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# Phosphatidylinositol-specific phospholipase C activity in *Lactobacillus rhamnosus* with capacity to translocate

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**Abstract**

Phosphatidylinositol-specific phospholipase C (PI-PLC) activity was investigated in 25 different lactic acid bacteria (LAB) strains belonging to the genera *Lactobacillus*, *Weisella*, and *Enterococcus*. PI-PLC activity was detected in 44% of the strains studied in culture medium without carbon source. From the PI-PLC positive strains, *Lactobacillus rhamnosus* ATCC 7469 was selected for translocation studies. Healthy mice were orally administered with a daily dose of $2.0 \times 10^9$ of viable *L. rhamnosus* suspension. Viable bacteria were detected in liver and spleen of mice fed with LAB for 7 days. Bacterial colonies isolated from liver were biochemically characterized, and further subjected to randomly amplified polymorphic DNA. Amplification patterns of five strains displayed identical profiles to *L. rhamnosus*. PI-PLC activity was determined in the strains recovered from liver. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

*Keywords:* Phospholipase; Translocation; Probiotic; *Lactobacillus*

## 1. Introduction

Lactic acid bacteria (LAB) are recognized as members of gastrointestinal healthy microflora from humans and animals, and also as major components of microflora involved in food fermentation [1]. During the last decades, there has been a renewed interest in developing probiotic foods using LAB as additives to enhance human and animal health [2]. Extensive, time-consuming, and expensive safety studies are necessarily performed in LAB strains for potential use in food products. It is important to confirm the safety of any identified probiotic strains, particularly due to the increased number of reports of their association with human infections, mainly in the last decade [3–5]. Thus, a number of in vitro and in vivo assays must be performed for the selection of microbial strains to be used as probiotics. One particularly important assay relates to the bacterial ability to translocate. Bacterial translocation is defined as the passage of viable bacteria from the gastrointestinal tract to mesenteric lymph nodes, spleen, liver, and other target organs [6]. Bacterial capability to translocate, survive, and proliferate in extra-intestinal tissues has been postulated as a potential source of sepsis in susceptible subjects [3,7]. Bacterial translocation process involves complex interactions between host defense mechanisms, and also the bacterial ability to invade host tissues [8], but the precise mechanism (or mechanisms) involved remains unclear.

Bacterial phospholipases are a large group of enzymes with a wide range of effects, described both in vivo and in vitro, from minor alterations in cell membrane composition to increased vascular permeability and lethality at low concentrations [9]. Phospholipase activity has been described in a number of microorganisms including *Bacillus cereus*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, and others [10–13]. Also, phospholipase $A_2$ activity was described in *Lactobacillus* sp. from human samples [13]. However, to our knowledge no references have been previously reported concerning other phospholipases in LAB.

Considering both the role of phospholipases in bacterial pathogenesis, and the capability of some LAB to trans-

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locate, the presence of phosphatidylinositol-specific phospholipase C (PI-PLC) in microorganisms belonging to the genera *Enterococcus*, *Lactobacillus*, and *Weisella* was investigated. The results of this report provide, for the first time, evidence of PI-PLC activity displayed by one *Lactobacillus rhamnosus* strain with ability to translocate.

## 2. Materials and methods

### 2.1. Bacteria and culture conditions

LAB strains used for this investigation are listed in Table 1. *Lactobacillus*, *Weisella*, and *Enterococcus faecalis* (from monkey vagina) strains were provided by CERELA Culture Collection. Other three *Enterococcus* strains isolated in our laboratory were used as positive controls of spleen and liver translocation since they induced translocation when they were administered to healthy mice (unpublished results). Three pathogenic strains of *L. monocytogenes*, strain Scott 'A', and strain 4ab (food origin, from IHT, Karlsruhe, Germany), and strain FBUNT (clinical isolate, from Facultad de Bioquímica, Química y Farmacia-UNT, Tucumán, Argentina) were used as positive controls of PI-PLC activity. The strains were grown in MRS medium [14], LAPTg medium [15], and TY medium containing 1.0% tryptone, 0.5% yeast extract, 1.0% NaCl. Microbial cultures were incubated at 37°C for 24 h.

