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Bacteriophage induction versus vaginal homeostasis: role of H₂O₂ in the selection of *Lactobacillus* defective prophages

Rebeca Martín,[1] Nora Soberón,[1] Susana Escobedo,[1] Juan E. Suárez[1,2]*

[1]Microbiology Area, University Institute of Biotechnology, University of Oviedo, Oviedo, Spain. [2]Institute of Dairy Products of Asturias (CSIC), Villaviciosa, Spain

Summary. Vaginal disorders associated with systemic chemotherapy arise by direct inhibition of the resident microbiota (dominated by lactobacilli) or, possibly, by induction of prophages harbored in their genomes, leading to cell lysis. In the present study, proficient *Lactobacillus* phages could not be isolated from vaginal exudates. However, lysogeny appeared to be widespread, although about half of the strains harbored prophage sequences that were not responsive to SOS activation. In other cases, prophage induction was achieved, but viable phages were not generated, despite the fact that the induced supernatants of some strains were bactericidal. In one case, this activity was accompanied by the production of a bacteriophage subsequently identified as a member of the family *Siphoviridae* (isometric capsid and long non-contractile tail). Most of the lactobacilli tested generated hydrogen peroxide, which acted as an inducer of the SOS response, suggesting that H₂O₂ selects for strains that harbor SOS-insensitive, defective prophages, which are thus unable to promote vaginal lactobacilli phage-induced lysis. [**Int Microbiol** 2009; 12(2):131-136]

Keywords: *Lactobacillus* · bacteriophages of *Lactobacillus* · prophages · vaginal microbiota · SOS response

Introduction

Lactobacilli are usually found as part of the normal microbiota that colonize the internal cavities of the body, especially the intestines and the vagina. Proof of their major role in the preservation of vaginal health is the fact that they account for more than 70% of all microorganisms isolated from vaginal exudates of healthy, fertile women, and frequently they are virtually exclusive [7,23]. Lactobacilli exert their protecting role through two main mechanisms: blockage of pathogen colonization and production of antimicrobial substances [15].

*Corresponding author: J.E. Suárez
Área de Microbiología, Facultad de Medicina
Universidad de Oviedo
Julián Clavería, 6
33006 Oviedo, Spain
Tel. +34-985103559. Fax +34-985103148
E-mail: evaristo@uniovi.es

Vaginal lactobacilli adhere specifically to the underlying epithelium and form a biofilm that masks the mucosal receptors. Furthermore, lactobacilli co-aggregate with vaginal pathogens, which enhances the antimicrobial effect of the lactic acid produced as a result of their obligate fermentative metabolism of sugars [3]. In addition, a substantial proportion of vaginal lactobacilli produce hydrogen peroxide, which likewise contributes to their antimicrobial action [2,7,19,28].

The beneficial role of lactobacilli is also evidenced by the fact that the development of pathology is almost always accompanied by their depletion. However, it is not clear whether this is a predisposing circumstance or a consequence of pathogen invasion [9,26]. The first hypothesis is backed by reports indicating that systemic treatment of infections and/or cancer increases the frequency with which vaginal disorders appear [5,29]. This suggests that the chemotherapeutic agents used to treat systemic or neoplastic disease should permeate the vaginal surface and inhibit the develop-

ment of the lactobacilli (most antineoplasic drugs are also active on gram-positive bacteria). However, there is an alternative explanation for the observed situation. Most anticancer drugs inhibit DNA replication, as quinolones and other antimicrobials do. Bacteria (in this case lactobacilli) react against the arrest of DNA metabolism by inducing the SOS response and they do so at drug concentrations that are usually lower than the minimum inhibition concentration (MIC) [12,27]. The SOS response includes the induction of resident prophages from their lysogenic hosts which, upon resumption of the lytic cycle, result in the lysis of the host and generation of a progeny able to infect neighboring cells [30]. The final outcome of this process is the vulnerability of the mucosa towards colonization by undesirable microorganisms. In fact, bacterial vaginosis is more frequent among smokers [10] due to the presence in their vaginal exudates of toxic tobacco components [25], one of which induces prophages harbored by lactobacilli [22].

Furthermore, the SOS response may also be triggered by β-lactams such as ampicillin and cephalexin [20], and it has been shown that treatment of lysogenic lactococci with a cell-wall-inhibiting bacteriocin induces the lytic cycle of resident prophage [16].

