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A SUCCESSFUL USE OF A NEW SHUTTLE CLONING VECTOR PA13 FOR THE CLONING OF THE BACTERIOCINS BACSJ and ACIDOCIN 8912

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Abstract – The aim of this paper was to research the molecular cloning of genes encoding the novel bacteriocin BacSJ from *Lactobacillus paracasei* subsp. *paracasei* BGSJ2-8 by using a newly constructed shuttle cloning vector pA13. A new shuttle-cloning vector, pA13, was constructed and successfully introduced into *Escherichia coli, Lactobacillus* and *Lactococcus* strains, showing a high segregational and structural stability in all three hosts. The natural plasmid pSJ2-8 from *L. paracasei* subsp. *paracasei* BGSJ2-8 was cloned in the pA13 using *Bam*HI, obtaining the construct pB5. Sequencing and *in silico* analysis of the pB5 revealed 15 open reading frames (ORF). Plasmid pSJ2-8 harbors the genes encoding the production of two bacteriocins, BacSJ and acidocin 8912. The combined N-terminal amino acid sequencing of BacSJ in combination with DNA sequencing of the *bacSJ2-8* gene enabled the determination of the primary structure of a bacteriocin BacSJ. The production and functional expression of BacSJ in homologous and heterologous hosts suggest that *bacSJ2-8* and *bacSJ2-8i* together with the genes encoding the ABC transporter and accessory protein are the minimal requirement for the production of BacSJ. Biochemical and genetic analyses showed that BacSJ belongs to the class II bacteriocins. The shuttle cloning vector pA13 could be used as a tool for genetic manipulations in lactobacilli and lactococci.

Keywords: Shuttle cloning vector, bacteriocin BacSJ, plasmid pSJ2-8

UDC 577.2:579.253

INTRODUCTION

Many lactic acid bacteria (LAB) are known as producers of bacteriocins, ribosomally synthesized antimicrobial peptides (Diep and Nes, 2002). Most bacteriocins from LAB are of small size, heat-stable, cationic, amphiphilic, membrane-permeabilizing molecules. The bacteriocins can have either a narrow inhibitory spectrum limited to closely related bacteria or, in some cases, a broad inhibitory spectrum which includes food-spoilage and food-born pathogenic bacteria (Field et al., 2007). Furthermore, Lüders and coauthors (2003) showed the synergistic effect between the eukaryotic antimicrobial peptide pleurocidin and bacteriocins from LAB that gave a new approach in their possible application.

Although classification of the bacteriocins from LAB is under reconsideration (Klaenhammer, 1993; Cotter et al., 2005; Heng and Tagg, 2006) they can be classified into three main classes. Post-translationally modified bacteriocins or lantibiotics form class I while non-modified, heat-stable bacteriocins comprise class II. Class II can be divided into subclasses: IIa (pediocin-like, antilisterial bacteriocins); IIb (two-peptide bacteriocins); IIc (cyclic bacteriocins), and IId (other peptide bacteriocins). Heat-labile bacteriocins with a higher molecular mass represent the class III. Most of class II bacteriocins are synthesized in LAB as biologically inactive peptides containing a double-glycine-type leader peptide at N-terminus. They are cleaved and exported across the cytoplasmatic membrane by ABC transporters and their accessory proteins

(Håvarstein et al., 1995). In addition, the ABC transporter system can be a part of the complex immunity mechanism that is characteristic for some lantibiotics (McAuliffe et al., 2001). However, some bacteriocins from class II contain N-terminal extensions of the sec type that are cleaved and exported by the general secretory pathway (van Wely et al., 2001).

Plasmids are very commonly associated with the majority of the lactic acid bacteria. At first sight, the wealth of naturally occurring plasmids in LAB would seem to offer endless opportunities for the development of cloning vectors. However, many of the endogenous plasmids turned out to be cryptic and without useful selection markers like antibiotic resistance that resulted in a limitation of their use. On the other hand, plasmids have attracted attention for several reasons: i) the analysis of their distribution in nature and their genetic relationship to host cells, ii) the elucidation of their relatedness and evolutionary origins, and iii) the analysis of horizontal gene transfer, a process with tremendous impact in risk assessment of the release of genetically modified organisms. In general, lactobacilli contain multiple plasmids that can vary in size (Mayo et al., 1989). The plasmid vectors most widely used in the genetic manipulation of lactobacilli belong to three types: i) plasmids based on rolling circle replication (RCR) replicons, ii) plasmids with two origins of replication, one for *E. coli* and one for Gram-positive bacteria, and iii) *Lactobacillus* vectors with an alternative replication origin for Gram-negative bacteria. This growing interest in the characterization of *Lactobacillus* replicons themselves as potential useful vectors led to the development of a *Lactobacillus/E.coli* shuttle vector (Alpert et al., 2003; Pavlova et al., 2002). Therefore, in this study we present the construction of a novel shuttle lactobacilli/lactococci/*E. coli* cloning vector that contained parts of the plasmids pIL253 and pA1 that will facilitate the cloning of new genes from LAB and improve the knowledge of their genetics.