### 2.2. Detection of PI-PLC activity

The strains were spot-inoculated in the media described above supplemented with 1.0% (w/v) agar. Cultures were incubated for 24–48 h at 37°C under anaerobic conditions. Thereafter, PI-PLC activity was detected by overlaying the plates with 20 mg $l^{-1}$ L-α-phosphatidylinositol substrate in 1.4% (w/v) agarose and incubated at 37°C up to 5 days. PI-PLC active strains showed a turbid halo around the colony by the release of the insoluble diacylglycerol from the substrate [16].

### 2.3. Animals and feeding procedures

Balb/c mice, each weighing between 23 and 25 g, were housed and treated at CERELA. Each experimental group consisted of six animals for each dose assayed. One group of animals was fed with *L. rhamnosus* ATCC 7469 (provided by CERELA Culture Collection as strain CRL 932). The microorganism was cultured on MRS broth for 18 h and then concentrated by centrifugation. The cell pellet was washed twice with sterile saline solution, resuspended in 5.0 ml of sterile non-fat milk and given at 15% (v/v) in drinking water. The number of viable cells (colony-forming units (CFU) per ml) was determined by the agar plate method. Mice were orally administered with $2.0\times10^9$ of *L. rhamnosus* ATCC 7469 daily suspensions for 2, 5, and 7 days. Another group of mice was fed with suspensions of *L. rhamnosus* CRL 534 without PI-PLC activity, given

Table 1
LAB strains used for detection of PI-PLC activity

| Species | Strains | Source | PI-PLC activity |
|---|---|---|---|
| *L. acidophilus* | CRL 1014 | infant feces | + |
| | CRL 745 | unknown | + |
| *L. animalis* | $L_3$ | chicken gastrointestinal tract | − |
| *L. brevis* | CRL 1038 | human feces | − |
| | CRL 772 | commercial flour | − |
| | CRL 376 | regional cheese | − |
| *L. buchneri* | CRL 578 | infant feces | − |
| | CRL 925 | tomato pulp | − |
| *L. casei* | CRL 431 | infant feces | − |
| | CRL 1110 | infant feces | + |
| *L. fermentum* | 27 A | chicken gastrointestinal tract | − |
| *L. fermentum* subsp. *cellobiosus* | 408 | chicken gastrointestinal tract | − |
| *L. paracasei* subsp. *paracasei* | CRL 575 | infant feces | − |
| | CRL 577 | infant feces | − |
| *L. rhamnosus* | CRL 932[a] | unknown | + |
| | CRL 534 | human feces | − |
| | CRL 576 | infant feces | + |
| | CRL 536 | human feces | + |
| | CRL 799 | unknown | − |
| *W. confusa* | CRL 570 | infant feces | + |
| | CRL 572 | infant feces | + |
| *E. faecalis* | LU204 | human feces | + |
| | LU223 | human feces | + |
| | LU224 | human feces | + |
| | CRL 41 | monkey vagina | − |

[a] *L. rhamnosus* ATCC 7469.

under the same conditions as those used for the *L. rhamnosus* ATCC 7469 assays. A third group of mice was used as non-probiotic treatment control and received sterile milk in the drinking water. All mice were fed ad libitum with a conventional diet.

### 2.4. Translocation of LAB to extra-intestinal tissue

Mice were sacrificed by cervical dislocation, and the spleen and liver were aseptically removed and homogenized in 5.0 ml 0.1% peptone solution. Serial dilutions of organs were plated in triplicate in the following agarized media: experiments using LBS for enumeration of lactobacilli, McConkey for analysis of enterobacteria, and blood-supplemented brain-heart infusion broth for enumeration of anaerobic and aerobic microorganisms were performed. Bacterial growth was observed after incubation for 48–72 h at 37°C. To identify the *Lactobacillus* detected in tissues, growing microbes in LBS agar plates were subcultured in MRS broth for 24–48 h at 37°C. Microorganisms were observed under a microscope to determine bacteria morphological characteristics, and Gram reaction. Also, the catalase activity, carbohydrate utilization and growth at 37 and 45°C tests were carried out. Microorganisms that possessed similar attributes to *L. rhamnosus* strain used in this study were selected for further identification by randomly amplified polymorphic DNA (RAPD) finger-printing method, as described below.

