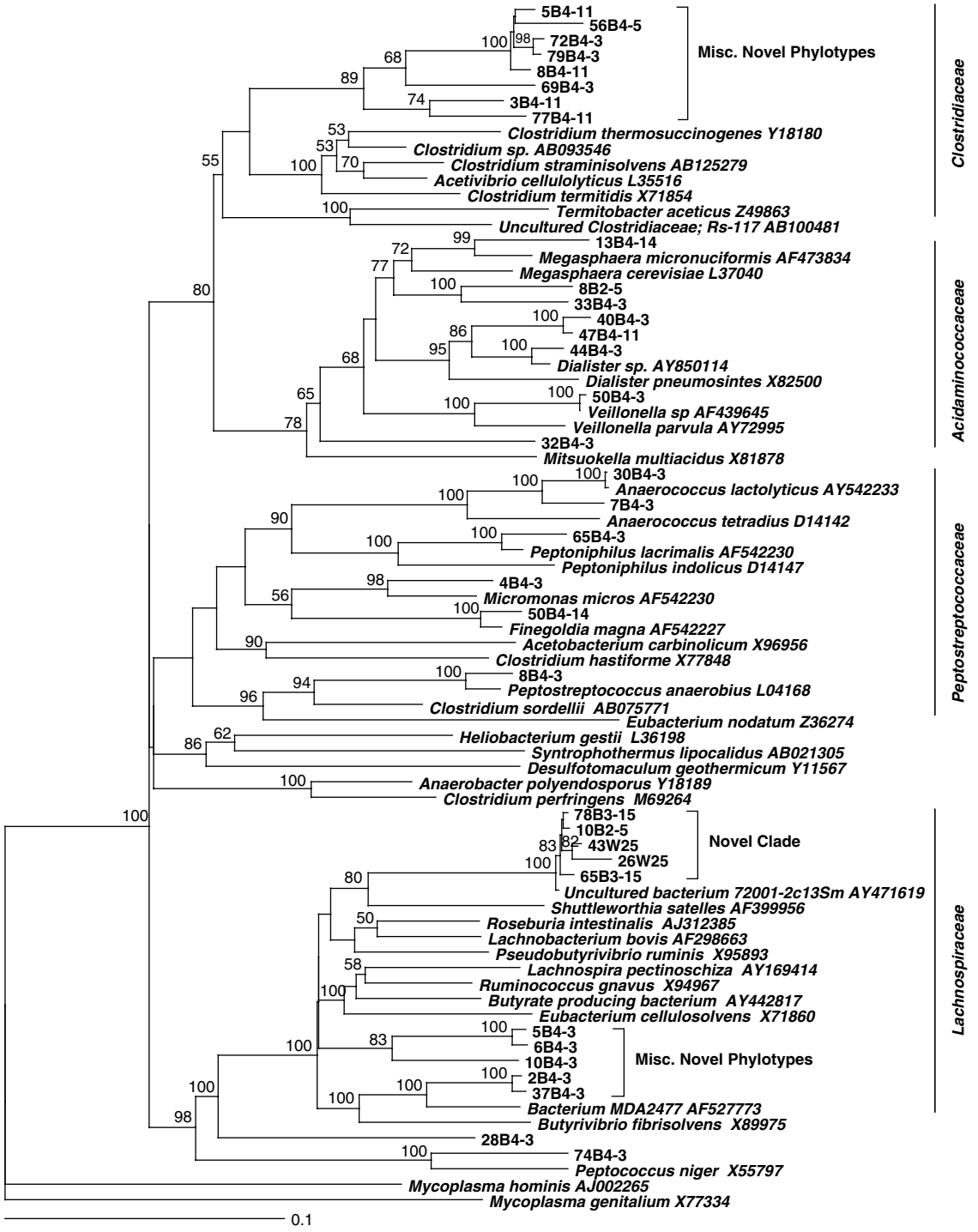

Figure 2 A phylogenetic tree showing the relationship of selected phylotypes from vaginal communities of Caucasian and black women to type strains from the order *Clostridiales*. The phylogenetic tree was constructed using a neighbor-joining algorithm, with *Mycoplasma* serving as an out-group. Phylotypes in bold font were from this study while phylotypes in italics were from GenBank. Bootstrap values (from 500 replicates) greater than 50% are shown at the branch points, and the bar indicates 10% sequence divergence.

and *L. jensenii* in vaginal communities. Most phylotypes of *L. crispatus* (~90%) were most closely related to *L. crispatus* DSM 20584T (GenBank sequence LCR17362), which is a type strain from Deutsche Sammlung von Mikroorganismen und Zellkulturen. The remainder were most closely related to *L. crispatus* NCTC4 (GenBank sequence AJ242969) from the National Collection of Type Cultures (London, UK). Thus, there appears to be two distinct lineages that have been classified as *L. crispatus*, each of which is found in the human vagina. In contrast to *L. crispatus* and *L. jensenii*, the

