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Cloning and Identification of the Lactococcin A and M Gene Cluster from *Lactococcus lactis* subsp. lactis biovar *diacetylactis* DRC1

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Abstract

The bacteriocin genes from the plasmid pDR1-6 of *Lactococcus lactis* subsp. *lactis biovar diacetylactis* DRC1 were cloned into pGKV21 and pGKV259 as 4.0 kb *HindIII* and 9.5 kb *EcoRI* fragments, and expressed in plasmid-free and bacteriocin-non-producing strain *L. l. lactis* DRC1021. The nucleotide sequences of the genes were identified as a part of operons of lactococcin A and M.

Discipline: Biotechnology
Additional key words: bacteriocin, dricin

Introduction

Bacteriocins are proteinaceous bactericidal compounds that are produced by some microorganisms, including lactic acid bacteria. The best-known bacteriocin of lactic acid bacteria origin is probably nisin, which is a lantibiotic type bacteriocin produced by certain strains of *Lactococcus lactis*. A large number of new bacteriocins in the group of lactic acid bacteria have been characterized mainly biochemically due to the development of efficient protocols for purification of these hydrophobic and cationic peptides in recent years. In several cases it has been demonstrated that bacteriocin production is associated with plasmid DNA. However, only a few genetic determinants for these substances have been cloned and sequenced[8,10].

Previously, Powell et al. reported that *L. lactis* DRC1 (Commonwealth Scientific and Industrial Research Organization, Dairy Research Laboratory collection) produced dricin which is a heat-stable proteinous bacteriocin and suggested that the gene involved in dricin production is carried on a conjugative plasmid[11]. We also found that an antagonistic substance against other lactococci was produced by *L. l. lactis* biovar *diacetylactis* DRC1 in our laboratory collection, formerly designated as *Streptococcus lactis* subsp. *diacetylactis* DRC1 originally obtained from the National Institute for Research in Dairying (now AFRC Institute of Food Research)[5].

In this report, we demonstrated that the inhibitory activity is due to the concerted action of at least 2 bacteriocins, lactococcin A and M in *L. l. lactis* DRC1 by cloning and sequencing of the genes coding for bacteriocin production.

Materials and methods

The bacterial strains and plasmids used in this study are listed in Table 1. Thirty-eight lactococcal strains in our laboratory collection[5] were examined for cross-antagonistic activity using the agar spot test. Colonies of bacteria that could produce bacteriocin were grown on M17G agar plate overnight[14]. A lawn of 4 mL of M17G soft agar (0.7%) containing 100 μL of a fresh culture of the indicator organism was poured over a plate. After incubation overnight at 30°C, the colonies were examined for zones of growth inhibition.

Plasmid DNA was isolated from lactococcal strains by the method of Anderson and McKay[1]. Transformation of plasmid-free strain *L. l. lactis* DRC1021 was performed by electroporation, with a Bio-Rad gene pulser (Bio-Rad Laboratories, Richmond, Calif.) according to the method of Holo and

The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases with the following accession numbers D86737 and D86738.

Table 1. Bacterial strains and plasmids

Bactrial strain or plasmid	Remarks	Reference or source
Strain		
E. coli		
XL-1Blue		Stratagene
DH11S		Bethesda Research Laboratory
L. l. lactis		
527	Sensitive indicator	4)
1061	Plasmid-free derivative of 527, sensitive indicator	This study
DRC1	Wild type (Lac^+, Cit^+, Bac^+)	4)
ATCC13675	Wild type (Lac^+, Cit^+, Bac^+)	4)
DRCg4	Plasmid-cured derivative of DRC1 (Lac^-, Cit^-, Bac^+)	This study
DRC1021	Plasmid-free derivative of DRC1 (Lac^-, Cit^-, Bac^-)	This study
Plasmid		
pBluescriptII	*E. coli* cloning vectors, Amp^r	Stratagene
pGKV21	*E. coli*, *B. subtilis* and *L. lactis* shuttle vector, Em^r	9)
pGKV259	*E. coli*, *B. subtilis* and *L. lactis* shuttle vector, Em^r	10)
pDR1-6	Bac^+ plasmid	This study
pDR403	SKII+; *Hin*dIII 4 kb	This study
pDR405	pGKV21; *Hin*dIII 4 kb (Bac^+)	This study
pDR407	SKII+; *Hin*dIII-*Sau*3AI 1.6 kb	This study
pDR413	SKII+; *Eco*RI 9.5 kb	This study
pDR422	SKII+; *Sca*I-*Cla*I 1.8 kb	This study
pDR430	pGKV259; *Eco*RI 9.5 kb (Bac^+)	This study

