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Characterization of Microbial Communities Found in the Human Vagina by Analysis of Terminal Restriction Fragment Length Polymorphisms of 16S rRNA Genes

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To define and monitor the structure of microbial communities found in the human vagina, a cultivation-independent approach based on analyses of terminal restriction fragment length polymorphisms (T-RFLP) of 16S rRNA genes was developed and validated. Sixteen bacterial strains commonly found in the human vagina were used to construct model communities that were subsequently used to develop efficient means for the isolation of genomic DNA and an optimal strategy for T-RFLP analyses. The various genera in the model community could best be resolved by digesting amplicons made using bacterial primers 8f and 926r with HaeIII; fewer strains could be resolved using other primer-enzyme combinations, and no combination successfully distinguished certain species of the same genus. To demonstrate the utility of the approach, samples from five women that had been collected over a 2-month period were analyzed. Differences and similarities among the vaginal microbial communities of the women were readily apparent. The T-RFLP data suggest that the communities of three women were dominated by a single phylotype, most likely species of *Lactobacillus*. In contrast, the communities of two other women included numerically abundant populations that differed from *Lactobacillus* strains whose 16S rRNA genes had been previously determined. The T-RFLP profiles of samples from all the women were largely invariant over time, indicating that the kinds and abundances of the numerically dominant populations were relatively stable throughout two menstrual cycles. These findings show that T-RFLP of 16S rRNA genes can be used to compare vaginal microbial communities and gain information about the numerically dominant populations that are present.

The microflora of the lower genital tract in women has been studied in a variety of human populations by using different bacterial isolation and identification techniques (4, 21, 30, 34, 53). In healthy women, vaginal communities are typically dominated by various species of lactobacilli that number approximately 10^8 CFU per ml (or g) of vaginal secretion. The remainder of the community varies among women in terms of both the kinds and abundances of species present (3, 29, 34, 38). Investigators have demonstrated the occurrence of coagulase-negative staphylococci, group B streptococci, and species of *Corynebacterium*, *Prevotella*, *Peptostreptococcus*, *Gardnerella*, enteric bacteria, and various other taxa (38). The typical abundance of these populations ranges from 10^4 to 10^6 CFU per ml. Other than a common presence of lactobacilli, it is not yet possible to rigidly define the structure of a normal vaginal community given the data that are available.

The active role of normal flora in preventing disease by precluding colonization or limiting the growth of pathogens is becoming increasingly recognized, and various efforts are being made to promote the maintenance of normal flora (5, 9, 11, 17, 18, 25, 43, 44). Previous studies on the microbial flora of the human vagina have provided compelling evidence that *Lactobacillus* spp. play a key role in determining the overall structure of the community and in preventing successful colonization by "undesirable" organisms, including those responsible for bacterial vaginosis, yeast infections, urinary tract infections, and sexually transmitted diseases (36, 48, 49). The ecological mechanisms by which *Lactobacillus* spp. work to exclude such organisms have not been unequivocally established, but they are believed to be linked to products of their metabolism, including organic acids, which create a low-pH environment that is unfavorable to many bacterial species, and H_2O_2, which is microbicidal in vitro (22, 23).

While the dominance of lactobacilli in the vaginal community has been widely reported and accepted, the kinds of species that constitute the lesser members of the community and their roles in preventing disease are not well understood. Moreover, there is a distinct possibility that various taxa that are indigenous to the human vagina may have been overlooked due to limitations in the methodologies that have been used. Prior efforts to characterize the vaginal flora have largely employed methods that are commonly used in clinical microbiology laboratories (14, 29). These methods are inherently limited because they require cultivation of organisms on selective and nonselective media in the laboratory, after which they are classified into broad taxonomic groups based on phenetic characters and microscopy. Slow-growing, strictly anaerobic, or fastidious organisms may not be recovered by these methods. Others may be

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overgrown or otherwise overlooked because investigators are unaware of their ability to grow on selective media. Finally, the coarse classification methods used probably do not distinguish ecotypically distinct populations in samples. While these studies have been invaluable because they provide at least some information about the kinds and abundances of organisms in the human vagina, they suffer from incompleteness and often fail to provide sufficiently detailed information.

Culture-independent methods based on the analysis of 16S and 18S rRNA gene sequences of microorganisms offer the possibility to overcome many of the limitations described above, and they have been successfully used in numerous studies to explore the microbial diversity in various habitats (7, 20, 56, 57). These methods consistently reveal the existence of novel taxa and result in a perception of diversity that is quite different from that provided by cultivation-dependent methods (42). Moreover, several of these methodologies lend themselves to the analysis of large numbers of samples and therefore may be useful in studies on the ecology of the human vagina. In one such method, profiles of microbial communities based on the terminal restriction fragments (T-RFs) of 16S rRNA genes are produced (31), and these profiles provide insight into the phylogeny of the populations present in the samples (33; C. Shyu et al., unpublished data). Briefly, rRNA genes are amplified from total community DNA by using PCR wherein one or both of the primers used are labeled with a fluorescent dye. The mixture of amplicons is then digested with one or more restriction enzymes that have 4-base-pair recognition sites, and the sizes and relative abundances of the fluorescently labeled T-RFs are determined using an automated DNA sequencer. Since differences in the sizes of T-RFs reflect differences in the sequences of 16S rRNA genes, phylogenetically distinct populations of organisms can be resolved. Thus, the pattern of terminal restriction fragments observed is a composite of DNA fragments with unique lengths that reflects the diversity and composition of the numerically dominant populations in the community. While this method shares problems inherent to any PCR-based method, it has been shown to provide a facile means to assess changes in microbial community structure on temporal or spatial scales, based on the gain or loss of specific fragments from the profiles (39).

The objectives of the present research were to adapt this methodology to the analysis of vaginal microbial community structures and to demonstrate its utility in a pilot study to assess differences in communities among women and changes in structure that may occur over time.