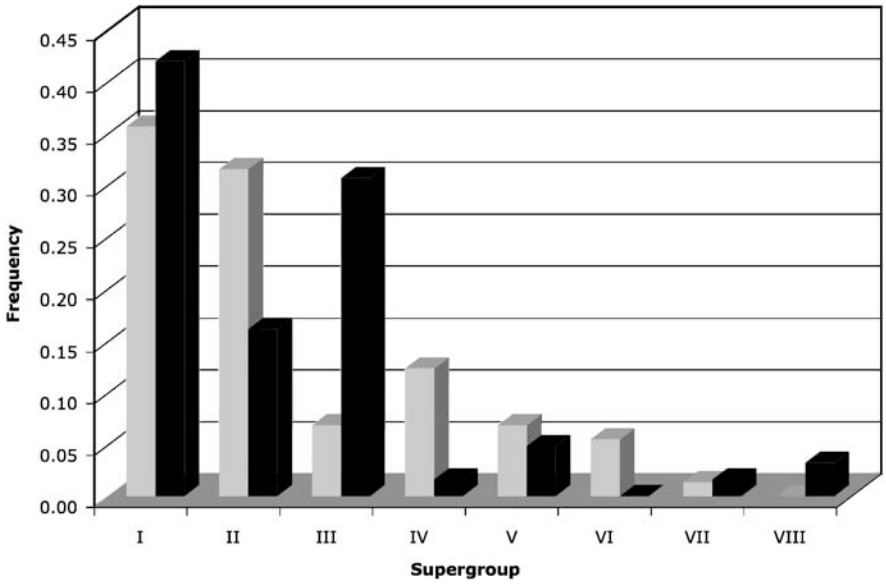

Figure 3 The rank abundances of vaginal community supergroups found in Caucasian and black women. The proportions of Caucasian women are shown as gray bars, and the proportions of black women are shown as black bars.

Table 3 Contingency table of Caucasian and black women organized in supergroups based on the similarity in the microbial community structures

	Supergroups								*Total*
	I	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*	
Caucasian (W)	26	23	5	9	5	4	1	0	73
Black (B)	26	10	19	1	3	0	1	2	62
Supergroup totals	52	33	24	10	8	4	2	2	135

Table 4 Pair-wise comparison between proportions of Caucasian women and black women belonging to the same supergroups to identify those groups that contributed most to rejecting the reduced multinomial test

	Supergroups								*Total*
	I	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*	
Caucasian (W)	0.36	0.32	0.07	0.12	0.07	0.05	0.01	0.00	1.00
Black (B)	0.42	0.16	0.31	0.02	0.05	0.00	0.02	0.03	1.00
z-score	0.75	2.14	3.62	2.57	0.50	2.06	0.12	1.44	
P-value	0.2266	0.0162	0.0001	0.0051	0.3085	0.0197	0.4522	0.0749	

clones of *L. iners* were highly related to one another and to a single reference strain, *Lactobacillus* sp. LSPY 16329 (data not shown). The occurrence of a virtually clonal lineage of *L. iners* in different women suggests there might be strong selection for specific phenotypic characteristics that are found in few strains.

Discussion

In this study, we found that discrete differences exist in the composition of vaginal microbial communities of Caucasian and black women in North America. In total, there were eight distinct types of communities (supergroups) that differed in terms of the kinds and relative abundances of bacterial species, and these accounted for all those that occur at a frequency of 0.05 ($P>0.99$) in the general populace. Importantly, there were also highly significant differences in the rank abundance of these communities among women in the two racial groups. We hypothesize that these differences in the structure and composition of microbial communities may underlie well-known differences in the susceptibility of women in these racial groups