Nes[7]. Recombinant DNA analysis was performed as described by Sambrook et al.[12]. DNA was sequenced by Taq Dye Deoxy Cycle sequencing on an Applied Biosystems 373A DNA sequencer (Applied Biosystems, Foster City, Calif.). For sequencing, stepwise deletion derivatives of cloned DNA fragments were constructed with ExoIII/Mung Bean deletion kit from Stratagene. The GENETYX-MAC program (Software Co., Japan) was used to analyze DNA sequences. For Southern hybridization, DNA was transferred to Hybond N (Amersham, Buckinghamshire, United Kingdom), using Vacu-gene (Pharmacia, Uppsala, Sweden) according to the manufacturer's specifications. Nonradioactive DNA probes were prepared using a random-primed labeling and detection kit (Boehringer Mannheim, GmbH, Germany). Hybridization and immunological detection were performed as recommended by the supplier.

Results and discussion

As a result of the cross-antagonistic test, *L. l. lactis* DRC1 and ATCC13675 inhibited the growth of the majority of the lactococcal strains such as *L. l. lactis* 527 and 1061. These strains exhibited a cross-immunity to each other and the antagonistic agent was heat-stable under acidic conditions and inactivated by incubation with pronase E, protease K, subtilisin and trypsin. These properties suggested that the strains produced the same polypeptide bacteriocin.

Several plasmid-cured derivatives from *L. l. lactis* DRC1 were isolated after growth in broth containing acridine orange and compared with their plasmid profile and phenotype. *L. l. lactis* DRC1 harbored 6 distinct plasmids originally. Plasmid-free derivatives *L. l. lactis* DRC1021 and *L. l. lactis* DRC104, which contained a cryptic 7.9 kb plasmid pDR1-1, were unable to inhibit the growth of the indicator strain *L. l. lactis* 1061, and were sensitive to the bacteriocin produced by *L. l. lactis* DRC1. Since *L. l. lactis* DRCg4, which contained the large (more than about 60 kb) plasmids pDR1-6 and pDR1-1, still displayed a bacteriocin activity, it was suggested that the presence of pDR1-6 was required for bacteriocin production and its immunity. Plasmid pDR1-6 extracted from *L. l. lactis* DRCg4 was digested with *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I and *Sal*I, respectively. These fragments were ligated into the multiple cloning sites of the pWV01-derived cloning vectors pGKV21[16] and pGKV259[17], and transformed into *L. l. lactis* DRC1021.

Erythromycin-resistant transformants were overlaid with indicator cells to screen the bacteriocin activity. Plasmid pDR405 (4 kb *Hin*dIII fragment in pGKV21) which contained the transformant inhibited

the growth of the indicator strain but did not inhibit the growth of the bactericin-producing strains DRC1 and ATCC13675.

The restriction endonuclease map and nucleotide sequence of the 4 kb *Hin*dIII fragment specifying antagonistic activity were determined (Fig. 1A). The 4093 bp nucleotide sequence was identical with the lactococcin A gene cluster described by Stoddard et al.[13], including 3 complete open reading frames, *lcn*D, *lcn*A and *lci*A. Incomplete open reading frame of *lcn*C, interrupted by the *Hin*dIII site, was also present upstream of *lcn*D. Downstream of *lci*A the sequence was identical with the promoter and N-terminal regions of *lcn*B, which was described by van Belkum et al.[4].

