Plasmid-encoded determinants for bacteriocin production and immunity in a *Lactococcus lactis* strain and purification of the inhibitory peptide

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Lactococcin, a bacteriocin produced by *Lactococcus lactis* subsp. *lactis* ADRIA 85LO30, was purified as a 2.3–2.4 kDa peptide. Six non-bacteriocin-producing (Bac[−]) and non-immune (Imm[−]) strains were isolated after curing experiments. These strains had in common the loss or modification of two plasmids: pOS4 (32 kb) and pOS5 (70 kb). By comparing pOS5 and several modified plasmids, a DNA region from pOS5 of about 10 kb, which was necessary for wild-type bacteriocin production and immunity, was identified.

Introduction

Many lactic acid bacteria produce bacteriocins or bacteriocin-like compounds (Geis *et al.*, 1983; Klaenhammer, 1988). Bacteriocins are secreted proteins with bactericidal activity directed against species that are usually closely related to the producer bacterium (Tagg *et al.*, 1976). Lactic acid bacteria are used extensively in the food industry and bacteriocins or bacteriocin-producing strains are of great interest since they may prevent growth of spoilage and pathogenic bacteria. Nisin, the best-known bacteriocin of lactic acid bacteria, is thus used in dairy products (Hurst, 1981). Moreover, the genetic determinants for bacteriocin production and immunity have great potential as genetic markers. Several bacteriocins from lactococci have been characterized: they appear to form a heterogeneous group of proteins with respect to their molecular mass, biochemical properties, activity spectrum and mode of action (Klaenhammer, 1988).

Lactococci characteristically harbour a number of plasmid species, varying from two to eleven. Some of these plasmids carry determinants for identified traits such as lactose metabolism or proteolytic activity (McKay, 1983). Bacteriocin production and immunity are often associated with plasmid DNA. Production of bacteriocin by *Lactococcus lactis* subsp. *lactis* WM4, diplococcin by *L. lactis* subsp. *cremoris* 346, and

bacteriocin by *L. lactis* subsp. *cremoris* 9B4 were demonstrated to be encoded by plasmids of 135 (pNP2), 83 and 60 kb (p9B4-6) (Scherwitz *et al.*, 1983; Davey, 1984; Neve *et al.*, 1984), respectively. The genetic determinant for nisin production by *L. lactis* was found to be located on a plasmid (Tsai & Sandine, 1987; Kaletta & Entian, 1989) but recent investigations indicated that the nisin determinant may be a component of a large region of transmissible DNA that also contains nisin-resistance and sucrose-metabolizing genes (Dodd *et al.*, 1990). Genetic determinants for nisin and bacteriocins from *L. lactis* WM4 and *L. cremoris* 9B4 have been cloned (Buchman *et al.*, 1988; Kaletta & Entian, 1989; Dodd *et al.*, 1990; Scherwitz Harmon & McKay, 1987; van Belkum *et al.*, 1989), and the nisin gene and two bacteriocin operons from *L. lactis* subsp. *cremoris* 9B4 (van Belkum *et al.*, 1991) have been sequenced.

This study was undertaken with the purpose of cloning the genetic determinant of a bacteriocin-like substance, designated lactococcin, produced by *Lactococcus lactis* subsp. *lactis* ADRIA 85LO30, one of the four strains of *L. lactis* that have been selected for their inhibitory activity against *Clostridium tyrobutyricum* (Thuault *et al.*, 1991). This bactericidal effect against *C. tyrobutyricum* is of great interest since this bacterium provokes a late swelling during ripening of Emmental-type cheese when milk is contaminated with it. This paper reports the

