

# Synthesis of lactococcin 972, a bacteriocin produced by *Lactococcus lactis* IPLA 972, depends on the expression of a plasmid-encoded bicistronic operon

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**Synthesis of lactococcin 972 is plasmid-encoded. An operon composed of two genes that encode pre-bacteriocin and a putative immunity protein has been identified. The first gene encodes a 91-residue polypeptide that is exported via a sec-dependent system to give the mature 66-aa bacteriocin. The immunity protein is a 563-residue polypeptide with seven potential transmembrane domains. Two transcripts were observed from this region: one comprises the whole operon and is synthesized during the exponential phase of growth while the other, which corresponds just to the bacteriocin structural gene, presents a maximum in exponential cultures but is still present in late-stationary-phase cells.**

**Keywords:** lactococcin 972, *Lactococcus lactis*, bacteriocin, plasmid-encoded genes

## INTRODUCTION

Among lactic acid bacteria (LAB), lactococci are the main components of the mesophilic starter cultures used in the manufacture of most dairy products. They contribute to the development of sensorial properties of fermented products and prevent the growth of food-borne pathogenic and food-spoilage organisms (Vanderbergh, 1993). Their antagonistic effect relies mainly on lactic acid excretion, but also on other antimicrobial compounds, such as bacteriocins (Daeschel, 1989).

Bacteriocins have been defined as proteins or protein complexes which are bactericidal against closely related bacteria (Tagg *et al.*, 1976). The concept was widened to include peptides of a fairly broad inhibitory spectrum (Klaenhammer, 1993). LAB bacteriocins have been recently classified into three classes (Nes *et al.*, 1996): I, lantibiotics; II, small heat-stable unmodified peptides; and III, large heat-labile proteins. Classes I and II

comprise the most abundant and best studied bacteriocins. The diversity of class II bacteriocins has motivated its division into three sub-groups: IIa, bacteriocins with antilisterial effect; IIb, two-peptide bacteriocins; and IIc, sec-dependent secreted bacteriocins.

The potential application of LAB bacteriocins as natural food preservatives, as well as the approval of nisin for that purpose, has encouraged interest in them. Most are plasmid-encoded (Davey, 1984; Dufour *et al.*, 1991) and even a single plasmid has been found to encode three different bacteriocins (van Belkum *et al.*, 1992). However, nisin is encoded by a 70 kb conjugative transposon (Rauch & de Vos, 1992). The structural genes are usually clustered with accessory genes that encode proteins associated with immunity and/or maturation (Kolter & Moreno, 1992). Finally, bacteriocin synthesis appears to be a cell-density-regulated process, dependent on interaction of peptide pheromones with devoted two-component signal transduction systems (Diep *et al.*, 1995; Kuipers *et al.*, 1995).

In this paper we report the cloning and characterization of the genetic determinant for lactococcin 972, a bacteriocin produced by *Lactococcus lactis* subsp. *lactis* IPLA 972, that is active on all lactococci tested to date (Martínez *et al.*, 1995). This would protect the producing strain from contamination by other lactococci when used as a unique starter in cheese fermentation. Ad-

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**Abbreviation:** LAB, lactic acid bacteria.

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ditionally, the bacteriocin would be used as a potential accelerator of maturation through lysis of the starters. Lactococcin 972 is composed of two copies of a single 7500-Da peptide whose amino-terminal sequence is E-G-T-W-Q-X-G-Y-G-V-, where the X stands for an undetermined residue. The isolated monomers do not show any antimicrobial activity (Martínez *et al.*, 1996).

## METHODS

**Bacterial strains, media and plasmids.** *Lactococcus lactis* subsp. *lactis* IPLA 972 (Martínez *et al.*, 1995) and *L. lactis* MG 1614 (Str<sup>r</sup>, Rif<sup>r</sup>) (Gasson, 1983) were used as the producer and lactococcin-972-sensitive strains, respectively. Cultures were grown in M17 medium (Biokar) supplemented with 0.5% lactose (*L. lactis* IPLA 972) or 0.5% glucose (*L. lactis* MG 1614). Incubation was at 32 °C. *Escherichia coli* HB101 (Bolivar & Backman, 1979) and XL-1 Blue were grown on 2 × TY (Sambrook *et al.*, 1989) at 37 °C with shaking. The vectors pACYC184 (Chang & Cohen, 1978), and M13mp18 and 19 (Yanisch-Perron *et al.*, 1985) were used in cloning experiments. The strains were maintained at -80 °C in growth medium containing 10% (v/v) glycerol and propagated twice before each experiment. When appropriate, chloramphenicol (50 µg ml<sup>-1</sup>), tetracycline (50 µg ml<sup>-1</sup>), spectinomycin (50 µg ml<sup>-1</sup>) or streptomycin (500 µg ml<sup>-1</sup>) were used.

