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Comparative Study of Vaginal *Lactobacillus* Phages Isolated from Women in the United States and Turkey: Prevalence, Morphology, Host Range, and DNA Homology

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Lactobacilli play an important role in maintaining vaginal health. However, during bacterial vaginosis lactobacilli decrease for unknown reasons. Our preliminary study showed that phages could infect vaginal lactobacilli. Therefore, the aim of this study was to analyze the distribution, virulence, and types of vaginal *Lactobacillus* phages isolated from women of two countries: the United States and Turkey. A total of 209 vaginal lactobacilli were isolated from reproductive-aged women in the United States ($n = 107$) and Turkey ($n = 102$). By analysis of 16S rRNA gene sequence and by comparison of protein profiles, most lactobacilli were identified as *L. crispatus*, *L. gasseri*, and *L. jensenii*. After mitomycin C induction, 28% of American lactobacilli and 36% of Turkish lactobacilli released phages. A total of 67 phages were isolated and further characterized by their host range, electron microscopy, and DNA homology. All 67 phages were infective against lactobacilli from both collections. The host ranges of most phages were broad, including multiple *Lactobacillus* species. Even though the phages were all temperate, they were able to cause lytic infection in various strains. The electron micrographs of these phages showed a hexagon-shaped head and a long tail with or without a contractile tail sheath. Based on their morphology, these phages belonged to Bradley's phage groups A and B, and could be further classified into four morphotypes. All four types were found among American phages, but only three were found among Turkish isolates. DNA hybridization with labeled probes of the four types of phages revealed that additional genetic types existed within each morphotype among these phages. The phage genomic sizes ranged between 34 and 55 kb. Many of the lysogenic *Lactobacillus* strains released phages spontaneously at a high frequency of 10^{-3} to 10^{-4} PFU/cell. In conclusion, lysogeny in vaginal lactobacilli is widely spread. Some lysogenic lactobacilli spontaneously release phages with a broad host range, which can be lytic against other vaginal lactobacilli regardless of their geographic origin.

Lactobacilli indigenous to the human vagina are beneficial to women's health (35). These bacteria can inhibit other potentially harmful microorganisms by producing lactic acid, hydrogen peroxide (H_2O_2), and antimicrobial substances (12, 23, 43). In most healthy women, lactobacilli are the dominant species in the vagina. Theoretically, the anaerobic bacteria are suppressed by lactobacilli (12, 23) and cannot replace lactobacilli unless the latter is first diminished. However, the group of anaerobic bacteria commonly outnumber lactobacilli, causing a microbial imbalance called bacterial vaginosis (BV) (3, 9, 10, 15, 38, 40).

BV is a clinical condition that is characterized by decreased lactobacilli and an increased number of anaerobic gram-negative rods, *Gardnerella* species, and genital mycoplasmas (10, 38, 40). Women who suffer from BV may have an increased discharge that often has an unpleasant fishy odor. BV has been associated with many health risks, including preterm birth of low-birth-weight infants, midtrimester pregnancy loss, amniotic fluid infection, postpartum endometritis, pelvic inflammatory disease, and gynecologic postoperative infections (14, 16, 17, 28, 29). Recently, a lack of vaginal lactobacilli or the presence of BV was found to promote human immunodeficiency virus transmission (8, 27, 37).

The cause of BV is currently unknown, and it is unclear what causes the decrease of vaginal lactobacilli. Several possible mechanisms by which vaginal lactobacilli decrease have been proposed. These include douching (13); the use of spermicide, such as nonoxynol-9 (18); and treatment with antibiotics for other infections. It is important to examine the possibility that vaginal lactobacilli may decrease due to natural causes, such as phages or viruses.

Lactobacillus phages have been isolated from various sources, including dairy products (22), sausage (30), human intestines (34), and sewage (24). Recently, we reported the isolation of phages from human vaginal lactobacilli and documented their infectivity in vitro against lactobacilli isolated from the same and/or different women (32, 41). This suggested that reduction of vaginal lactobacilli may be caused by phages. It is important to further study and characterize these phages. In this study, we analyzed 67 vaginal *Lactobacillus* phages isolated from women in the United States and in Turkey based on their morphology, host range, spontaneous induction rate, DNA homology, and prevalence.

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TABLE 1. Morphotype-specific primers for vaginal *Lactobacillus* phage classification

Type	Strain	Primer	Sequence	Product size (bp)
A1	ϕkc5a	Forward	5′-ATGCTGACGGAAGGTGTGGTCAATGCT-3′	480
		Reverse	5′-AGTGCTACAACAGCCCTTGCACCGT-3′	
A2	ϕkc12a	Forward	5′-GCGGTTTATCTGGAAGTATAGCCCT-3′	326
		Reverse	5′-CTGATGCCAACCTTCACCATGAAGCCT-3′	
B1	ϕkc39	Forward	5′-CGAACTGGCGAATTTGTACCATCT-3′	237
		Reverse	5′-GTCGCCAGTTGTTGAAGCAGTGATGT-3′	
B2	ϕTL76	Forward	5′-CACCTCCGAGTGACATGGGCACAGCT-3′	250
		Reverse	5′-GCAATTGCAAATACTGCACCA-3′	

