Microbiology (2004), **150**, 2565–2573 DOI 10.1099/mic.0.26905-0

Characterization of vaginal microbial communities in adult healthy women using cultivation-independent methods

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Received 12 November 2003
Revised 5 March 2004
Accepted 10 May 2004

The normal microbial flora of the vagina plays an important role in preventing genital and urinary tract infections in women. Thus an accurate understanding of the composition and ecology of the ecosystem is important to understanding the aetiology of these diseases. Common wisdom is that lactobacilli dominate the normal vaginal microflora of post-pubertal women. However, this conclusion is based on methods that require cultivation of microbial populations; an approach that is known to yield a biased and incomplete assessment of microbial community structure. In this study cultivation-independent methods were used to analyse samples collected from the mid-vagina of five normal healthy Caucasian women between the ages of 28 and 44. Total microbial community DNA was isolated following resuspension of microbial cells from vaginal swabs. To identify the constituent numerically dominant populations in each community 16S rRNA gene libraries were prepared following PCR amplification using the 8f and 926r primers. From each library, the DNA sequences of approximately 200 16S rRNA clones were determined and subjected to phylogenetic analyses. The diversity and kinds of organisms that comprise the vaginal microbial community varied among women. Species of *Lactobacillus* appeared to dominate the communities in four of the five women. However, the community of one woman was dominated by *Atopobium* sp., whereas a second woman had appreciable numbers of *Megasphaera* sp., *Atopobium* sp. and *Leptotrichia* sp., none of which have previously been shown to be common members of the vaginal ecosystem. Of the women whose communities were dominated by lactobacilli, there were two distinct clusters, each of which consisted of a single species. One class consisted of two women with genetically divergent clones that were related to *Lactobacillus crispatus*, whereas the second group of two women had clones of *Lactobacillus iners* that were highly related to a single phylotype. These surprising results suggest that culture-independent methods can provide new insights into the diversity of bacterial species found in the human vagina, and this information could prove to be pivotal in understanding risk factors for various infectious diseases.

INTRODUCTION

Previous studies on the microbial flora of the human vagina indicate that micro-organisms normally present in the human vagina play a key role in preventing successful colonization by 'undesirable' organisms, including those responsible for bacterial vaginosis, yeast infections, sexually transmitted diseases and urinary tract infections (Donders *et al.*, 2000; Gupta *et al.*, 1998; Sobel, 1999; van De Wijgert *et al.*, 2000). Moreover, epidemiologic studies have clearly established that abnormal vaginal microbial communities and lower genital tract infections are significantly associated with an increased risk of HIV infection (Cohen *et al.*, 1995; Martin *et al.*, 1999; Sewankambo *et al.*, 1997). Based on this, researchers have speculated that the normal vaginal microbial ecosystem may play a pivotal role in lowering the probability of heterosexual HIV transmission (Hillier, 1998; Schwebke, 2001). Clearly, an accurate understanding of the composition and ecology of the vaginal microbial ecosystem in normal healthy women is essential to understanding how the normal flora reduces the risk of acquiring these communicable diseases.

The GenBank accession numbers for the sequences reported in this paper are AY269020–AY269034 (*Atopobium vaginae*), AY271931–AY271953 (*Megasphaera* sp.), AY267541–AY267542 (*Leptotrichia* sp.), AY283264–AY283275 (*Lactobacillus iners*) and AY335493–AY335504 (*Lactobacillus crispatus*). The remaining sequences were assigned AY344130–AY344131.

The vagina and its unique microflora form a finely balanced ecosystem, with the vaginal environment controlling the microbial types present and the microflora in turn controlling the vaginal environment (Pybus & Onderdonk, 1999). This ecosystem is dynamic with changes in structure and composition being influenced by age, menarche, time in the menstrual cycle, pregnancy, infections, methods of birth control, frequency of sex, number of sexual partners, as well as various habits and practices such as douching (Burton & Reid, 2002; Clarke *et al.*, 2002; Eschenbach *et al.*, 2000; Ness *et al.*, 2002; Schwebke *et al.*, 1999) and sexual behaviours (Schwebke *et al.*, 1999). In the past 100 years since the first microbiological study of the human vagina (Döderlein, 1892), lactobacilli have been thought to be the predominant members of normal postpubertal vaginal microflora (Antonio *et al.*, 1999). A diverse array of other bacteria such as *Staphylococcus*, *Ureaplasma*, *Corynebacterium*, *Streptococcus*, *Peptostreptococcus*, *Gardnerella*, *Bacteroides*, *Mycoplasma*, *Enterococcus*, *Escherichia*, *Veillonella*, *Bifidobacterium* and *Candida* (Larsen & Monif, 2001; Marrazzo *et al.*, 2002; Redondo-Lopez *et al.*, 1990) can be present but in much lower numbers. It has been postulated that lactobacilli play a critical role in maintaining the normal vaginal ecosystem by preventing overgrowth by pathogens and other opportunistic organisms by producing lactic acid, hydrogen peroxide (H_2O_2), bacteriocins and other antimicrobial substances (Hillier, 1998). Given this, it is not surprising that various efforts are being made to promote the maintenance of normal flora (Hughes & Hillier, 1990; McLean & Rosenstein, 2000; Reid & Burton, 2002). Unfortunately, these have not proven to be very successful (Nyirjesy *et al.*, 1997). This could be because about 10–42 % of women whose vaginal microbial communities lack appreciable numbers of lactobacilli apparently maintain 'normal' vaginal ecosystems (Hillier, 1998, 1999; Larsen & Monif, 2001; Marrazzo *et al.*, 2002; Redondo-Lopez *et al.*, 1990). Obviously microbial populations other than lactobacilli are dominant in a rather large proportion of normal vagina microbial communities, and alone or in some combination work to suppress the growth of pathogens. However, the identity and diversity of these populations remain largely obscure and the complex interactions of the various members of the vaginal flora are still poorly understood.

