

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

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## 20 **Summary**

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 etiology of these diseases. Common wisdom is that lactobacilli dominate the normal vaginal microflora of post-pubertal women. However, this  
25 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. We used cultivation-independent methods to analyze 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  
30 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* dominated the communities in 4 of the 5 women. However, the  
35 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  
40 clones that were related to *L. crispatus*, whereas the second group of two women had clones of *L. iners* that were highly related to a single phylotype. These surprising results suggest that culture-independent methods can provide new insights to 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.

## 45 Introduction

Previous studies on the microbial flora of the human vagina indicate that microorganisms 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; 50 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 55 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 floras reduce the risk of acquiring these communicable diseases.

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 60 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 behaviors 65 (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*  
70 *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<sub>2</sub>O<sub>2</sub>), bacteriocins and  
other antimicrobial substances (Hillier, 1998). Given this, it is not surprising that various efforts  
75 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; Hillier, 1999; Larsen & Monif, 2001; Marrazzo *et*  
80 *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.

85 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 to the  
composition of these communities, they suffer from incompleteness, often lack statistical rigor,  
90 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  
95 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 microorganisms 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  
100 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).

105 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  
110 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

115 The study population and sampling methodology have been previously described (Coolen *et al.*, 2003). Mid-vaginal swabs from five premenopausal, nonpregnant, white women between the ages of 28 and 44 y were obtained. The bacterial cells retrieved on swabs were resuspended in 3 ml liquid dental transport medium (LDTM, Anaerobe Systems, Morgan City, CA), and genomic DNA was isolated as previously described (Coolen *et al.*, 2003).

### 120 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  $\mu$ l (total volume) reaction mixtures that contained 100 ng (1  $\mu$ l) of vaginal sample DNA, 2U of AmpliTaq DNA polymerase (Roche Molecular System, Inc., Branchburg, 125 New, Jersey, USA), 1  $\times$  AmpliTaq reaction buffer, 3mM MgCl<sub>2</sub>, 200  $\mu$ M each deoxyribonucleotide triphosphate, 5% DMSO and 0.1  $\mu$ M each primer. Initial DNA denaturation was performed at 94°C in a PTC-100 Programmable Thermal Controller (MJ Research, Inc.) 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 130 production, the mixture was analyzed by electrophoresis in 1.5 % agarose gel followed by staining with ethidium bromide and visualization under ultraviolet light.

## 16S rRNA gene clone library construction

16SrDNA genes were cloned into pCR2.1-TOPO (Invitrogen Corporation, Carlsbad, NM, USA) using a vector/insert ratio of 1:1 and procedures recommended by the Invitrogen. Ligations mixtures were used to transform *Escherichia coli* TOP 10 cells (Invitrogen Corporation, Carlsbad, NM, USA) that were subsequently plated onto Luria-Bertani agar plates containing 100 µg/ml kanamycin and incubated overnight at 37°C. Approximately 200 white, well-isolated colonies were randomly selected from each of library and grown 200 µl of Luria-Bertani broth containing 100 mg/ml kanamycin in 96-well microtiter plates for 24~48 h. These cultures were re-inoculated into Hogness buffer containing 3% glycerol, and cultured overnight. The cells from 800 µl were harvested and the plasmid DNAs were isolated using QIAprep96 Turbo Miniprep Kits (QIAGEN Inc, Valencia, CA, USA) using standard operation procedures by a QIAGEN BioRobot 3000 workstation. The remainders of the cultures were archived at -80°C.

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## Sequencing and Sequence Analysis

Approximately 1200 isolated plasmids with cloned inserts (approximately 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, Foster City, California), and resolved on an automatic sequencer (3100 PRISM Genetic Analyzer, Applied Biosystems). Sequences were edited to exclude the PCR primer binding sites and manually corrected with Chromas 2 (Chromas Version 2.22; <http://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 [<http://www.ncbi.nlm.nih.gov/>] databases using standard

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nucleotide-nucleotide BLAST program [blastn]; [<http://www.ncbi.nlm.nih.gov/>] to ascertain their closest relatives.