MATERIALS AND METHODS

Bacterial strains and growth media. Vaginal samples were obtained from reproductive-aged women visiting obstetrics and gynecology clinics at the Truman Medical Center in Kansas City, Mo., and at the medical schools of Karadeniz Technical University, Trabzon, Turkey, and Firat University, Elazig, Turkey. These included healthy women and women with vaginal infections, such as BV and candidiasis. Both the Amsel criteria (3) and Nugent scoring system (31) were used for diagnosis of vaginosis. Vaginal pH was measured with pH paper (Fisher Scientific). Microscopic examination of the Gram-stained vaginal sample slides was used to confirm the initial clinical diagnosis. During sampling, two sterile cotton swabs were inserted into the vagina, rotated a few turns along the vaginal sidewall, and allowed to absorb for a few seconds before being withdrawn. One swab was used for Gram staining. The other swab was placed into a test tube containing the RTF-glycerol transport buffer and sent to the laboratory for analysis. The transport buffer included (wt/vol) 0.045% K_2HPO_4, 0.045% KH_2PO_4, 0.09% NaCl, 0.09% $(NH_4)SO_4$, 0.018% $MgSO_4$(or $MgCl_2$), 0.038% EDTA, 0.04%Na_2CO_3, 0.02% dithiothreitol, and 10% glycerol. Samples were either analyzed immediately or kept at −20°C for several weeks before processing. To isolate lactobacilli, the samples were streaked onto *Lactobacillus* Rogosa (Difco, Detroit, Mich.) agar plates (pH 5.2) and incubated at 37°C for 48 h under anaerobic conditions. The MRS medium (Difco) was subsequently used to grow lactobacilli. Lactobacilli were initially identified by their ability to grow on the selective Rogosa agar, gram-positive staining, rod shape, and catalase-negative phenotype. Purified cultures were maintained at −80°C in MRS broth with 10% glycerol. Biochemical analyses, including sugar fermentation profile and gas production in MRS broth, were conducted as described in *Bergey's Manual of Systematic Bacteriology* (21). *Lactobacillus* type strains used in the study included *Lactobacillus acidophilus* ATCC 4356 and 4357, *Lactobacillus brevis* ATCC 14869, *Lactobacillus buchneri* ATCC 4005, *Lactobacillus casei* subsp. *casei* ATCC 393 and 27139, *Lactobacillus crispatus* ATCC 33197 and 33820, *Lactobacillus fermentum* ATCC 14931 and 23271, *Lactobacillus gasseri* ATCC 9857, *Lactobacillus jensenii* ATCC 25258, *Lactobacillus johnsonii* ATCC 33220, *Lactobacillus plantarum* ATCC 8014 and 14917, *Lactobacillus reuteri* ATCC 23272, *Lactobacillus rhamnosus* ATCC 7489, *Lactobacillus ruminis* ATCC 25644, *Lactobacillus salivarius* subsp. *salivarius* ATCC 11741, and *Lactobacillus vaginalis* ATCC 49540.

Whole-cell protein analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of whole-cell proteins of lactobacilli was performed to help identify bacterial species. Approximately 50 mg of cells (wet weight)/ml was lysed by boiling in SDS sample buffer (25) for 10 min and then centrifuged at 10,000 × *g* for 15 min to remove any precipitates. The gel system of Laemmli (25) was used. Proteins were visualized by staining with Coomassie blue. Marker proteins were obtained from Sigma (St. Louis, Mo.).

16S rRNA gene sequence analysis. The extraction of the genomic DNA of lactobacilli was performed as described by Chassy et al. (6). The amplification of the 16S ribosomal DNA (rDNA) by PCR and the determination of the sequences were described previously (Pavlova et al., Abstr. 100th Gen. Meet. Am. Soc. Microbiol., abstr. C-94, 2000). Analysis of genes encoding 16S rRNA of vaginal lactobacilli from women in different countries reveals multiple novel species (unpublished data). The sequences were used for comparison with data from GenBank.

Phage induction. Mitomycin C (Sigma) was used to induce phages from vaginal lactobacilli as previously described (22, 32). The induction of *Lactobacillus* prophages was indicated by the lysis of a *Lactobacillus* culture 4 to 7 h after the addition of mitomycin C. These lysates were then centrifuged, filtered through a 0.45-μm-pore-size filter, and maintained at 4°C with a drop of chloroform.

Spontaneous phage induction. Each lysogenic vaginal *Lactobacillus* strain was grown in 2 ml of MRS broth to mid-exponential phase without mitomycin C treatment. One milliliter of the culture was diluted and plated on MRS agar plates for cell count. Another 1 ml was centrifuged to harvest the supernatant, which was filtered through a sterile 0.45-μm-pore-size filter. The supernatant was diluted and used to infect its indicator strain by the soft-agar overlay method as described before (32). Plaques were enumerated after 24 h of incubation at 37°C. The frequency of spontaneous phage induction was calculated as the total number of phage plaques per milliliter of culture divided by the number of CFU and the burst size of the phage, which was calculated by one-step growth curves as described before (22, 32).

Phage infectivity assay. Phage infectivity was determined by the agar spot method as previously described (32). All of the 67 phages were used to infect the two collections of vaginal *Lactobacillus* strains of a total of 209 isolates. The positive results were verified by single plaque formation.