**Determination of lactococcin 972 activity.** Production of the bacteriocin was tested in filter-sterilized culture supernatants throughout the growth cycle of *L. lactis* IPLA 972. The inhibitory activity of lactococcin 972 was quantified using the agar diffusion test of twofold serial lactococcin 972 dilutions (Reddish, 1929), using *L. lactis* MG 1614 as an indicator. The highest dilution that produced a clear zone of growth inhibition was defined as one arbitrary unit of activity (AU) ml<sup>-1</sup>. Concentration and partial purification of lactococcin 972 was performed from supernatants of late-exponential-phase cultures of *L. lactis* IPLA 972 which 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 50 mM sodium phosphate buffer, pH 7 (Martínez *et al.*, 1996). The bacteriocin was visualized through Tricine/SDS-PAGE (Schägger & von Jagow, 1987).

**DNA techniques.** Lactococcal plasmids were extracted by the method of O'Sullivan & Klaenhammer (1993). *L. lactis* MG 1614 cells were electroporated by the method of Leenhouts *et al.* (1990). Selection of the transformants was made by including serial dilutions of the transformed cultures in top-agar overlays (GM17, 0.7% agar) containing streptomycin (500 µg ml<sup>-1</sup>) and partially purified lactococcin 972 (250 AU ml<sup>-1</sup>). Electroporation of *E. coli* was performed as described by Dower *et al.* (1988). Standard DNA manipulations such as *E. coli* plasmid isolation, restriction endonuclease analysis, ligations and gel electrophoresis were performed according to Sambrook *et al.* (1989). To localize the genetic determinant of lactococcin 972, a degenerate 17-mer oligonucleotide (5'-TGGCARTGYGGNTAYGG-3', R = A or G, Y = C or T, N = A, T, C or G) was designed from its amino-terminal sequence (EGTWQXGYGV) (Martínez *et al.*, 1996), assuming that X corresponded to cysteine. The oligonucleotide was labelled with [ $\gamma$ -<sup>32</sup>P]ATP using T4 polynucleotide kinase (Boehringer Mannheim). Southern hybridization was carried out in Rapid-Hyb buffer (Amersham) at 37 °C for 16 h. Washes were performed under low stringency conditions [2 × SSC (0.3 M sodium chloride, 0.03 M sodium citrate)/0.1 %

SDS, at 37 °C]. PCR amplifications were done with the proofreading *Pwo* polymerase (Boehringer Mannheim). Nucleotide sequencing was performed according to the dideoxy chain-termination method (Sanger *et al.*, 1977) using sequenase 2.0 (Amersham). Sequences were analysed using the GCG software package (Devereux *et al.*, 1984).