### 2.5. DNA preparation

Microbial cultures were grown overnight at 37°C in MRS medium supplemented with 0.5% (w/v) calcium carbonate [14]. Cells from 1.5 ml culture were collected by centrifugation at 3000 rpm, and washed twice in 1.0 ml of TE buffer (10.0 mM Tris–1.0 mM EDTA, pH 8.0). The cells were resuspended in 100 μl of proteinase K (Sigma, USA) in TE buffer (400 ng $\mu l^{-1}$) and incubated at 55°C for 1 h. In order to denature proteolytic activity, the samples were incubated at 85°C for 15 min. The cell debris was removed by centrifugation, and the supernatant was kept at 4°C. Five-μl samples were used for PCR reactions (see below).

### 2.6. Identification of translocated microorganisms by RAPD

RAPD finger-printing method [17] was used to identify the microorganisms isolated from liver and spleen. RAPD patterns of suspected strains were compared with that of *L. rhamnosus* ATCC 7469.

Amplification was performed in a reaction mixture containing *Taq* buffer, 2.0 mM $MgCl_2$, 0.2 mM of the four deoxynucleoside triphosphates, 2.5 ng DNA, 1 U of *Taq* polymerase (Sigma), and 1.0 μM of the following primers (in 5′–3′ order): A1 (CCC AAG GTC C), A2 (GGT GCG GGA A), A7 (CCC GAT TCG G), B06 (GTG ACA TGC C), B07 (AGA TGC AGC C), B09 (ATG GCT CAG C), B10 (CAG GCA CTA G), and UBC#4 (CCT GGG CTG G) (Biodynamics). Twenty-five μl of reaction mix was cycled through the following temperature profile: one 5-min cycle at 94°C to denature, followed by one 1-min cycle at 94°C, one 1-min cycle at 32°C, thirty 2-min cycles at 72°C for amplification, and one 7-min cycle at 72°C. RAPD products were electrophoresed for 120 min at 45 V in a 1.2% TAE (Tris–acetate–EDTA buffer, pH 8.5) agarose gel. The gels were stained in ethidium bromide, and photographed under UV transillumination.

### 2.7. Statistical analysis

Experiments were performed in triplicate. Student's *t* test was performed in translocation experiments, taking $P < 0.01$ as significant.

## 3. Results and discussion

Twenty-five LAB isolated from different sources and belonging to the genera *Lactobacillus*, *Enterococcus*, and *Weisella* were assayed for PI-PLC activity (Table 1). Using the phosphatidylinositol overlay assay, PI-PLC activity was detected in 44% of the strains screened: *L. rhamnosus* (three positive from five strains tested), *Lactobacillus acidophilus* (two positive from two strains tested), *Lactobacillus casei* (one positive from two strains tested), *E. faecalis* (three positive from four strains tested), *Weisella confusa* (two positive from two strains tested). A typical reaction is

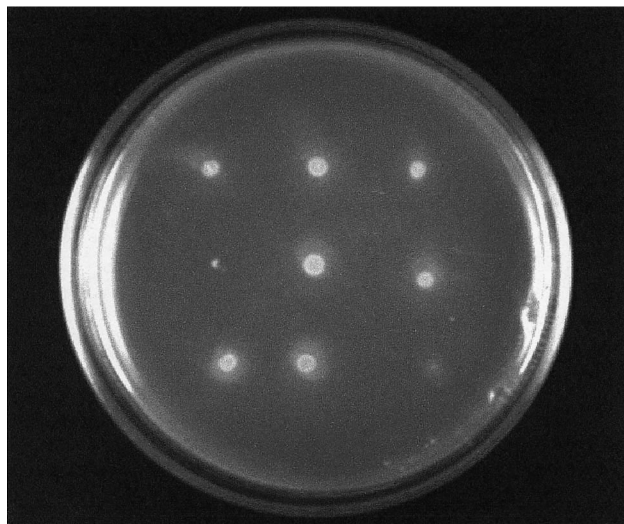

Fig. 1. Detection of PI-PLC activity in LAB strains. Bacteria were spot-inoculated on TY agar plates in three rows, listed from the top to the bottom of each row from left to right as follows: row 1, *E. faecalis* Lmc1, *L. brevis* CRL 376, *E. faecalis* Lmc2; row 2, *L. rhamnosus* ATCC 7469, *L. rhamnosus* CRL 536, *W. confusa* CRL 572; row 3, *L. monocytogenes* Scott 'A' (positive control), *L. acidophilus* CRL 745, *L. rhamnosus* CRL 576.

shown in Fig. 1. None of the *Lactobacillus* strains including *L. animalis*, *L. brevis*, *L. buchneri*, *L. fermentum*, and *L. paracasei* tested showed any PI-PLC activity under the experimental conditions assayed.

