Lactococcin 972: a homodimeric lactococcal bacteriocin whose primary target is not the plasma membrane

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***Lactococcus lactis* subsp. *lactis* IPLA 972 was shown to produce a bacteriocin which had a bactericidal effect on sensitive lactococci. Production of lactococcin 972 reached a maximum during the late-exponential phase of growth. The bacteriocinogenic activity was heat-sensitive, active in the pH range 4.0–9.0 and showed low susceptibility to proteases. Purification of the bacteriocin rendered a single polypeptide of 7.5 kDa (monomer) as shown by SDS-PAGE. Gels overlaid with a lawn of sensitive bacteria showed inhibitory activity at a point corresponding to 15 kDa. Changes in the electrophoretic conditions allowed the detection of a band at a position corresponding to that expected for a hypothetical dimer. Sequencing of the NH₂-terminal end of lactococcin 972 revealed the sequence NH₂-EGTWQHGYGV, which is not related to any other bacteriocin sequence present in the databases. Finally, lactococcin 972 did not induce the efflux of compounds previously incorporated into the cytoplasm of sensitive cultures nor did it inhibit macromolecular synthesis, suggesting that, in contrast to other bacteriocins, its primary target is not the plasma membrane.**

Keywords: *Lactococcus*, bacteriocin, structure, mode of action

INTRODUCTION

Lactic acid bacteria are used extensively as starters in the manufacture of dairy products. They contribute simultaneously to the sensory attributes and to the preservation of fermented foods. Public concern about chemical food preservatives has attracted research interest in this last property, which is based on metabolites accumulating to inhibitory levels. These include organic acids, ethanol, hydrogen peroxide and bacteriocins (Piard & Desmazeaud, 1991, 1992). In particular, bacteriocins (peptides or proteins that are bactericidal to sensitive strains) are believed to have great potential as natural food preservatives. Their production has been described in bacteria belonging to all main genera of the lactic acid bacteria group. Klaenhammer (1993) has placed the bacteriocins in four classes according to their chemical, structural and functional properties. The majority belong to classes I and II. Class I comprises the lantibiotics, small peptides that are post-translationally modified and thus contain unusual amino acids such as lanthionine. They have a wide spectrum of activity, nisin being the best known member of the group. Nisin is a lactococcal bacteriocin which has been licensed to be used as a food preservative (Food and Drug Administration, 1988). Class

II is made up of small, heat-stable peptides, active on membranes, with either narrow or wide spectra of activity. Some of them depend on the complementary action of two different peptides (Nissen-Meyer *et al.*, 1992; Allison *et al.*, 1994). The third class includes large proteins and the fourth, peptides that depend on non-protein moieties to undertake their activities.

In this report, we describe a new bacteriocin that cannot be properly included in any of the classes described above since its active form seems to be a homodimer and its primary target is not the cytoplasmic membrane. This bacteriocin is produced by *Lactococcus lactis* subsp. *lactis* IPLA 972. This strain was identified in a previous screening for antagonistic properties among the wild lactococcal strains present in ripening home-made cheeses manufactured from raw milk in the absence of any starters in Asturias (northern Spain) (Martínez *et al.*, 1995).

METHODS

Bacterial strains and culture conditions. *Lactococcus lactis* subsp. *lactis* IPLA 972 is a wild strain isolated from a home-made cheese and was used as a lactococcin 972 producer (Martínez *et al.*, 1995). *L. lactis* MG 1363, its derivative *L. lactis* MG 1614 (Str^r Rif^r) (Gasson, 1983) and *Lactobacillus sake*

LMG 13558 were used as indicators. Cultures were grown in M17 broth (Biokar) supplemented with 0.5% lactose (LM17) for *L. lactis* IPLA 972 or with 0.5% glucose (GM17) for *L. lactis* MG 1363 and MG 1614. *Lb. sake* LMG 13558 was grown in MRS broth (Biokar). Solid media were prepared by adding 2.0% (w/v) agar to the broths. All strains were incubated at 32 °C. When needed, streptomycin (500 µg ml⁻¹) or rifampicin (300 µg ml⁻¹) was used. Strains were stored at -80 °C in broth containing 10% (v/v) glycerol and propagated twice before each experiment.