Electron microscopy. One drop of the purified phage in 0.1 M ammonium acetate (pH 7.0) was spotted on grids with a carbon-coated Formvar film (Ladd Research Industry, Burlington, Vt.). After drying for 30 s, the sample was negatively stained with 2% uranyl acetate (pH 4.2). Electron microscopy was performed with the CM12 transmission electron microscope (Philips Electronic Instruments, Inc., Mahwah, N.J.) at 80 kV.

Phage DNA isolation and restriction analysis. The *Lactobacillus* phages were purified from 1 liter of mitomycin-induced lysate by a procedure described by Maniatis et al. (26). The phage DNA was extracted with the QIAGEN (Chatsworth, Calif.) lambda phage DNA isolation kit. Restriction enzyme (*Eco*RI) digests of the phage DNA were subjected to gel electrophoresis on a 0.8% agarose gel at 40 V for 3 h. The gel was stained with ethidium bromide and photographed under a UV light.

Phage genomic DNA hybridization. The genomic DNA from representative phages was isolated and labeled with the nonradioactive biotinylated labeling kit from GIBCO-BRL as probes (Life Technologies, Inc., Rockville, Md.). The DNA from target phages was processed by two methods. The first method was to digest the DNA with restriction enzymes. The digested DNA was then subjected to agarose gel electrophoresis and Southern hybridization with the labeled probes. The second method was to perform a simple dot hybridization with undigested DNA.

Phage classification by PCR. To obtain sequence data for the PCR analysis, the genomic DNA of four phages representing each morphotype was digested with *Sau*3A1. The digested DNA fragments were cloned into the pUC18 plasmid. A pUC18 plasmid that carries a random insert of about 1 to 2 kb was selected for each phage. The sequence of the cloned DNA was determined by the automated sequencing facility at the University of Missouri—Kansas City. The sequence data were analyzed by the BLAST program and used to design PCR primers. The primers used are listed Table 1. The DNA of target phages was isolated and used as template DNA. PCR was performed by using a thermal cycler (Techne, Princeton, N.J.). The reaction mixture (final volume of 50 μl) contained 100 ng of template DNA; 1 U of *Taq* DNA polymerase (Biolase; Bioline, Reno, Nev.); 1× reaction buffer (buffer J; pH 9.5, from the Invitrogen PCR optimizer kit; Invitrogen, Carlsbad, Calif.); 2 mM $MgCl_2$; deoxynucleoside triphosphates, 0.1 mM each; primers, 50 pmol each; and bovine serum albumin, 2 μg. The thermal cycling program used was as follows: initial denaturation at 94°C for 2 min and 35 cycles of 94°C for 1 min, 50°C for 2 min, and 72°C for 3 min. Finally, there was an extension step at 72°C for 7 min. The PCR DNA products were analyzed for correct sizes and for purity by agarose gel electrophoresis.

RESULTS

Isolation and identification of vaginal lactobacilli. About 200 vaginal samples were obtained from reproductive-aged

TABLE 2. *Lactobacillus* lysogens among different species and anaerobic groups in vaginal isolates from women in the United States and Turkey

Species or group	No. in U.S. collection		No. in Turkish collection		Total no.		% Lysogen
	Strain	Lysogen	Strain	Lysogen	Strain	Lysogen	
L. crispatus	27	4	34	7	61	11	18
L. gasseri	30	11	33	14	63	25	40
L. jensenii	30	12	30	14	60	26	43
L. fermentum	10	2	0	0	10	2	20
L. vaginalis	1	1	2	2	3	3	100
Other *Lactobacillus* spp.	9	0	3	0	12	0	0
Facultative anaerobes	97	23	92	31	189	54	29
Obligate anaerobes[a]	10	7	10	6	20	13	65
Total	107	30	102	37	209	67	32

[a] Among Turkish lactobacilli, the obligate anaerobes were *L. jensenii* and *L. crispatus*, but among U.S. lactobaclli, the obligate anaerobes were *L. gasseri* and *L. jensenii*.

women in Turkey and about 100 were obtained from the United States. While the Turkish women were all Caucasian, the American group included black (55%), white (35%), Asian (5%), Hispanic (3%), and Native American (2%) women. Some Turkish isolates did not survive the oversea shipping, so only 102 *Lactobacillus* strains were obtained. From American women, 107 strains were obtained. Among the Turkish women, 43 cases of BV were diagnosed, but only 22 had culturable lactobacilli. Among the American women, 14 cases of BV were diagnosed, but only 4 had culturable lactobacilli. Storage of samples in the RTF-glycerol buffer at −20 to −70°C did not result in loss of *Lactobacillus* viability. Each collection had 10 obligate anaerobic strains (about 10%). All of the remaining strains were facultative anaerobes (Table 2).