MATERIALS AND METHODS

Construction of vaginal model communities. Sixteen clinical isolates (Table 1) were obtained from A. Onderdonk (Harvard Medical School), cultivated on various media, and used to prepare cell suspensions that were subsequently combined to produce model vaginal communities. Obligate anaerobes were cultivated on prereduced brucella blood agar with 5% sheep blood, 0.001% (wt/wt) hemin, and vitamin K_1 (Becton Dickinson, Franklin Lakes, NJ). Prior to inoculation, the medium was prereduced by overnight incubation in an anaerobic chamber with an atmosphere of 80% N_2, 10% CO_2, and 10% H_2. All manipulations were carried out in the same anaerobic chamber. Afterwards, the plates were sealed in anaerobic jars and incubated at 37°C. Aerobic or facultatively anaerobic bacterial strains were cultured either on brain heart infusion agar or in brain heart infusion broth and incubated over night at 37°C.

Model communities were prepared in 3 ml of liquid dental transport medium (pH 7.2) (Anaerobe Systems, Morgan City, CA) by adding the appropriate amounts of cell suspensions needed to give the final numbers indicated in Table 1. The numbers of bacteria in cell suspensions of each strain were enumerated by counting DAPI (4′,6′-diamidino-2-phenylindole)-stained cells (41). Model community A consisted of an equal number of cells (10^7 cells ml^{-1}) of each of the 16 strains, whereas in model community B the abundance of each strain approximated that found in the vaginas of healthy women.

TABLE 1. Bacterial populations commonly found in the human vagina and compositions of model communities A and B

Species or strain	Typical incidence (%)	Log_{10} cells ml^{-1} in model community:	
		A	B[k]
Lactobacillus crispatus[a]	100	7.0	8.6
Lactobacillus sp. strain 2	90–100	7.0	8.6
Lactobacillus sp. strain 3	60	7.0	7.4
Streptococcus agalactiae[b]	30	7.0	5.8
Corynebacterium coyleiae[c]	40	7.0	4.9
Lactobacillus panis[d]	60	7.0	4.9
Peptostreptococcus vaginalis[e]	70	7.0	4.8
Peptostreptococcus harei[f]	80	7.0	4.5
Peptostreptococcus magnus	30	7.0	4.7
Corynebacterium minutissimum	35	7.0	4.3
Corynebacterium singulare[g]	30	7.0	4.5
Enterococcus faecalis[h]	50	7.0	4.8
Prevotella melaninogenica	35	7.0	4.7
Staphylococcus haemolyticus[i]	30	7.0	4.3
Staphylococcus epidermidis strain 1	70	7.0	4.2
Staphylococcus epidermidis strain 2[j]	30	7.0	4.0

[a] Originally identified as *Lactobacillus* sp. The 16S rRNA gene sequence had 99.6% similarity to that of *Lactobacillus crispatus*.

[b] Originally identified as group B *Streptococcus*. The 16S rRNA gene sequence had 99.8% similarity to that of *Streptococcus agalactiae.*

[c] Originally identified as *Corynebacterium afermentans*. However, the 16S rRNA gene sequence had 98.9% similarity to that of *Corynebacterium coyleiae* but only 97.6% similarity to that of *Corynebacterium afermentans*.

[d] Originally identified as *Prevotella bivia*, but the 16S rRNA gene sequence had 97.1% similarity to that of *Lactobacillus panis*.

[e] Originally identified as *Peptostreptococcus magnus*. However, the 16S rRNA gene sequence had 97.3% similarity to that of *Peptostreptococcus vaginalis* but only 85.9% similarity to that of *Peptostreptococcus magnus*.

[f] Originally identified as *Peptostreptococcus asaccharolyticus*. However, the 16S rRNA gene sequence had 98.2% similarity to that of *Peptostreptococcus harei* but only 92.7% similarity to that of *Peptostreptococcus asaccharolyticus*.

[g] Originally identified as *Corynebacterium striatum*. However, the 16S rRNA gene sequence had 98.2% similarity to that of *Corynebacterium singulare* but only 97.6% similarity to that of *Corynebacterium striatum*.

[h] Originally identified as group D *Streptococcus*. The 16S rRNA gene sequence had 98.8% similarity to that of *Enterococcus faecalis*.

[i] Originally identified as *Staphylococcus warnerii.* However, the 16S rRNA gene sequence had 100.0% similarity to that of *Staphylococcus haemolyticus* but only 98.0% similarity to that of *Staphylococcus warnerii*.

[j] Originally identified as *Staphylococcus capitis*. However, the 16S rRNA gene sequence had 99.9% similarity to that of *Staphylococcus epidermidis* and 99.3% similarity to that of *Staphylococcus capitis*.

[k] Abundance in model community B was based on typical abundance in vaginal samples (A. Onderdonk, personal communication).

Clinical study. Study protocol and informed consent documents were reviewed and approved by The Procter & Gamble Corporate Institutional Review Board. Informed consent was obtained from all subjects prior to participation in the study. The study population consisted of five premenopausal, nonpregnant, white women between the ages of 28 and 43 years who were in good general health. They were recruited for the study via advertisement and screened for study eligibility. To be included in the study, the women had to have regular menstrual cycles, be willing to provide informed consent, and refrain from douching during the entire course of the study, bathing within 2 h of the clinic visits, and having sexual intercourse for 48 h prior to study visits. They also agreed to exclusively use Olay moisturizing bar soap for cleansing needs during the study and Always pads (Procter and Gamble, Cincinnati, OH) during menstruation. Women were excluded from the study if they had used antibiotics (orally or by topical appli-

TABLE 2. Predicted lengths of terminal restriction fragments expected following PCR amplification of 16S rRNA genes from vaginal strains by using primers 8f and 926r and digestion with HaeIII