Bacteriocin detection and bioassay. Bacteriocin production was assayed by the agar diffusion test (Reddish, 1929). Plates were prepared by adding approximately 10⁸ sensitive cells of an overnight culture to molten M17 medium containing 1.2% (w/v) agar. After solidification, wells (6.0 mm diameter) were made with a sterile cork borer and filled with 30 µl of neutralized and filter-sterilized supernatants of *L. lactis* IPLA 972. After incubation, the diameter of the inhibition zone was measured. In order to quantify the bacteriocin activity, twofold serial dilutions of active supernatants were tested and the highest dilution that produced a clear zone of growth inhibition of the indicator lawn was defined as 1 arbitrary unit of activity ml⁻¹ (AU ml⁻¹).

Preliminary characterization of lactococcin 972. Overnight supernatants of *L. lactis* IPLA 972 or purified bacteriocin preparations were subjected to heat treatments (40, 45, 50, 55, 60 and 100 °C for 15 min) and enzyme digestions using α -chymotrypsin, trypsin, proteinase K, pronase E and α -amylase, all at a final concentration of 1 mg ml⁻¹ in 20 mM Tris/HCl, pH 8.0. The incubations were performed at 37 °C for 1 h. The remaining activity was measured as described before and untreated samples were used as controls. Aliquots of active supernatant were also adjusted to various pH values (2–12) with NaOH or HCl, incubated at 21 °C for 12 h, and neutralized prior to testing the remaining activity. Finally, the supernatants were treated with catalase (65 U ml⁻¹) to exclude inhibition by hydrogen peroxide.

Kinetics of lactococcin 972 production. LM17 broth was inoculated with overnight cultures of the producer strain (1%, v/v) and incubated at 32 °C without agitation for 24 h. Samples were removed at regular intervals and analysed for growth (c.f.u. ml⁻¹), bacteriocin activity and pH.

Purification and NH₂-terminal amino acid sequence analysis. Active supernatants (80 ml) obtained after centrifugation and neutralization of late-exponential-phase cultures of *L. lactis* IPLA 972 (fraction I) were precipitated with 5 vols acetone at -20 °C for 30 min. After centrifugation at 4 °C, the pellet was dried under vacuum and resuspended in 40 ml 50 mM sodium phosphate buffer, pH 7 (fraction II). Samples (20 ml) were applied to a 5 ml cation exchanger chromatographic column (High-S; Bio-Rad), previously equilibrated with 50 mM sodium phosphate buffer, pH 7. The column was washed with 0.1 M NaCl in the same buffer and lactococcin 972 was eluted at 0.4 M NaCl. The protein concentration was determined by the method of Bradford with the Bio-Rad Protein Assay kit. The purified bacteriocin preparation was stored at -20 °C without appreciable loss of activity.

To determine the NH₂-terminal amino acid sequence of lactococcin 972, 10 µg was spotted onto an Immobilon-P membrane (Millipore) and subjected to automated Edman degradation analysis (Edman & Begg, 1967).

SDS-PAGE and bioassay. Tricine/SDS-PAGE (Schägger & von Jagow, 1987) was used to determine the molecular mass of lactococcin 972 with a 16.5% T/3% C separating gel and a 4% T/3% C stacking gel. After electrophoresis at 75 V, the gels

were cut in two pieces; one half was stained with Coomassie Brilliant Blue R-250 and the other half was washed and overlaid with an indicator lawn of *L. lactis* MG 1614 in streptomycin/semisolid GM17 (0.7% agar) (Bunhia *et al.*, 1987).

Mode of action. Early-exponential-phase cultures (OD₆₀₀ 0.2) of *L. lactis* MG 1614 and *Lb. sake* LMG 13558 were harvested by centrifugation and resuspended in fresh medium containing purified lactococcin 972 (6 µg ml⁻¹), or 50 mM sodium phosphate buffer, pH 7, as a negative control, and the incubation was continued. At different times, aliquots were removed to determine viable cell counts and the OD₆₀₀ was measured in a spectrophotometer (Uvikon 930; Kontron Instruments).