Species identification of lactobacilli. Since the traditional biochemical and physiological methods could not effectively classify these lactobacilli to the species level, we applied genetic and molecular methods. First, we grouped these strains based on their sugar fermentation pattern and whole-cell protein profiles. Then, we determined the sequence of the 16S rDNA of some representative strains from each group. Based on the sequence data, we identified their species. Finally, the whole cell protein profiles were analyzed among all of the remaining strains. Several representative strains from each group that shared the same cell morphology, sugar fermentation pattern and whole cell protein profile were selected to analyze their 16S rDNA sequences. The sequence data of 23 strains (9 from Turkey and 14 from the United States) have been deposited into GenBank with accession numbers from AF243150 to AF243166 and from AF243170 to AF143175. These data were compared to those for *Lactobacillus* type strains already in GenBank using the BLAST program (2). Once the species of the representative strains were identified, the identification for the remaining strains was achieved by comparison of their total protein profiles with those of the representative strains. The results of species designation of these strains are listed in Table 2. Figure 1 shows the result of one of the SDS-PAGE gels. Based on the 16S rDNA analysis and the protein profile comparison, most clinical vaginal strains belonged to three *Lactobacillus* species, *L. crispatus*, *L. gasseri*, and *L. jensenii*. The protein profiles of *L. gasseri* and *L. jensenii* were highly consistent among all isolates tested. Although a major band of *L. crispatus* was variable among different isolates (between 40 and 60 kDa on the SDS-PAGE gel), all of the other bands were consistent within the same species. Additional species included *L. fermentum*, *L. vaginalis*, and several unknown species. Interestingly, the fourth largest species among American isolates was *L. fermentum* (9%), while the Turkish isolates did not have any *L. fermentum* strains. As shown in Table 2, the majority of vaginal lactobacilli were facultative anaerobes.

Phage isolation. Phage induction was performed by the mitomycin C method for 209 clinically isolated vaginal strains. The lysates were used to interact with these *Lactobacillus* strains to screen for phage-sensitive indicator strains. Sixty-seven lysates were confirmed to contain phages, because they formed single plaques on the agar plates of sensitive strains. Additionally, these phages were confirmed by DNA hybridization with labeled phage DNA probes and observation under an electron microscope to rule out possible bacterial inhibition effects due to bacteriocins, H_2O_2, and organic acids. Among the 67 phages, 30 were isolated from the American collection, while 37 were isolated from the Turkish collection.

Table 2 shows that the obligate anaerobes were more likely to carry a phage (65%) than the facultative anaerobic lactobacilli (29%). The difference was significant ($P < 0.01$). About 36% of vaginal lactobacilli from Turkish women released phages, while about 28% of lactobacilli from American women released phages. The difference was not statistically significant between the two groups ($P > 0.05$). Among six *Lactobacillus* strains isolated from the four American women with BV, four strains from two patients were lysogens. Among 22 *Lactobacillus* strains from Turkish women with BV, 11 were infected by phages (lysogens). Overall, about 50% of lysogenic lactobacilli were isolated from women with BV, but only about 30% of lysogens were isolated from women without BV. The difference was statistically significant ($P < 0.05$).

Spontaneous phage induction and burst size. Phages can be spontaneously released without any inducing agent due to random errors during the host bacterial DNA replication (20). In this study, the lysogenic lactobacilli released infective phages at different rates, which were detected by observation of phage plaques on the indicator *Lactobacillus* plate cultures. Among American lactobacilli, 17% of lysogenic strains spontaneously released phages at a higher frequency of 10^{-3} to 10^{-4} PFU/cell, while 27% of lysogenic strains from the Turkish collection

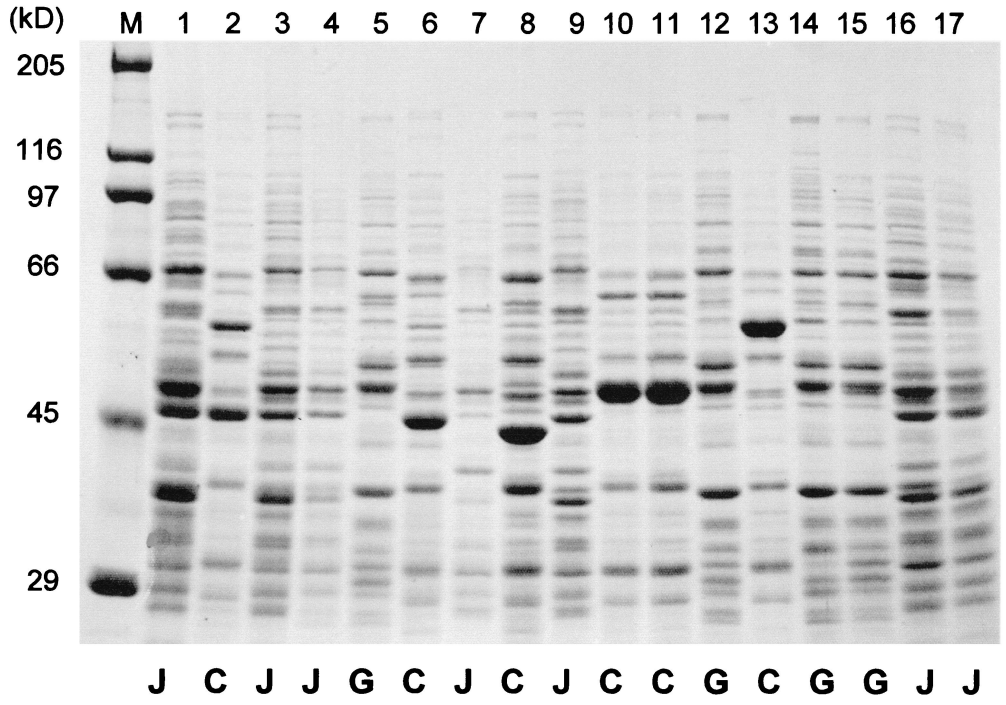

FIG. 1. Protein profiles of some representative *Lactobacillus* strains on SDS-PAGE(10% polyacrylamide). Lane M contains protein molecular weight markers. Lanes 1 to 17 contain the indicated vaginal *Lactobacillus* strains: 1, KC23T; 2, TL152; 3, TL145a; 4, TL143b; 5, TL114; 6, TL127a; 7, TL109b; 8, TL60a; 9, TL27; 10, TL23b; 11, TL23a; 12, TL33a; 13, TL13; 14, TL102; 15, TL76; 16, TL74c; 17, TL34c. At the bottom of the gel, the species identification of each strain is indicated by a letter. C; *L. crispatus*; G; *L. gasseri*; J; *L. jensenii*.