In our previous work, purification of the bacteriocin BacSJ produced by *Lactobacillus paracasei* subsp. *paracasei* BGSJ2-8 resulted in a single peptide that in electrospray ionization-mass spectroscopy (ESI-MS) analysis showed a major mass peak of 5372 Da, suggesting that the BacSJ was purified to homogeneity (Lozo et al., 2007). Furthermore, plasmid curing experiments showed that the production of BacSJ depended on the presence of the plasmid pSJ2-8 (14442 bp) in *L. paracasei* subsp. *paracasei* BGSJ2-8, indicating that the complete set of genes necessary for bacteriocin production are located on this plasmid (Topisirović et al., 2006). In this work, we present the cloning and expression of the genes responsible for bacteriocin BacSJ production by using a novel shuttle cloning vector pA13.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. The strains of lactobacilli were cultivated in MRS medium (Merck, GmbH, Darmstadt, Germany) at 30°C, while the strains of lactococci were cultivated in M17 medium (Merck) supplemented with D-glucose (0.5% w/v)(GM17) at 30°C. *E. coli* DH5α, used for the cloning and propagation of constructs, was grown in Luria-Bertani (LB) broth (Miller 1972) aerobically at 37°C. Agar plates were made by adding 1.5% (w/v) agar (Torlak, Belgrade, Serbia) to the liquid media. Transformants of *L. paracasei* subsp. *paracasei* BGHN14, BGSJ2-83 and *Lactococcus lactis* subsp. *lactis* BGMN1-596, were selected on MRS or GM17 plates containing 5 μg ml^{-1} of erythromycin (Sigma Chemie GmbH, Deisenhofen, Germany). *E. coli* transformants were selected on LB plates containing 250 μg ml^{-1} of erythromycin. Isopropylthio-β-D-galactoside (IPTG) (Fermentas, Vilnius, Lithuania) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) (Fermentas) were added to the LB medium plates for blue/white color selection of colonies at final concentration of 0.1 mmol l^{-1} and 40 μg ml^{-1}, respectively. The cell-free supernatant of *L. paracasei* subsp. *paracasei* BGSJ2-8 was obtained by centrifugation (16 000 g for 10 min) of a 16

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)*	Source or reference
Strains		
Lactobacillus paracasei subsp. *paracasei*		
BGHN14	Natural isolate from home-made semi hard cheese	Kojic *et al.,* 1991
BGSJ2-8	Natural isolate from home-made semi hard cheese; harbours pSJ2-8	Topisirovic *et al.,* 2006
BGSJ2-83	Plasmid free derivative of *L. paracasei* subsp. *paracasei* BGSJ2-8	This study
BGSJ2-8/pB5	Derivative of *L. paracasei* subsp. *paracasei* BGSJ2-83 with pB5	This study
Lactococcus lactis subsp. *lactis*		
BGMN1-596	Plasmid free derivative of *L. lactis* subsp. *lactis* BGMN1-5	Gajic *et al.,* 1999
Escherichia coli		
DH5α	*supE44 ΔlacU169 (ø80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Hanahan1983
Plasmids		
pA1	2.8 kb RCR replicon	Vujcic and Topisirovic 2003
pIL253	4.9 kb high-copy vector with theta replicon, Em^r	Simon and Chopin 1988
pA13	4.6 kb lactobacilli/lactococci/*E. coli* shuttle cloning vector; Em^r	This study
pSJ2-8	14.4 kb wild-type plasmid from *L. paracasei* subsp. *paracasei* BGSJ2-8	This study
pB5	19 kb derivative of pA13 carrying pSJ2-8 (14.4 kb) into *Bam*HI site, Em^r	This study
pBP3	15.93 kb derivative of pA13 carrying *Bam*HI/*Pst*I (11331 bp) fragment of pSJ2-8, Em^r	This study
pBSC5	14.4 kb derivative of pA13 carrying *Bam*HI/*Sac*I (9809 bp) fragment of pSJ2-8, Em^r	This study
pBE7	11.1 kb derivative of pA13 carrying *Bam*HI/*Eco*RI (6486 bp) fragment of pSJ2-8, Em^r	This study
pEP3	8 kb derivative of pA13 carrying *Eco*RI/*Pst*I (3374 bp) fragment of pSJ2-8, Em^r	This study
pEST3	6.3 kb derivative of pA13 carrying *Eco*RI/*Stu*I (1701 bp) fragment of pSJ2-8, Em^r	This study
pSTP1	6.27 kb derivative of pA13 carrying *Stu*I/*Pst*I (1673 bp) fragment of pSJ2-8, Em^r	This study
pBML9	12.1 kb derivative of pA13 carrying *Bam*HI/*Mlu*I (7516 bp) fragment of pSJ2-8, Em^r	This study

*Em^r, erythromycin resistant

hour-old culture and subsequent filtration through a 0.45 μm filter. The antagonistic activity of BGSJ2-8 and recombinant strains was evaluated by agar well diffusion assay as previously described (Lozo et al., 2004). Briefly, the wells were made in soft MRS agar containing indicator strains. Aliquots (50 μl) were poured into the wells and the plates were incubated overnight at 30°C. Bacteriocin activity and sensitivity was quantified by measuring the diameters of the inhibition zone (in mm) from the edge of the well to the end of the halo around the well.