Prior efforts to characterize microbial populations found in the vagina have largely employed methods commonly used in clinical microbiology laboratories that involved plating of samples on selective media, semi-quantitative estimates of their abundance and classification based on phenetic criteria into broad taxonomic groups. While these studies have provided insight into the composition of these communities, they suffer from incompleteness, often lack statistical rigour and do not provide sufficiently detailed information. Studies on many habitats have demonstrated the limitations of cultivation-dependent methods to assess microbial community composition. In most instances, this is because readily cultivated populations represent a small fraction of the extant community (McCaig *et al.*, 1999). In recent years, culture-independent methods based on the analysis of 16S and 18S rRNA gene sequences have been used to overcome many of these limitations (Ward *et al.*, 1998). These molecular techniques provide the most powerful tools currently available to reveal the phylogenetic diversity of micro-organisms found within complex ecosystems and are widely employed to explore microbial diversity and understand community dynamics. These studies have often included construction and analysis of 16S rRNA gene clone libraries to provide precise information as to the phylogeny of the constituent populations. In addition to being widely used for studies on the ecology of terrestrial and aquatic habitats (Dunbar, 1999; Eilers *et al.*, 2000; McCaig *et al.*, 1999), they are increasingly being used to study human and animal flora, including that of the colon and subgingival crevice (Burton & Reid, 2002; Hold *et al.*, 2002; Kazor *et al.*, 2003; Kroes *et al.*, 1999; Paster *et al.*, 2001; Suau *et al.*, 1999).

The aim of this study was to characterize the structure of microbial communities found in five normal, healthy women of reproductive age using culture-independent methods. 16S rRNA gene libraries were prepared from total community DNA and phylogenetic analyses of 16S rRNA gene sequences were done. To our knowledge, this is the first report describing the use these approaches to characterize the composition and diversity of normal vaginal communities. The results showed that heretofore unknown populations are abundant in certain women, that *Lactobacillus iners* may be more common than previously thought and that the within-species diversity of lactobacilli in the vagina can vary significantly between individuals.

METHODS

Sample collection and genomic DNA extraction. Mid-vaginal swabs from five premenopausal, non-pregnant, white women between the ages of 28 and 44 years were obtained. The study protocol was approved by the company's Internal Review Board and included a number of inclusion and exclusion criteria. Annually, a gynaecologist examined the women, and at each visit they were asked to complete a questionnaire that included several questions pertaining to their health, and were examined by a nurse. At the annual visit, a gynaecologist performed thorough physical examinations of each woman to detect abnormal conditions. This included examining the external genitalia, vulva, vagina, cervix, uterus and adnexae. Any abnormalities, including inflammation, pain or pressure, discharge, swelling or ulceration would be reported and result in disqualification from the study. In addition, at each visit, the women were asked to self-report vaginal symptoms, including vaginal discharge or dryness, itching, burning sensations and non-menstrual bleeding, as well as any open sores, rashes or lesions on genitalia or the inner thigh. While there are clinical means to diagnose bacterial vaginosis, including Amsel criteria and the Nugent test, these are none more reliable than self-reporting of vaginal discharge or vaginal odour (Yen *et al.*, 2003). Based on these clinical examinations and the lack of self-reported abnormalities, it was concluded that the women were apparently healthy.