In this work we addressed the question of whether prophage induction plays a significant role in the increase in vaginal disorders observed after systemic treatment of unrelated pathologies. We therefore asked how common lysogeny among vaginal lactobacilli was and whether the prophages were responsive to SOS activation. Finally, we tested whether H_2O_2 was an inducer of the SOS response for vaginal lactobacilli, which might contribute to the selection of strains devoid of proficient prophages.

Materials and methods

Microorganisms and culture conditions. All 45 strains of lactobacilli used in this work were collected from vaginal swabs of reproductive-aged women. These strains consisted of: *Lactobacillus crispatus* (21 isolates), *L. jensenii* (17), *L. gasseri* (6), and *L. plantarum* (1) [19]. The lactobacilli were grown on MRS agar (Oxoid) supplemented with either 1% hemoglobin (Sigma, Madrid, Spain) (MRS-H) or 20 μg hemin/ml (Sigma) and incubated at 37°C under a 10% CO_2 atmosphere. Liquid cultures in MRS broth were incubated at 37°C without agitation.

Analysis of vaginal exudates for the presence of *Lactobacillus* phages. The enrichment procedure for phage isolation was established as follows: vaginal exudates were incubated overnight in liquid MRS with mixtures of the 45 above-described strains of lactobacilli as possible hosts [19]. The cultures were centrifuged and the enrichment procedure was repeated using the culture supernatants as the phage source. The resulting supernatants were filtered, and drops were placed on top of lawns of each of the bacterial strains on MRS-H agar. When growth became visible, the plates were inspected for the presence of clearings.

Prophage induction. Exponential cultures of all lactobacilli (OD_{600} = 0.4) were treated with 1.25 μM mitomycin C [27], and incubation was then continued for up to 5 h. Aliquots of the supernatants of these cultures were transferred to lawns of each vaginal isolate growing in soft MRS-H (0.75% agar) placed on top of solid MRS-H plates. After incubation for 24 h, the cultures were inspected for inhibition halos or plaques of lysis. In addition, cultures of strains harboring mitomycin-C-inducible prophages were tested for induction with H_2O_2 at concentrations ranging from 0.1 to 5 mM (final concentration).

Polymerase chain reactions. PCR was performed with the primers and conditions specified for tailed *Lactobacillus* phages in [14]. Real-time PCR was carried out as described previously [27] using the PCR Q SYBR Green Supermix (Bio-Rad). The data obtained were recorded as Ct values; i.e., the cycle number during which the fluorescence signal crossed the threshold set by the manufacturer of the thermocycler.

Purification and visualization of virions. Liquid cultures of three representative strains were set in 1 l of MRS liquid medium and induced as indicated above. The medium was centrifuged at low speed to eliminate the cells and debris, and the phages were precipitated with polyethylene glycol (8000 Da; Sigma). The pellet was suspended in SM buffer, layered on top of a preformed CsCl gradient and centrifuged for 3 h at 35,000 rpm in a SW40 Beckman rotor. The resulting band was subjected to continuous CsCl gradient centrifugation for 24 h under the same conditions [24]. For electron microscopy, drops of purified phage suspensions, dialyzed against SM buffer, were placed onto Formvar-coated copper grids shadowed with carbon, and then negatively stained with 2% (w/v) uranyl acetate. Micrographs were taken in a Jeol 1200 EXII electron microscope at an acceleration of 80 kV. Photographs were taken on Kodak SO-163 plates. The dimensions of the phages are the mean ± standard deviation obtained after measuring at least 25 particles.

Results

Search for *Lactobacillus* phages in vaginal exudates. A two round enrichment procedure was set up using vaginal exudates as sources of viruses, and mixtures of 45 strains of lactobacilli previously isolated from the vaginas of healthy, fertile women, as possible hosts [19]. Drops of the culture supernatants from the second enrichment round were placed on top of lawns of all 45 isolates, but no inhibition halos or plaques of lysis were observed.

Testing for lysogenic bacteria. The search for lysogenic bacteria was performed by treating exponential cultures of all vaginal lactobacilli with mitomycin C to induce the lytic cycle of prophages that respond to activation of the SOS response. The culture supernatants of *L. crispatus* Lv3 and of *L. jensenii* Lv39 consistently gave rise to zones of inhibition on lawns of other strains. Three other strains of *L. crispatus* and one of *L. gasseri* also produced halos, although sporadically. However, upon dilution, these supernatants never gave rise to plaques of lysis, indicative of infection by proficient phages able to generate a progeny. These bactericidal activities were lost upon heating of the supernatants to

Table 1. Number of strains of each *Lactobacillus* species that supported amplification with probes specific for the phage morphotypes A1 to B2 [14]

	A1	A2	B1	B2	B1 + B2	Total
L. crispatus	1	2	0	0	1	4
L. gasseri	3	0	0	0	0	3
L. jensenii	0	1	3	0	4	8
L. plantarum	0	0	0	0	0	0

60ºC for 15 min. These data excluded bacteriocins, the other antimicrobial agents found in lactic acid bacteria, which are usually extremely heat resistant and not responsive to SOS activation [8,21], and suggested that some of the strains might harbor defective temperate phages.