Molecular techniques

Mini-prep isolation of plasmids from lactobacilli was performed by using the method of O'Sullivan and Klaenhammer (1993). Plasmid isolation from

E. coli was carried out by using a QIAprep Spin Miniprep kit according to the manufacturer's recommendations (QIAGEN, Hilden, Germany). Digestion with restriction enzymes was conducted according to the supplier's instructions (Fermentas). Agarose gel electrophoresis, end filling of DNA fragments with the Klenow fragment of the DNA polymerase and dephosphorylation were performed by using standard methods (Sambrook et al., 1989). Plasmids were introduced into *Lactococcus* and *Lactobacillus* by electroporation (Holo and Nes, 1989; Walker et al., 1996) using Eppendorf Electroporator (Eppendorf, Hamburg, Germany). The standard heat-shock transformation was used for plasmid transfer into *E. coli* (Sambrook et al., 1989). Purification of the DNA fragments was carried out using a QIAqick Gel extraction kit as described by the manufacturer (QIAGEN). DNA ligation was performed using T4 DNA ligase (New England, BioLabs, USA) according to the manufacturer's instructions.

Construction of a shuttle-cloning vector

The pA13 shuttle-cloning vector was constructed in order to perform the molecular analysis of plasmid pSJ2-8 (Fig. 1). The smallest cryptic RCR plasmid pA1 of *L. plantarum* A112 (Vujčić and Topisirović, 1993) was digested with *Eco*RI and fused with the pIL253 (Simon and Chopin, 1988), digested with the same enzyme. The new construct, designated pA-IL/*Eco*RI, was digested with *Hha*I and the obtained mixture of DNA fragments were ligated. The ligation mixture was used to transform *E. coli* DH5α competent cells and the obtained Em^r transformants were screened for the smallest construct possible to replicate in *L. paracasei* subsp. *paracase*i BGHN14. The plasmid named pA1-6, containing two of three *Hha*I DNA fragments of the plasmid pA1, and one *Hha*I fragment carrying the *ermAM* gene from pIL253, was used for the next step of plasmid construction. The *Ava*II-*Cla*I DNA fragment from the M13mp18 phage containing the *lac*Z gene was blunted by Klenow DNA polymerase and inserted into the blunted unique *Eco*RI restriction site of plasmid pA1-6. The blue Em^r transformants were selected in *E. coli* DH5α and the obtained plasmid, designated pA13, was characterized by restriction enzyme analysis. The analysis showed that plasmid pA13 contained two *Eco*RI restriction sites, because the junction of blunted *Cla*I and *Eco*RI sites caused the regeneration of the *Eco*RI site. To obtain the vector with a single *Eco*RI restriction site, the plasmid pA13 was linearized by partial digestion with *Eco*RI restriction enzyme, end filled with Klenow DNA polymerase, and self-ligated. After restriction analysis of several blue Em^r transformants, one pA13 derivative containing a unique *Eco*RI site at the polycloning site was purified, sequenced and used in further work.

Plasmids constructions

Plasmid pSJ2-8 was digested with *Bam*HI and the obtained fragments were cloned into the *Bam*HI site of the pA13 giving construct designated pB5 that was used for further analyses. Derivatives of plasmid pB5 were constructed as follows: plasmid pB5 was digested with various restriction enzymes and the obtained fragments were circularized by intramolecular ligation (directly or after treatment with Klenow enzyme) and used for the transformation of *E. coli* DH5α. The obtained constructs were designated as pBP3 (*Bam*HI/*Pst*I - 11331 bp) (part of *repB1*, ORF3, *repB*, *bacSJ2-8*, *orf2*, *tnpIS30*, *abcT*, *acc*, *acdT*, *orf1*, *tnp6*, *mobC*, part of *mobA*), pBSC5 (*Bam*HI/*Sac*I – 9809 bp) (part of *mobA*, *orf5*, *orf4*, *repB1*, *orf3*, *repB*, *bacSJ2-8*, *orf2*, *tnpIS30*, *abcT*), pBE7 (*Bam*HI/*Eco*RI - 6486 bp) (part of *mobA*, *orf5*, *orf4*, *repB1*, *orf3*, *repB*, *bacSJ2-8*, *orf2*), pEP3 (*Eco*RI/*Pst*I – 3374 bp) (part of *repB1*, *orf3*, *repB*, *bacSJ2-8*, *orf2*), pEST3 (*Eco*RI/*Stu*I – 1701 bp) (*bacSJ2-8*, *orf2*), pSTP1 (*Stu*I/*Pst*I – 1673 bp) (part of *repB1*, *orf3*, *repB*), pBML9 (*Bam*HI/*Mlu*I – 7516 bp) (part of *tnpIS30*, *abcT*, *acc*, *acdT*, *orf1*, *tnp6*, *mobC*, and a part of *mobA*) (see Results). To confirm that the anticipated final plasmid constructions were obtained, the restriction enzyme digestion and sequencing of the constructs were performed. The constructs were reisolated from *E. coli* and then transferred to *L. paracasei* subsp. *paracasei* BGHN14 and BGSJ2-83 by electroporation