The PI-PLC activity markedly depends on the amount of carbohydrate in the culture media. When LAB were grown in media containing higher amounts of carbohydrate, MRS supplemented with 20.00 g $l^{-1}$, and LAPTg with 10.00 g $l^{-1}$ of glucose, PI-PLC activity was undetected (data not shown). However, when microorganisms were changed to TY medium containing vestiges of glucose (less than 0.03 g $l^{-1}$), PI-PLC activity was observed (Fig. 1). These results are in agreement with previous reports where bacteria showing PI-PLC, including *B. cereus*, *Bacillus thuringiensis*, *L. monocytogenes*, and *Staphylococcus aureus*, required glucose-free media for PLC activity to be detected [18–20]. Carbon source can affect the expression of virulence factors in many bacterial models manifested by changes in growth rate, metabolic pools and pH [21]. PI-PLC activity was specifically repressed by cellobiose in *L. monocytogenes* [22]. It is possible that the PI-PLC activity detected in some strains of *Lactobacillus*, and *Enterococcus*, which occurred after transfer from either MRS or LAPTg to TY medium, was a stress-induced response caused by the cells transfer to a carbon source-restricted medium. Adaptation to stress conditions was accompanied by different bacterial responses such as induction of distinctive protein sets which are essential for cell survival against environmental changes and expression of catabolic enzymes [23,24]. In particular, over-expression of proteins of various molecular masses was reported in *L. acidophilus* and *Streptococcus thermophilus* PB18 under stress growth conditions including starvation [25–27]. This kind of response could be similar to LAB adaptation to major challenges in environmental conditions observed in the intestine, where bacteria must survive to bile and pancreatic juices and interactions with other microorganisms belonging to the indigenous flora [28]. Because stress responses may play an important role in pathogenesis [21], PI-PLC activity could be regulated by these stresses that LAB encounters in the intestine.

*L. rhamnosus* was selected to perform translocation studies, because *L. rhamnosus* strains have been reported as the most common *Lactobacillus* species isolated in human infective endocarditis and bacteremia, suggesting that it may have a greater pathogenic potential than other *Lactobacillus* species [3–5]. After feeding for 7 days with *L. rhamnosus* strain CRL 534 without PI-PLC activity, no viable cells were detected in spleen and liver of mice. However, after the same period of time viable bacteria were detected in spleen and liver of mice fed with *L. rhamnosus* strain ATCC 7469 with PI-PLC activity. While Gram (+) cocci, anaerobic microorganisms and *Lactobacillus* sp. were detected in liver (Table 2), only Gram (+) cocci (3.85 log CFU $g^{-1}$ organ) were found in spleen. Fifty-one isolates from liver, cultured in LBS agar medium, were sub-cultured for identification. In order to select microorganisms with similar attributes to *L. rhamnosus*, identification tests were performed, as described in Section 2. The isolated strains were microaerophilic, catalase negative, Gram (+) rods. However, only eight out of 51 grew at 37 and 45°C. The eight strains were further tested for rhamnose fermentation: five of them were positive, and were further subjected to RAPD analysis. The five selected strains showed the same RAPD profiles (data not shown). Two from eight primers, UBC#4 and B6, displayed more than one band. Fig. 2 shows the RAPD patterns of representative bacterial cells isolated from tissue samples. Isolated strains with translocation ability displayed identical amplification pattern profiles to *L. rhamnosus* ATCC 7469. Finger-printing profiles suggest that all strains are genetically identical, in agreement with morphological and biochemical assays. The results indicated that recovered strains were identical to the reference *L. rhamnosus* ATCC 7469 strain. The phosphatidylinositol overlay assay was also used to detect PI-PLC activity in the eight translocated strains described above; enzymatic activity was detectable in all of them.