Species or strain	Size of T-RF (bp)	
	5′ (8f)	3′ (926r)
Lactobacillus crispatus	245	459
Lactobacillus sp. strain 2	327	459
Lactobacillus sp. strain 3	327	458
Streptococcus agalactiae	311	87
Corynebacterium coyleiae	65	23
Lactobacillus panis	35	617
Peptostreptococcus vaginalis	320	334
Peptostreptococcus harei	272	582
Peptostreptococcus magnus	320	586
Corynebacterium minutissimum	65	23
Corynebacterium singulare	65	23
Enterococcus faecalis	288	5
Prevotella melaninogenica	267	501
Staphylococcus haemolyticus	310	613
Staphylococcus epidermidis strain 1	310	613
Staphylococcus epidermidis strain 2	310	613

cation in vulvar/vaginal area) during the prior 6 weeks or if they were immunocompromised (self-reported); were pregnant (self-reported); had body piercings in the vulvar, thigh, or buttock areas; or had a sexually transmitted disease, AIDS, or hepatitis (self-reported). All women completed all five of the study visits. All submitted vaginal swabs were acceptable for further vaginal microbial community analyses.

After enrollment (visit 1), subjects completed a total of four additional visits. Enrolled subjects were given a supply of Always pads (Procter and Gamble, Cincinnati, OH), with instructions to use the pads exclusively while menstruating. This was done in order to standardize the menstrual habits and practices of subjects while they were enrolled in the study. Two of the visits coincided with days 20 to 24 of the menstrual cycle. The other two visits coincided with days 2 to 5 of the menstrual cycle. Each study subject was interviewed before each of the four study visits to ensure that all of the inclusion and none of the exclusion criteria were met. A vaginal swab was obtained from each study participant. Vaginal swab samples were obtained from the mid-vagina by using a speculum lubricated with sterile saline and a swab (Pur-Wraps Harwood Medical Company). Swabs were immediately placed into 3 ml liquid dental transport medium (Anaerobe Systems, Morgan City, CA) and shipped on the same day to A. Onderdonk at Harvard Medical School, Boston, MA. Cells collected on the swab were suspended by vortexing. A small amount of each cell suspension was removed for studies done in his laboratory (data not shown), and the remainder was archived at −80°C. Batches of samples were shipped on dry ice to the University of Groningen and delivered within 24 h, where they were stored at −80°C. Experiments showed that samples shipped to the University of Groningen via Harvard University were no different than those obtained from a replicate sample that was shipped directly to the University of Groningen (data not shown). Once all the samples had been gathered, genomic DNAs were isolated as described below.

Genomic DNA extraction. 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 enzymatically 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 of incubation at 100°C, which 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, Hilden, Germany) during 1 h of incubation at 55°C. Further isolation and purification of the total DNA extract was performed using the Wizard DNA purification kit (Promega, Madison, WI).

T-RFLP analyses. To prepare samples for terminal restriction fragment length polymorphism (T-RFLP) analyses, regions of the 16S rRNA genes in each sample were amplified using combinations of four fluorescently labeled primers: 8f (5′-AGA GTT TGA TCC TGG CTC AG-3′) (27), 341f (5′-CCT ACG GGA GGC AGC AG-3′) (27), 926r (5′-CCG TCA ATT CCT TTR AGT TT-3′) (1), and 1406r (5′-ACG-GGC GGT GTG TRC-3′) (27). Reaction mixtures for PCR contained 50 ng of genomic DNA, 5 μl of 10× buffer (500 mM KCl; 100 mM Tris-HCl, pH 9.0; and 15 mM $MgCl_2$) (Amersham Biosciences, Piscataway, NJ), bovine serum albumin (20 μg), each deoxynucleoside triphosphate at a concentration of 200 μM (Amersham Biosciences), each primer at a concentration of 0.4 μM, and 1 U of *Taq* polymerase (Amersham Biosciences) in a final volume of 50 μl. If PCR products were used for subsequent T-RFLP analysis, the forward primers were labeled with 5-carboxy-fluorescein at the 5′ termini, and the reverse primers were labeled with tetrachlorofluorescein at the 5′ termini (Eurogentec, Seraing, Belgium). The same primers without fluorescent labels were used for PCRs to generate target DNA for subsequent cycle sequencing reactions as described below. DNA amplification was performed with a Geneamp 9700 thermocycler (Perkin-Elmer, Norwalk, CT), using the following program: a 5-min initial denaturation at 94°C, followed by 30 cycles consisting of denaturation (1 min at 94°C), primer annealing (1 min at 49.5°C for the primer combination 341f-926r, and 1 min at 55°C for the primer combinations 8f-926 and 8f-1406r), and primer extension (2 min at 72°C). A final extension was performed at 72°C for 10 min. Salts, nucleotides, and primers were removed from PCR products by using Qiaquick PCR purification kits (QIAGEN). Amplification of DNA was verified by electrophoresis of each PCR product in 1.5% agarose in 1× Tris-acetate-EDTA buffer, followed by staining with ethidium bromide and visualization under UV illumination.

Reaction mixtures for the enzymatic digestion of amplified rRNA genes contained 100 ng of PCR product, 1× restriction buffer, 20 μg of bovine serum albumin, and 10 units of restriction enzyme. The mix was adjusted to a final volume of 20 μl with water, and the DNA was digested at 37°C for 3 h. The restriction enzymes used to evaluate model microbial communities were AluI, HhaI, HaeIII, RsaI, MspI, and HinfI (all from Amersham Pharmacia Biotech, Uppsala, Sweden) and MvnI (Roche Applied Science, Indianapolis, IN) with buffers recommended by the manufacturers.

For analysis of terminal restriction fragment length polymorphisms, 1 μl of digested PCR product was mixed with 0.5 μl internal size standards (Tamra 2500; ABI) and deionized formamide. After 3 min of denaturation at 95°C, the lengths of the various T-RFs were analyzed using an ABI 310 Prism automated sequencer (ABI). The sizes of T-RFs are designated by the color used in the electropherogram traces, i.e., green (G) for 5′ T-RFs and blue (B) for 3′ T-RFs, followed by the size of the fragment in base pairs.