### **Phylogenetic analysis**

160 Sequence data were edited and combined with ContigExpress from InforMax Vector NTI Suite 8 (<http://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; <http://inn-prot.weizaman.aci/software/ClustalX.html/>) and AlignX from InforMax Vector NTI Suite 8 (<http://www.informaxinc.com>). These alignments were

165 manually adjusted to reduce errors before the sequences were further used. Phylogenetic trees were reconstructed using Neighbor-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.htm/>), a software package for the Microsoft Windows environment, was used to graphically represent the phylogenetic trees. Only representative

170 sequences and sequences at least that were 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.

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### **Nucleotide sequence accession number**

The 16S rDNA sequences determined in this study have been deposited in GenBank. The accession numbers for *Atopobium vaginae* sequences are AY269020 to AY269034, those of

*Megasphaera sp.* are AY271931-271953, and those of *Leptotrichia sp.* were AY267541-267542.  
180 The accession numbers of *Lactobacillus iners* sequences are AY283264-283275, and those of  
*Lactobacillus crispatus* are AY335493-335504. The remaining sequences were assigned  
AY344130-AY344131.

## Results

### Structure of Vaginal Microbial Communities

185 To evaluate the relative abundances of microbial populations in normal vaginal microbial communities, the 16S rRNA genes were amplified from samples taken from 5 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 *A. 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 of five 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 % to 100.0 % of the clones sequenced in 4 of 5 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 analyzed, 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, 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 apparently absent 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.*, *Hydrogenophaga palleroni*, *Comamonas terrigena*, 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. 1-4). 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 4.) 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. 5). 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 previously sequenced. Given the

230 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  
235 *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  
*Hydrogenophaga sp.*, *Comamonas sp.*, *Aerococcus sp.*, and other phylotypes with low similarity  
to known species is curious. Their abundance in the communities (>0.5%) suggests they are  
nontrivial, but it is unknown if they are truly indigenous members of the community or simply  
240 transient populations.

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

## Discussion

245 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 the role in colonization resistance – protection against invasion by overt pathogens or against overgrowth and dominance by potentially pathogenic species among the normal flora (Stahl & Hill, 1986); and secondly, perturbations of the community structure may  
250 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  
255 clinicians to understand the etiology of various diseases, and hindered the development of strategies to maintain normal flora. The results of the present study constitute the first in-depth effort to characterize the phylogenetic diversity of numerically dominant bacterial populations in the human vagina using cultivation-independent methods. Although the small number of women sampled limits the present study, a number of important observations were made that may lead  
260 one to question common wisdom.

Consistent with previous studies (Antonio *et al.*, 1999; Hillier *et al.*, 1993) the data showed that lactobacilli were the most abundant organisms in the vaginal communities in most women. However, it is noteworthy that 2 of these women were colonized only with *L. crispatus*, while 2 others were only colonized with *L. iners*. Antonio *et al.* (Antonio *et al.*, 1999) surveyed 215  
265 sexually active women and reported that most were colonized with either *L. crispatus* (32%), *L. jensenii* (23%), *Lactobacillus* sp. 1085V (15%), or *L. gasseri* (5%). Rarely (1.5%) were other species

of *Lactobacillus* isolated, while 29% of the women apparently lacked lactobacilli. Recently, *Lactobacillus* sp. 1085V has been found to have (DNA) homology to *L. iners* (S. Hillier, personal communication). The occurrence of *L. iners* as a member of vaginal communities has also been  
270 demonstrated by Vasquez et al. (Vasquez et al., 2002) who 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<sub>2</sub>O<sub>2</sub> is commonly produced by  
275 strains of *L. crispatus* and *L. jensenii* (95 and 94% of strains tested) (Antonio et al., 1999), it is an uncommon characteristic among strains of *L. iners* and *L. gasseri* (9 and 7% of strains tested) (Antonio et al., 1999).

Interestingly, one of the women sampled in this study apparently lacked species of *Lactobacillus* in her vagina but instead was colonized with *Apotobium* sp., organisms that also produce lactic acid.  
280 The production of lactic acid is also characteristic of *Megasphaera* sp. and *Leptotrichia* sp., organisms that were found to be numerically dominant in 2 of the 5 women in this study. 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  
285 women (Cu-Uvin et al., 1999; Skarin & Sylwan, 1986; Taha et al., 1998) and risk to acquiring HIV. The findings of this study indicate that although the structure of vaginal microbial communities varied between women, the function of these communities (lactic acid production) was apparently conserved.

