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Bacteriocins produced by wild *Lactococcus lactis* strains isolated from traditional, starter-free cheeses made of raw milk

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ABSTRACT

Sixty bacterial strains were encountered by random amplification of polymorphic DNA (RAPD) and repetitive extragenic palindromic (REP) typing in a series of 306 *Lactococcus lactis* isolates collected during the manufacturing and ripening stages of five traditional, starter-free cheeses made from raw milk. Among the 60 strains, 17 were shown to produce bacteriocin-like compounds in both solid and liquid media. At a genotypic level, 16 of the strains were identified by molecular methods as belonging to *L. lactis* subsp. *lactis* and one to *L. lactis* subsp. *cremoris*. Among the *L. lactis* subsp. *lactis* strains, phenotypic and genetic data determined that eleven produced either nisin A (nine strains) or nisin Z (two strains), and that five produced lactococcin 972. Variable levels of the two bacteriocins were produced by different strains. In addition, nisin was shown to be produced in inexpensive, dairy- and meat-based media, which will allow the practical application of its producing strains in industrial processes. Specific PCR and nucleotide and deduced amino acid sequence analysis identified the inhibitor produced by the single *L. lactis* subsp. *cremoris* isolate as a lactococcin G-like bacteriocin. Beyond the use of bacteriocins as functional ingredients for the biopreservation of foods, the newly identified bacteriocin-producing *L. lactis* strains from traditional cheeses may also be useful for designing starter cultures with protective properties and/or adjunct cultures for accelerating cheese ripening.

1. Introduction

Many microbial groups produce bacteriocins—peptides and proteins with bactericidal activity. Bacteriocins of some bacteria inhibit growth of closely related microbes, while others inhibit a much wider range of microorganisms, including food-borne pathogens and spoilage microorganisms such as *Listeria monocytogenes*, *Bacillus cereus*, *Staphylococcus aureus* and *Clostridium tyrobutyricum* (Gálvez et al., 2008).

From a biochemical point of view, two types of bacteriocins have been identified in lactic acid bacteria (LAB), those characterized by the presence of dehydrated (dehydroalanine and dehydrobutyrine) and/or thioether amino acids (lanthionine and β-methyllanthionine), usually referred to as lanthibiotics (or class I), and those containing unmodified amino acids (non-lanthibiotics) (Jack et al., 1995). Non-lanthibiotics are divided into classes II through IV depending on their size and the presence of non-protein moieties. Both lanthibiotics and non-lanthibiotics are synthesized via a ribosomal pathway, but the former are later modified enzymatically. In the last 25 years, intensive research into the bacteriocins produced by LAB has been undertaken with the aim of improving the microbial quality and safety of fermented products (de Vuyst and Leroy, 2007).

Lactococcus lactis strains are the majority LAB components of commercial starter cultures used by the dairy industry for the manufacture and ripening of cheese and fermented milk (Limsowtin et al., 1995). Lanthibiotic and non-lanthibiotic bacteriocins produced by *L. lactis* from different sources have been identified and characterized (Venema et al., 1995). The first bacteriocin isolated from *L. lactis* was nisin (Mattick and Hirsch 1947), a 34-amino acid lanthibiotic. This is currently approved and exploited in over 50 countries as a food additive (code E234) (Delves-Broughton et al., 1996). To date, five natural nisin variants (A, Z, Q, U, and F) have been identified (de Kwaadsteniet et al., 2008). Other lanthibiotics produced by *L. lactis* include the single peptide lacticin 481 and the two-component system lacticin 3147 (de Vuyst and Leroy 2007). Non-lanthibiotic bacteriocins from *L. lactis* include pediocin-like bacteriocins (class IIa) such as lactococcin MMFII, two-peptide component bacteriocins (class IIb) such as lactococcin G and M, thiol-activated bacteriocins (class IIc) such as lactococcin B, and heat-labile, lactococcus-specific bacteriocins (class IId) such as

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lactococcin A (diplococcin) and lactococcin 972 (Venema et al., 1995; Oppegård et al., 2007).

The incorporation of bacteriocin-producing lactococci as starter or adjunct cultures in the manufacture of fermented food provides an attractive and economic alternative to the addition of purified bacteriocins (indeed, metabolic compounds produced during fermentation are no longer considered as additives). Bacteriocin-producing *L. lactis* has therefore been experimentally tested in the manufacture of several cheese varieties (Ryan et al., 1996; Martínez-Cuesta et al., 2001; O'Sullivan et al., 2003; Rilla et al., 2003; Garde et al., 2006) and other fermented products (Diop et al., 2009). Following its addition, starter lysis is increased (O'Sullivan et al., 2003) and peptidolytic and transamination activities, key factors in the formation of aroma and taste compounds, may also be enhanced (Martínez-Cuesta et al., 2003; Fernández de Palencia et al., 2004). In addition to its technological applications, bacteriocin-producing *L. lactis* has been assayed for the treatment of mastitis in cows (Ryan et al., 1999; Twomey et al., 2000; Klostermann et al., 2009), and is being evaluated as an antipathogenic agent in human gastrointestinal infections (O'Connor et al., 2006; Millette et al., 2008).

The aim of the present work was to screen for bacteriocin production in a large number of *L. lactis* strains isolated during the manufacturing and ripening stages of different batches of five traditional, Spanish, starter-free cheeses made from raw milk. Efforts were also made to identify these antimicrobial compounds by searching for bacteriocin-encoding genes. Of the 17 bacteriocin producers detected, phenotypic and genetic analyses identified eleven as nisin producers, five as lactococcin 972 producers, and a single producer of lactococcin G.