To test the potential damage of the cell membrane, exponentially growing cultures of *L. lactis* MG 1363 were treated with rifampicin (300 µg ml⁻¹) (at this concentration RNA synthesis is completely inhibited) and 5 min later, [5-³H]uridine (0.9 Bq mmol⁻¹, 37 kBq ml⁻¹) was added. Incubation proceeded for 20 min and was followed by addition of partially purified preparations of lactococcin 972 or nisin as a positive control. Samples were taken at different time intervals, filtered through nitrocellulose filters (0.45 µm), extensively washed with warm GM17, and the radioactivity inside the cells was measured as described previously by González *et al.* (1994).

The possible effect of lactococcin 972 on macromolecular synthesis was tested. The continuous incorporation of radio-labelled precursors into trichloroacetic-acid-insoluble material was measured in exponential cultures of *L. lactis* MG 1363 treated with a partially purified preparation of lactococcin 972. [methyl-³H]Thymidine (925 GBq mmol⁻¹, 37 MBq ml⁻¹), [5-³H]uridine (0.9 Bq mmol⁻¹, 37 MBq ml⁻¹) and a mixture of ³H-labelled amino acids (37 MBq ml⁻¹) were used at a final concentration of 37 kBq ml⁻¹. For control purposes, lactococcin 972 preparations were replaced by the same volume of 2 × M17 broth. The samples were collected at different time intervals and processed as described by Rodríguez *et al.* (1986).

RESULTS AND DISCUSSION

Production of lactococcin 972 and preliminary characterization

The inhibitory activity produced by *L. lactis* subsp. *lactis* IPLA 972 was detected in cell-free supernatants of LM17 and GM17 cultures, indicating that its production was not

Fig. 1. Kinetics of production of lactococcin 972 in LM17 broth at 32 °C without agitation. At different time intervals samples were taken and c.f.u. ml⁻¹ (●), AU ml⁻¹ (○) and pH (□) were measured. *L. lactis* MG 1614 was used as an indicator.

Table 1. Effect of different treatments on active supernatants of *L. lactis* IPLA 972

Relative activity was measured by an agar diffusion test against *L. lactis* MG 1614. —, No inhibition; +, slight inhibition; ++, moderate inhibition; + + +, strong inhibition.

Enzymic treatment	Relative activity	pH	Relative activity	Heat treatment (15 min)	Relative activity
Pronase E	++	4	—	40 °C	+ + +
Trypsin	+ + +	5	+ + +	45 °C	++
Proteinase K	+ + +	6	+ + +	50 °C	+
α -Chymotrypsin	+ + +	7	+ + +	55 °C	—
α -Amylase	+ + +	8	++	60 °C	—
Catalase	+ + +	9	+	100 °C	—
		10	—	121 °C	—
Control	+ + +		+ + +		+ + +

Table 2. Purification of lactococcin 972

Fraction	Volume (ml)	Total protein (μ g)	Total activity (AU)	Specific activity (AU μ g ⁻¹)	Yield (%)	Purification (-fold)
I. Supernatant	80	16000	10640	0.665	100	1
II. Acetone precipitation	40	9000	5320	0.591	50	0.89
III. Cation exchange	16	200	2128	10.640	20	16

repressed by glucose. The activity was first observed in the very-early-exponential phase and continued increasing until the late-exponential phase to a maximum activity of 266 AU ml⁻¹. Afterwards, the production apparently stopped but the amount of active bacteriocin in the supernatant remained constant. Once the culture entered into the stationary phase the activity abruptly decreased (Fig. 1).

To discover the chemical nature of the inhibitory compound produced by *L. lactis* IPLA 972, cell-free supernatants were subjected to different treatments. The results are summarized in Table 1. The absence of inactivation by catalase suggested no involvement of hydrogen peroxide in the inhibition. Bacteriophages were excluded because serial twofold dilutions onto an indicator lawn did not form individual plaques and because the activity partially passed through filters with a cut-off limit of 10 kDa. Organic acids were also discarded because the inhibition was detected in neutralized supernatants. Furthermore, irreversible inactivation occurred at pH values < 4.0 and > 9.0. Lack of sensitivity to α -amylase indicated that glucosidic moieties were not required for its biological activity. In contrast, although the activity was quite resistant to protease digestion it was partially inactivated by pronase E. Since the peptidic nature of lactococcin 972 was proven after purification (see below), the reason for the resistance to proteases is still unsolved. However, it is important to note that this characteristic will be an advantage for its application in food systems

where proteases are abundant. The activity was heat-labile, as indicated by its irreversible inactivation after treatment at 50 °C for 15 min. As far as we know, this is the first bacteriocin produced by a lactococcal strain which is heat-sensitive at neutral pH (Geis *et al.*, 1983; Nettles & Barefoot, 1993). In fact, this feature is restricted to large-complex bacteriocins produced by lactobacilli, such as helveticin J (Joerger & Klaenhammer, 1986), acidophilucin A (Toba *et al.*, 1991a) and the lacticins A and B (Toba *et al.*, 1991b). However, the fact that lactococcin 972 passed through filters of known pore diameter indicated that it was between 3 and 10 kDa.