The bacterial cells retrieved on swabs were resuspended in 3 ml liquid dental transport medium (LDTM; Anaerobe Systems) and stored either on dry ice or at −80 °C until the samples were thawed and analysed. Genomic DNA was isolated from 0·5 ml aliquots of the cell suspensions using a two-step cell lysis procedure. First, bacterial cell walls were disrupted enzymically by the addition of mutanolysin (50 μg) and lysozyme (500 μg) followed by incubation for 1 h at 37 °C. Second, the cells were mechanically disrupted by six freeze–thaw cycles. Each cycle consisted of 2 min incubation at 100 °C that was immediately followed by 2 min in liquid nitrogen. Between each freeze–thaw cycle, the cell suspensions were incubated for 1 min in an ultrasonic bath. Proteins in the disrupted cell suspension were digested with proteinase K (Qiagen) during a 1 h incubation at 55 °C. Further isolation and purification of total DNA extract were performed using the Wizard DNA purification kit (Promega).

PCR amplification. Universal bacterial primers 8f and 926r (based on *Escherichia coli* positions) were used to amplify internal fragments of 16S rRNA genes in the genomic DNA obtained from samples. Amplification was performed in 100 μl (total volume) reaction mixtures that contained 100 ng (1 μl) vaginal sample DNA, 2 U AmpliTaq DNA polymerase (Roche), 1× AmpliTaq reaction buffer, 3 mM $MgCl_2$, 200 μM each deoxyribonucleotide triphosphate, 5 % DMSO and 0·1 μM each primer. Initial DNA denaturation was performed at 94 °C in a PTC-100 programmable thermal controller (MJ Research) for 5 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and elongation at 72 °C for 2 min, which was followed by a final extension at 72 °C for 10 min. To confirm amplicon production, the mixture was analysed by electrophoresis in 1·5 % agarose gel followed by staining with ethidium bromide and visualization under ultraviolet light.

16S rRNA gene clone library construction. 16S rDNA genes were cloned into pCR2.1-TOPO (Invitrogen) using a vector/insert ratio of 1 : 1 and procedures recommended by the manufacturer. Ligation mixtures were used to transform *E. coli* TOP 10 cells (Invitrogen) that were subsequently plated onto Luria–Bertani agar plates containing 100 μg kanamycin ml^{-1} and incubated overnight at 37 °C. Approximately 200 white, well-isolated colonies were randomly selected from each of library and grown in 200 μl Luria–Bertani broth containing 100 μg kanamycin ml^{-1} in 96-well microtitre plates for 24–48 h. These cultures were reinoculated into Hogness buffer containing 3 % glycerol and cultured overnight. The cells from 800 μl of culture were harvested and the plasmid DNAs were isolated using QIAprep96 Turbo Miniprep Kits (Qiagen) using standard operation procedures by a Qiagen BioRobot 3000 workstation. The remainder of the cultures was stored at −80 °C.

Sequencing and sequence analysis. Approximately 1200 isolated plasmids with cloned inserts (approx. 920 bp length for all libraries) were sequenced with both M13R and M13F primers. The sequences of the inserts were determined using Big Dye version 3 cycle sequencing reactions (Applied Biosystems) and resolved on an automatic sequencer (3100 PRISM Genetic Analyser; Applied Biosystems). Sequences were edited to exclude the PCR primer binding sites and manually corrected with Chromas 2 (Chromas Version 2.22; www.technelysium.com.au/chromas.html). For identification of closest relatives, newly determined sequences were compared to those available in the Ribosomal Database Project (RDP) (Maidak *et al.*, 2001) and GenBank (www.ncbi.nlm.nih.gov) databases using the standard nucleotide–nucleotide BLAST program (BLASTN; www.ncbi.nlm.nih.gov) to ascertain their closest relatives.

Phylogenetic analysis. Sequence data were edited and combined with ContigExpress from InforMax Vector NTI Suite 8 (www.informaxinc.com). The sequence data for reference strains were obtained from the GenBank and RDP databases (Maidak *et al.*, 2001). Similar sequences were aligned by using the CLUSTAL X program (version 1.81; www-igbmc.u-strasbg.fr/BioInfo/ClustalX/Top.html/) and ALIGNX from the InforMax Vector NTI Suite 8 (www.informaxinc.com). These alignments were manually adjusted to reduce errors before the sequences were used further. Phylogenetic trees were reconstructed using neighbour-joining/minimum evolution, maximum-parsimony and maximum-likelihood algorithms using the PAUP program. TREEVIEW 1.6.6 (Win32) (http://taxonomy.zoology.gla.ac.uk/rod/rod.html), a software package for the Microsoft Windows environment, was used to graphically represent the phylogenetic trees. The trees calculated with these three different algorithms were almost identical in topology. Only representative sequences and sequences that were at least 90 % complete were used for tree construction. Bootstrap analyses for 500 resamplings were performed to provide confidence estimates for tree topologies. Alignment positions at which less than 50 % of sequences of the entire set of data had the same residues were excluded from the calculations to prevent uncertain alignment within highly variable positions of 16S rRNA genes to avoid errors in tree topology.