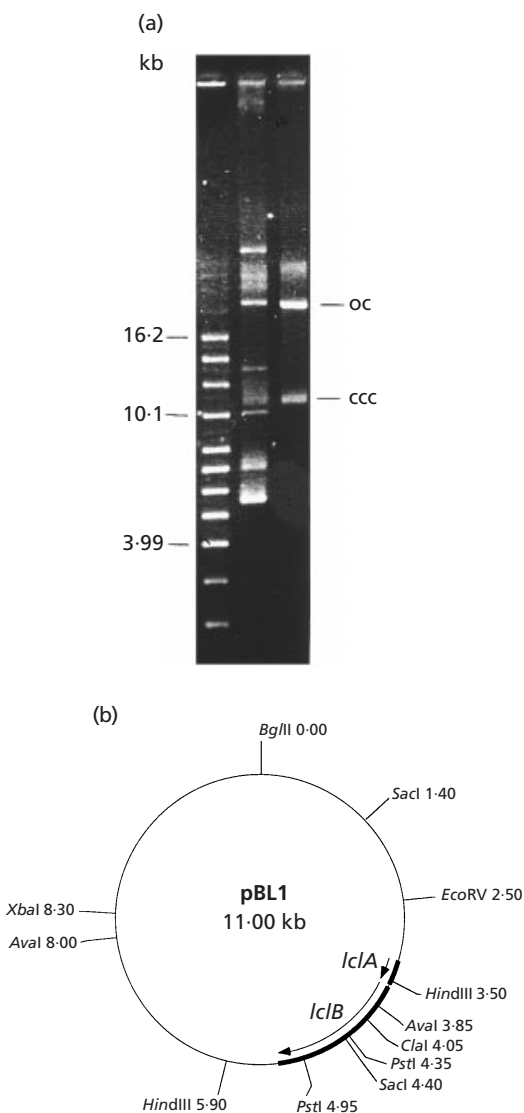
**RNA isolation, Northern hybridization and primer extension analysis.** RNA extraction was done by a modification of the method of Chomczynski & Sacchi (1987). In brief, samples of *L. lactis* IPLA 972 grown in M17 broth were harvested at different time intervals, resuspended in denaturing solution and disrupted with 4.5–5.5 mm glass balls by vigorous vortexing. After several phenol/chloroform extractions, nucleic acids were treated with DNase (0.05 U µl<sup>-1</sup>, Boehringer Mannheim) and stored as ethanol precipitates. For Northern analysis, 5 µg RNA was fractionated on agarose gels (Sambrook *et al.*, 1989) and transferred to Hybond-N membrane (Amersham). After addition of the probe, hybridization was performed at 42 °C in Rapid-Hyb buffer and the membrane was washed according to the manufacturer's instructions. Double-stranded DNA probes were labelled with [ $\alpha$ -<sup>32</sup>P]dCTP using the Rediprime labelling kit (Amersham).

Primer extension experiments were performed with a 15-mer oligonucleotide (5'-ATAATTTGAATATGC-3') complementary to the coding sequence in the region of the amino terminus of the mature bacteriocin, end-labelled with [ $\gamma$ -<sup>32</sup>P]ATP (Amersham) using T4 polynucleotide kinase. The primer (10 pmol) was annealed to 20 µg DNase-treated total RNA and cDNA synthesis was performed by using a cDNA synthesis kit (Promega) following the manufacturer's protocol. The product was analysed on a 6% polyacrylamide/urea sequencing gel next to standard sequencing reactions as size markers.

## RESULTS AND DISCUSSION

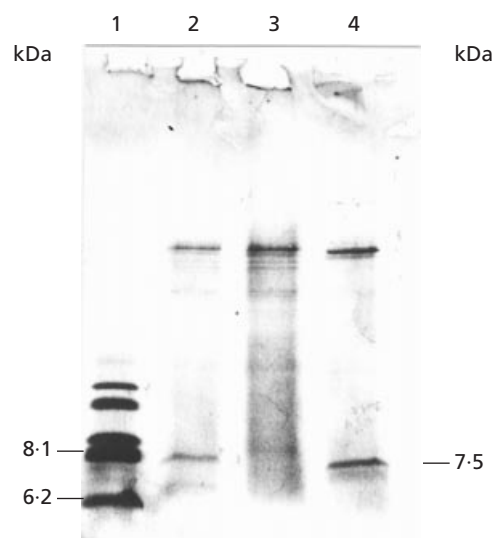
### Lactococcin 972 is a plasmid-encoded bacteriocin

The degenerated oligonucleotide designed to localize the genetic determinant of lactococcin 972 was hybridized with the total DNA or plasmid complement of *L. lactis* IPLA 972. Positive hybridization was obtained with a plasmid of about 11 kb (data not shown), which was unexpected because bacteriocin (Bac) production appeared to be very stable (all attempts to isolate Bac<sup>-</sup> derivatives failed). To confirm the location of the lactococcin 972 structural gene (*lclA*) the plasmid complement of *L. lactis* IPLA 972 was extracted and used to electroporate the streptomycin-resistant, plasmid-free, lactococcin 972-sensitive strain *L. lactis* MG 1614. The transformants were selected on plates containing 500 µg streptomycin ml<sup>-1</sup> and 250 AU lactococcin 972 ml<sup>-1</sup>, i.e. assuming that production and immunity would be linked. Overnight supernatants of the transformants inhibited the parental strain MG 1614. They harboured an 11 kb plasmid (pBL1), also detected in the bacteriocin-producer IPLA 972 (Fig. 1). Further confirmation of the linkage between the Bac<sup>+</sup> phenotype and pBL1 was reached after electroporation of MG 1614 with the isolated plasmid. One of these latter transformants, MG 1614.2, was used for further characterization and as source of pBL1. As a final test to ensure that the inhibitory activity detected in