Purification and NH₂-terminal amino acid sequence

Purification of lactococcin 972 was achieved through a two-step protocol comprising acetone precipitation and cation-exchange chromatography. The purification steps and recoveries are summarized in Table 2. Fig. 2 shows the chromatogram resulting from the last purification step. At 0.4 M NaCl, the elution of a single peak of protein that coincides with the inhibitory activity can be observed. The purification procedure resulted in recovery of 20 % of the initial activity.

Confirmation of the purity of the bacteriocin was obtained after NH₂-terminal end sequencing, which rendered just one amino acid per cycle after the Edman degradation. The sequence obtained was NH₂-Glu-Gly-Thr-Trp-Gln-His-Gly-Tyr-Gly-Val-. No homology to lactococcal

Fig. 2. Elution of lactococcin 972 at 0.4 M NaCl by cation-exchange chromatography. The sample (20 ml) was from acetone precipitation of the supernatant of a *L. lactis* IPLA 972 culture grown in LM17 broth. Fractions (1 ml) were collected. Protein concentration (○) was determined by the Bradford assay and activity (●) was measured as the diameter of the inhibition halo on *L. lactis* MG 1614. —, NaCl elution gradient.

bacteriocins or other proteins was found in the SWISS-PROT database.

Bioautography of the culture supernatant (not shown) and of the solubilized acetone precipitate showed an inhibition zone at a level corresponding to a 15 kDa polypeptide (Fig. 3b, lane 1). When a sample of pure bacteriocin was boiled and subjected to tricine/SDS-PAGE, a single band of approximately 7.5 kDa was observed (Fig. 3a, lane 2). As expected, this band did not show any inhibitory activity (Fig. 3b, lane 2). These data suggested that the active form of the bacteriocin was a homodimer that became denatured upon heat treatment of the samples prior to electrophoresis. To test this possibility, unheated samples were run and either stained

(Fig. 3a, lane 3) or subjected to bioassay (Fig. 3b, lane 3). In this first case, bands of 15 and 7.5 kDa were observed and, as expected, only the upper band rendered the inhibitory activity.

This finding was further confirmed when it was observed that the sensitivity of lactococcin 972 to acid was also due to the dissociation of the two component peptides (the gels showing the bacteriocin after treatment at pH 2 had the same appearance as those run after heating it). This seems to indicate that formation of the dimer is based on rather weak forces, perhaps of electrostatic or hydrophobic nature, although this question cannot be solved until the complete sequence of lactococcin 972 is available.

The dependence of the inhibitory activity on two peptides was first reported for lactococcin G (Nissen-Meyer *et al.*, 1992) and later extended to other bacteriocins, such as lactacin F (Allison *et al.*, 1994) and plantaricin S (Jiménez-Díaz *et al.*, 1995) among others, although in all cases, the inhibition depended on the concerted action of two different peptides. In addition, these activities were heat-resistant, indicating that strong, probably covalent, links existed between the two component peptides.

Inhibitory spectrum of lactococcin 972 and mode of action

Lactococcin 972 has a narrow spectrum of activity: it only inhibits other lactococcal strains and *Lb. sake* LMG 13558 (Martínez *et al.*, 1995). Of 50 lactococcal strains isolated from natural dairy environments and from different bacterial collections, all were found to be sensitive, including nisin A or nisin Z producers. In contrast, all the members of a set of other Gram-positive bacteria, which included *Clostridium*, *Listeria*, *Enterococcus* and *Bacillus* strains, were resistant. Most of the lactococcal bacteriocins have a narrow spectrum of activity, with the notable exception of the lantibiotics, such as nisin and lactacin 481

Fig. 3. Tricine/SDS-PAGE of lactococcin 972 and direct detection of its antimicrobial activity. *L. lactis* MG 1614 was used as indicator. (a) Gel stained with Coomassie Brilliant Blue R-250; (b) bioassay of inhibitory activity on a washed gel. Lanes: 1, solubilized acetone precipitate of culture supernatant; 2, boiled purified lactococcin 972; 3, unheated purified lactococcin 972; 4, low-molecular-mass protein markers (16950, 14400, 10600, 8160, 6210, 3460 and 2510 Da; Sigma).