RESULTS

Structure of vaginal microbial communities

To evaluate the relative abundances of microbial populations in normal vaginal microbial communities, the 16S rRNA genes were amplified from samples taken from five women using universal bacterial primers, and the sequences of ~1200 clones were determined and compared to reference sequence data in the RDP and GenBank databases. Sequence similarity searches were used to assign each clone to major bacterial phylotypes. The distributions of phylotypes in each library are listed in Table 1. The number of phylotypes found in each healthy woman ranged from 2 to 7. Most clones were closely related to *L. crispatus*, *L. iners* and *Atopobium vaginae*, while *Megasphaera* sp., *Leptotrichia* sp. and other phylotypes were less common. It should be noted that universal bacterial primers were used in this study; hence only numerically abundant populations (>1 % of the communities) were represented in the libraries, and less abundant, yet possibly ecologically important populations remain unknown. Clones related to *Lactobacillus* species previously isolated and characterized using culture-dependent methods were recovered from all five of the clone libraries. However, this is the first instance where *A. vaginae*, *Megasphaera* sp. and *Leptotrichia* sp. have been found to be members of the vaginal normal flora.

The predominant microbial populations in the vagina differed among women (Table 1). Species of *Lactobacillus* constituted 70·8–100·0 % of the clones sequenced in four of the five women. Clones with 96·8–99·0 % sequence similarity to *L. iners* were numerically dominant in the vaginas of W-4 and W-5, and comprised 98·8 and 70·8 % of the clones analysed, respectively. In contrast, libraries prepared from vaginal samples from W-2 and W-3 yielded clones with 96·4–99·0 % 16S rRNA sequence similarity to *L. crispatus*. These constituted 98·3 % of the clones in W-2 and 100·0 % of the clones from W-3. No clones with similarity to *L. crispatus* were recovered from W-1, W-4

Table 1. Abundance of phylotypes in 16S rRNA gene clone libraries of normal vaginal microbial communities and percentage similarity to reference sequences

Phylotype*	Clones (%)†					Sequence identity (%)
	W-1 ($n=190$)	W-2 ($n=181$)	W-3 ($n=182$)	W-4 ($n=176$)	W-5 ($n=250$)	
Lactobacillus crispatus	0·0	98·3	100·0	0·0	0·0	97·9 ± 1·1
Lactobacillus iners	2·6	0·0	0·0	98·8	70·8	97·2 ± 1·8
Atopobium vaginae	92·1	0·0	0·0	0·0	5·2	96·8 ± 2·2
Megasphaera sp. 3.1	0·0	0·0	0·0	20·4	90·9 ± 5·1	
Leptotrichia sp. 0.0	0·0	0·0	0·0	2·4	93–98	
Gardnerella sp. 0.0	0·0	0·0	0·0	0·8	93–96	
Peptostreptococcus sp. 0.7	0·0	0·0	0·0	0·0	93	
Veillonella sp. 0.0	0·0	0·0	0·0	0·4	90	
Enterococcus faecalis	0·0	0·0	0·0	0·6	0·0	98
Aerococcus sp. 1.5	0·0	0·0	0·0	0·0	97–98	
Novel phylotypes	0·0	1·7	0·0	0·6	0·0	

*Phylogenetically related clones which on average had ⩾90 % sequence similarity to a reference strain were presumed to be of the same genus, and clones which on average had ⩾97 % sequence similarity were designated with the corresponding epithet.

†W-1 to W-5 represent the women sampled. *n*, Number of clones analysed.

or W-5, and no clones with similarity to *L. iners* were found in libraries prepared from W-1, W-2 or W-3. In sharp contrast to the other women, lactobacilli were not predominant in the vagina of W-1, and instead, 92·1 % of the clones had 95·5–99·0 % sequence similarity to the 16S rRNA sequence of *A. vaginae*. Sequences related to *A. vaginae* also accounted for 5·2 % of the clones from W-5. Clones with sequences related to *Megasphaera* sp. were recovered in samples from W-1 and W-5 and constituted 3·1 and 20·4 % of the totals, respectively. Sequences most closely related to *Leptotrichia* sp., *Gardnerella* sp., *Enterococcus* sp., *Peptostreptococcus* sp. and *Veillonella* sp. were encountered less frequently in libraries from various women except W-3. Overall, the composition, species-richness and -evenness varied considerably among the women sampled.