released phages at this level. About one-third of both collections released phages at an intermediate frequency (about 10^{-6} PFU/cell). Approximately one-half of the culture collections from both countries spontaneously released phages at a frequency of less than 10^{-8} PFU/cell. These data were repeated observations, and the frequency of phage release from each strain was highly stable. The burst sizes were between 60 and 300 phages per cell.

Phage host ranges and infection characteristics. All 67 temperate phages isolated from vaginal lactobacilli infected vaginal lactobacilli in vitro by forming clear plaques on agar plates. As shown in Table 3, the 30 phages from the United States and 37 phages from Turkey infected most vaginal lactobacilli from both collections, including lysogenic strains. Overall, fewer lactobacilli isolated from Turkish women resisted phage infection than lactobacilli isolated from U.S. women. A group of vaginal lactobacilli sensitive to multiple phages was identified. They were used as indicator strains to display clear single plaques after the infection and used to screen for new phages. There were no apparent differences in phage sensitivity between lactobacilli isolated from healthy women and those from women with vaginal infections.

TABLE 3. Infection of vaginal lactobacilli by 67 phages from the United States and Turkey

Infection category	No. of vaginal lactobacillus strains from[a]:		Total
	U.S. women	Turkish women	
Infected by both phage collections	71 (20)	86 (25)	157 (45)
Infected only by American phages	7 (5)	2 (0)	9 (5)
Infected only by Turkish phages	3 (1)	13 (11)	16 (12)
Resisted all phages	26 (4)	1 (1)	27 (5)
Total	107 (30)	102 (37)	209 (67)

[a] The number in parentheses represents lysogenic strains in each group.

Many phages had a broad host range and infected vaginal *Lactobacillus* strains of multiple species, including *L. crispatus*, *L. jensenii*, *L. gasseri*, *L. fermentum*, and *L. vaginalis*. Among the obligate anaerobic lactobacilli, the American collection had mostly *L. gasseri* strains, while the Turkish collection had mostly *L. jensenii* strains. They were equally high in the rate of phage lysogeny. After infection of 100 million *Lactobacillus* cells by these phages (multiplicity of infection, 1:10), no survival colonies or lysogens could be observed, indicating lytic infection. Nearly all temperate phages in the two collections lytically infected other sensitive lactobacilli.

Phage morphology. The electron micrograph (Fig. 2) showed two major morphotypes, Bradley (5) type A and B, among the 67 phages studied to date. Bradley type A is characterized by a hexagonal head and a tail with a contractile sheath. The first type, represented by ϕkc21T and ϕkc12a, belongs to Bradley phage type A (5), because both phages had a contractile tail sheath. However, there was a difference between the two phages in the head size and tail length. Additionally, ϕkc12a had a tail plate. Bradley type B is characterized by a hexagonal head and a tail without a contractile sheath. The second type, represented by ϕkc39 and ϕkc7a,

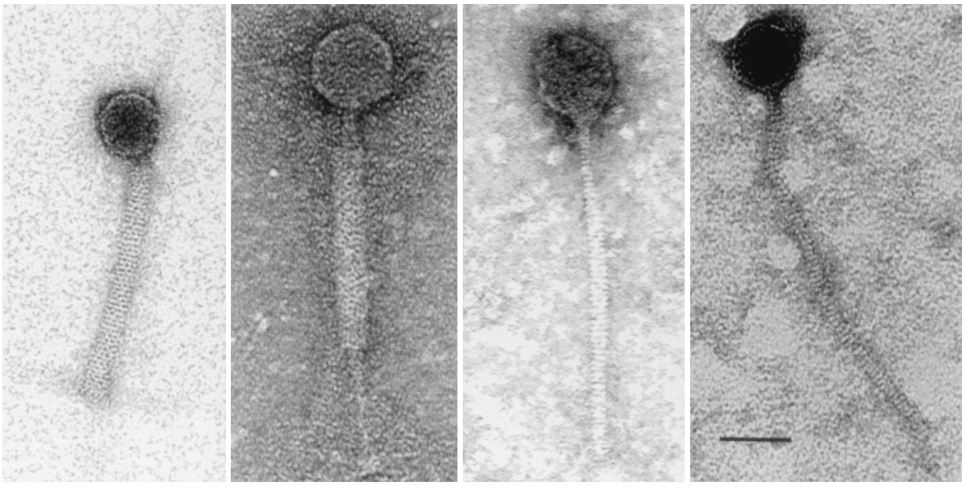

FIG. 2. Electron micrograph of vaginal *Lactobacillus* phages. A1, ϕkc21T; A2, ϕkc12a; B1, ϕkc39; B2, ϕkc7a. Bar = 50 nm.