Table 1. *Bacterial strains and plasmids*

<i>L. lactis</i> subsp. <i>lactis</i> strain	Derivation	Phenotype*	Size of resident plasmids (kb)	Plasmid designation	Reference
ADRIA 85LO30	Wild-type strain	Bac ⁺ Imm ⁺	2.9, 3.0, 3.5, 32, 70	pOS4 (32 kb), pOS5 (70 kb)	Thuault <i>et al.</i> (1991)
C3015	Plasmid-cured derivative of ADRIA 85LO30	Bac ⁺ Imm ⁺	2.9, 3.0, 3.5, 83	pES1 (83 kb)	This work
C2102	Plasmid-cured derivatives of C3015	Bac ⁺ Imm ⁺	2.9, 3.0, 3.5, 74	pES2 (74 kb)	This work
C2122		Bac ⁺ Imm ⁺	2.9, 3.0, 3.5, 90	pES3 (90 kb)	
C2109		Bac ⁽⁻⁾ Imm ⁻	2.9, 3.0, 3.5, 62	pRS4 (62 kb)	
C2111		Bac ⁻ Imm ⁻	2.9, 3.0, 3.5		
C2128		Bac ⁻ Imm ⁻	2.9, 3.0		
IL1403		Imm ⁻	None		Chopin <i>et al.</i> (1984)

* Bac, bacteriocin; Imm, immunity.

purification of lactococcin and the putative assignment of the gene to a high-molecular-mass plasmid as determined by curing experiments and variant studies.

Methods

Bacterial strains, plasmids and culture conditions. The strains used and their resident plasmids are listed in Table 1. *L. lactis* IL1403 (Chopin *et al.*, 1984) was inhibited by the bacteriocin (Imm⁻) and was used as an indicator strain of bacteriocin activity. Bacteria were propagated at 30 °C in glucose M17 broth (M17G) (Terzaghi & Sandine, 1975). For subsequent bacteriocin purification, the producing strain was grown at 30 °C in a 2-litre fermenter (Setric Génie industriel) in which the pH was maintained at 5.2 by addition of NH₄OH. The medium used in this case was CG broth (Bhunja *et al.*, 1987) modified as follows: glucose and magnesium sulphate were used at 2 and 0.06% (w/v), respectively; 0.02% K₂HPO₄, 0.02% KH₂PO₄ and 0.003% iron sulphate were added, and disodium phosphate was omitted.

Bacteriocin production and immunity. In order to test for bacteriocin production (Bac⁺), two methods were used. The first one was a solid medium test. Decimal dilutions of bacteria were plated onto M17G agar (1.5%, w/v) and overlaid with M17G soft agar (0.7%, w/v). After 24 h incubation at 30 °C, the plates containing up to 20 colonies were overlaid with M17G agar (1.5%, w/v) seeded with a culture of the indicator organism (Imm⁻) and incubated for an additional 24 h at 30 °C. Colonies surrounded by a clear zone of inhibition were Bac⁺. In the second method, culture supernatant was assayed for the presence of the bacteriocin. Bacteria were grown to late exponential phase and cells removed by centrifugation. The supernatant was sterilized by filtration through a 0.45 µm membrane filter and 100 µl aliquots were poured into wells of M17G agar (1.5%, w/v) plate previously seeded with a culture of the indicator strain. After overnight incubation at 30 °C, wells surrounded by clear zones of inhibition indicated bacteriocin activity (Tagg & McGiven, 1971). To quantify the activity, twofold dilutions of supernatant culture fluid were tested in the same way. The reciprocal of the greatest inhibitory dilution was used to calculate arbitrary activity units (AU) per ml (Mayr-Harting *et al.*, 1972).

Bacteriocin immunity (Imm⁺) was examined by using the second method, in which the indicator strain was replaced by the strain to be characterized.