Complete lactococcin A gene cluster contained

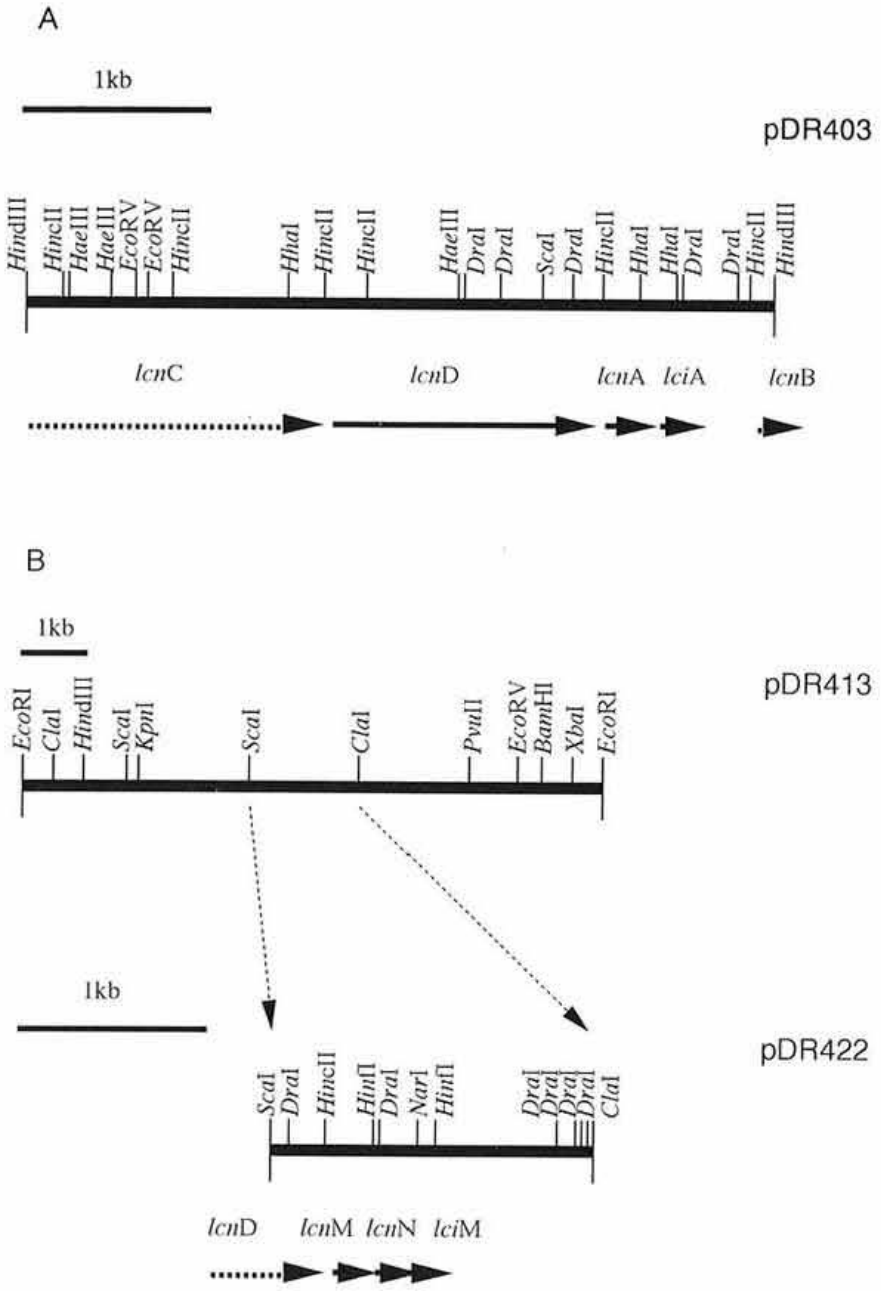

Fig. 1. Physical map of the cloned lactococcin operons from *L. l. lactis* DRC1

A: Restriction map of the recombinant plasmid, pDR403. The DDBJ accession number of this sequence is D86737.