**Fig. 1.** Identification of the genetic determinants of lactococcin 972. (a) Agarose gel electrophoresis of the *L. lactis* IPLA 972 plasmid complement (lane 2) and of the *L. lactis* MG 1614.2 Bac<sup>+</sup> Bac<sup>-</sup> electrotransformant (lane 3). Lane 1, cccDNA ladder (Gibco-BRL). (b) Physical map of plasmid pBL1 showing the location and direction of transcription of the structural (*lclA*) and putative immunity (*lclB*) genes involved in lactococcin 972 production.

MG 1614.2 cultures was due to the production of lactococcin 972, late-exponential-phase supernatants were treated with acetone, and the precipitate was resuspended and subjected to Tricine/SDS-PAGE (Fig. 2). A 7.5 kDa band, corresponding to the monomer of lactococcin 972, was found in both MG 1614.2 and IPLA 972 but was absent in the supernatants from the parent MG 1614 used as a negative control. Furthermore, the activity detected in MG 1614.2 presented the properties previously reported for lactococcin 972, inhibition restricted to lactococci, partial resistance to protease digestion and heat sensitivity (Martínez *et al.*, 1996).

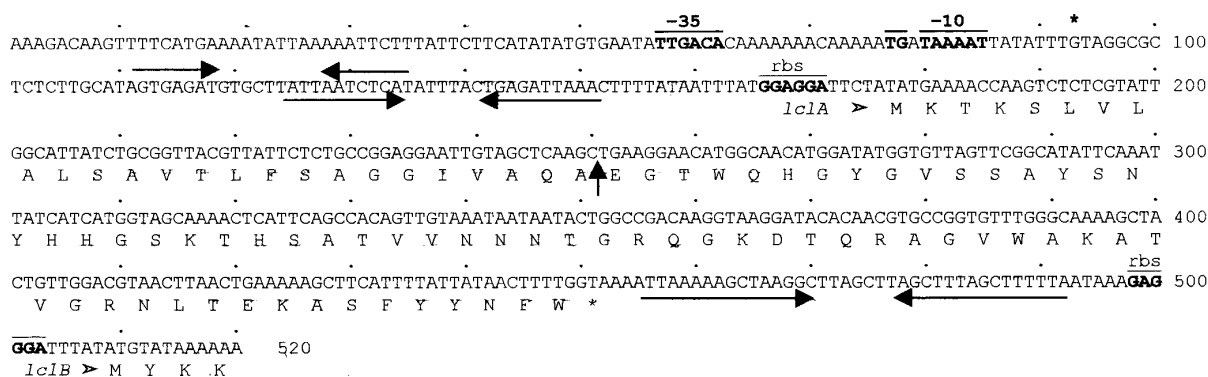


**Fig. 2.** Tricine/SDS-PAGE of acetone-concentrated supernatants from overnight cultures of *L. lactis* IPLA 972 (lane 2), parental *L. lactis* MG 1614 Bac<sup>+</sup> Bac<sup>-</sup> (lane 3) and the transformant *L. lactis* MG 1614.2 Bac<sup>-</sup> Bac<sup>+</sup> (lane 4). Lane 1, mass standards (MW-SDS-175 kit; Sigma).

Plasmid pBL1 did not alter the morphology nor the metabolic capacities of *L. lactis* MG 1614 (lactose utilization, proteolysis, sugar fermentation pattern, etc.).