Purification of bacteriocin. The supernatant of *L. lactis* ADRIA 85LO30 bacteria grown for 24 h in modified CG broth was recovered

by centrifugation (6000 *g* for 10 min) and extensively dialysed (cut-off 6.0 kDa) against sodium acetate buffer (50 mM, pH 4.5). Cation-exchange chromatography (CM-Trisacryl, IBF) was performed as recommended by the supplier. Positively charged proteins were eluted by a sodium chloride gradient (0–0.4 M). Fractions (12 ml) were collected and tested for bacteriocin activity as above. The active fractions were dialysed (cut-off 2.0 kDa) against potassium phosphate buffer (10 mM, pH 6.5) and lyophilized. The proteins were dissolved in 50 mM-ammonium bicarbonate pH 7.8 and applied to a gel filtration column (Sephadex G-50, Pharmacia). Fractions (4 ml) containing bacteriocin activity were lyophilized. The column was calibrated using gel filtration molecular mass markers ranging from 6.5 to 66 kDa (MW-GF-70 kit, Sigma). Protein concentrations were determined by the method of Bradford (1976) with bovine serum albumin as standard. Proteins were analysed by urea sodium dodecyl sulphate polyacrylamide gel electrophoresis (urea-SDS-PAGE) as described by Swank & Munkres (1971). The gel was stained by the method of Neuhoff *et al.* (1988). Size standards were five myoglobin chains of molecular mass 2.5–16.9 kDa (LKB).

Curing experiments. The method described by Sinha (1989) was used. The strains were grown at 30 °C in an unbuffered medium (M17: M17 without phosphate) and maintained at the same temperature for an extended period (at least 96 h). The bacteria were plated and screened either for the loss of plasmid species by plasmid pattern studies, or for the loss of the Bac⁺ phenotype by the overlay method described above. In the second case, colonies not surrounded by a clear zone of inhibition were picked for further investigation.

Plasmid DNA isolation and analyses. Plasmids were extracted as described by Anderson & McKay (1983). Purification of a specific plasmid from a preparation containing several plasmid species was performed by sucrose-gradient purification (Anderson & McKay, 1984). Preparations containing plasmids from 2 to 90 kb were centrifuged in a Beckman SW41 rotor at 36000 r.p.m. for 3 h at 20 °C through a 15–35% (w/v) sucrose gradient prepared in 1.0 M-NaCl, 20 mM-Tris/HCl pH 8.0, 5 mM-EDTA pH 8.0. Gradients were fractionated and analysed by agarose gel electrophoresis. Fractions of interest were dialysed against TE buffer (10 mM-Tris/HCl pH 8.0, 1 mM-EDTA pH 8.0). DNA was concentrated by n-butanol extractions, precipitated with ethanol and dissolved in TE buffer.

Restriction endonucleases were used as recommended by the suppliers. Plasmids and DNA restriction fragments were analysed by electrophoresis in 0.7–1.0% (w/v) agarose gels (Maniatis *et al.*, 1982). Sizes of supercoiled plasmid DNA were determined by using plasmids from *E. coli* or *L. lactis* as standards. Linear DNA fragment sizes were

Fig. 1. Elution of lactococcin activity from chromatography columns. (a) Cation-exchange column (CM-Trisacryl). (b) Gel filtration column (Sephadex G-50) loaded with pooled fractions 5–20 from (a). $\blacktriangle$, Absorbance at 280 nm (arbitrary units); $\bullet$, lactococcin activity; ---, linear NaCl gradient; $\downarrow$, elution peaks of molecular mass markers.

determined using bacteriophage λ DNA digested with *Bst*EII or *Eco*RI as standards. Hybridization experiments were performed as described by Maniatis *et al.* (1982), using nylon membranes (Hybond-N, Amersham): DNA probes were purified from agarose gels by using Gene Clean (Bio 101) and labelled by the random-priming method (Feinberg & Vogelstein, 1984).

Results and Discussion

Purification of lactococcin

The purification of the bacteriocin-like protein was undertaken to further characterize it and to determine an amino acid sequence from which an oligonucleotidic probe could be constructed to analyse the corresponding gene.