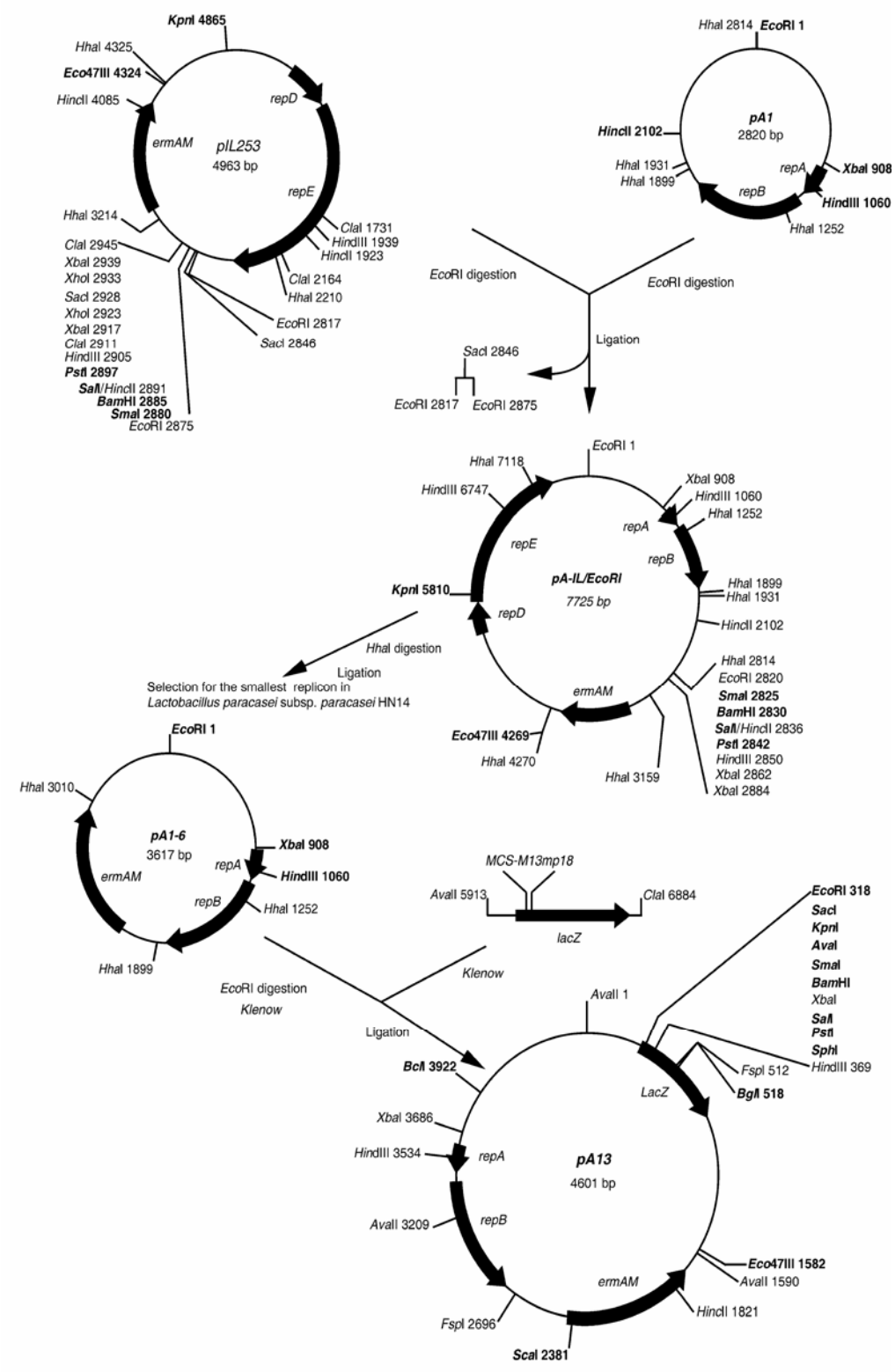


Figure 1.