Our observation that women 4 of 5 women were colonized by a single species (or group of closely  
290 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.* (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, preemptive  
295 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,  
300 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  
305 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 normal flora. While *A. vaginae* has rarely been isolated from any environment, the species has been isolated from the vagina of a health individual in Sweden (Jovita *et al.*, 1999), and species of *Atopobium* have also been implicated in halitosis (Kazor *et al.*,  
310 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-flavors, and off-odors (Doyle *et al.*, 1995; Ziola *et al.*, 1999). *Leptotrichia sp.* is an anaerobic gram-negative rod is reportedly part of normal oral flora and has rarely been isolated from clinical material (Konomen *et al.*, 1994; Kroes *et al.*, 1999; Tee *et al.*, 2001).

315 However, there are reports of *Leptotrichia* spp. associated with infections, and the organism has been isolated from a neutropenic patient with bacteremia, 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.

320 The occurrence of 2 genera that produce 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 odor that is not indicative of bacterial vaginosis or any other disease condition. Amsel *et al.* (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 malodor (fishy odor).  
325 Moreover, new diagnostic tests based on amine production and odor 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 odor, then diagnostic criteria should be  
330 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  
335 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.

340 In summary, data in this study suggest that the structure of vaginal microbial communities varied between women with respect to number as well as kinds of numerically dominant populations. Despite these differences, all communities were dominated by species of either *Lactobacillus* or *Apotobium* 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  
345 undesirable organisms – may be conserved despite differences in community structure.

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575 Table 1. Abundance of microbial populations in normal vaginal microbial communities and percent similarity to reference sequences.

580	Phylotype	% clones					Sequence identity (%)
		W-1*	W-2	W-3	W-4	W-5	
		N=190	N=181	N=182	N=176	N=250	
	<i>Lactobacillus crispatus</i>	0.0	98.3	100.0	0.0	0.0	97.9 ± 1.1
	<i>Lactobacillus iners</i>	2.6	0.0	0.0	98.8	70.8	97.2 ± 1.8
	<i>Atopobium vaginae</i>	92.1	0.0	0.0	0.0	5.2	96.8 ± 2.2
	<i>Megasphaera sp.</i>	3.1	0.0	0.0	0.0	20.4	90.9 ± 5.1
585	<i>Leptotrichia sp.</i>	0.0	0.0	0.0	0.0	2.4	93-98
	<i>Gardnerella sp.</i>	0.0	0.0	0.0	0.0	0.8	93-96
	<i>Peptostreptococcus sp.</i>	0.7	0.0	0.0	0.0	0.0	93
	<i>Veillonella sp.</i>	0.0	0.0	0.0	0.0	0.4	90
	<i>Hydrogenophaga palleroni</i>	0.0	0.5	0.0	0.0	0.0	97
590	<i>Comamonas terrigena</i>	0.0	0.0	0.0	0.6	0.0	97
	<i>Enterococcus faecalis</i>	0.0	0.0	0.0	0.6	0.0	98
	<i>Aerococcus sp.</i>	1.5	0.0	0.0	0.0	0.0	97-98
	Novel phylotypes	0.0	1.1	0.0	0.0	0.0	

595 \* Woman sample

- Number of clones analyzed

‡ Clones with ≥90% sequence similarity were given the name of the most closely related genus, and those with ≥97% sequence similarity were designated with the corresponding epithet.

## Legends

600 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 sequences in bold font were found in this study (and 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  
605 points. The sequence of *Methanococcus jannaschii* was used to root the tree. The bar indicates 10% sequence divergence.

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-2 to various closely related  
610 organisms. The sequences in bold font were found in this study (and 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.