To minimize the amount of contaminating proteins, lactococcin was purified from supernatants of broth cultures prepared in modified CG medium. Since lactococcin was shown to be produced during the exponential phase of bacterial growth (Thuault *et al.*, 1991), the *Lactococcus* was cultured in a fermenter at a constant pH of 5.2 to avoid growth inhibition caused by acidification of the medium due to the production of lactic acid. The culture supernatant was equilibrated at pH 4.5 by dialysis and directly applied to a cation-exchange column. No protein concentration step was performed before chromatography since the proteins retained by the column were thus concentrated. A single protein peak containing bacteriocin activity was eluted between 0 and 0.4 M-NaCl (Fig. 1a). Urea-SDS-PAGE analysis (Fig. 2, lane 1) showed a very heterogeneous

Fig. 2. Urea-SDS-PAGE of proteins from culture supernatant of *L. lactis* ADRIA 85LO30 during the purification of lactococcin. Lanes: 1, Bac⁺ fractions after cation-exchange chromatography; 2, Bac⁺ fractions after gel filtration chromatography; 3, molecular mass markers. The arrow indicates the purified peptide.

protein population. When active fractions were concentrated and applied to a Sephadex G-50 column, several protein peaks were obtained; the inhibitory activity was contained in the most prominent one (Fig. 1b). The molecular mass of proteins in the major active fractions was estimated as approximately 2.3 kDa by comparison with gel filtration size standards. Urea-SDS-PAGE analysis (Fig. 2, lane 2) showed a single protein band of around 2.4 kDa, confirming this estimate.

Table 2. Purification of lactococcin

Purification step	Volume (ml)	Lactococcin activity (AU ml ⁻¹)	Total lactococcin activity (AU)	Protein concentration (mg ml ⁻¹)	Amount of protein (mg)	Specific activity (AU mg ⁻¹)	Purification (-fold)
Culture supernatant	850	80	68 800	0.3	255	270	1
Dialysis (cut-off 6 kDa)	900	75	67 500	0.25	225	300	1.1
Cation-exchange chromatography	190	45	8 550	0.03	5.7	1 500	5.5
Gel-filtration chromatography	39	80	3 100	0.02	0.8	3 900	14.5

A scaled-down purification of lactococcin is represented in Table 2. The final purification was 14.5-fold. It is noteworthy that no significant inhibitory activity was lost during the dialysis step with a 6.0 kDa cut-off, although the molecular mass of lactococcin was found to be around 2.4 kDa. A possible explanation is that lactococcin may be in a multimeric form in the culture supernatant. We were not able to determine an amino acid sequence of the purified peptide by Edman degradations. This may have been due to contaminating proteins or to the presence of lanthionines in lactococcin. These unusual amino acids are characteristic of a class of small bactericidal proteins termed lantibiotics, of which nisin, subtilin and epidermin are representatives (Buchman *et al.*, 1988). Lanthionines are obtained by post-translational modifications of serine, threonine and cysteine residues, resulting in thioether cross-linkages which may explain the sequencing difficulties.

Plasmid localization of the bacteriocin gene

The wild-type Bac⁺ strain *L. lactis* ADRIA 85LO30 was shown to harbour the following plasmids: 2.9, 3.0, 3.5, 32 and 60–70 kb (Fig. 3, lane 4). The 2.9 and 3.0 kb plasmids were not separated in these electrophoretic conditions (0.75% agarose gel, 3.75 V cm⁻¹) but they could be distinguished by electrophoresis on a higher percentage agarose gel. Restriction analysis showed that the bands of 60–70 kb were due to a unique plasmid species of about 70 kb (see below). The 32 and 70 kb plasmids were designated pOS4 and pOS5, respectively. Curing experiments were undertaken to investigate whether bacteriocin production could be correlated with a specific plasmid. The method of Sinha (1989) was chosen because of the described high frequency of plasmid elimination and of its apparent non-mutagenicity compared with methods involving mutagenic chemicals. The plasmid content and the phenotype of the strains described below are summarized in Table 1.