The obtained Em^r transformants were tested for bacteriocin production and sensitivity to it.

Analysis of segregational and structural stability of plasmid pA13 and construct pB5

The structural and segregation stability of plasmid pA13 (4 601 bp) and the largest construct pB5 (19 044 bp) was studied in *E. coli*, *L. paracasei* subsp. *paracasei* BGHN14 and *L. lactis subsp. lactis* BGMN1-596. A single transformant of each host carrying either plasmid pA13 or pB5 was grown in a corresponding antibiotic-free medium for up to 50 generations. During the propagation of the cultures, the percentage of cells carrying plasmids pA13 or pB5 was determined by plating the samples on the corresponding medium with and without erythromycin until the appearance of colonies.

The structural stability of plasmid pA13 in *E. coli* was monitored in the medium containing erythromycin for 50 generations. The percentage of erythromycin-resistant blue colonies in *E. coli* was taken as the measure of the structural stability of the plasmid pA13. The structural stability of plasmid pB5 was studied in lactobacilli and *E. coli* in the following manner: cultures in MRS medium containing erythromycin were incubated at an appropriate temperature; after 50 generations of propagation in lactobacilli several dilutions were plated on MRS plates containing erythromycin. The percentage of erythromycin-resistant Bac^+ colonies was taken as the measure of structural stability. After the propagation of 50 generations in *E. coli,* several dilutions were plated on LB plates containing erythromycin. The plasmid DNA from one hundred colonies was isolated and used for transformation of *L. paracasei* subsp. *paracasei* BGHN14 competent cells by electroporation. The percentage of erythromycin-resistant Bac^+ transformants was taken as the measure of structural stability of plasmid pB5 in *E. coli.*

Protein and DNA sequence analysis

The bacteriocin BacSJ was purified as described previously (Lozo et al., 2007). Briefly, BacSJ was purified using cation exchange chromatography and reverse phase chromatography, followed by the final step of purification on reverse phase HPLC. The purified bacteriocin BacSJ was subjected to N-terminal amino acid sequencing by Edman degradation using a service of Alta Bioscience, University of Birmingham, UK. For DNA sequencing, pB5 and other constructs were isolated from either *E. coli* or *L. paracasei* subsp. *paracasei* BGHN14 using a QIAprep Spin Miniprep Kit (QIAGEN) as described by the manufacturer, with the addition of lysozyme in resuspension buffer (40 μg ml^{-1}), and lysis was performed for 15 min at 37°C for lactobacilli. Sequencing of plasmid pB5 was performed by the primer-walking method of both strands in the Macrogen's sequencing service, Seoul, Korea. Sequence annotation and database search for similar sequences were performed using the BLAST site of programs at the National Center for Biotechnology Information (Altschul et al., 1997). The DNA Strider program was used for open reading frame (ORF) prediction.

Nucleotide sequence accession number

The nucleotide sequences for the plasmids pA13 and pSJ2-8 were submitted to EMBL GenBank under accession numbers FM212242 and FM246455, respectively.

RESULTS

Segregational and structural stability of pA13 and pB5

The pA13 vector contains a *lacZ* gene with a polycloning site for blue/white color screening of the inserts in *E. coli*, and M13 sequencing primers available for the direct sequencing of cloned fragments. In addition, the erythromycin resistance gene of pIL253 and RCR origin from the pA1 plasmid allow replication and selection in *E. coli,* lactobacilli and lactococci. This shuttle-cloning vector was successfully introduced into *E. coli* DH5α, *L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris* and *L. paracasei* subsp. *paracasei* strains. Plasmid analysis of transformants confirmed that it was

Table 2. General features of putative ORFs from pSJ2-8 with best matches to sequences in the public database

Protein, gene or ORF	Position	Size (aa)	Proposed function	Source strain	Identity* (%)	GenBank accession no.
MobA	296-13345	468	Mobilization protein	*Lactobacillus plantarum*	40	AAZ13606
MobC	589-278	103	Mobilization protein	*Lactobacillus plantarum*	48	AAZ13605
Tnp6	1103-1786	227	Transposase	*Lactobacillus casei* ATCC334	99	YP_796441
ORF1	2272-1895	125	Hypothetical protein	*Lactobacillus acidophilus* TK8912	100	NP_604415
acdT	2743-2603	46	Acidocin 8912	*Lactobacillus acidophilus* TK8912	100	NP_604414
Accessory protein	4581-3493	362	Accessory protein	*Lactobacillus acidophilus* TK8912	99	NP_604413
ABC transporter	6685-4592	697	ABC transporter	*Lactobacillus acidophilus* TK8912	98	NP_604412
Transposase IS30 family	6921-7937	338	Transposase	*Lactobacillus casei* ATCC334	99	YP_807205
bacSJ2-8i	8644-8333	103	BacSJ immunity protein	*Lactobacillus acidophilus* TK8912	100	NP_604411
bacSJ2-8	8850-8644	68	Bacteriocin BacSJ	*Lactobacillus acidophilus* TK8912	97	BAB86318
RepB	9772-10281	169	Replication protein	*Lactobacillus paracasei* NFBC338	44	AAW81290
ORF3	10706-10356	116	Hypothetical protein			
RepB-1	11892-10960	310	Replication protein	*Lactobacillus paracasei subsp. paraacsei* ATCC 25302	99	ZP_03964311
ORF4	12871-12248	207	Hypothetical protein			
ORF5	13332-12853	159	Hypothetical protein			