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Fig. 3. Phylogenetic tree showing the relationship based on the analysis of 16S rRNA gene sequences of clones from the vaginal microbial community of W-4 to various closely related organisms. The sequences in bold font were found in this study (and N is the number of clones with virtually the same sequence). Sequences in italics were selected from public databases  
620 (Genbank). Bootstrap values (from 500 replicates) greater than 50% are shown at the branch

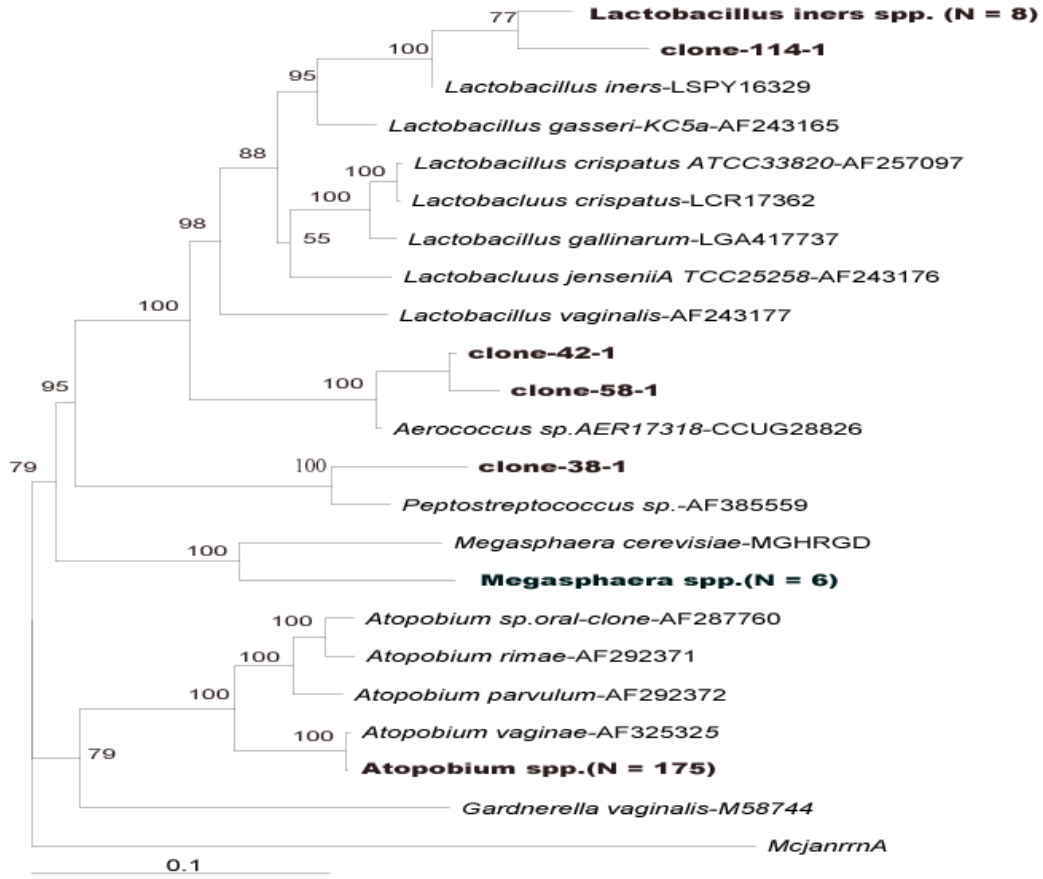
points. The sequence of *Methanococcus jannaschii* was used to root the tree. The bar indicates 10% sequence divergence.

625 Fig. 4. 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 sequences in bold font were found in this study (and 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  
630 10% sequence divergence.

Fig. 5. 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 sequences with colored font were found in this study; sequences in black font were selected from Genbank. Bootstrap values (from 500 replicates) greater than 50% are shown at the branch points. *Enterococcus faecalis* served as an out-group. The bar indicates 10% sequence divergence.

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Fig.1.



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Fig.2.