DNA sequencing and analysis. The sequences of the 16S rRNA genes (positions 8 to 926) of reference strains were determined. Each sequencing reaction mixture contained 4 μl of 5× sequencing buffer, 2 μl of Ready Reaction Mix (Applied Biosystems Inc., Foster City, CA), 20 ng of template DNA, and a final concentration of 0.2 μM of primer. Sterile water was added to a final volume of 20 μl. Each cycle sequencing reaction consisted of 25 cycles, and each cycle included a melting step at 96°C for 10 s, followed by primer annealing at 50°C for 5 s and extension at 60°C for 4 min. Prior to sequence analysis, the products were purified using the isopropanol precipitation method as described by the manufacturer. Sequence data were collected using an ABI Prism 310 Genetic Analyzer and analyzed using the AutoAssembler version 2.0 software package (Applied Biosystems Inc.). The 16S rRNA gene sequences obtained were matched with all sequences presently available from the databases of the Ribosomal Database Project (32) and GenBank (www.ncbi.nlm.gov) to identify their closest relatives.

Construction and analysis of 16S rRNA gene clone libraries. To construct 16S rRNA gene clone libraries, 3 μl of cleaned PCR product was cloned in a TOPO TA vector (Invitrogen, San Diego, CA), using the method recommended by the manufacturer. Competent cells of *Escherichia coli* (One Shot *E. coli*; Invitrogen, Inc., San Diego, CA) were transformed with ligated plasmids, and 50 μl of each transformation mixture was spread onto Luria-Bertani (LB) agar plates that contained X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), IPTG (isopropyl-β-D-thiogalactopyranoside), and 50 μg/ml kanamycin. After incubation overnight at 37°C, 100 white colonies were picked and inoculated into 5-ml aliquots of LB broth containing 50 μg/ml kanamycin. After overnight incubation, the cells were harvested from each culture and plasmid DNAs were extracted. The 16S rRNA gene inserts were individually amplified by PCR using the conditions described above and subjected to terminal restriction fragment analysis as described above. Clones yielding T-RFs that corresponded to those in the T-RFLP profile were sequenced, and the data were analyzed as described above. Ten clones representing each T-RF were sequenced, and all were sequenced if there were fewer than 10 clones for a given T-RF.

RESULTS

Optimization of T-RFLP. An optimal strategy for characterizing microbial communities found in the human vagina by

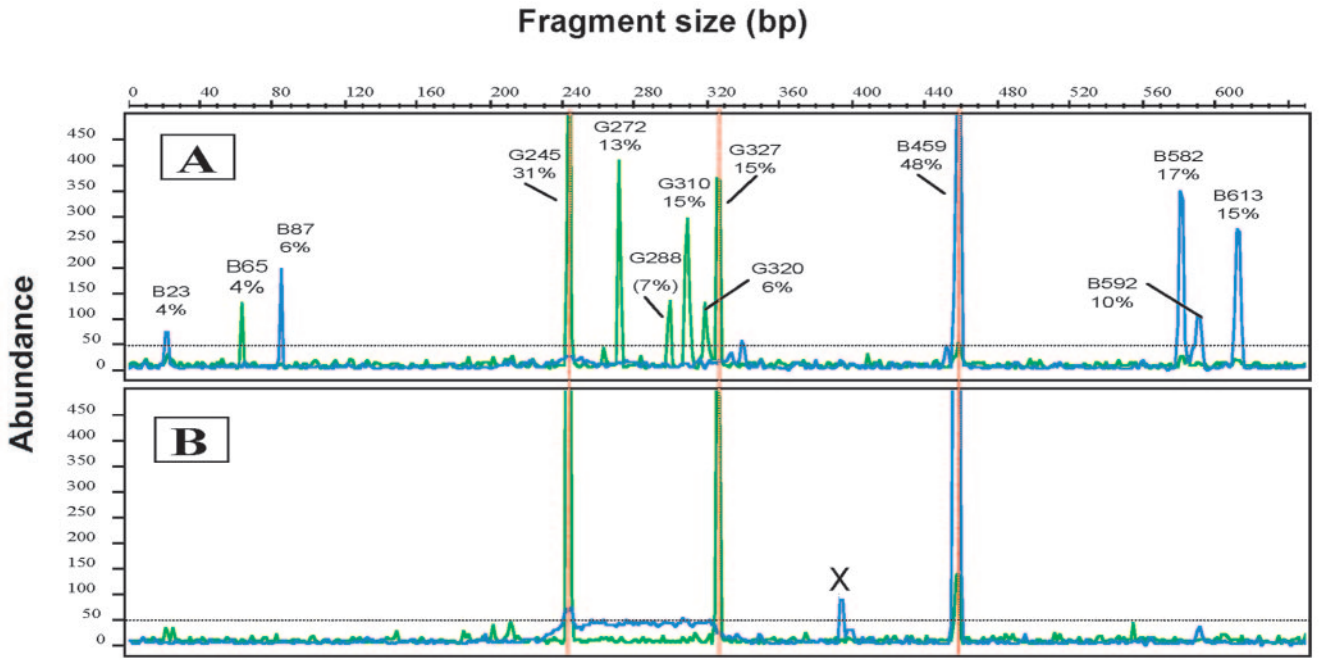

FIG. 1. T-RFLP analyses of model communities comprised of known numbers of the 16 vaginal isolates. (A) Profile from model community A (Table 1), which contained equal numbers of all the strains. (B) Profile from model community B, in which the numbers of various strains were approximately those found in the vaginas of healthy women. The lengths of 5′ T-RFs are shown in green and preceded by G, whereas 3′ T-RFs are shown in blue and preceded by B, along with the percentage of total peak area. An unexpected T-RF is marked with ×. Any peaks of less than 50 fluorescence units (horizontal dotted line) were excluded from calculations of the total peak area.

