Prophage induction in *Lactococcus lactis* by the bacteriocin Lactococcin 972

Running title: Prophage induction by Lactococcin 972

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Abstract

Lactococcin 972 (Lcn972) is a non- pore forming bacteriocin with a narrow spectrum of activity restricted to *Lactococcus*. Lcn972 inhibits the incorporation of cell wall precursors in the septum area, thereby inhibiting cell division. In this work, an additional inhibitory effect is described, namely, the induction of the lytic cycle of resident prophages in the lysogenic strain *L. lactis* IPLA 513. Lcn972 triggered the release of prophages in a concentration-dependent fashion. The extent of prophage induction was influenced by the physiological status of the cultures, being maximal at the early exponential growth phase. A microtiter based protocol was designed and the induction ability of several antimicrobials was compared. Prophages were activated by all cell wall biosynthesis inhibitors tested, although the levels of induction were lower than those obtained after activation of the SOS response. As far as we know, this is the first report of prophage induction by an antimicrobial peptide. Since Lcn972 is active against *L. lactis* strains currently used in commercial starters, promising applications for dairy fermentations are discussed.

Keywords: Dairy Starters, Bacteriocin, Prophage, *L. lactis*

1. Introduction

Lactococcus lactis is widely used in the production of dairy products and is the main component of the starter cultures used in cheese manufacture. The key function of *L. lactis* as a starter is to produce enough lactic acid through the fermentation of lactose, the main carbohydrate of milk. This leads to a pH decrease relevant for milk clotting and for preventing spoilage and/or pathogen development. *L. lactis* also contributes greatly to the textural and organoleptic quality of the fermented products by the synthesis of flavour compounds and texturing agents. The hygienic quality is also enhanced by the production of several antimicrobial compounds, including bacteriocins (Mäyra-Mäkinen and Bigret, 2005). Therefore, the fermentation process relies, basically, on the optimal performance of the starter.

One of the major leading causes of food fermentation failures is the presence of phages. Prophages in *Lactococcus* seem to be quite common and many strains possess one or more prophages integrated in their genome (Bolotin et al. 2001; Ventura et al. 2007). Prophages may be induced under several stress conditions. The most studied is the activation of the SOS response by DNA damage. However, other environmental conditions were shown to activate the lytic cycle of temperate phages although the molecular pathways remain largely unknown for phages infecting *L. lactis* (Feirtag and McKay, 1987; Lunde et al. 2005; Meijer et al. 1998).

Bacteriocins are ribosomally synthesised antimicrobial peptides which usually kill the target bacteria by forming pores in the cytoplasmic membrane (for a review see Cotter et al. 2005). Bacteriocin production is a common feature among lactic acid bacteria and bacteriocin producers are often isolated from dairy products (Cleveland et al. 2001; Stiles, 1996). Lactococcin 972 is a non-modified 66-aa hydrophilic bacteriocin synthesised by *L. lactis* IPLA 972 and bactericidal to lactococci. In contrast to most

64 LAB bacteriocins described so far, Lcn972 does not form pores in the cytoplasmic
65 membrane of susceptible cells. Cells treated with Lcn972 do not suffer either leakage of
66 cytoplasmic solutes or significant inhibition of their macromolecular synthesis.
67 However, incorporation of cell wall precursors in actively growing cells shifts from an
68 exponential to a linear mode and subsequently becomes arrested (Martínez et al. 1996,
69 2000). Treated cells suffer a gross change in cell size and shape and no septum is
70 formed. Recently, it has been demonstrated that Lcn972 tightly binds to the cell wall
71 precursor lipid II (Martínez et al. 2008).

72 While it is widely recognized that bacteriocin production may enhance the hygienic
73 quality of the fermented products by inhibiting undesirable bacteria, little is known
74 about putative adverse effects on starter performance. In this work we have assayed the
75 ability of the Lcn972 to induce prophages in *L. lactis.* We show that Lcn972 and other
76 cell wall antimicrobials act as prophage inducers in a concentration dependent fashion
77 in early exponentially growing *L. lactis*.