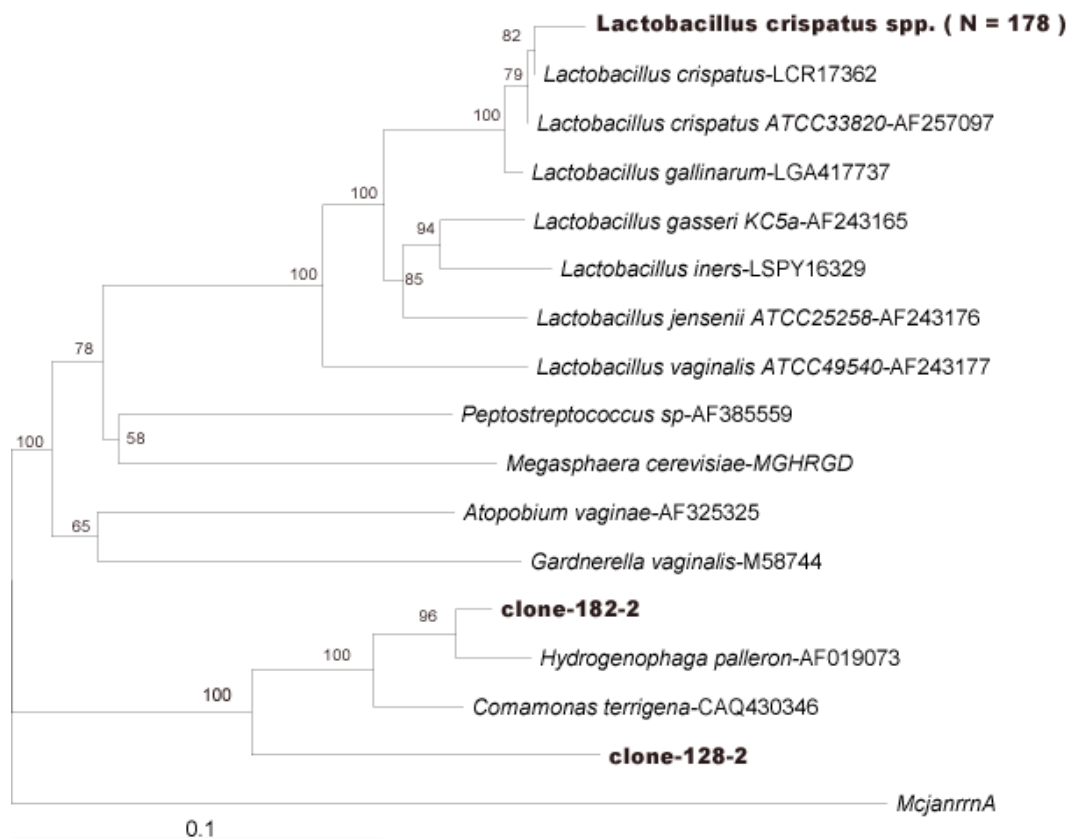
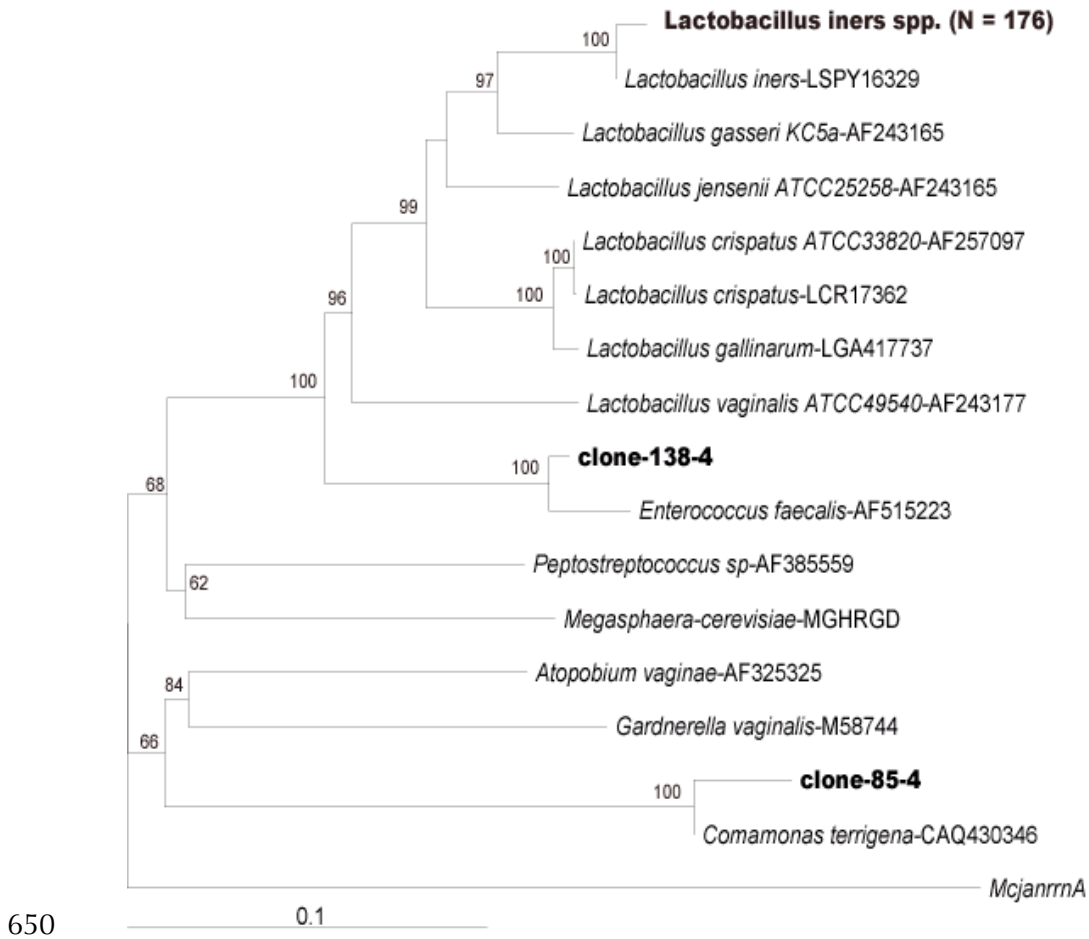