T-RFLP would employ the fewest combinations of PCR primers and restriction enzymes needed to resolve all the populations likely to be encountered in samples from healthy women. The optimal combination of primers and restriction enzyme(s) would be that which produces 5′ or 3′ T-RFs that differ in size, thus providing a means to resolve all members of the community. Pairs of four commonly used bacterial primers, namely, 8f, 341f, 926r, and 1406r, were used. Seven restriction enzymes that are frequently used in T-RFLP analyses (13, 31) were evaluated; these were AluI, HhaI, HaeIII, MvnI, RsaI, MspI, and HinfI. Combinations of primers and restriction enzymes were tested on axenic cell suspensions of 16 bacterial strains commonly found in the vaginal tracts of healthy women. Primers 8f and 926r were superior to 341f and 1406r when used in combination with HaeIII, HhaI, and MspI, which were equally effective in their ability to resolve populations found in the vagina. The predicted sizes of 5′ and 3′ HaeIII fragments from vaginal bacteria are shown in Table 2.

We opted to use the 8f-926r primer pair for PCR, followed by digestion with HaeIII, for the analysis of model communities and clinical samples. All of the strains tested could be resolved to at least the genus level. For *Peptostreptococcus*, three species could be distinguished based on difference in the sizes of both 5′ and 3′ fragments. In contrast, *Staphylococcus* and *Corynebacterium* could not be resolved at the species level. The predicted 5′ T-RF from *Lactobacillus crispatus* (245 bp) differed from that of the other two *Lactobacillus* spp. (327 bp), allowing resolution of this species. The predicted sizes of the 3′ T-RFs of *Lactobacillus* sp. strains 1 and 2 differed by 1 bp, and thus in theory these two strains could be resolved, since current technology provides resolution of DNA fragments that are ±1 bp in size.

Model vaginal microbial communities. Model communities (Table 1) were used to determine whether each member of the community could be detected by T-RFLP when the populations were present in equal numbers (community A) or when the abundance of each population was comparable to that found in the vaginas of healthy women and lactobacilli outnumbered other species by at least 2 orders of magnitude (community B). Both model communities were constructed by addition of known amounts of cells that had been quantified by direct microscopic counts of DAPI-stained cells, rather than by addition of known amounts of DNA. Analyses done on communities constructed on the basis of equal amounts of DNA could be misleading, since genome sizes and 16S rRNA gene copy number vary among bacterial species (15, 16). Furthermore, various factors (including cell wall composition and architecture) can markedly affect the efficiency of cell lysis. For these reasons, it was preferable to construct the model communities by addition of known amounts of intact cells.

According to the T-RFLP profile of model community A (Fig. 1A), strains of *Corynebacterium* (T-RF pair, G65/B23), *Lactobacillus crispatus* (G245/B459), *Lactobacillus* sp. strains 1 and 2 (G327/B458 or B459), *Peptostreptococcus harei* (G272/B582), *Staphylococcus* (G310/B613), *Streptococcus agalactiae* (G311/B87), and *Enterococcus faecalis* (G288/B592) could be resolved based on differences in the sizes of their 5′ and/or 3′ T-RFs. This was consistent with the outcome predicted based on the 16S rRNA gene sequences (Table 2). For unknown reasons, the 3′ T-RFs from *Peptostreptococcus vaginalis* and

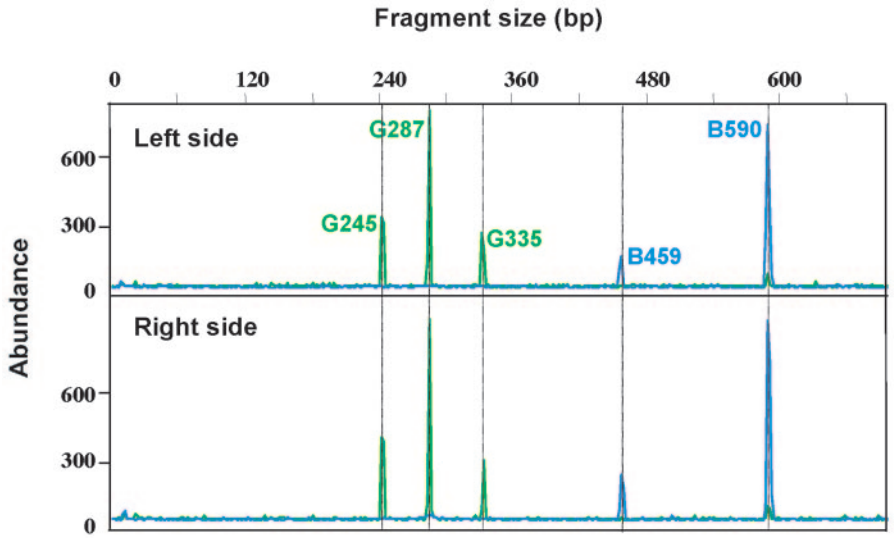

FIG. 2. T-RFLP profiles of vaginal communities sampled from the left and right sides of the mid-vagina. The lengths of 5′ T-RFs are shown in green and preceded by G, whereas the lengths of 3′ T-RFs are shown in blue and preceded by B.

Peptostreptococcus magnus were absent from the profile. Digestion of PCR products from the 16S rRNA genes of *Peptostreptococcus vaginalis* and *Peptostreptococcus magnus* resulted in the 210-bp-long 5′ T-RF; however, the 3′ T-RFs were missing. The variation in the quantity (peak area) of the various T-RFs as well as the absence of T-RFs derived from both *Prevotella* species could be explained by differences in the efficiency of cell lysis or 16S rRNA gene copy number between various bacterial strains (15, 16), preferential amplification of specific 16S rRNA genes, or other unknown causes (26, 50).