To test the defective prophage possibility further, total DNA of the 45 strains was subjected to PCR, using primers specific for the morphotypes A1 and A2 (*Myoviridae*) and B1 and B2 (*Siphoviridae*) of vaginal lactobacilli [14]. PCR amplification was obtained in 20 out of the 45 reactions, which corresponded to 15 strains, the distribution among species being non-random (Table 1). A1-related sequences were detected mainly in *L. gasseri* isolates (3 of 4 cases) while B1 and B2 amplicons were almost exclusive to *L. jensenii* (7 of 8). Note that no strain harbored sequences specific for B2-like phages exclusively, but five isolates gave positive amplifications with the B1 and B2 primer pairs. In contrast, neither A and B nor A1 and A2 related sequences were found together in any one strain.

One of the two strains (*L. jensenii* Lv39) that consistently showed antimicrobial activity (see above) was positive by PCR with the B1 and B2 primer pairs, while the other (*L. crispatus* Lv3) did not yield any amplification at all. In addition, only *L. gasseri* Lv36, out of the other four strains that sporadically produced bactericidal supernatants, generated an A1-specific amplicon.

Inducibility of the resident prophages. To determine how many of the prophage specific sequences found were still responsive to activation of the SOS response, an analysis of the induction kinetics was undertaken through real-time PCR, using the primers that had previously rendered a positive amplification. Samples were taken at intervals from mitomycin-C-treated cultures and the data obtained compared with those from control cultures without the drug. Seven of the fifteen strains showed amplification, among which was *L. jensenii* Lv39, as expected based on the antimicrobial effect of its mitomycin-C-treated cultures. The other strains positive for prophage sequence amplification never produced inhibition halos, although the DNA of some, such as *L. jensenii* Lv59,
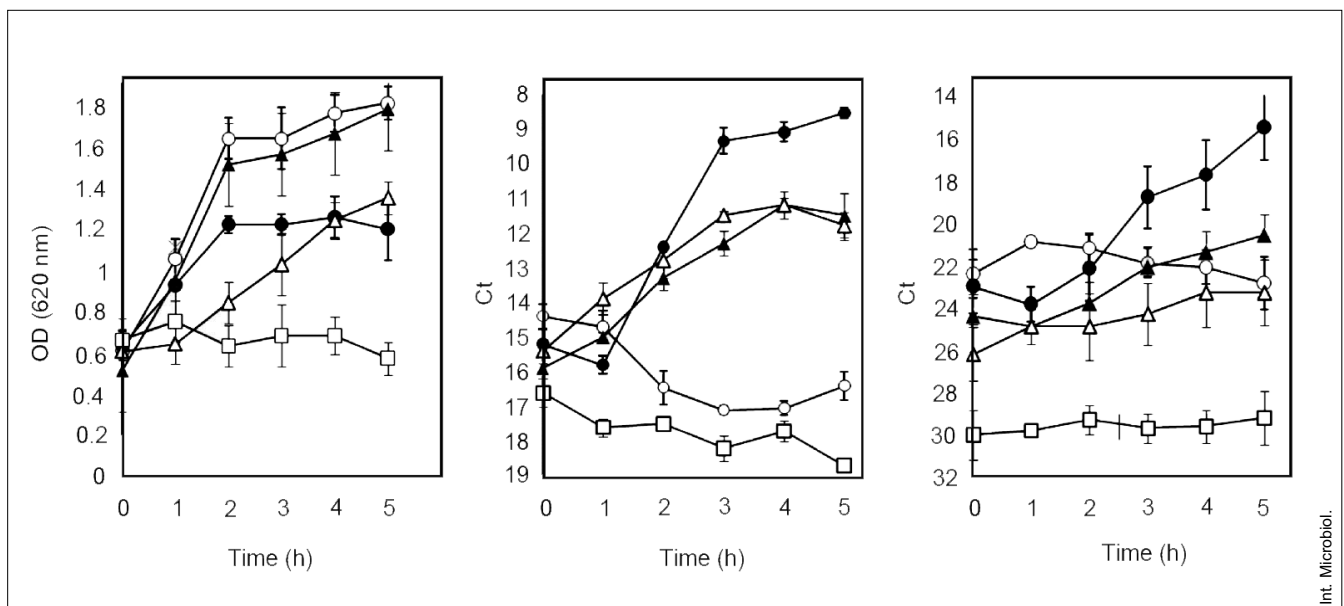