Fig. 4. Effect of purified lactococcin 972 on exponentially growing cells of *L. lactis* MG 1614. OD₆₀₀ measurements (a) and viable counts (b) of both treated (●) and control (○) cultures are shown. The arrow shows the addition of the bacteriocin (66 AU ml⁻¹).

(Jack *et al.*, 1995). Narrow-spectrum bacteriocins probably do not have a clear role as food preservatives; however, they can be used in, for example, accelerated maturation of cheese through promotion of the controlled lysis of sensitive lactococci present in the starter, which would allow direct interaction between the intracellular components and the fermenting substrate (Morgan *et al.*, 1995).

The addition of lactococcin 972 to exponential cultures of *L. lactis* MG 1614 resulted in an immediate arrest of the increase in optical density of the cultures (Fig. 4a), which was accompanied by a sharp decrease in the number of viable cells (Fig. 4b). These data indicate that lactococcin 972 is a bactericidal peptide that does not induce cell lysis as a secondary effect. Similar results were obtained when *L. lactis* MG 1363 and *Lb. sake* LMG 13558 were tested.

All bacteriocins whose mode of action is known produce complexes that insert into the plasma membrane of sensitive bacteria, creating pores through which the cell material leaks (van Belkum *et al.*, 1991; Nissen-Meyer *et al.*, 1992; Abee *et al.*, 1994). To test if this was also the case with lactococcin 972, two sets of experiments were devised. In the first of them, we analysed the effect of lactococcin 972 on cytoplasmic [5-³H]uridine, previously accumulated by *L. lactis* MG 1363 cells treated with rifampicin to inhibit the incorporation of the labelled precursor into RNA. No release of uridine was observed (Fig. 5d), whereas addition of nisin, a bacteriocin that forms membrane pores (Jack *et al.*, 1995), resulted in a fast and complete leakage of the intracellular uridine. This clearly suggests that lactococcin 972 does not create pores in the cytoplasmic membrane. The second experiment was carried out to analyse the effect of lactococcin 972 on

Fig. 5. Macromolecular synthesis in cultures of *L. lactis* MG 1363 treated (●) or untreated (○) with partially purified lactococcin 972 (150 AU ml⁻¹) as measured by incorporation of exogenous labelled precursors into trichloroacetic-acid-insoluble material. (a) DNA; (b) RNA; (c) protein. (d) Efflux of [5-³H]uridine, previously accumulated inside rifampicin-treated cells, after addition (at time zero) of lactococcin 972 (●) or nisin (■) (200 AU ml⁻¹) as a positive control; ○, control cultures without bacteriocin. Samples were taken in duplicate. The data shown are the means of three independent experiments.

macromolecular synthesis in sensitive cells. No inhibition of the incorporation of precursors into DNA (Fig. 5a), RNA (Fig. 5b) or proteins (Fig. 5c) was observed, even

after 1 h of addition of the bacteriocin. This result contrasts with those obtained with plantaricin C (González *et al.*, 1994). In that case, addition of the bacteriocin to sensitive cultures caused an immediate cessation of all macromolecular synthesis and the efflux of labelled precursors previously accumulated into the cell.

We can conclude that lactococcin 972 forms a new class of lactococcal bacteriocin with a narrow spectrum of activity and whose primary target is not the plasma membrane. It is suggested that this activity relies on an unstable homodimeric structure, easily destroyed by heat and low pH. The strong bactericidal activity against lactococcal strains shown by the lactococcin 972 producer could be used to design a single- or mixed-strain starter in which it is combined with leuconostoc strains. These starters would compete efficiently in food fermentations and would protect themselves against any potential contamination by other lactococcal strains.

Finally, it has been suggested that wild lactic acid bacteria isolated from natural dairy environments, in which no commercial starters are used, would be a good source of new bacteriocins which might have novel properties: lactococcin 972 is one such example.

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