*Em^r, erythromycin resistant

introduced without any structural rearrangements (data not shown).

The percentage of erythromycin resistant colonies (the number of cells that retained plasmid) was taken as the measure of plasmid stability. In *E. coli*, after 50 generations 53% of the colonies retained the plasmid pA13 and 30% pB5; in lactobacilli the percentage of erythromycin resistant colonies after 50 generations was over 90%.

Evaluation of the structural stability of pA13 in *E. coli* was monitored after 50 generations and 99.6% of the colonies retained plasmid with a whole *lacZ* gene. The structural stability of pB5 in lactobacilli showed that between five thousand colonies it was not possible to detect Bac^- colonies, while in *E. coli* during 50 generations more than 90% of cells retained the plasmid with a whole bacteriocin operon. Analysis of the segregational and structural stability of pA13 in lactococci revealed a similar stability of plasmids as in lactobacilli.

Analysis of the nucleotide sequence of plasmid pSJ2-8

The complete sequence of plasmid pSJ2-8 consisted of 14 442 bp, with a G+C content of 42.2 %. In addition, *in silico* analysis of this nucleotide sequence with the DNA Strider program revealed 15 open reading frames (ORF) (Table 2 and Fig. 2). At the same time, bacteriocin BacSJ was purified, and the sequence of the first ten amino acids obtained by Edman degradation was YSYFGGSNGY.

Determination of the N-terminal sequence of the purified bacteriocin BacSJ permitted the identification of a DNA sequence that contains the *bacSJ2-8* gene encoding bacteriocin synthesis. The polypeptide encoded by *bacSJ2-8* is 68 amino acids long and contains a double-glycine (GG)-leader

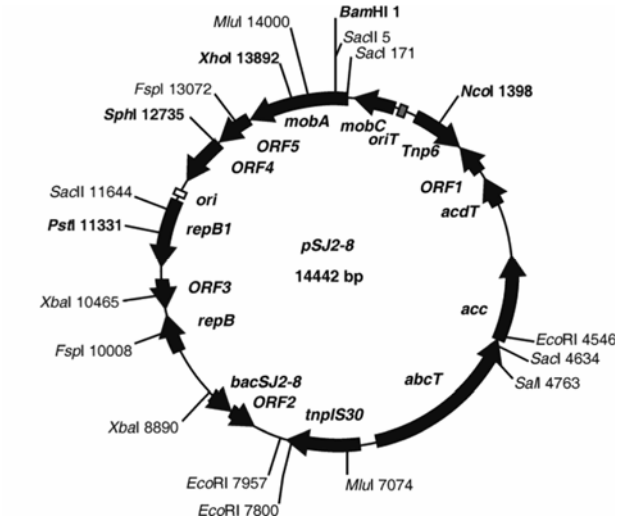


Figure 2.

consensus at the N-terminus, while the predicted mature peptide consists of 50 amino acids. Comparative analysis of the leader peptide revealed the conserved amino acids that are characteristic for leader peptides from class II bacteriocins. The TTG start codon is preceded by a potential RBS and a -10 and -35 consensus promoter region (Fig. 3). BLAST protein sequence homology search revealed that BacSJ showed a very high homology with acidocin M (accession No. BAB86318, see Table 2), a protein of unknown function with an unidentified start codon that was located on plasmid pLA103 from *L. acidophilus* TK8912 (Kanatani et al., 1995). The *bacSJ2-8* gene is followed by *orf2*, the sequence that encodes a polypeptide of 103 amino acids. The position and the size suggested that the product of this gene serves as an immunity protein (Motlagh et al., 1992). The location of the *orf2* gene indicated that it has a common promoter with the *bacSJ2-8* gene, and that they are most probably transcribed as one transcription unit.