Lactobacilli are one of the most commonly used probiotic genera in human food [29]. It has been demonstrated that most *Lactobacillus* species show no pathogenicity, and no acute oral toxicity to animals [1] or humans [30]. Commercialization of probiotic strains such as *L. acidophilus* (LA-1), *L. casei* CRL 431, and *L. acidophilus*

Table 2
Translocation in liver of healthy mice after feeding with *L. rhamnosus*

| Experimental group | Log CFU $g^{-1}$ liver | | | |
|---|---|---|---|---|
| | Gram (−) rods | Gram (+) cocci | Anaerobic microorganisms | *Lactobacillus* |
| Control | N | N | N | N |
| *L. rhamnosus*[a] CRL 534 | N | N | N | N |
| *L. rhamnosus*[b] ATCC 7469 | N | 3.43 ± 0.51[c] | 3.21 ± 0.44[c] | 3.79 ± 0.40[c] |

Animals were orally administered with *L. rhamnosus* ATCC 7469 for 7 days; another group of mice was fed with suspensions of *L. rhamnosus* CRL 534. A third group of mice was used as non-probiotic treatment control and received sterile milk in the drinking water as explained in the text. Values are the mean of $n = 9 \pm$ S.D. N = Negative translocation.

[a] *L. rhamnosus* strain CRL 534 without PI-PLC activity.

[b] *L. rhamnosus* strain ATCC 7469 with PI-PLC activity.

[c] Significant differences related to the control values $P < 0.01$.

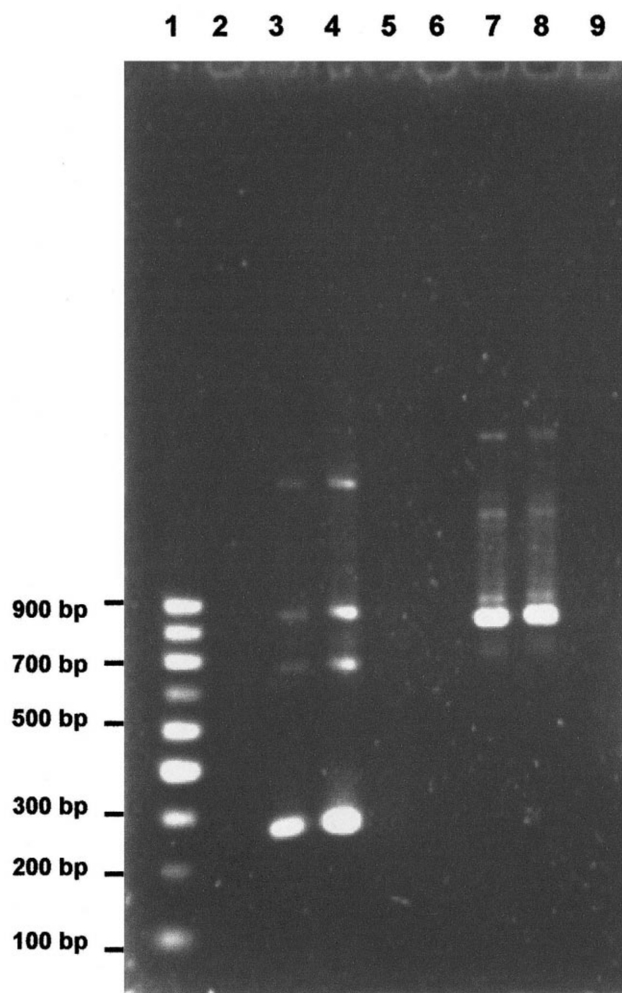


Fig. 2. RAPD patterns of strains recovered from translocation experiments, and reference strain *L. rhamnosus* ATCC 7469. Lanes: 1, ladder 100 bp; 2, negative controls without DNA; 5 and 9, controls with unamplified *L. monocytogenes* strain Scott 'A' DNA; 3 and 7, *L. rhamnosus* ATCC 7469; 4 and 8, isolated samples. Primer UBC#4 in lanes 3–5; and primer B6 in lanes 7–9.

730 into the food market has resulted in no adverse reports, which confirms that these strains are safe for human consumption [31,32]. However, little information is available concerning potential side effects such as bacterial translocation of some LAB. Perdigón and coworkers previously reported that *L. rhamnosus* administered at a dose of $10^{11}$ cells day$^{-1}$ per mouse during 7 consecutive days induced translocation in healthy mice after the second feeding days [33]. Our results showed the capability for translocation of *L. rhamnosus* ATCC 7469 administered to healthy mice. Also, presence of PI-PLC activity in this strain and other translocating strains was detected. Since bacterial translocation can be associated with potential bacterial infectivity and pathogenic degree [29], the ability for translocation should be determined in every LAB strain with probiotic attributes, before its introduction in food products. The PI-PLC activity detected in the translocated LAB tested could play an important role in the translocation process. Further research will be required to provide information concerning the specific relationship between PI-PLC activity and bacterial translocation. This work is currently underway in our laboratory.

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