#### Identification and sequencing of *lclA*

The oligonucleotide mix corresponding to the amino-terminal end of lactococcin 972 was used to localize *lclA* in the plasmid map (Fig. 1). Sequencing of that region revealed two ORFs (Fig. 1). The first ORF corresponds to *lclA* and encodes a predicted polypeptide of 91 aa that presents a potential ribosome-binding site (RBS; GGAGGA) 6 bp upstream of the translation start codon ATG (Fig. 3). The previously determined N-terminal amino acid sequence of lactococcin 972 starts at Glu-26. This indicates that *lclA* encodes a pre-bacteriocin with a 25-residue N-terminal extension and a 66-aa C-terminal portion that would yield the mature lactococcin 972. The calculated molecular mass of this second part (7381 Da) fits with the estimation obtained by Tricine/SDS-PAGE of the pure bacteriocin (Martínez *et al.*, 1996). The presumptive leader has characteristics typical of signal peptides, such as a positively charged amino end, a hydrophobic central region and a sec-dependent processing signal, Ala-Gln-Ala, that conforms to the -3, -1 rule of von Heijne (1983). Curiously, this amino-terminal stretch also shows features that resembles those of double glycine leaders, which are frequently found in Class II bacteriocins, namely, the dimers G-G and L-S at positions 19-20 and 10-11, respectively (Havarstein *et al.*, 1994). However, in the purification experiments of lactococcin 972 we never observed a second peak of antimicrobial activity, nor did we find a double signal



**Fig. 3.** Partial nucleotide and deduced amino acid sequence of the lactococcin 972 operon. Inverted repeats are indicated by converging arrows. The  $-35$  and  $-10$  sites and the RBS (rbs) are indicated. The origin of transcription is marked with an asterisk. The processing point of LclA to give the lactococcin 972 monomer is indicated by a vertical arrow.

during sequencing of the bacteriocin. Of course this does not rule out the possibility that some processing of lactococcin 972 occurs at the double glycine motif but, in that case, the resulting polypeptide would not have any appreciable growth-inhibitory activity. Three nucleotides downstream of the stop codon (TAA) of *lclA* a 16 bp inverted repeat sequence was found, which could act as a  $\rho$ -independent terminator with a  $\Delta G$  value of  $-35$  kcal mol $^{-1}$ . A search of protein sequence databases revealed no polypeptides with homology to lactococcin 972. In contrast with most bacteriocins, the hydrophobicity plot of lactococcin 972 indicates that it is a hydrophilic peptide that presents up to 53% polar amino acids. It is also quite basic with a predicted pI of 10.57.

The transcription start site of *lclA* was determined, by primer extension analysis, to be a G at position  $-85$  from the start codon. Upstream of this residue, a  $-35$  (TTGACA) and a  $-10$  extended (TGNTAAAAT) promoter consensus region, separated by a canonical distance of 14 bp, were identified. Between the transcription start point and the putative RBS two partially overlapping inverted sequences, with  $\Delta G$  values of  $-15$  and  $-20$  kcal mol $^{-1}$ , were present (Fig. 3). They might act in the regulation of the expression of *lclA*. Synthesis of most bacteriocins depends on external inducers (Diep *et al.*, 1995) and indeed the expression of *lclA* is not constitutive (see below).