The first experiment was performed with a 96 h incubation time. Of 30 colonies screened by plasmid extractions, one had a modified plasmid content. This

Fig. 3. Agarose gel electrophoresis of plasmid DNA from *L. lactis* ADRIA 85LO30 and derived strains. Lanes: 4, ADRIA 85LO30 (pOS4, pOS5); 5, C3015 (pES1); 6, C2102 (pES2); 7, C2122 (pES3); 8, C2109 (pRS4); 9, C2111; 10, C2128. Molecular size markers: plasmid DNA from *L. lactis* or *E. coli*: Lanes: 1, pAMβ1 (27 kb); 2, pR483 (93 kb); 3, pRP1 (60 kb); 11, pIL253 (5.0 kb); 12, pBluescript (3.0 kb, Stratagene). Chr, chromosomal DNA.

strain, designated C3015, had lost pOS4 (32 kb) but an additional plasmid species of 83 kb appeared, named pES1 (Fig. 3, lane 5). *L. lactis* C3015 presented a Bac⁺ Imm⁺ phenotype. pOS4 was therefore not needed for bacteriocin production or immunity, unless pOS4 or a part of it was included in pES1.

Fig. 4. Inhibitory activity of various strains derived from *L. lactis* ADRIA 85LO30 tested against *L. lactis* IL1403 (Imm^-) by (a) culture supernatant diffusion or (b) solid agar medium test. A, D, C2102; B, E, C2109; C, F, C2111. Inhibitory activity is shown by a clear zone.

Subsequent curing experiments were performed with C3015. The incubation time had to be increased to 480 h to isolate new variant strains. The screening was performed either by Bac phenotype analyses or by plasmid preparations. By the first method, six Bac^- colonies were isolated in three experiments. The Bac^- character of one of these strains, designated C2111, is shown in Fig. 4(C, F). The six Bac^- strains were all sensitive to lactococcin (Imm^-). Each strain was cured of all high-molecular-mass plasmids. Four strains harboured the 2.9, 3.0 and 3.5 kb plasmids (for example C2111, Fig. 3, lane 9) while the two other strains contained only the 2.9 and 3.0 kb ones (for example C2128, Fig. 3, lane 10). These observations suggested that the plasmids pOS4 or pOS5 were involved in bacteriocin production and immunity. By the second screening method, the plasmid content of 58 colonies was analysed after an incubation of 192 h. With respect to C3015, all colonies showed a modified plasmid electrophoretic pattern. The differences were exclusively located in the high-molecular-mass plasmid region. Three types of plasmid patterns were observed (Fig. 3, lanes 6 to 8). The strains C2102 and C2122, representative of two plasmid patterns, harboured high-molecular-mass plasmids of 74 (pES2) (Fig. 3, lane 6) to 90 kb (pES3) (Fig. 3, lane 7) and had a $\text{Bac}^+ \text{Imm}^+$ phenotype (the Bac^+ character of C2102 is shown in Fig. 4, A, D). One strain, designated C2109, harboured a unique high-molecular-mass plasmid of reduced size (62 kb), pRS4 (Fig. 3, lane 8). Strain C2109 was found to be Bac^- when the bacteriocin activity of the culture supernatant was tested (Fig. 4, B), but was shown to induce a small inhibition halo when the test was done by the overlay method (Fig. 4, E). This inhibitory activity could be concentrated from a liquid medium culture by ultrafiltration and may result from a residual bacteriocin production. The Bac phenotype of C2109 was denoted $\text{Bac}^{(-)}$. *L. lactis* C2109 could be inhibited by lactococcin (Imm^-), but must have a sufficient immunity level to

survive its own bacteriocin production. The $\text{Bac}^{(-)} \text{Imm}^-$ phenotype of C2109 may result from the reduced-size plasmid pRS4. This hypothesis is in agreement with the association of lactococcin production and immunity with the large plasmid species.

Identification of a region of pOS5 involved in bacteriocin production and immunity

To further characterize and compare the large plasmids from the different Bac^+ strains and C2109 ($\text{Bac}^{(-)}$), DNA restriction analysis of these purified plasmids was performed. The results from *Bam*HI digests are presented in Fig. 5(a). By sucrose-gradient centrifugation we were able to separate the plasmids of around 70 kb from the 2.9, 3.0 and 3.5 kb ones, but not from pOS4 (32 kb), only present in *L. lactis* ADRIA 85LO30. Thus, when digesting large plasmids from this strain, fragments of greater intensity arising from pOS4 were observed. For example, restriction of pOS4 by *Bam*HI generated two DNA fragments of 20 and 13 kb (Fig. 5a, lane 1).