Phylogenetic analyses

The phylogenies of populations in the vaginas of the women sampled were determined by comparing the 16S rRNA gene sequences from this study to those of previously described species (Fig. 1 and Fig. 2). The diversity of populations was greatest in W-1 and W-5. Clones from W-1 (Fig. 1) belonged to five clades, including *A. vaginae* ($n=175$), *L. iners* ($n=8$), *Megasphaera* sp. ($n=6$), *Aerococcus* sp. ($n=2$) and *Peptostreptococcus* sp. ($n=1$), while clones from W-5 (Fig. 2) were related to *L. iners* ($n=177$), *Megasphaera* sp. ($n=51$), *A. vaginae* ($n=13$) and *Leptotrichia* sp. ($n=6$). Interestingly, clones of *Megasphaera* sp. and *A. vaginae* were coincident, with one being found only when the other was also present.

Numerous clones from W2, W-3, W-4 and W-5 were highly similar to 16S rRNA sequences of *L. crispatus* and *L. iners*, with 96·4–99·0 % and 96·8–99·0 % similarity, respectively (Fig. 3). Likewise, clones designated as being derived from *A. vaginae* were 95·5–99·0 % similar to 16S rRNA genes of *A. vaginae* (GenBank accession no. AF325325) that had been sequenced previously. Given the high level of similarity, these clones were designated with the corresponding epithet. The sequence heterogeneity among clones of *L. crispatus* was greater than that of *L. iners*, suggesting the existence of evolutionarily divergent subpopulations of *L. crispatus*. In contrast, the libraries of W-1, W-4 and W-5 yielded clones of *L. iners* that were highly related to one another and to a single reference strain, *Lactobacillus* sp. LSPY 17362. In contrast, clone sequences referred to as *Megasphaera* sp. and *Leptotrichia* sp. had low similarity (83·5–96·0 % and 93·0–98·0 %, respectively) to species of these genera that have been sequenced. The occurrence in the vagina of *Aerococcus* sp. and other phylotypes with low similarity to known species is curious. Their abundance in the communities (>0·5 %) suggests they are not trivial, but it is unknown if they are truly indigenous members of the community or simply transient populations.

Overall, there appeared to be three classes of normal microbial communities; those dominated by *L. crispatus* (W-2 and W-3), by *L. iners* (W-4 and W-5) or by *A. vaginae* (W-1), and there was a high diversity of abundant bacterial populations present in four of the five women sampled.

DISCUSSION

It is important to understand the structure of normal vaginal communities for at least two reasons. First, some of these microbes may have physiological functions that directly affect the health of women. They play a role in colonization resistance providing protection against invasion by overt pathogens or against overgrowth and

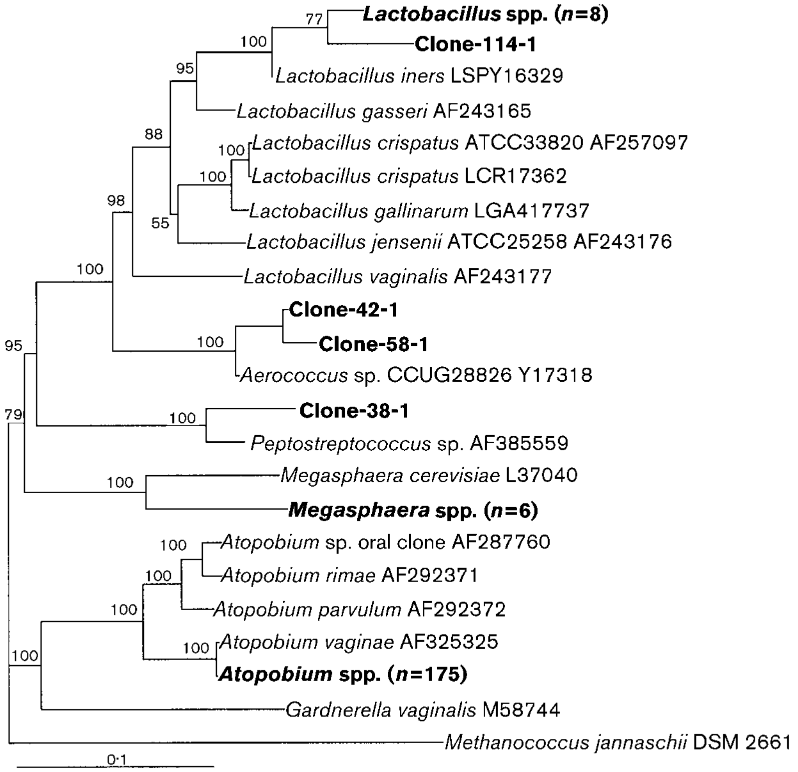

Fig. 1. Phylogenetic tree showing the relationship based on the analysis of 16S rRNA gene sequences of clones from the vaginal microbial community of W-1 to various closely related organisms. The tree was constructed using neighbour-joining algorithms based on 16S rRNA sequences. The sequences in bold type were found in this study (*n* is the number of clones with virtually the same sequence). Sequences in italics were selected from public databases (GenBank). Bootstrap values (from 500 replicates) greater than 50 % are shown at the branch points. The sequence of *Methanococcus jannaschii* was used to root the tree. The bar indicates 10 % sequence divergence.