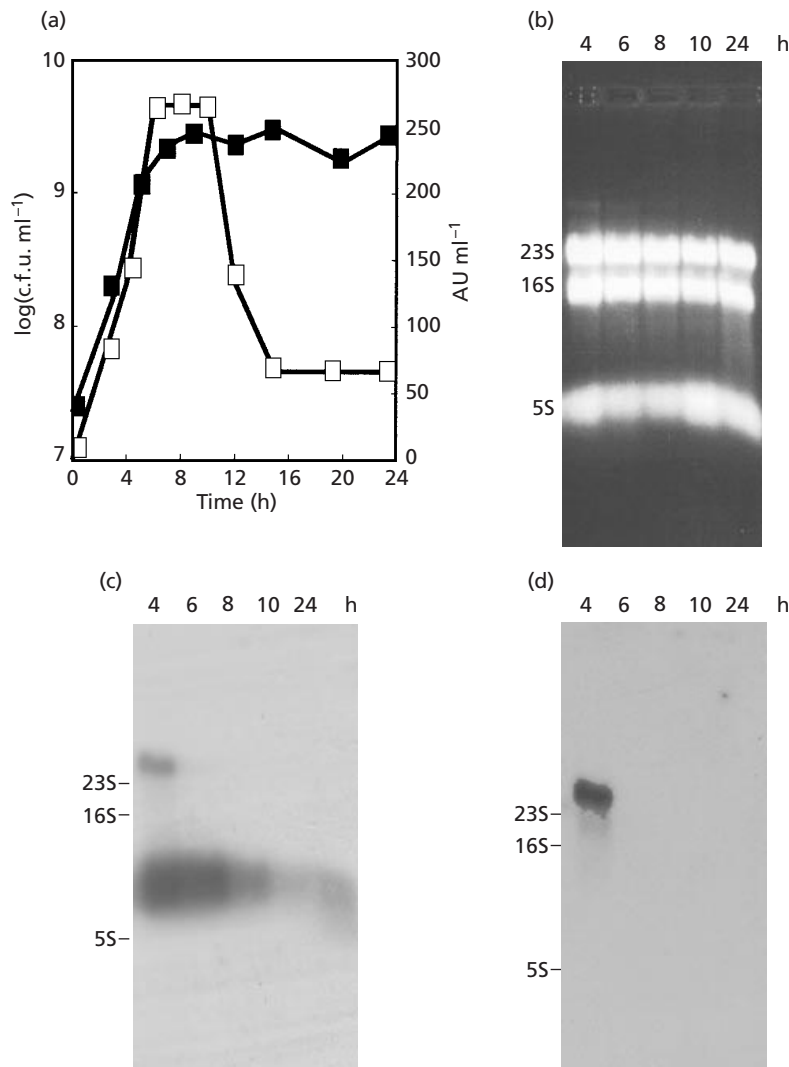
Downstream of *lclA* a second ORF, corresponding to *lclB* and with coding capacity for a polypeptide of 563 aa, was found. They are separated by a stretch of 57 nt that includes the putative  $\rho$ -independent terminator described above. Analysis of the primary sequence of this second polypeptide (LclB) revealed seven hydrophobic zones, each composed of 14–20 aa, indicating that the putative protein might span the plasma membrane. Five of the seven hydrophobic domains were clustered in the central region of the polypeptide and were well separated from the other two that occupied the extreme amino and carboxy ends of the protein. A potential RBS (GAGGGA) was found 6 nt before the

start codon (ATG). An inverted repeat with a  $\Delta G$  of  $-20$  kcal mol $^{-1}$  was located 152 nt after the stop codon (TGA). No homologies were found between LclB and proteins stored in databases.

#### Production of lactococcin 972 depends on expression of the operon formed by *lclA-lclB*

Lactococcin 972 is produced by *L. lactis* IPLA 972 during the exponential phase of growth that lasts about 6 h under standard conditions. The concentration of the bacteriocin in culture supernatants remains constant during early stationary phase and is abruptly lowered after 10 h cultivation (Martínez *et al.*, 1996; Fig. 4a). To determine whether *lclA* and *lclB* were independently transcribed or not and if the kinetics of accumulation of lactococcin 972 was dependent on the rate of transcription of *lclA*, total RNA was extracted from *L. lactis* IPLA 972 cultures periodically through its life cycle (Fig. 4b) and hybridized to (i) a PCR-amplified segment spanning the putative RBS of *lclA* to the end of this gene and (ii) a 600 bp *Pst*I segment internal to *lclB*. In the first case two bands were observed (Fig. 4c). One of them, of about 2.5 kb, was only present in exponential-phase cultures, while the other, of about 400 bp, showed maximum levels in exponentially growing cells but was evident even in late-stationary-phase cultures. When the *lclB* internal segment was used as probe, only the 2.5 kb transcript became evident (Fig. 4d). The size of this band corresponds to the length of the segment that comprises *lclA* plus *lclB* which, together with the hybridization data with the probes specific for each gene, indicates that both form a single bicistronic operon. Furthermore, the presence of the largest transcript only in exponential-phase cultures may be a consequence of a non-absolute stop of transcription at the level of the terminator that lies between *lclA* and *lclB*. Since the accumulation of specific RNA and, presumably, its synthesis is maximal in exponential-phase cultures, it may well be that the stop signal was missed more frequently at this time of the cycle, producing a detectable 2.5 kb band.