Downstream of the *orf2*, a divergently oriented gene was located. It exhibited homology to the transposase *IS30* family from *L. casei* ATCC334. The following *orf* located 1 959 bp downstream of the *bacSJ2-8* was the *abcT* gene for the ABC transporter with the highest homology to those located in plasmid pLA103 from *L. acidophilus* TK8912 (Kanatani et al., 1995). Immediately after it is the *orf* encoding putative accessory protein. Interestingly, the gene encoding accessory protein on the plasmid pSJ2-8 was 102 amino acids shorter in comparison to the accessory protein present in pLA103. The *acdT* gene encoding bacteriocin acidocin 8912 (Kanatani et al., 1995), was positioned 4,063 bp downstream of the *bacSJ2-8* gene. The convergently-oriented *orf1* located downstream of the *acdT* gene encodes a putative protein of unknown function. The region from plasmid pSJ2-8 that contains the *orf*'s mentioned above exhibited a very high sequence similarity with the part of the plasmid pLA103. The homology starts approximately 1 000 bp upstream of *bacSJ2-8* gene and ends after *orf*1. However, it was shown that on plasmid pSJ2-8 this region contains the transposase *TnpIS30* which is not present in plasmid pLA103 (Fig. 2). The other part of plasmid pSJ2-8 (carrying *repB*, ORF3, *repB1*, ORF4, ORF5, *mobA*, and *mobC* genes) does not show homology with the other plasmids from lactobacilli. *In silico* analysis showed that the *ori* sequence for the *repB* gene is not present whereas upstream of the *repB1* gene, *ori* was identified with a four-times repeated sequence of 22 nucleotides (ATGTATATCAAAAAGGTCCGTC). Sequence analysis of plasmid pDOJH10L from *Bifidobacterium longum* DJO10A showed a structure of four direct repeats in front of the *repC* gene (Lee and O'Sullivan, 2006). Two mobilization proteins are located in the plasmid pSJ2-8 and the putative *oriT* sequence was located upstream of the *mobC*.

Heterologous production and functional expression of bacteriocins BacSJ and acidocin 8912

In order to examine whether the genes responsible for BacSJ and acidocin 8912 synthesis and immunity can be expressed in a different host, the plasmid pB5 that contains a complete set of genes required for the production of both bacteriocins was introduced into two *Lactobacillus* strains. *L. paracasei* subsp. *paracasei* BGHN14 was used for heterologous expression, whereas the Bac$^-$ deri-