Similar *Bam*HI restriction patterns were obtained with the large plasmids from the various strains (Fig. 5a), leading us to conclude that the various high-molecular-mass plasmids were derived from pOS5. The sum of the sizes of the DNA restriction fragments was in good agreement with the estimated size of the native plasmids and corresponded to a unique 60–90 kb plasmid in each strain. When comparing the *Bam*HI restriction fragments (Fig. 5a), we observed in the $\text{Bac}^{(-)}$ pRS4 plasmid (lane 5) the loss of three fragments of 4.5, 3.1 and 2.1 kb which were present in pOS5 and the pOS5-derived plasmids of all Bac^+ strains (lanes 1–4). These fragments were cloned in *E. coli* and used as probes in Southern hybridization experiments. The results of hybridizations with DNA of Fig. 5(a) are presented in Fig. 5(b–d). Each probe hybridized with its corresponding *Bam*HI fragment in lanes 1–4, but there was no similar hybridization

Fig. 5. (a) Agarose gel electrophoresis of *Bam*HI-digested high-molecular-mass plasmids from *L. lactis* ADRIA 85LO30 and derived strains. (b, c, d) Autoradiograms resulting from Southern hybridizations of panel (a) with the 4.5 kb (b), 3.1 kb (c) or 2.1 kb (d) fragments of pES2 as ³²P-labelled probes. Lanes: 1, pOS4 and pOS5 (ADRIA 85LO30); 2, pES1 (C3015); 3, pES2 (C2102); 4, pES3 (C2122); 5, pRS4 (C2109). All strains are Bac⁺ Imm⁺, except C2109 (Bac⁻ Imm⁻). Lanes 6 and 7, molecular size markers: bacteriophage λ DNA digested with *Bst*EII or *Eco*RI, respectively. The arrows indicate the DNA fragments deleted in pRS4 and used as probes in panels (b), (c) and (d).

in lanes 5, confirming that the three fragments used as probes were present in all Bac⁺ high-molecular-mass plasmids, and absent in the Bac⁻ plasmid pRS4. It is noteworthy that the 3.1 and 2.1 kb probes strongly cross-hybridized with other *Bam*HI restriction fragments. By hybridizing these probes with *Eco*RI or *Hind*III digests of the various plasmids, the three *Bam*HI fragments were shown to be contiguous, forming a single plasmid region (data not shown). A region of the Bac⁺ plasmids pOS5, pES1, pES2 and pES3, which was deleted in the Bac⁻ plasmid pRS4, was thus identified. This region probably contains genetic information which is required for lactococcin production and immunity in the wild-type Bac⁺ Imm⁺ strain.

Conclusions

Curing experiments indicated that production of lactococcin, a bacteriocin-like peptide of about 2.4 kDa, and immunity are probably associated with a plasmid from *L. lactis* subsp. *lactis* ADRIA 85LO30. The curing method used resulted not only in elimination of plasmid

species (six Bac⁻ Imm⁻ strains were cured of all high-molecular-mass plasmid species), but also in modifications of the plasmid pOS5 (70 kb). No similar observation has previously been reported with this method. When comparing pRS4, a deleted Bac⁻ Imm⁻ plasmid, with Bac⁺ plasmids, we identified in pOS5 a DNA region of about 10 kb probably necessary for lactococcin production and immunity.

These results suggest, without formal proof, that genetic determinants for bacteriocin production and immunity are localized on pOS5, and are at least partially included in the 10 kb region. Work currently in progress is aimed at isolating the genetic determinant for lactococcin production and immunity by cloning fragments of the Bac⁺ plasmid into lactic acid bacteria and searching for a clone able to express the gene.

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