belonged to Bradley phage type B, because both phages were lacking a contractile tail sheath, although they differed in head size and tail length. While all four types existed in the American phage collection, only three types (all but A2) were found among the phages in the Turkish collection. All four types had hexagonal heads but were of two sizes. The smaller one, type A1 and B2, was about 45 nm in diameter, and the larger one, type A2 and B1 was about 67 nm in diameter. The length and appearance of their tails were quite different. The type A1 phages had a shorter tail, about 160 nm long, which could be completely covered by a sheath with about 50 horizontal bands. The type A2 phages had a longer tail about 260 nm long and a tail plate. The sheath was about the same size as that in type A1 phages, but it had a dotted pattern instead of horizontal bands. The tail of the type B1 phages was about 250 nm in length with about 60 disks. The type B2 phages had the longest tails, about 300 nm long with about 80 disks, and also a tail fiber about 40 nm long.

Phage DNA restriction analysis. To further characterize these phages, DNA from phages representing different morphotypes were isolated, digested with *Eco*RI, and subjected to agarose gel electrophoresis (Fig. 3). The phage genomes ranged from 34 to 55 kb and were all double stranded and linear as determined by the DNA-heating agarose gel electrophoresis assay (22). The DNA fingerprints showed that most of the phages were genetically different, even among phages with the same morphotype. One identical pattern, however, was found among three phages isolated from different women. According to the protein profile analysis, the three lysogenic lactobacilli belonged to two different species. They were *L. jensenii* TL34 and TL74c and *L. gasseri* TL76.

Phage classification by DNA hybridization and PCR. DNA probes were made of complete genomic DNA of four phages, each representing different morphotypes as shown in Fig. 2. The PCR primers were designed according to the sequence data from the shotgun-cloned phage DNA fragments representing four morphotypes. The BLAST analysis of these sequences did not yield any homology with existing data in GenBank. By Southern hybridization, we found that the genome of ϕkc5a was homologous to those of ϕkc21T, ϕTL32, and ϕTL138, representing phage type A1, and the genome of ϕTL76 was homologous to those of ϕTL34, ϕTL74c, and ϕTL75a, representing phage type B2. Several homology groups were identified by additional Southern hybridization and dot blot hybridization, as well as by PCR. The results are shown in Table 4. No correlations were found between these phage types and the vaginal health status of these women, because most women who suffered from BV had no detectable lactobacilli in their vaginal samples.

DISCUSSION

BV is the most common vaginal disorder affecting women worldwide (38, 40). Since it can increase the risk of preterm delivery of low-birth-weight infants (14, 16, 17) and the risk of contracting human immunodeficiency virus in women (8, 27, 37), treatment and prevention of BV become an important issue (29). Unfortunately, the exact cause of BV is unknown. It has been well documented, however, that during BV, the normally predominant *Lactobacillus* vaginal flora is replaced with anaerobic bacteria (3, 9, 10 38, 40). Therefore, the question was raised of whether bacteriophages could inhibit lactobacilli in the vagina. We have previously reported the identification of phages in vaginal lactobacilli (32, 41). In this work, we report the study on the prevalence, genetic diversity, and infectivity of these phages from women in two geographically distant countries: the United States and Turkey.

To study whether the phage infection in vaginal lactobacilli was species specific, we first classified the species of these lactobacilli. By comparing the protein profiles of the strains of unknown species with those of known species and *Lactobacillus* type strains, most of the strains were characterized to the species level. The majority of strains from both countries belonged to three species, *L. gasseri*, *L. jensenii*, and *L. crispatus*,