2. Material and methods

2.1. Strains, media and culture conditions

A series of 306 lactococcus-like isolates collected during the manufacture and ripening of five Spanish traditional, starter-free cheeses made from raw milk were grouped by typing and identified by partial ARDRA, sequencing and sequence comparison. These isolates came from *Casín* (80), *Cabrales* (106), *Genestoso* (63), *Peñamellera* (44), and *Valle del Narcea* (13) cheeses. Representative isolates of the 60 different strains found were tested for the production of antimicrobial compounds against a series of Gram-positive indicator bacteria. The indicator strains included *L. lactis* subsp. *cremoris* MG 1363, *L. lactis* subsp. *lactis* NCDO 497 (nisin producer), *L. lactis* subsp. *lactis* IPLA 972 (lactococcin 972 producer), *Lactobacillus sakei* CECT 906^T, *Lactobacillus plantarum* LL 441 (plantaricin C producer), *Listeria innocua* 86/26 and *S. aureus* CECT 86^T. Cryopreserved cultures of cheese isolates and control strains in glycerol were recovered on M17 agar plates (lactococci), de Man, Rogosa and Sharpe (MRS) agar plates (lactobacilli), or in tryptone soy broth (TSB) (*L. innocua* and *S. aureus*), and incubated at the corresponding optimum temperature for 24 h. *Micrococcus luteus* CECT 245 (=ATCC 10240) was used as the indicator strain for measuring nisin activity. This strain was grown in nutrient broth (NB) with shaking at 37 °C for 24 h.

2.2. Identification and typing of isolates

Total genomic DNA from isolates was purified from overnight cultures using the GenElute™ Bacterial Genomic DNA kit (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's recommendations. Electrophoresis was performed in 1% agarose gels, and the bands were stained with ethidium bromide (0.5 μg/mL) and photographed under UV light. Isolates were grouped by repetitive extragenic palindromic (REP) fingerprinting employing the polymerase chain reaction (PCR) and the primer BoxA2-R (Table 1), as reported by Koeuth et al. (1995), followed by random amplification of polymorphic DNA (RAPD) typing with the primer M13 (Table 1), as reported by Rossetti and Giraffa (2005). Reproducibility studies of the combined REP and RAPD techniques showed a percentage similarity of over 95%.

Representative isolates of the REP and RAPD groups were identified by partial ARDRA, followed by sequencing of representative amplicons and comparison of the sequences obtained against those in databases. For ARDRA, 16S rRNA genes were almost completely amplified using universal primers 27-F and 1492-R (Table 1). Amplicons were purified using GenElute™ PCR Clean-Up columns (Sigma-Aldrich), digested with restriction enzymes *Hae*III and *Hin*fI (Invitrogen Ltd., Paisley, UK), and electrophoresed as above. When required, amplicons were sequenced by cycle extension in an ABI 373 DNA sequencer (Applied Biosystems, Foster City, CA, USA). Sequences were compared to those in the GenBank database using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/), and to those held by the Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp).

2.3. Antimicrobial activity

Antimicrobial activity was successively examined by an agar spot test and a well-diffusion assay. For the former, overnight cultures of the isolates were spotted (5 μL) on the surface of M17, MRS and TSB agar plates and incubated at 30 °C for 24 h. Spots were then covered with 10 mL of soft agar (0.75%) inoculated at 0.25% with indicator

Table 1
Primers used throughout this study.

Name	Sequence (5′→3′)	Technique/Amplification	Reference/GenBank accession no
BoxA2-R	ACGTGGTTTGAAGAGATTTTCG	REP-PCR typing	Koeuth et al. (1995)
M13	GAGGGTGGCGGTTCT	RADP typing	Rossetti and Giraffa (2005)
27-F	AGAGTTTGATCCTGGCTCAG	16S rRNA gene	S-D-Bact-0008-a-S-20
1492-R	GGTTACCTTGTTACGACTT	16S rRNA gene	S-*-Univ-1492R-b-A-21
Nis-F	CGGCTCTGATTAAATTCTGAAG	Nisin genes	M65089
Nis-R	GGATTAGCTAGTAGTAACTGTTC	Nisin genes	M65089
Lact3147-F	GTCTTTGTGTTGTTTGGAGATG	Lacticin 3147 gene	AE001272
Lact3147-R	CAACTCCCGAAATAAATCATCG	Lacticin 3147 gene	AE001272
Lact481-F	CCAATGTCATTGCATCTGCAC	Lacticin 481 gene	X71410
Lact481-R	GTCCTTATGTTGCTATTCATC	Lacticin 481 gene	X71410
Lcn972-F	TTGTAGCTCCTGCAGAAGGAACATGG	Lactococcin 972 gene	Martínez et al. (1999)
Lcn972-R	GCCTTAGCTTTGAATTCTTACCAAAAG	Lactococcin 972 gene	Martínez et al. (1999)
LactABM-F	GAAGAGGCAATCAGTAGAG	Lactococcin A, B, and M genes	M90969, S38128, van Belkum et al. 1991
LactA-R	GTGTTCTATTTATAGCTAATG	Lactococcin A gene	M90969
LactB-R	CCAGGATTTTCTTTGATTTACTTC	Lactococcin B gene	S38128
LactM-R	GTGTACTGGTCTAGCATAAG	Lactococcin M gene	van Belkum et al. (1991)
LactGQ-F	GAAAGAATTATCAGAAAAAG	Lactococcin G and Q genes	FJ938036, AB182406
LactGQ-R	CCACTTATCTTTATTTCCCTCT	Lactococcin G and Q genes	FJ938036, AB182406