2. Material and methods

2.1. Bacterial strains and culture conditions.

Lactococcus lactis IPLA 513 (Cuesta et al. 1995) and *L. lactis* MG1614 (Gasson, 1983) were used as lysogenic and phage susceptible strains, respectively. *L. lactis* strains from commercial starters were previously isolated by Madera et al. (2004). The lysogenic *L. lactis* IMN-C1814 carrying ΦLC3 as a prophage (Lunde *et al*., 2003) was kindly provided by I.F. Nes (Agricultural University of Norway). Cultures were grown in M17 broth (Scharlab, Barcelona, Spain) supplemented with 0.5% (v/v) glucose (GM17) at 32 °C. Stock cultures were maintained at -80 °C in broth containing 20% (v/v) glycerol and propagated from an isolated colony before each experiment.

2.2. Bacteriocin purification and quantification.

Lactococcin 972 (Lcn972) was extracted from supernatants of late exponential phase cultures of *L. lactis* IPLA 972 as previously described (Martínez et al. 1996). The bacteriocin activity was quantified by the agar diffusion test. Twofold serial dilutions of Lcn972 in 50 mM sodium phosphate buffer, pH 6.8, were tested. The antimicrobial activity was defined as the reciprocal of the highest dilution that produced a clear zone of growth inhibition on the indicator lawn (Arbitrary Units, AU/ml).

2.3. Minimum Inhibitory Concentration (MIC).

The susceptibility of *L. lactis* strains was determined by the agar diffusion test as described in 2.2. Plates were inoculated with 10^5 cfu/ml from overnight cultures. MICs were defined as the lowest concentration that produced a clear inhibition halo.

2.4 Prophage induction and plaque assay.

The lysogenic strain *L. lactis* IPLA 513 was grown to the early exponential phase (Optical Density, $OD_{600\ nm}$=0.2) in GM17 broth. Cells were washed with ¼ Ringer solution (Merck, Damstadt, Germany) and resuspended in the same volume of fresh broth. Different concentrations of Lcn972 were added, followed by further incubation at 32 ºC for 2 h. Samples were taken and the evolution of viable bacteria and the production of a viral progeny were determined. Viable cells were enumerated on GM17 agar 2%. Culture supernatants were sterilized by using 0.2-µm-pore-diameter cellulose acetate filters (VWR International, Barcelona, Spain). For plaque assays, serial dilutions of the supernatants were mixed with 100 µl of an overnight culture (10^9 cfu/ml) of the sensitive strain *L. lactis* MG1614 in top GM17 agar 0.7% and immediately poured onto GM17 plates containing 10 mM $CaCl_2$ and 10 mM $MgSO_4$. The plates were inspected for plaques of lysis after incubation for 24 h at 32 ºC. To test prophage induction from cells at different physiological status, *L. lactis* IPLA 513 was grown at the desired $OD_{600\ nm}$, namely, at 0.2 (early-), 1.0 (middle-) and 2.0 (late-exponential growth phase). Cells were collected, washed and adjusted to an $OD_{600\ nm}$ of 0.2 in fresh broth in order to treat the same number of cells with Lcn972 (20 AU/ml), independently of the initial OD of the culture. Controls without Lcn972 were also carried out. Prophage induction factor was defined as pfu/ml of treated culture divided by pfu/ml of untreated culture.

2.5. Microtiter-based prophage induction.

Mitomycin C, bacitracin, penicillin G, chloramphenicol and mutanolysin were purchased from Sigma-Aldrich (St Louis, MO, USA) and lysozyme from USB (Cleveland, OH, USA). Plantaricin C was purified as previously described (Wiedemann et al. 2006). Stocks were made fresh in sterile distilled water at four-times the desired

128 concentration: 2 mg/ml for lysozyme, 40 µg/ml for bacitracin and mutanolysin, 20
129 µg/ml for chloramphenicol, 10 µg/ml for plantaricin C, and 2 µg/ml for penicillin G.
130 100 µl aliquots were dispensed in the microtiter wells containing 100 µl of GM17 broth
131 and twofold dilutions were made. The microtiter plates were inoculated with 100 µl of
132 an exponentially growing culture of *L. lactis* IPLA 513 at an $OD_{600\ nm}$=0.2. Plates were
133 incubated at 32 ºC and the $OD_{600\ nm}$ was monitored every 15 min in a Benchmark
134 Microtiter plate reader (BioRad Laboratories, Hercules, CA, USA). After 210 min, the
135 plates were centrifuged for 10 min at 1000 x*g* to collect the supernatants. Phage release
136 was quantified by the plaque assay.