Only the T-RFs of the *Lactobacillus* species were evident in the T-RFLP profile of model community B (Fig. 1B). The total number of *Lactobacillus* cells (8.2×10^9) in model community B exceeded that of other species by at least 2 orders of magnitude. In experiments in which DNA from 10^8 cells of *Lactobacillus* strain 1 was mixed with DNA from a known number of cells from *Staphylococcus epidermidis* strain 1, the limit of detection of the latter organism was roughly 1% of the total number of organisms (data not shown). This was in agreement with experiments done by Dunbar (10), who found that only populations that constituted between 0.1 and 1% of a bacterial community could be detected in T-RFLP profiles. Thus, it was expected that T-RFs from strains other than lactobacilli would be absent from the T-RFLP profile of model community B.

T-RFLP of vaginal communities. Samples taken from the left and right sides of the vagina of each woman were used to evaluate the methodology and to compare the outcomes of T-RFLP analyses with those obtained from sequences from 16S rRNA clone libraries prepared from the same samples. The T-RFLP profiles of swab samples taken from the left and right sides of the vagina (Fig. 2) were virtually the same, suggesting that the methodology was reproducible. The simplicity of the profiles was consistent with the notion that comparatively few populations of bacteria dominate vaginal communities. Sequencing and phylogenetic analyses of 16S rRNA clones prepared from the same samples showed that these samples had high numbers of populations closely related to *Lactobacillus crispatus* (T-RF pair G245/B459) and three other species (T-RF pairs G287/B590, G287/B459, and G335/B590) and that all of the fragments in the T-RFLP profile could be accounted for in the library (data not shown). The three populations were only distantly related to *Lactobacillus acidophilus* and *Lactobacillus johnsonii* and were phylogenetically distinct from *L. crispatus*, *Lactobacillus jensenii*, and *Lactobacillus gasseri*, three species of *Lactobacillus* known to commonly occur in the human vagina (2, 24, 40). The lack of sequence similarity to known species of *Lactobacillus* suggests these may be phylotypes that were not previously known to reside in the human vagina.

Comparisons of vaginal communities among women. The T-RFLP profiles of microbial communities in different women and within an individual over a 2-month period of time were compared. Differences and similarities among women were readily apparent (Fig. 3). The vaginal communities of woman 2 and woman 3 had identical simple profiles that contained a single 5′ fragment and a single 3′ fragment, with sizes of 244 and 452 bp, respectively. This suggests that these communities had a limited number of numerically dominant populations, and perhaps only a single population. The profiles of woman 4 and woman 1 differed from each other and from those of women 2 and 3. Like for women 2 and 3, a single 5′ fragment and a single 3′ fragment, with sizes of 220 and 179 bp, respectively, dominated the T-RFLP profile of woman 1. The community of woman 4 had at least two phylogenetically distinct populations, which yielded two 5′ T-RFs that were 286 and 331 bp and a single 3′ T-RF that was 588 bp. Comparisons of the 5′ and 3′ T-RFs found in the communities of women 2, 3, and 4 to those predicted from in silico analyses of known 16S rRNA gene sequences indicated that the numerically dominant populations in these women were species of *Lactobacillus*. This is in contrast to the results of comparable analyses done on the numerically dominant population(s) present in the vaginal community of woman 1. None of the 16S rRNA gene sequences from species of *Lactobacillus* would yield the 220-bp