B: Restriction map of the recombinant plasmids, pDR413 and pDR422. The DDBJ accession number of this sequence is D86738. Arrows show the putative open reading frames.

*lcn*C and *lcn*D genes as well lactococcin A structural and immunity genes. It is now generally accepted that *lcn*C and *lcn*D are required for the secretion via a system oriented to bacteriocin export. In this experiment, *L. l. lactis* DRC1021 was able to produce externalizing active lactococcin A in the absence of the plasmid-encoded *lcn*C gene. It was suggested that the chromosomal *lcn*C gene may be present in *L. l. lactis* DRC1021 as in the case of *L. l. lactis* IL1403[15]. A 1.6 kb *Hin*dIII-*Sau*3AI fragment encompassing internal *lcn*C was used as the probe in Southern hybridization. A signal was indeed found on the chromosomal DNA of *L. l. lactis* DRC1021.

Lactococcin A which belongs to class II bacteriocin, was purified from *L. l.* subsp. *cremoris* LMG2130 by Holo et al.[6] and is produced by several strains of *L. lactis*. The conjugative 60 kb plasmid p9B4-6 isolated from *L. l. cremoris* 9B4 contained 3 operons coding for lactococcins A, B, and M as well as for the corresponding immunity proteins[2–4]. Recently, Morgan et al. have reported that the genes encoding lactococcin A, B, and M are located on a 72- and a 78-kb nonmobilizable plasmid in *L. l. lactis* DPC938 and DPC3286, respectively[9]. This finding prompted us to determine whether *L. l. lactis* DRC1 carries genes homologous to the other lactococcins. By using pDR403 encompassing incomplete *lcn*A operon as a probe, 2 signals, 9.5 kb and 24 kb, were obtained in *Eco*RI fragments of pDR1-6.

The 9.5 kb *Eco*RI fragment was cloned into competent *E. coli* XL-1 Blue by using the vector pBluescript II, designated as pDR413, and the 9.5 kb fragment was then religated into the *Eco*RI site of pGKV259 and transformed into *L. l. lactis* DRC1021. Resultant transformants also exhibited bacteriocin activity. This recombinant plasmid was designated as pDR430 (9.5 kb *Eco*RI fragment in pGKV259). However, *L. l. lactis* DRC1021 harboring pDR430 did not confer a resistance against *L. l. lactis* DRC1. The restriction map of 9.5 kb *Eco*RI fragment presented in Fig. 1B revealed a similarity to the restriction map of pMB200, which encoded the low antagonistic activity of p9B4-6 described by van Belkum et al., and it was shown that the lactococcin M operon was located on the internal 1.8 kb *Sca*I-*Cla*I fragment[2,3]. Therefore, pDR413 was subcloned in *E. coli*, and the following 1.8 kb *Sca*I-*Cla*I fragment was obtained as pDR422. The nucleotide sequence of this 1782bp fragment was determined and identified with lactococcin M operon completely, including a part of *lcn*D and 3 open reading frames of *lcn*M, *lcn*N and *lci*M (Fig. 1B)[2,3].

In this experiment, we were not able to clone the 24 kb *Eco*RI fragment of pDR1-6, which contains the operons of lactococcin A and B. *Lcn*B operon was located near *lcn*A operon on p9B4-6[4] and pSM72[9]. The nucleotide sequence analysis showed that downstream of *lci*A on pDR403 a sequence was present which was identical with the N-terminal sequence of *lcn*B. These findings suggest that pDR1-6 also encoded the third bacteriocin gene.

Initially, it was considered that the inhibitory activity of *L. l. lactis* DRC1 was due to the action of a single bacteriocin (dricin) as described by Powell et al.[11]. However, cloning and sequencing revealed that the observed activity was probably due to the combination of at least 2 different bacteriocins, namely lactococcin A and M.

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