3. Results and Discussion

Defective cell wall synthesis was revealed as an inducer of the bacterial SOS response in *E. coli* (Miller et al. 2004). A well-known consequence of the activation of the SOS response is the activation of prophages (Little and Mount, 1982). Our hypothesis was that cell wall synthesis inhibition by Lcn972 might trigger a similar response and, thereby, Lcn972 could behave as a prophage inducer in *L. lactis*.

Viable phages were detected in the supernatants of early exponentially growing cultures of the lysogenic strain *L. lactis* IPLA 513 after treatment with Lcn972 (Fig. 1). The number of infecting virions correlated well with the increasing concentrations of Lcn972. Prophage induction became evident at Lcn972 concentrations of 20 AU/ml and over. Below this threshold, phage numbers around 3.3±0.1 log pfu/ml, which would correspond to the spontaneous induction levels, were found.

The extent of Lcn972-mediated prophage induction was also tested on cells from middle and late exponential growth phase (Table 1). Under these conditions, no effect of Lcn972 was observed. Similar phage numbers were detected in the supernatants of treated and control cultures as revealed by induction factors close to 1. The lack of phage induction at these growth stages could be related to the lower antimicrobial activity of Lcn972 against late exponentially growing cells (Martínez et al. 2000). The loss of viability after Lcn972 treatment in middle and late exponential cells was half of that observed for early exponential cells (Table 1). It was also noted that the level of spontaneous induction was higher at the end of the exponential growth phase (data not shown). This has been already reported in the literature and thought to be due to a loose chromosomal status and a higher instability of the prophage genome integrated in it (Lunde et al. 2003).

In additional experiments, phage induction by different antimicrobials was studied in order to know whether this effect was specific for Lcn972. The well known prophage

inducer mitomycin C was used as a reference. After treatment with this drug, induction factors of 102.75±20.9) were obtained, which are five times higher than those obtained by Lcn972 (data not shown). Induction factors similar to those achieved by Lcn972 were obtained with other cell wall interacting antimicrobials such as the bacteriocin plantaricin C, and the antibiotics bacitracin and penicillin G (Fig. 2). Remarkably, prophage release was not observed after treatment of lysogenic cultures with the muramidases lysozyme and mutanolysin nor with the protein biosynthesis inhibitor chloramphenicol. The former antimicrobials have in common that they inhibit cell wall biosynthesis either by interfering with lipid II (PlnC and bacitracin) or by inactivating the penicillin-binding-proteins (PBPs) which carry out the ultimate transpeptidation steps. In contrast, lysozyme and mutanolysin hydrolyze the pre-existing cell wall. Therefore, it is tempting to speculate that the stimulus that induces temperate phage excision relies on the disturbance of the lipid II cycle. Recently, we have shown that Lcn972 activates the two component system CesSR that triggers the response of *L. lactis* to cell envelope stress (Martínez et al. 2007). One of the members of this regulon is the membrane-bound protease FtsH (or HflB) which is involved in regulating the lysogenic state of phage λ (Herman et al. 1997; Ito and Akiyama, 2005). Whether FtsH plays a similar role in *L. lactis* remains to be investigated. This molecular network could act independently of the bacterial SOS response and, based on our results, the signalling cascade would lead to a weaker induction compared to SOS.

As far as we know, this is the first description of prophage release induced by a bacteriocin. This effect may be restricted to specific prophage/host systems because attempts to induce the prophage ΦLC3 present in *L. lactis* IMN-C1814 with Lcn972 failed. Exponentially growing cultures of *L. lactis* IMN-C1814 were treated with 20 up to 200 AU/ml Lcn972 but similar phage numbers were detected in the supernatants of

188 treated and control cultures (data not shown). On the contrary, mitomycin C effectively
189 induced the prophage with induction factors of 20 to 40. Other lysogenic *L. lactis*
190 strains should be tested to determine which prophages may be induced by Lcn972.

191 The presence of bacteriophages is envisaged as detrimental to industrial
192 fermentations due to premature lysis of the starters. However, it has also been suggested
193 that lysogenic lactococcal strains and bacteriocin producers could be useful to promote
194 programmed lysis of starter cells and the release of intracellular enzymes into the cheese
195 matrix to accelerate ripening (Lepeuple et al. 1998; Morgan et al. 1996). It has been
196 reported that Lcn972 inhibits several *L. lactis* strains (Martínez et al. 1996). Moreover,
197 we have tested the susceptibility to Lcn972 of 16 representative strains isolated from
198 commercial starters. The MICs ranged from 50 to 1600 AU/ml and, roughly, half of
199 them were as susceptible as *L. lactis* IPLA513 (Table 2). Thus, the inclusion of the
200 Lcn972 producer strain in starter formulations might be useful to promote starter lysis.
201 Experiments to evaluate this strategy to accelerate cheese ripening are currently in
202 progress.