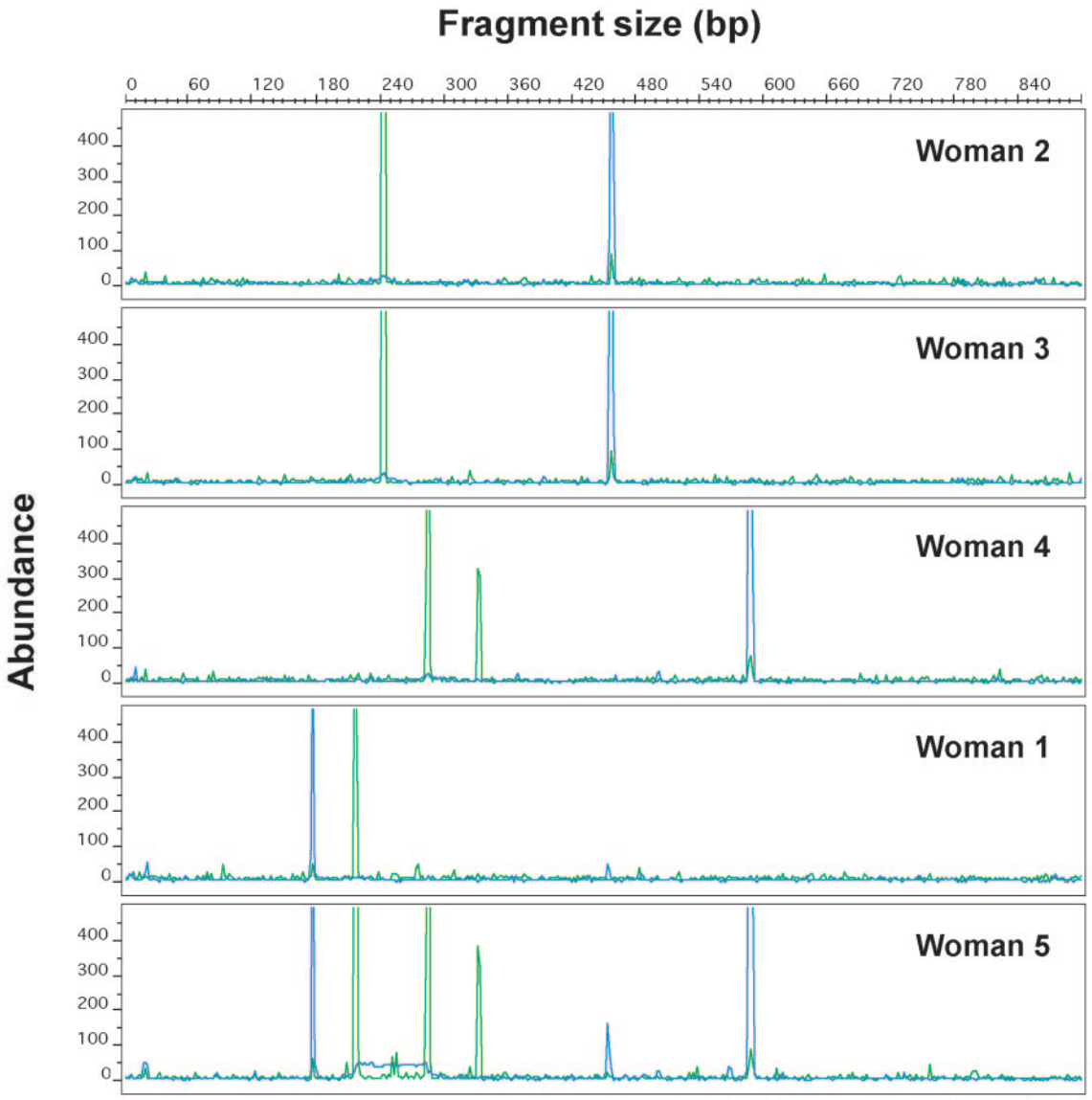

FIG. 3. T-RFLP profiles of microbial communities in vaginas of five women. All the samples were collected on the third visit of the study. The 5′ T-RFs are shown in green, whereas the 3′ T-RFs are shown in blue.

5′ fragment or a 179-bp 3′ fragment. Analyses done using algorithms available on the Microbial Community Analysis website (http://mica.ibest.uidaho.edu/) suggest that these fragments might be produced by *Atopobium fossor*, which would be expected to yield 221-bp 5′ and 180-bp 3′ fragments, while data obtained by Zhou et al. (55) suggest that the population could be *Atopobium vaginae*, which would produce 220-bp 5′ and 179-bp 3′ fragments. Of course, the possibility that it is some as-yet-uncharacterized phylotype cannot be excluded. Interestingly, the community of woman 5 appeared to be a composite of those found in women 1 and 4 and contained all the 5′ and 3′ fragments found in these communities. Given this, it appeared that woman 5 also had numerically dominant phylotypes that were not species of *Lactobacillus*. Analyses of samples collected from each woman four times over a 2-month period indicate that the numerically dominant populations in these communities remained remarkably constant (Fig. 4) even though the samples were collected from each woman during menses as well as at other times during their menstrual cycles, from the left and right sides of the vagina, and with scrapers as well as cotton swabs (data not shown).

Taken in sum, these data suggest the following: (i) there are distinct differences in the identities of the numerically dominant populations, thus suggesting that there is more than one kind of "normal" microbial community found in healthy women; (ii) the structures of the communities (at least as reflected by the most abundant populations) were comparatively invariant over time; and (iii) some communities (women 1 and 5) have phylotypes other than lactobacilli among the dominant populations. Conclusions i and ii are consistent with those of previous studies (3, 19, 30, 53), whereas this represents the first instance where populations other than lactobacilli have been