dominance by potentially pathogenic species among the normal flora (Stahl & Hill, 1986). Second, perturbations of the community structure may predispose individuals to various infectious diseases (Redondo-Lopez *et al.*, 1990). Unfortunately, much of what is known about the composition of the microbial flora of the female genital tract is derived from qualitative and descriptive studies that relied on characterization of readily cultivated bacterial populations (Chow & Barlett, 1989; Johnson *et al.*, 1985; Larsen & Monif, 2001; Marrazzo *et al.*, 2002). These technical limitations have unwittingly affected the ability of clinicians to understand the aetiology of various diseases and have hindered the development of strategies to maintain normal flora. The results of the present study constitute the

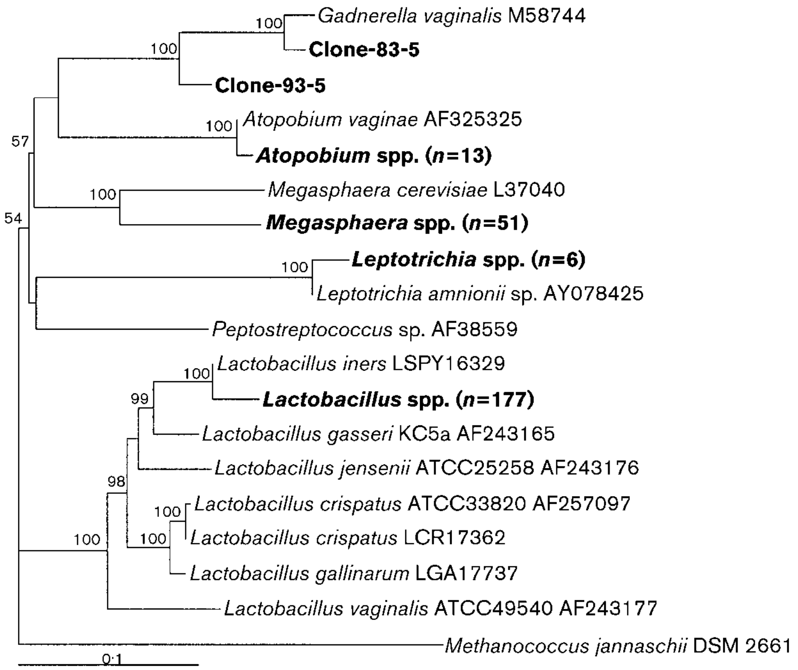

Fig. 2. Phylogenetic tree showing the relationship based on the analysis of 16S rRNA gene sequences of clones from the vaginal microbial community of W-5 to various closely related organisms. The tree was constructed using neighbour-joining algorithms based on 16S rRNA sequences. The sequences in bold type were found in this study (*n* is the number of clones with virtually the same sequence). Sequences in italics were selected from public databases (GenBank). Bootstrap values (from 500 replicates) greater than 50 % are shown at the branch points. The sequence of *Methanococcus jannaschii* was used to root the tree. The bar indicates 10 % sequence divergence.