203

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Figure legends.

Figure 1. Prophage induction in early exponentially growing cultures of the lysogenic strain *L. lactis* IPLA 513 in response to increasing Lcn972 concentrations. Lcn972 was added to cultures at an $OD_{600\ nm}$=0.2 and incubated at 32 ºC. Viable cells (bars) and phage (line) counts are expressed as cfu/ml and pfu/ml, respectively.

Figure 2. Prophage induction of *L. lactis* IPLA 513 by several antimicrobials. Exponentially growing *L. lactis* IPLA 513 cells were treated with different concentrations of PlnC (plantaricin C), Bac (bacitracin), PenG (penicillin G), Cm (chloramphenicol), Lys (lysozyme) and Mut (mutanolysin). Lcn972 was included as a positive control. Induction factors were calculated as pfu/ml in the treated samples divided by pfu/ml in the untreated samples. The inducer of the SOS response mitomycin C (0.5 µg/ml) was used and induction factors of 102.75±20.9 were achieved (data not shown). Values are mean of three experiments. Standard deviations are shown.

Table 1. Viability and prophage induction after 2 h treatment with Lcn972 of *L. lactis* IPLA513 at different physiological status.

Growth phase (OD_{600nm})[a]	Log cfu/ml		Prophage induction factor
	Control	Lcn972	
Early exponential (0.2)	8.76	4.49	5.61
Middle exponential (1.0)	8.72	6.09	1.55
Late exponential (2.0)	8.66	6.09	1.38

[a]Cells from the different growth phases were adjusted to OD_{600nm}=0.2 that corresponds to 7.8 log cfu/ml.

Table 2. Susceptibility to Lcn972 of *L. lactis* IPLA 513, *L. lactis* MG1614 and several *L. lactis* strains isolated from commercial starters.

Commercial Starter[a]	Strain	Lcn972 MIC (AU/ml)[b]
Starter 1	1.1	50
Starter 5	5.1.	50
Starter 6	6.1	50
	6.2	25
	6.3	50
	6.4	50
Starter 7	7.1	50
	7.2	800
	7.3	>3200
Starter 8	8.1	1600
	8.2	200
Starter 9	9.1	400
	9.2	200
	9.3	800
	9.4	800
	9.5	200
L. lactis	IPLA 513	50
L. lactis	MG 1614	50

[a]Strains from commercial starters were described by Madera *et al*. (2004).
[b]MIC, Minimum Inhibitory Concentration; AU, Arbitrary Units.

Fig. 1. Madera et al.

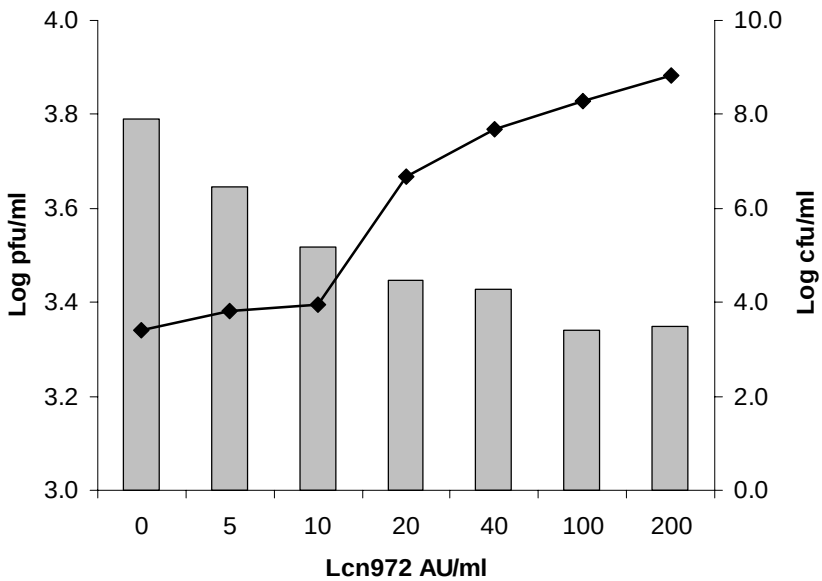

Fig. 2. Madera et al