Fig. 1. Effect of mitomycin C and H$_2$O$_2$ on the growth and activation of the SOS response of exponentially growing cultures of *Lactobacillus jensenii* Lv59. (**A**) Evolution of the absorbance of the cultures. (**B,C**) Kinetics of induction of, respectively, B1- and B2-specific sequences, as measured by the increase in the DNA concentration detected through real-time PCR. Note that in the ordinate axis the Ct values are depicted in descending order to provide a more intuitive representation (the lower the Ct value the higher the initial concentration of the DNA segment being amplified). Error bars represent the standard deviations of three independent experiments. Symbols: untreated cultures (open circles); cultures with 1.25 μM mitomycin C (closed circles); and cultures with 0.1 mM (closed triangles), 1 mM (open triangles), and 5 mM H$_2$O$_2$ (open squares).
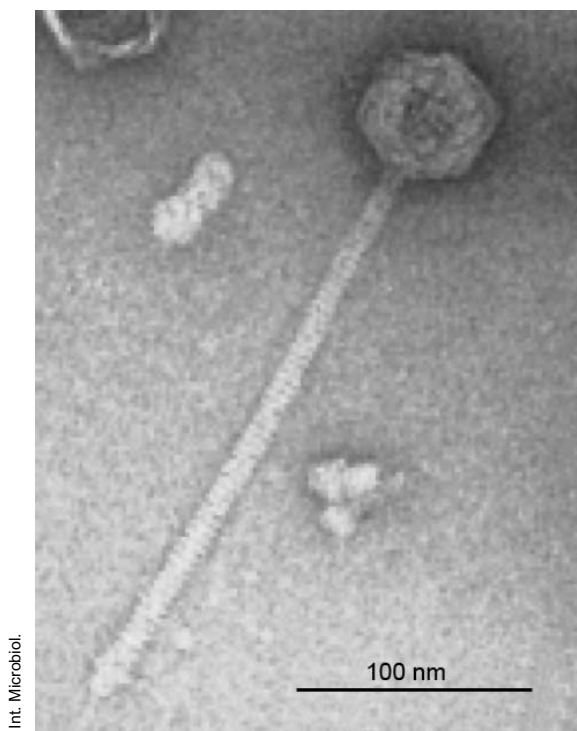
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100 nm

Fig. 2. Electron micrograph of phage Lv1 obtained upon treatment of its host, *L. jensenii* Lv39, with mitomycin C.

gave induction with the B1- and B2-specific primers (Fig. 1). Hydrogen peroxide has been previously found to induce the lytic cycle of prophages resident in *Escherichia coli* and in *Lactobacillus casei* lysogens [11,27]. Since 70% of the strains used in this work produced the compound [19], we tested whether its addition to the cultures resulted in activation of their SOS response. It was found that H_2O_2, when used at concentrations between 0.1 and 1 mM, induced replication of the resident prophages, although to a lesser extent than mitomycin C (see Fig. 1 for comparison in the case of *L. jensenii* Lv59). No phage-specific DNA synthesis was detected at 5 mM H_2O_2, the growth of the cultures being immediately inhibited.

Phage isolation. To better understand the relationship between the antimicrobial activities observed and the induction data, a protocol for phage extraction and purification was applied to cultures of three representative strains treated with mitomycin C: *L. crispatus* Lv3, that was inhibitory but that did not render a positive PCR with any of the primer pairs used; *L. jensenii* Lv39 from which, in addition to being inhibitory, B1 and B2 related sequences were amplified; and *L. jensenii* Lv59 that, in spite of generating positive PCRs with both primer pairs, did not show any antimicrobial activity.