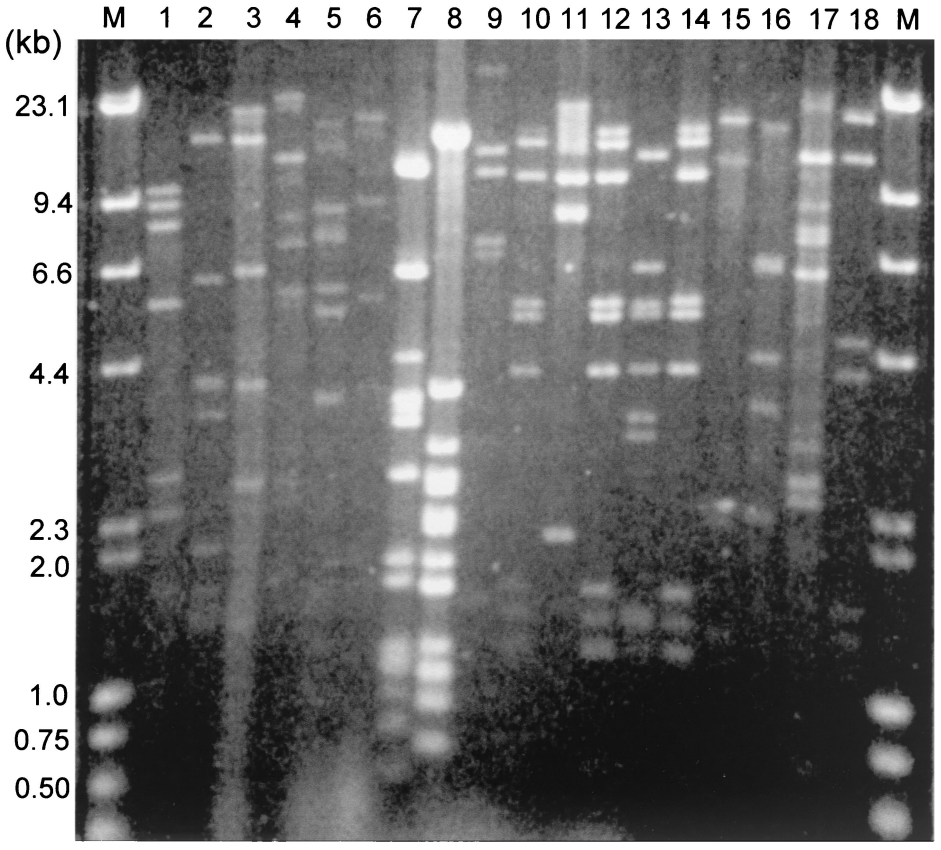

FIG. 3. DNA fingerprinting of vaginal *Lactobacillus* phages. The phage DNA were digested by *Eco*RI. Lanes: M, molecular weight DNA markers; 1, ϕkc5a; 2, ϕkc7a; 3, ϕkc12a; 4 ϕkc21T; 5, ϕkc23T; 6, ϕkc31; 7, ϕkc39; 8, ϕTL32b; 9, ϕTL33a; 10, ϕTL34; 11, ϕTL72a; 12, ϕTL74c; 13, ϕTL75a; 14, ϕTL76; 15, ϕTL122b; 16, ϕTL125, 17, ϕTL138; 18, ϕTL141. Note: lanes 10, 12, and 14 show identical DNA patterns.

with almost equal proportions. These data largely agreed with previous studies performed by DNA-DNA hybridization (4, 11, 39). The protein patterns for *L. gasseri* and *L. jensenii* were mostly consistent and reliable. *L. crispatus* was distinguished from *L. gasseri* and *L. jensenii* by having a thick band, with sizes between 40 and 60 kDa among different isolates (Fig. 1). This thick band appeared to represent its S-layer protein (19). Not only could it serve as a potential marker to differentiate *L. crispatus* from *L. gasseri* and *L. jensenii*, but it may also be used to identify different strains within the species of *L. crispatus* due to its size variability. The overall correlation between 16S rDNA data and the protein profiles was strong. The combination of these two methods offered a reliable approach to identify species of a large number of lactobacilli.

By analyzing phage host ranges and *Lactobacillus* species data, we found that many phages infected multiple *Lactobacillus* species. However, some strains remained uninfected. This implied that the phage host range in vaginal lactobacilli might not be determined by species-specific markers. Instead, certain characteristic receptors on the cell surface might determine phage host ranges. Although we do not know what may be the phage receptor on these vaginal lactobacilli, our study (data not shown) revealed that it was not the rhamnose residue of the polysaccharide on the cell surface as in the case of *L. casei* (42). Further studies are needed to identify these phage receptor molecules. Normally, a lysogenic strain is immune from infection by the same phage or the same type of phages. This is called superinfection immunity (20). However, in this study, we found that many lysogenic strains were superinfected by different phages, and some were even infected by the same phage. This suggested that the superinfection immunity might not always function in the group of vaginal *Lactobacillus* lysogens.

Our phage classification studies included electron microscopy and DNA analysis. Based on current knowledge about phage taxonomy (1), phages with similar morphology may be genetically different, but phages with different morphology are usually different in their genomics. The differences in genomic sizes and restriction patterns among the four phages (Fig. 3: lane 3, ϕkc7a, 34.5 kb; lane 4, ϕkc12a, 47 kb; lane 5, ϕkc21T, 38 kb; and lane 8, ϕkc39, 41 kb) further indicate that these four phages may be genetically different species. Although only four phage morphotypes were noticed among the 67 phages studied, additional genetic types may exist within each morphotype, because many phages did not hybridize with the probes made of the genomic DNA of these four phages. Clearly, none of these phages displayed a prolate-shaped head like that of the dairy *Lactobacillus* phage ϕy8, which was released by a *Lactobacillus* starter strain in one of the name brand American yogurts (22). The most prevalent phage morphotype was type B. Three phages showed an identical DNA fingerprinting pattern (Fig. 3), suggesting that a prevalent phage might be trans-

TABLE 4. Phage classification by electron microscopic (EM) morphology, DNA hybridization, and PCR