A higher accumulation of *lclA* transcripts with respect



**Fig. 4.** Time-course expression of the *lclA-lclB* operon. (a) Growth curve of *L. lactis* IPLA 972 (■) and bacteriocin concentration in the supernatant (□) of the culture used for RNA extraction. (b) Total RNA of *L. lactis* IPLA 972 obtained at 4, 6, 8, 10 and 24 h incubation. (c) Northern blot of the gel shown in (b) hybridized with a probe that spans *lclA*. (d) The same blot hybridized with a *lclB*-specific probe.

to those covering *lclB* as well fits with the respective functions ascribed to their products. The first encodes the pre-bacteriocin which, upon maturation, will be exported, making a high continuous supply necessary to obtain a significant inhibitory effect in the vicinity of the producing cell. The second putatively encodes a plasma membrane protein, the requirement for which will only be determined by the rate of growth of the membrane. The situation might be similar to that found in the nisin operon. In this case, the first gene, *nisA*, that encodes the pre-nisin peptide is followed by a terminator that, at least under certain conditions, greatly attenuates the expression of the other genes, which encode intracellular or membrane-bound proteins involved in maturation, export and immunity (Kuipers *et al.*, 1995; Ra *et al.*, 1996).

The abundance of the transcripts paralleled the accumulation rate of lactococcin 972 in culture supernatants (Fig. 4). This was high throughout exponential phase, coinciding with a maximum in the concentration of both RNA species. The stationary phase was ac-

companied by a plateau in the concentration of bacteriocin and by a decrease in the abundance of specific transcripts. Finally, the concentration of active lactococcin 972 decreased, possibly as a consequence of the resolution of the peptide dimers that form the active bacteriocin, to reach a new plateau, which might represent an equilibrium between synthesis – the transcript was evident even in 24-h-old cultures – and degradation upon export.

Therefore, it appears that *lclA* and *lclB* form a single transcriptional unit. The first gene would encode the pre-bacteriocin which, upon export, probably through the general sec-secretion system of the cell, would reach the external medium, being processed at the same time to give the final 66 aa polypeptide. This forms a dimer to produce the active lactococcin 972. The product of *lclB* might be the immunity protein that protects the producing cell from suicide. This hypothesis is based (i) on its location, immediately downstream of the structural bacteriocin gene, (ii) on the fact that the two genes form an operon and (iii) on the possible location of *LclB*

spanning the plasma membrane. All these properties are typical of bacteriocin immunity systems (Nes *et al.*, 1996). Furthermore, all the attempts to disrupt *lclB* have failed, possibly indicating that its product is necessary to protect the cell from the potential toxicity of lactococcin 972 (B. Martínez, A. Rodríguez & J. E. Suárez, unpublished data). However, *lclB* would encode a 563-aa polypeptide while the usual Class II bacteriocin immunity proteins are fairly small, in all cases less than 200 aa and in some cases as small as around 50 aa.

To date, four other bacteriocins have been reported to have signal peptides matching the requirements of the sec-export system, but none were isolated from lactococci: acidocin B and divergicin A from *Lactobacillus acidophilus* and *Carnobacterium divergens*, respectively (Leer *et al.*, 1995; Worobo *et al.*, 1995), and two pediocin-like peptides isolated from enterococci (Cintas *et al.*, 1997; Tomita *et al.*, 1996). In all cases production was dependent on the expression of a single putative operon that comprised the structural and putative immunity genes, without the need of dedicated secretion or maturation proteins. It is tempting to speculate that production of active lactococcin 972 will also be achieved by the expression of *lclA* and *lclB*. In any case, the information needed is probably encoded by plasmid pBL1, since its transformants produced the bacteriocin and were immune to it.

Lactococcin 972 presents a series of features that make it difficult to include it in any of the existing bacteriocin classes. It was previously shown that its active form is a homodimer and that it does not affect plasma membrane integrity (Martínez *et al.*, 1996). The data reported here further confirm these two peculiar findings. First, there is only one structural gene; all two-component bacteriocins described so far are encoded by two adjacent ORFs, each encoding one of the polypeptides that constitute the active antimicrobial compound. Second, the monomer of lactococcin 972 is hydrophilic, which confirms that the membrane would not be its primary target. In fact, preliminary data seem to indicate that lactococcin 972 inhibits the synthesis of the cell wall (B. Martínez, A. Rodríguez & J. E. Suárez, unpublished data). This might justify the large size of the putative immunity protein LclB and its location, with several domains facing the outside of the plasma membrane. It is becoming clear that lactococcin 972 may be considered to be a hybrid between Classes IIb and IIc, although it presents some peculiarities that may force the definition of a new class of bacteriocins. Further studies, some of which are under way, will help in its definitive classification among LAB antimicrobial peptides.

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