Bands were observed from all three strains after CsCl gradient centrifugation. Recovery of the bands followed by elec-

tron microscopy showed that only the band corresponding to *L. jensenii* Lv39 revealed recognizable virions (Fig. 2). The resulting phage, named Lv1, had an isodiametric head and a non-contractile tail, which allowed its classification into the family *Siphoviridae*, and a spike at the tip of the tail. The capsids had a diameter of 58.8 ± 0.7 nm, while the tails were 239.3 ± 2.3 nm long by 10.76 ± 0.4 nm wide. When the viral DNA was subjected to PCR, only the B1-specific primers produced amplification. Finally, the purified phage suspensions maintained the antimicrobial activity shown by the culture supernatants of the host, *L. jensenii* Lv39, without rendering single plaques upon dilution, which strongly suggested that Lv1 is a defective bacteriophage.

Discussion

Oral administration of antibiotics frequently results in disturbance of the intestinal microbiota and in the establishment of opportunistic infections such as pseudomembranous colitis and enteritis of various etiologies [6,17]. Direct instillation of antimicrobials into the vaginal cavity to treat urogenital infections does not usually produce similar side-effects because the resident lactobacilli are intrinsically resistant to metronidazole and clindamycin, the antibiotics most commonly used to treat bacterial vaginosis and trichomoniasis, as

well as to the antimycotics used to treat candidiasis [19]. However, the extreme susceptibility of lactobacilli towards other drugs, such as β-lactams, macrolides, and tetracyclines [19], might lie behind the observed increase in vaginal disorders associated with the treatment of systemic infections and tumors [5,29]. Permeation by the drugs of the vaginal exudates might inhibit growth of the resident lactobacilli and/or induce prophages harbored in their genomes through activation of the SOS response, thus allowing colonization by undesirable microorganisms. To test whether prophage induction played a significant role in the vaginal pathology observed after systemic chemotherapy, we examined the frequency of lysogeny among vaginal lactobacilli, bearing in mind its high occurrence among environmental strains [4]. Since prophage induction would render free phages, the associated question on their abundance in the vaginal ecosystem was also addressed.

Forty-five strains of vaginal lactobacilli, whose probiotic properties had been previously determined [19], were used as sources and as hosts of phages. In spite of having carried out two successive enrichment rounds, we were unable to isolate from vaginal exudates phages capable of generating progeny on any of the strains. Nonetheless, lysogeny appeared to be common among these vaginal lactobacilli, since 15 of them contained sequences amplifiable with phage-specific primer pairs and one even produced bacteriophage particles. Of these 15, less than half were responsive to induction with SOS promoting agents, indicating that the prophages harbored by the rest had lost genes essential for development of a lytic cycle. However, the primers used probably did not reveal the entire prophage diversity of the population, because some strains that did not support phage-DNA amplification inhibited the growth of others upon induction of the SOS response. This was the case with *L. crispatus* Lv3. Its induced supernatants were bactericidal but did not render any virion-like structures, thus suggesting that the residing prophage had lost the morphogenetic cluster but remained responsive to SOS activation. At the end of lytic development, the production of two enzymes, holin and lysin, is a prerequisite for cell lysis [31]. These enzymes might have acted on the test bacteria and generated the inhibitory halos observed. On the other hand, Lv1, which resulted from induction of *L. jensenii* Lv39 cultures, might be a proficient virus for which no host was found or, more probably, a defective phage unable to produce infective virions. In either case, its lytic activity on other strains would have been a consequence of "lysis from without," promoted by disruption of the peptidoglycan layer by muralytic activities located at the tip of phage tails [1,13], in addition to the already quoted generation of soluble lytic enzymes.

The absence of any proficient prophage in the vaginal lactobacilli analyzed might have been a consequence of their frequent generation of H_2O_2. After cultivation of the 45 strains tested for 8–16 h, a substantial proportion generated H_2O_2, the upper limit for the strongest producers being 3 mM (unpublished data). If this also happened in the vaginal environment, the prophages of any lysogens reaching the vagina would enter the lytic cycle, causing lysis of the host and the infection of non-lysogenic neighbors. These events would represent a strong selection mechanism leading to the survival of cells that harbor defective prophages. In this respect, the different situations encountered in this study might represent successive steps in the bacteriophage's "masking evolution": the production of non-infective virions; DNA replication in response to SOS activation, but with no generation of recognizable viral structures; and the absence of any reaction to SOS induction.

It appears then, that far from our initial hypothesis, the induction of resident prophages, by systemic chemotherapy, irradiation, or other means, does not play a significant role in elimination of the vaginal microbiota. This represents an unexpected benefit to the human host, derived from H_2O_2 production by the resident lactobacilli, which adds to the already known antiseptic effect of this compound.

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