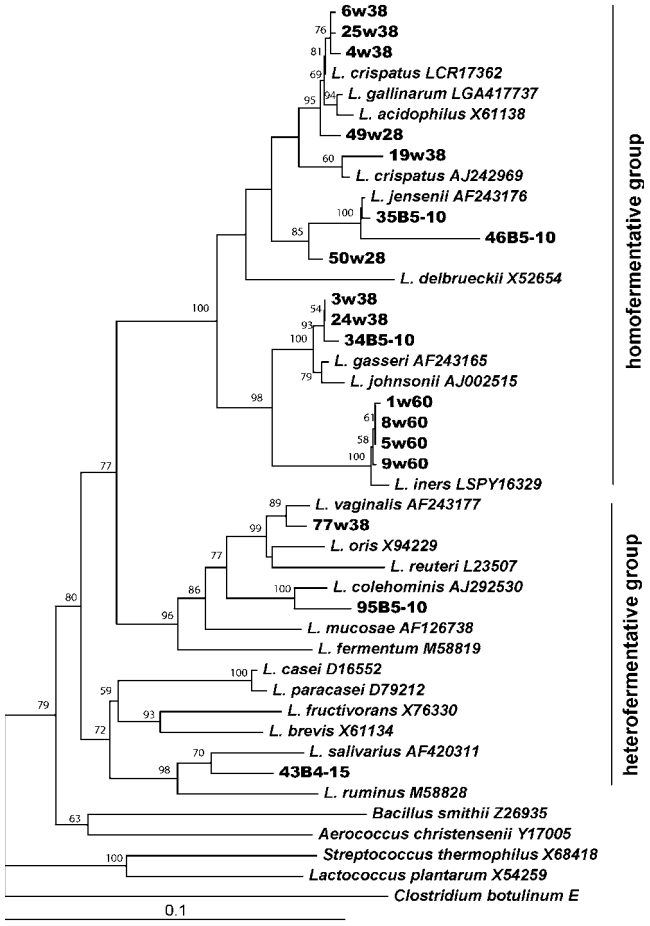

Figure 4 A phylogenetic tree based on 16S rRNA gene sequences that shows the relationship of phylotypes recovered in samples of vaginal microbial communities to organisms closely related to *Lactobacillus* species. *Clostridium botulinum* E served as an out-group. The phylogenetic tree was constructed using a neighbor-joining algorithm, with *Mycoplasma* serving as an out-group. Phylotypes in bold font were from this study while phylotypes in italics were from GenBank. Bootstrap values (from 500 replicates) greater than 50% are shown at the branch points, and the bar indicates 10% sequence divergence.

to BV and various vaginal infections (Rajamanoharan *et al.*, 1999; Royce *et al.*, 1999; Whitmore *et al.*, 2005). We specifically postulate that these differences in vaginal communities are accompanied by differences in community resilience, as defined by its ability to resist or absorb perturbations and return to a stable equilibrium state (Peterson *et al.*, 1998). It is largely determined by the ability of indigenous species to adapt to stresses and disturbances, as well as the existence and nature of ecological interactions among species. This notion is consistent with ecological theory and empirical data that indicate not all communities are equally resilient. Thus, if the resilience of a vaginal community is low then transitory changes in the structure of these communities may occur more readily in response to disturbances of various kinds, including menses, sexual intercourse, douching and contraceptive practices. These changes in community structure may include shifts in population densities or species loss, and concomitant changes in community function may occur. These disturbed communities may be more susceptible to invasion by species that are not indigenous to the human vagina including transient species of fecal origin and opportunistic pathogens including *Candida*. Moreover, we speculate that the disturbed state may itself constitute the clinical syndrome of BV.

There is no information available on the relative resilience of different vaginal microbial communities. However, since the prevalence of BV in black women is higher than in Caucasian women, it would make sense to focus attention on those community types that more commonly occur in black women, such as supergroups III and VIII because they may have lower resilience. The dominant members of these supergroups were *Atopobium* and various genera in the order *Clostridiales*. A second hypothesis is that vaginal communities more commonly found in Caucasian women may be more resilient, and therefore more resistant to enduring ecological upsets or invasion by nonindigenous organisms. Such communities are exemplified by supergroups II, IV and VI. Of these, supergroups IV and VI were characterized by the presence of roughly equal numbers of more than one species of *Lactobacillus*, which may confer increased stability owing to functional redundancy.

It is generally accepted that maintenance of a low pH in the vagina is important for precluding pathogenic organisms, and this function is most often attributed to lactic acid produced by species of *Lactobacillus*, which are commonly found in the vaginas of healthy women. Consistent with this, we found that lactobacilli constituted $\geqslant$10% of vaginal communities in 79% (107/136) of women. However, *Atopobium vaginae* also metabolizes glucose via homolactic fermentation (Rodriguez Jovita *et al.*, 1999), and phylotypes related to this species were important constituents of communities in supergroup III. Thus, if one includes members of this genus in the tally, the vaginal communities of all Caucasian women, and all but two black women had high numbers of lactic acid bacteria. The vaginal communities of these two black women were dominated by a novel phylotype that is only distantly related to known genera so there is no basis for speculation about its physiology. Nonetheless, it is clear that the ecological function of vaginal bacterial communities – the formation of lactic acid and maintenance of a low pH environment – was highly conserved among women of both racial groups despite the variations seen in community composition. While this has not been rigorously demonstrated for the human vagina before now, the conservation of function by communities that vary in composition is a common theme in microbial ecology that has been observed in numerous other habitats. Moreover, we found it interesting that only four species of *Lactobacillus* were numerically

important in the communities of women in both racial groups: *L. iners, L. crispatus, L. gasseri* and *L. jensenii.* Our results confirm those of Pavlova *et al.* (2002), who also found two lineages of *L. crispatus* in the human vagina, raising the specter that one of the two reference strains has been misclassified, or that there is sequence heterogeneity among the 16S rRNA genes in strains of *L. crispatus.* Nonetheless, the limited number of *Lactobacillus* phylotypes found in human vagina is somewhat surprising, which leads to the possibility that there are host factors that select for specific organisms, that these species have unusual characteristics that allow them to successfully colonize the vagina, or both.