Phage	EM	DNA hybridization[a]				PCR[a]			
		A1 ϕkc5a	A2 ϕkc12a	B1 ϕkc39	B2 ϕkc7a	A1 ϕkc5a	A2 ϕkc12a	B1 ϕkc39	B2 ϕTL76
U.S. phages									
ϕkc5a	A1	+	−	−	−	+	−	+	−
ϕkc6a, -b[b]		−	−	+	−	−	−	+	−
ϕkc7a, -b, -c[b]	B2	−	−	−	+	+	−	+	−
ϕkc12a	A2	−	+	−	−	−	+	+	−
ϕkc13[b]		−	−	+	−	−	−	+	−
ϕkc19[b]		−	+	−	−	−	+	−	−
ϕkc31	B1	−	−	+	−	−	−	+	−
ϕkc39	B1	−	−	+	−	−	−	+	−
ϕkc58a[b]		+	−	−	+	−	−	−	−
ϕkc59a[b]		+	−	−	+	+	−	−	−
ϕkc60a[b]		+	−	−	+	+	−	−	−
ϕkc102b[b]		−	−	+	−	−	−	+	−
ϕkc109a[b]		−	−	−	+	−	−	−	−
ϕkc148[b]		−	−	+	−	−	−	+	−
ϕkc149[b]		−	−	+	−	−	−	+	−
Turkish phages									
ϕkc21T	A1	+	−	−	−	+	−	+	−
ϕkc23T	B1	−	−	−	−	−	−	+	−
ϕkc26T		+	−	−	−	+	−	−	−
ϕTL16		−	−	+	−	−	−	−	−
ϕTL32		+	−	+	−	+	−	+	−
ϕTL33a	A1	+	−	−	−	−	−	−	−
ϕTL34	B2	−	−	−	+	−	−	+	+
ϕTL56b	A1	+	−	−	−	+	−	−	−
ϕTL74c	B2	−	−	−	+	−	−	+	−
ϕTL75a		−	−	−	+	−	−	−	+
ϕTL76	B2	−	−	−	+	−	−	−	+
ϕTL87[b]		+	−	−	−	+	−	−	−
ϕTL102[b]	A1	+	−	−	+	+	−	−	−
ϕTL109a, -c[b]		−	−	−	+	−	−	−	−
ϕTL110[b]		+	−	−	−	+	−	−	−
ϕTL122b	B1	−	−	+	−	−	−	+	−
ϕTL125	B2	−	−	−	+	−	−	−	−
ϕTL138		+	−	−	−	+	−	−	−
ϕTL139a, -c[b]		+	−	−	−	+	−	−	−
ϕTL141	B2	−	−	−	+	+	−	−	−

[a] Phages that showed negative results included ϕkc36b, ϕkc38, ϕkc48, ϕkc55a, ϕkc58b, ϕkc72, ϕkc74, ϕTL25a, ϕTL35, ϕTL39b, ϕTL56c, ϕTL59c, ϕTL61a, ϕTL61b, ϕTL65, ϕTL72, ϕTL109c, ϕTL113, and ϕTL134.

[b] Hybridization was performed with the lysogenic *Lactobacillus* chromosomal DNA.

mitting among different women. Further studies will be needed to study phage transmissions.

Normally, a bacteriophage may be spontaneously released at a frequency of 10^{-6} per cell (20). A high-frequency spontaneous phage release by many lysogenic vaginal lactobacilli (about 10^{-3} to 10^{-4} per cell) is of particular interest. It suggested that a large number of free phages can be spontaneously released from these strains and found present in the vaginal secretion. This characteristic may be clinically significant, because free phages can infect other lactobacilli in the same woman or be transmitted to different women to infect their lactobacilli. This matched the clinical observation that BV, or the lack of vaginal lactobacilli, is associated with sexual transmission (38, 40). Since many vaginal lactobacilli spontaneously released phages, it suggests that lysogenic *Lactobacillus* strains may be a source of potentially infectious phages.

Among lysogenic lactobacilli that had a low spontaneous induction frequency, phages were induced by mitomycin C. Some of these phages infected other *Lactobacillus* strains under in vitro conditions. These lysogenic strains might coexist with other phage-sensitive *Lactobacillus* strains in the same vaginal environment, because they rarely released phages. However, this condition could change when the vaginal environment encounters a phage-inducing agent. We have recently reported that trace amounts of cigarette smoke chemical benzo[*a*]pyrene diol epoxide promoted phage release from lysogenic vaginal lactobacilli (33). Among women who smoke, the cigarette-associated mutagenic chemicals could reach their vaginal secretions and cause phage induction in lysogenic lactobacilli.

All phages in the present study were temperate phages released from lysogenic strains. We have so far not been able to isolate lytic phages directly from women. Truly lytic or virulent phages are usually short lived. Once they appear, the virulent phages can rapidly eliminate their host bacteria; as a result, they lose their living shelter for self-reproduction. Therefore, phages that are temperate to some bacteria but lytic to others are of concern. It is well known that some temperate phages

can become virulent due to genetic mutations (36), but it is unknown why so many temperate phages from vaginal lactobacilli can become lytic against other vaginal *Lactobacillus* strains. Probably, certain differences in the bacterial host background prohibit these phages from integrating their DNA into the chromosome of their new hosts to form lysogens (7).

In conclusion, we studied phages from vaginal lactobacilli of women in Turkey and the United States. We have determined that most of these *Lactobacillus* strains belonged to three species, *L. crispatus*, *L. gasseri*, and *L. jensenii*. Phages isolated from vaginal lactobacilli of some women lytically infected vaginal lactobacilli of other women regardless of their countries of origin. Four morphotypes were identified among these phages, and their host range was broad and beyond any particular *Lactobacillus* species. Most lysogenic lactobacilli spontaneously released phages into the environment at varied frequencies. This suggested that lysogenic lactobacilli could be a source of infective phages. Although the phage infection observed in vitro may not necessarily indicate that the same situation could happen in vivo, the results imply that vaginal lactobacilli may be eliminated or repressed by phages. This implication may be important for studying the etiology of BV due to its association with a decrease in vaginal lactobacilli. Apparently, further studies with an increased number of clinical samples will be needed to associate phage infections in vaginal lactobacilli with women's vaginal health.

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