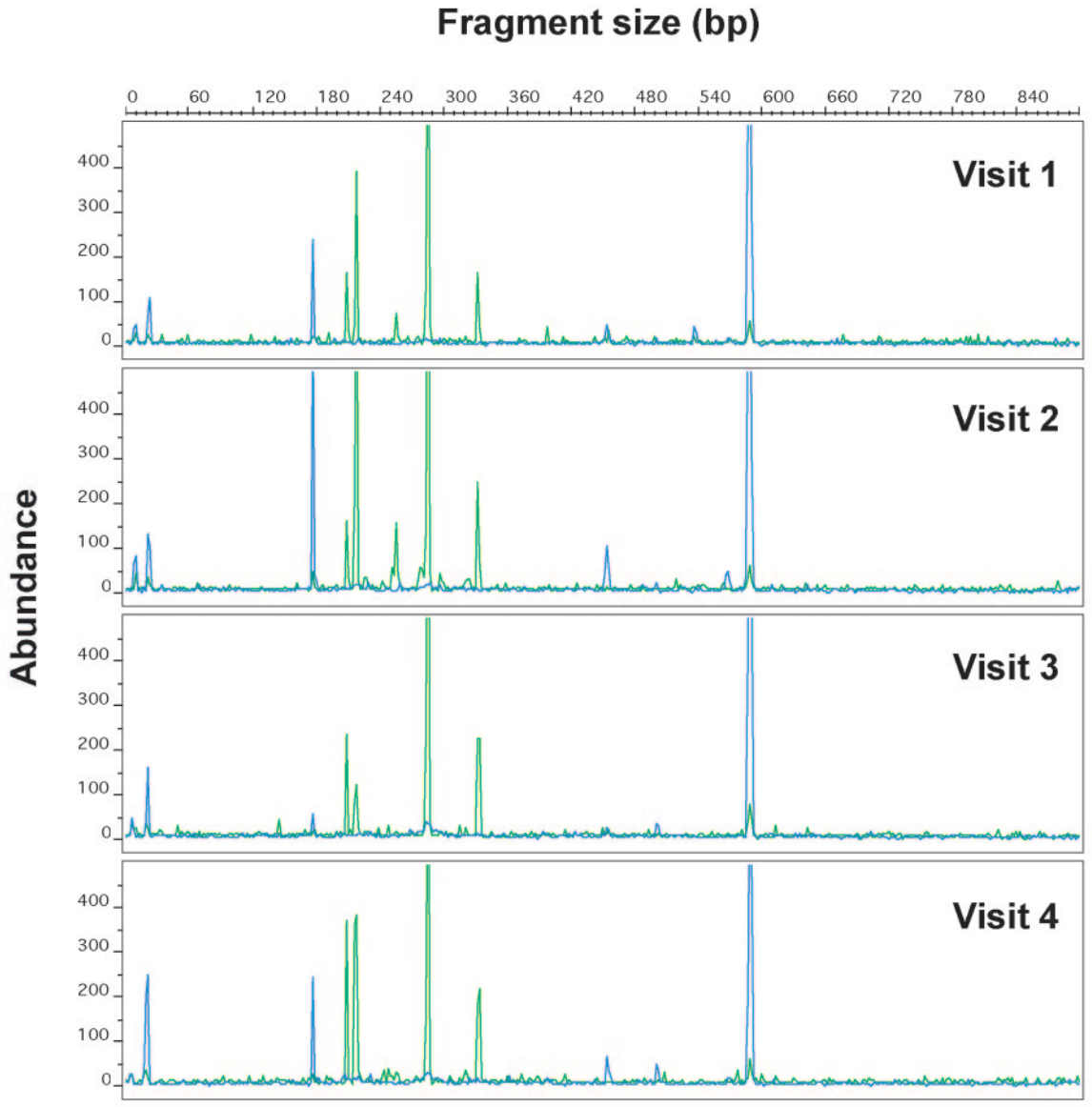

FIG. 4. T-RFLP profiles of the vaginal microbial community of woman 5 sampled on four consecutive visits over a 2-month period. Samples 1 and 3 were collected on day 1 or 2 during menses, while samples 2 and 4 were collected on day 22 or 23 of her menstrual cycle. The 5′ T-RFs are shown in green, whereas the 3′ T-RFs are shown in blue.

shown to be numerically dominant in the vaginal communities of healthy women. Additional studies are needed to verify this observation, since only a small number of women were included in this study.

DISCUSSION

The results show that T-RFLP analysis of 16S rRNA genes can be used to characterize microbial communities of the human vagina. The skewed species abundance of vaginal communities gives rise to a deceptively simple T-RFLP profile when universal bacterial primers are used to amplify 16S rRNA genes. Since populations of lactobacilli often outnumber others by 10- to 100-fold, they can easily mask the presence of other taxa that may be present in the community. This is expected, given that 16S rRNA genes from lactobacilli would constitute the majority of template sequences in total community DNA used in the PCRs. In analogous studies done on other habitats, investigators have overcome this problem though the use of group primers that selectively amplify genes from phylogenetic groups that were less abundant (35, 52). It is very likely that one could use a similar approach to study the ecology of the human vagina. The instrumentation and methodologies used to analyze samples based on T-RFLP, namely, PCR and the analysis of DNA fragments, can readily be scaled up to accommodate large numbers of samples. This provides the means to conduct studies in which large numbers of individuals are sampled or to assess the temporal and spatial dynamics of vaginal communities. The T-RFLP data obtained from such studies can readily be analyzed using various statis-

tical algorithms to quantitatively ascertain similarities and differences among communities and to infer plausible community structures (http://mica.ibest.uidaho.edu/). More-detailed information on the composition of any given community (or representatives of a group of samples) can be obtained by the construction and phylogenetic analyses of 16S rRNA gene clone libraries. Thus, the use of these methods provides a facile means to study the microbial ecology of the human vagina, while avoiding many of the limitations of culture-dependent methods.

A more complete and detailed understanding of vaginal microbial ecology is needed to define the degree of variability that occurs in healthy women so that shifts in these communities that are indicative of abnormal conditions can be discerned. This is important, since previous studies have shown that women with abnormal flora are at higher risk to acquire various sexually transmitted diseases, including human immunodeficiency virus infection (8, 45, 46, 51). Moreover, the means to identify women whose vaginal communities are more susceptible to upset is an essential step if we hope to intervene and employ preventive measures that reduce this risk. Conceivably, it may be possible to identify specific shifts in the abundance or species of organisms that foretell an impending upset in the community structure.

In studies of quite different ecosystems, the term "sentinel species" has been used to refer to particular species of organisms that are indicative of an impending or existing upset caused by some disturbance or change in conditions (6, 28, 37). A sentinel species is commonly an indigenous species that has been found to be particularly sensitive to changes in biological, physical, or chemical characteristics of the environment and responds through either an increase or (more commonly) a decrease in population size. The chain of events that cause an increase or decrease in the abundance of a sentinel species can be simple, wherein the environmental change directly affects the reproductive success of the species, or it may be complex and arise indirectly through a series of ecological events that connect the causes and changes in the population size of the sentinel species. The "sentinel species" concept is not widely employed in efforts to prevent or diagnose human diseases. Instead, the focus has been on understanding the composition of so-called normal flora and on the detection and identification of single species that are causative agents of various diseases. However, studies done more recently have shown that unhealthy conditions cannot always be attributed to single microbial species and sometimes arise from consortia of organisms (12, 54). This may well be the case for bacterial vaginosis (47), a condition that is clinically diagnosed based on various symptoms and general characteristics but not on the presence of any particular species of bacteria. Instead, bacterial vaginosis seems to reflect an upset in the normal community structure that is manifest in the outgrowth of various other populations, some of which may normally be present but at much lower numbers. In addition, the occurrence of disease is not entirely a function of a pathogen's virulence or ability to colonize a host, but it is also dependent on a variety of host factors that determine the susceptibility of an individual to infection, such as qualities of their immune system. Resistance to infection can also require the existence of a robust microbial community that can competitively exclude pathogenic organisms or create conditions that are unfavorable for the expression of virulence determinants. Given this, a desirable objective would be to develop the means to predict whether there is an increased risk of an individual contracting an infection due to shifts in the composition of the microbial community or changes in the abundance of specific populations. However, it is impractical to rely on exhaustive species inventories as a means to achieve this end. Instead, it would be more useful to identify one or more species whose numbers are responsive to and indicative of an impending or existing unhealthy condition—in other words, sentinel species of the vaginal ecosystem. The first step toward identification of potential sentinel species is to better define the species composition of the vaginal microbial community by using methods that provide more-detailed and more-comprehensive information than those previously used. The second step is to better define variations in community composition and population sizes that occur in healthy women so that disturbances that cause abnormal fluctuations can readily be identified. With these data in hand, rational predictions can be made concerning which members of the normal flora might best serve as sentinel species for the vaginal ecosystem.

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