The strong linkage between high numbers of lactic acid bacteria and a normal microbial community is consistent with Walker's 'drivers and passengers' hypothesis of community structure (Walker, 1992). This hypothesis posits that ecosystem function is largely determined by individual 'driver' species or guilds (functional groups) of such species and that other species in the community are 'passengers' that have minor ecological impact. Driver species strongly influence the species composition and structure of the biological communities in which they and passenger species exist. Under this driver–passenger model, lactic acid bacteria would be considered drivers because they strongly influence the ecosystem by maintaining a low pH through lactic acid production, whereas the other species present would be less influential and constitute passengers with little influence on the ecology of the system. The consideration of such models may prove useful to studies of vaginal microbiology since they provide a framework for understanding the ecology of the vagina, and the possible causes and prevention of ecological upsets such as BV.

Other recent studies also documented the occurrence of *Atopobium* in vaginal communities of reproductive age and postmenopausal women. For example, Ferris and Masztal (2004) reported that *A. vaginae* was present in a significant proportion (55%) of women diagnosed as having BV, but in only two of 24 women that had normal vaginal communities. Likewise, Burton *et al.* (2004) screened 35 postmenopausal women for the occurrence of BV and found *A. vaginae* in 44% of women with (asymptomatic) BV, while the organism was absent from subjects deemed healthy. Verhelst *et al.* (2004) and Fredricks *et al.* (2005) discovered that *A. vaginae* was frequently detected in subjects with BV. Based on these findings, investigators have implicated *A. vaginae* in BV. Our results, however, suggest that *Atopobium* is a commonly encountered constituent of vaginal communities in healthy women. In all of the studies cited above (except ours), the diagnosis of BV was based on the Nugent criteria in which a vaginal smear is Gram-stained and examined to determine the relative abundance of *Lactobacillus* morphotypes (large Gram-positive rods), and examined for the occurrence and number of lactobacilli that adhere to vaginal epithelial cells. Low numbers or the absence of lactobacilli is the criterion used for the diagnosis of BV. Although *Atopobium* is a lactic acid bacterium, its cellular morphology is distinctly different from that of *Lactobacillus (*Rodriguez Jovita *et al.*, 1999). Thus, it seems entirely plausible to us that the women whose communities are dominated by *Atopobium* and lack appreciable numbers of lactobacilli may be misdiagnosed as having BV. This may partly explain the high incidence of so-called asymptomatic BV that has been reported by many investigators (Schwebke, 2000; Yen *et al.*, 2003). Indeed, given the occurrence in the vagina of lactic acid bacteria that are not species of *Lactobacillus*, it could be that some patients with reoccurring BV may simply be reflecting their normal vaginal bacterial flora, which are mischaracterized as abnormal. This postulate should be rigorously tested in future studies.

The data obtained in this study showed that a fair proportion of healthy Caucasian and black women also host several fastidious, strictly anaerobic microorganisms that belong to the order *Clostridiales*. These include appreciable numbers of *Lachnospiraceae*, *Megasphaera*, *Dialister*, *Peptoniphilus*, *Peptostreptococcus* and *Anaerococcus,* as well as many novel bacteria. These populations probably may not have been recovered from samples by using the cultivation methods commonly employed in previous studies of vaginal flora, or routinely used in clinical microbiology laboratories. Given that they appear to constitute more than 5% of bacterial communities in 8% of Caucasian and 32% of black women, efforts are needed to isolate and characterize these organisms to obtain clues to their function in vaginal communities. Importantly, the common occurrence of species classified in the *Clostridiales* may also have important implications for the clinical diagnosis of BV. Members of this order are notorious for the production of malodorous compounds that include organic acids, amines and thiols through the metabolism of glucose, other carbohydrates and amino acids (Madigan *et al.*, 1997). Complaints of vaginal odor in the absence of infection are not uncommon. For example, Landers *et al.* (2004) studied 598 women with genital complaints and found that 23% women reported vaginal odor even though there were no outward signs of disease. Till now, the occurrence of odor has been considered abnormal. However, the high number of *Clostridiales* in many vaginal communities suggests that some degree of odor may be normal for some women, although it could lead to false positives in the diagnosis of BV. Indeed, the presence of amines in vaginal secretions is one of the four criteria used for the diagnosis of BV based on the recommendations on Amsel *et al.* (1983) that are commonly used in clinical settings. This also suggests that new diagnostic tests for BV that are based on amine production and odor formation

should be used with caution (Sonnex, 1995; Hay *et al.*, 2003).

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