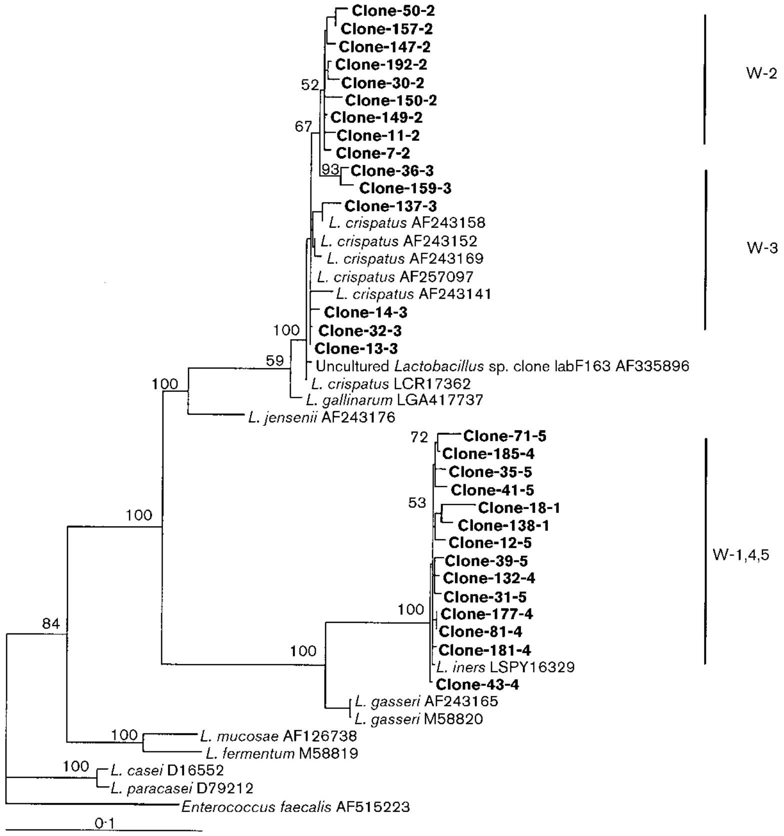

Fig. 3. Phylogenetic tree showing the relationship of clones closely related to *Lactobacillus* species from W-1 to W-5 vaginal microbial communities and reference sequences of lactobacilli obtained from GenBank. The tree was constructed using neighbour-joining algorithms based on 16S rRNA sequences. The sequences in bold type were found in this study; sequences in bold type were selected from GenBank. Bootstrap values (from 500 replicates) greater than 50 % are shown at the branch points. *Enterococcus faecalis* served as an outgroup. The bar indicates 10 % sequence divergence.

first in-depth effort to characterize the phylogenetic diversity of numerically dominant bacterial populations in the human vagina by analysis of 16S rRNA clone libraries. Although the small number of women sampled limits the present study, a number of important observations were made that may lead one to question common wisdom.

Consistent with previous studies (Antonio *et al.*, 1999; Hillier *et al.*, 1993), the data showed that lactobacilli were probably the most abundant organisms in the vaginal communities in most of the women. However, it is noteworthy that two of these women were colonized only with *L. crispatus*, while two others were only colonized with *L. iners*. Antonio *et al.* (1999) surveyed 215 sexually active women and reported that most were colonized with *L. crispatus* (32 %), *Lactobacillus jensenii* (23 %), *Lactobacillus* sp. 1085V (15 %) or *Lactobacillus gasseri* (5 %). Rarely (1·5 %) were other species of *Lactobacillus* isolated, while 29 % of the women apparently lacked lactobacilli. Recently, the occurrence of *L. iners* as a member of vaginal communities has also been demonstrated by others (Burton *et al.*, 2003; Burton & Reid, 2002; Vasquez *et al.* 2002). For example, Vasquez *et al.* (2002) used temperature gradient gel electrophoresis (TTGE) of 16S rRNA genes to show that *L. iners* was commonly found in healthy Swedish women. Although *L. crispatus*, *L. jensenii*, *L. iners* and *L. gasseri* are phylogenetically closely related to one another (Ennahar *et al.*, 2003), they differ in ways that may be important to the microbial ecology of the human vagina. For example, while H_2O_2 is commonly produced by strains of *L. crispatus* and *L. jensenii* (95 and 94 % of strains tested, respectively; Antonio *et al.*, 1999), it is an uncommon characteristic among strains of *L. iners* and *L. gasseri* (9 and 7 % of strains tested, respectively; Antonio *et al.*, 1999).

Interestingly, two of the women sampled had high numbers of *Atopobium* sp., organisms that also produce lactic acid (Dewhirst *et al.*, 2001; Downes *et al.*, 2001). The production of lactic acid is also characteristic of *Megasphaera* sp. and *Leptotrichia* sp., organisms that were found to be numerically dominant in two of the five women in this study. Although species of *Atopobium* have been found in human faeces and subgingival plaque (Harmsen *et al.*, 2000; Paster *et al.*, 2001), there is only one previous report of their isolation from the human vagina (Jovita *et al.*, 1999). This is the first time that a *Megasphaera* species has been reported as a member of the vaginal microbial community. The production of lactic acid by members of the normal flora and maintenance of vaginal acidity is widely recognized as important to sustaining an environment that is inhospitable to many bacteria (Schwebke, 2001), and it is negatively correlated with the incidence of bacterial vaginosis among women (Cu-Uvin *et al.*, 1999; Skarin & Sylwan, 1986; Taha *et al.*, 1998) and risk of acquiring HIV. The findings of this study indicate that although the structure of vaginal microbial communities varied between the women, the function of these communities (lactic acid production) was apparently conserved.