Fig.3.



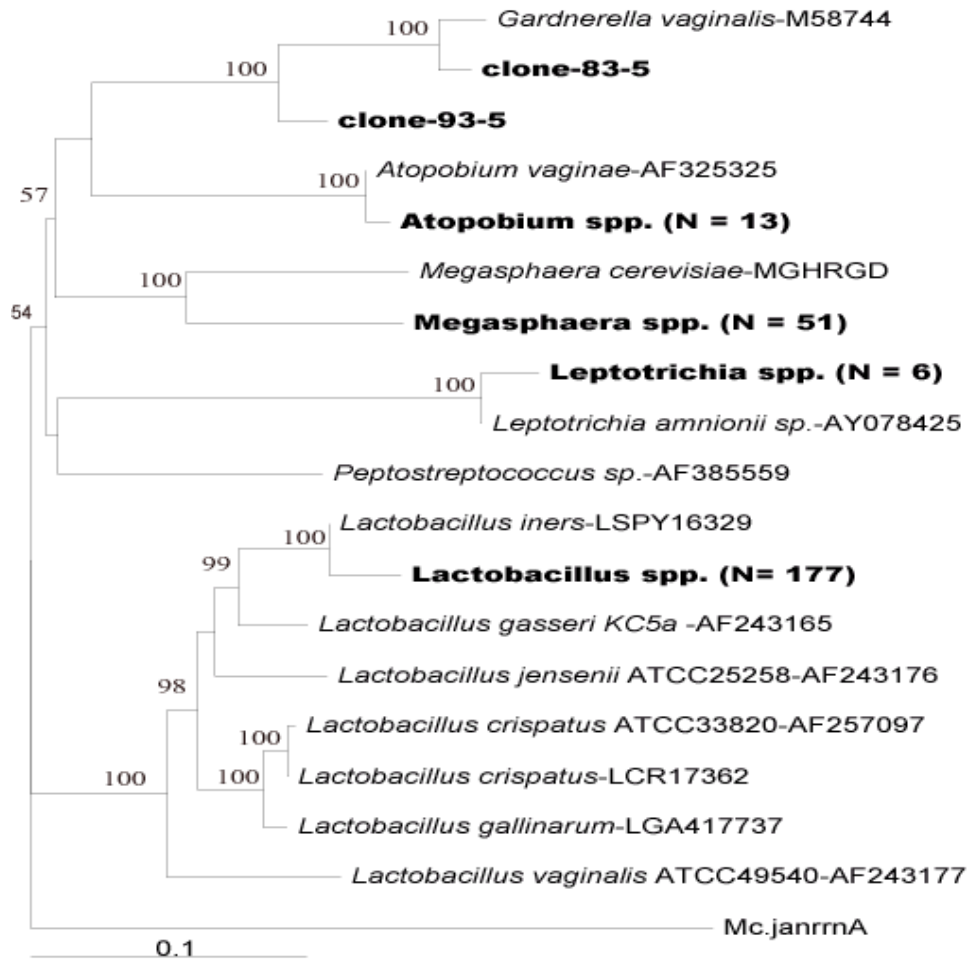
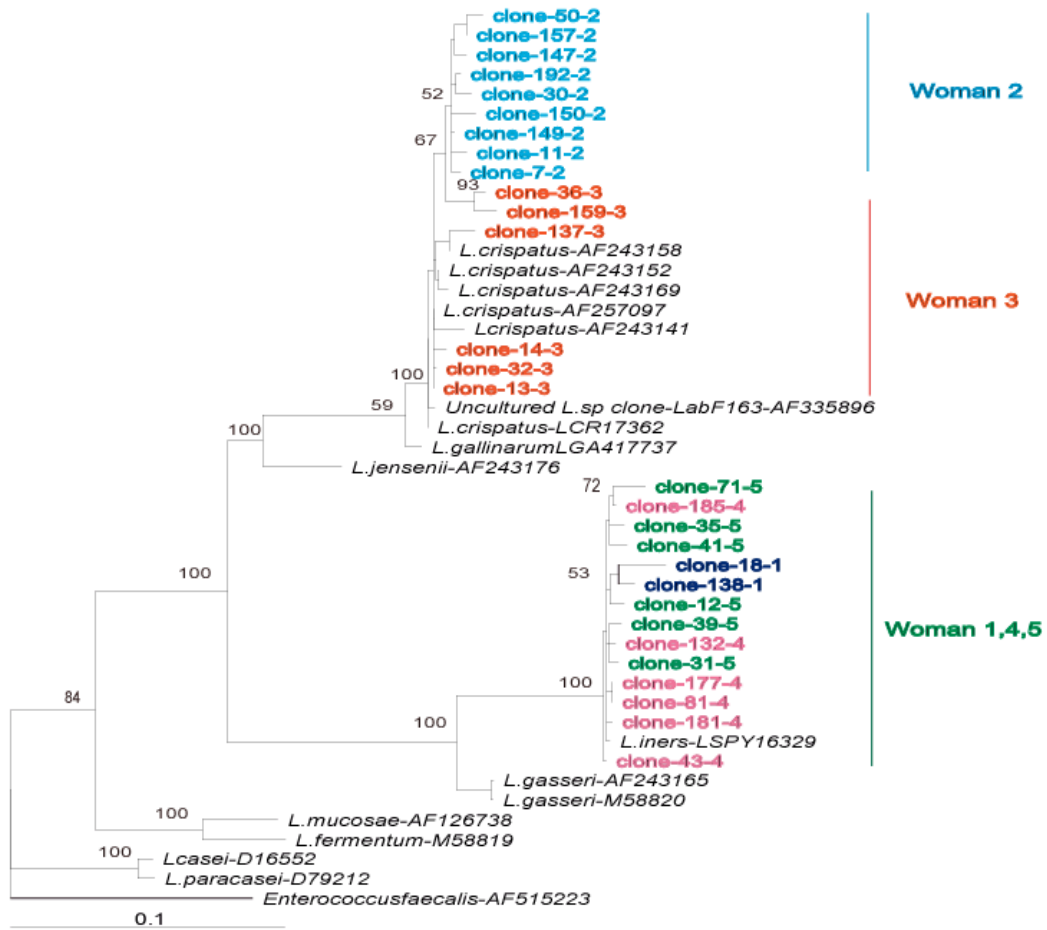


Fig. 5.



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