Our observation that the women were colonized by a single species (or group of closely related strains) of *Lactobacillus* is consistent with the findings of other studies (Antonio *et al.*, 1999; Hillier *et al.*, 1993; Reid *et al.*, 2003). For example, of the 215 women sampled by Antonio *et al.* (1999), only 8 % were found to have more than one species of *Lactobacillus* present in the vaginal community. The rare coexistence of multiple species of lactobacilli in vaginal communities could be caused by competitive exclusion of one species by another, pre-emptive colonization by a particular species or host factors that strongly influence which species are able to colonize the environment. Support for the latter notion can be inferred from the observation that white women are more likely to be colonized by *L. crispatus* and/or *L. jensenii* than by other species of lactobacilli (Antonio *et al.*, 1999), and similar findings that the composition of the vaginal flora differs among racial groups (Pavlova *et al.*, 2002). Any of the three mechanisms, either alone or in some combination, would account for the lack of *Lactobacillus* species diversity found in vaginal communities. It seems unwise to presume that differences between species of *Lactobacillus* are inconsequential or ecologically irrelevant since there is a near complete lack of information on the nature of possible host–bacterium interactions in the vagina and the ecology of the microbial community. Future efforts to develop probiotics should take these differences in the species composition of the vaginal community into account.

In the present study three taxa, namely *A. vaginae*, *Megasphaera* sp. and *Leptotrichia* sp., were found to be constituents of the normal flora of some women. While *A. vaginae* has rarely been isolated from any environment, the species has been isolated from the vagina of a healthy individual in Sweden (Jovita *et al.*, 1999), and species of *Atopobium* have also been implicated in halitosis (Kazor *et al.*, 2003). The clones of *Megasphaera* sp. from the vagina had modest similarity (89–95 %) to *Megasphaera cerevisiae* a Gram-negative, obligate anaerobe that is associated with beer spoilage by causing turbidity, off-flavours and off-odours (Doyle *et al.*, 1995; Ziola *et al.*, 1999). *Leptotrichia* sp., an anaerobic Gram-negative rod, is reportedly part of the normal oral flora and has rarely been isolated from clinical material (Konomen *et al.*, 1994; Kroes *et al.*, 1999; Tee *et al.*, 2001). However, there are reports of *Leptotrichia* spp. associated with infections, and the organism has been isolated from a neutropenic patient with bacteraemia and from the amniotic fluid of a woman after intrauterine fetal demise (Midolo & Kerr, 2001; Patel *et al.*, 1999; Shukla *et al.*, 2002). Little is known about the ecology of *Leptotrichia* sp., but they do produce lactic acid as the primary fermentation product from glucose (Tee *et al.*, 2001) and may represent opportunistic pathogens.

The occurrence of two genera that have previously been linked to the production of malodorous metabolites (Kazor *et al.*, 2003; Ziola *et al.*, 1999) in normal vaginal communities could mean that certain normal flora could be responsible for vaginal odour that is not indicative of bacterial vaginosis or any other disease condition. Amsel *et al.* (1983) proposed criteria for the clinical diagnosis of bacterial vaginosis that are, in part, based on the strong correlation that exists between bacterial vaginosis and malodour (fishy odour). Moreover, new diagnostic tests based on amine production and odour formation have been developed to help clinic doctors to quickly diagnosis bacterial vaginosis (O'Dowd *et al.*, 1996; Wolrath *et al.*, 2001). These tests can result in false-positives, since in two studies (Chen *et al.*, 1982; Kubota *et al.*, 1995) amines were found in samples from women without bacterial vaginosis. If future studies show that normal flora may cause odour, then diagnostic criteria should be amended to take this into account lest the condition be misdiagnosed and antibiotics be unnecessarily prescribed.

Several bacterial populations recovered in 16S rRNA clone libraries prepared in this study are not readily cultivated and may have been overlooked in previous studies. *L. iners* does not grow on certain selective media commonly used for the isolation of *Lactobacillus*, namely MRS and Rogosa media (Falsen *et al.*, 1999). Likewise, *A. vaginae*, *Megasphaera* sp. and *Leptotrichia* are strict anaerobes, require specialized media and often grow slowly. The finding of these organisms as members of normal vaginal flora illustrates how cultivation-based studies can be misleading. Further studies are needed to develop detection methods and approaches to determine the prevalence of these organisms and to recover them from clinical samples.

In summary, data in this study suggest that the structure of vaginal microbial communities varies between women with respect to number as well as kinds of numerically prominent populations. Despite these differences, all communities were dominated by species of either *Lactobacillus* or *Atopobium* that produce lactic acid. Thus, the ecological function of the flora – maintenance of a low pH environment that precludes the colonization and growth of pathogens and other undesirable organisms – may be conserved despite differences in community structure.

ACKNOWLEDGEMENTS

We would like to thank Holger Heuer, Celeste Brown and Sander Heijs for their valuable advice on phylogenetic analyses of sequence data, and Mayee Wong for her technical support. Financial support for this research was provided by The Procter & Gamble Co. and the National Institutes of Health (1-P20 RR16448-01) to L. J. F.

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