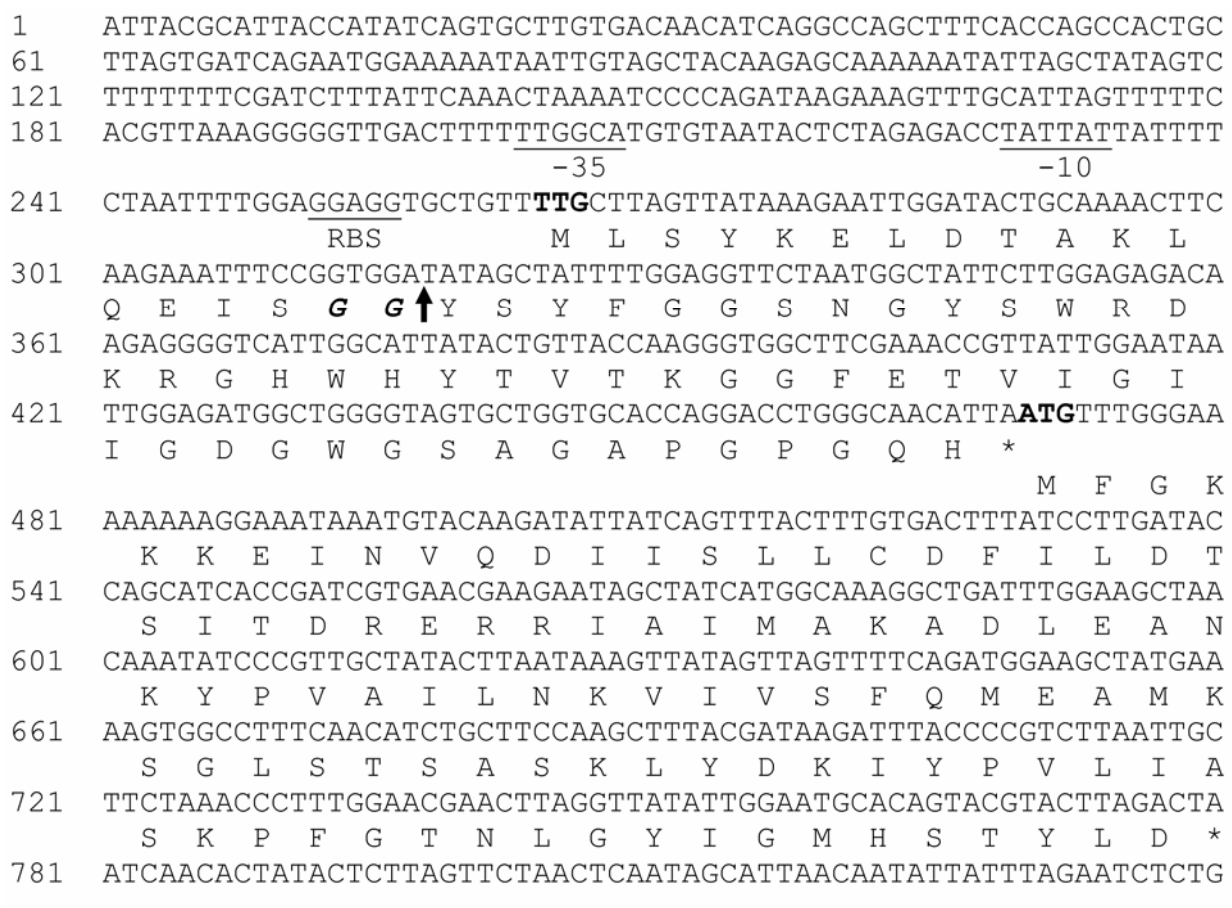
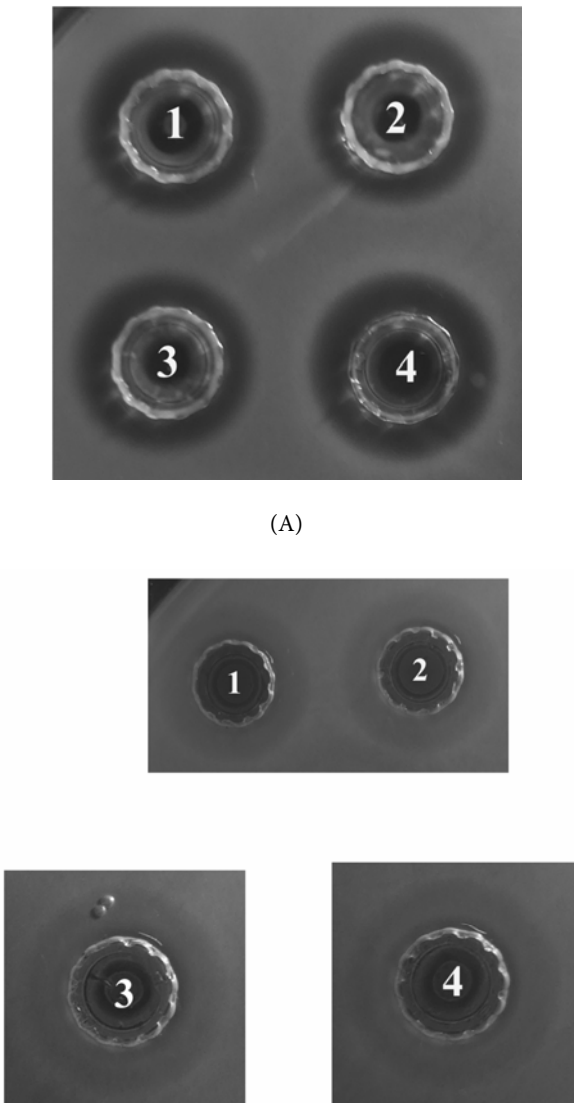


Figure 3.

vative of the parental strain, *L. paracasei* subsp. *paracasei* BGSJ2-83, was used as a homologous host. It was observed that, in both cases, supernatants of the obtained transformants gave the same size of zone of inhibition on the sensitive strain BGHN14 as the original strain *L. paracasei* subsp. *paracasei* BGSJ2-8. Furthermore, when these transformants were used as indicator strains in the test for antimicrobial activity of *L. paracasei* subsp. *paracasei* BGSJ2-8, it was observed that they were immune to the bacteriocin BacSJ (Fig. 4).

Various derivatives of plasmid pB5, designated as pBP3, pBSC5, pBE7, pEP3, pEST3, pSTP1 and pBML9 (Fig. 5A) were constructed. When *L. paracasei* subsp. *paracasei* BGHN14 was transformed with each of these constructs, it was observed that the production of bacteriocin BacSJ could be detected as a clear zone of inhibition of the indicator strain BGHN14 only if the transformant harbors the pBP3 construct (Fig. 4A). However, transformants carrying pBP3, pBSC5, pBE7, pEP3 or pEST3 constructs showed immunity to BacSJ when used as indicator strains (Fig 5B). Since the *orf2* is a common feature for all constructs including the smallest one (pEST3), it could be inferred that *orf2* (*bacSJ2-8i*) encodes the immunity protein for BacSJ (Fig. 4B). Moreover, the obtained results strongly indicate that the ABC transporter and accessory protein are necessary for the production of bacteriocin BacSJ. On the other hand, the ABC transporter does not contribute to the immunity against the BacSJ.

The transformants of *L. paracasei* subsp. *paracasei* BGSJ2-83 harboring either pB5 or pBML9



(A)

(B)

Figure 4.

constructs were used to test whether they are able to produce bacteriocin acidocin 8912. For this purpose, BGHN14 carrying the constructs pBSC5, pBE7, pEP3, pEST3 or pSTP1 (Fig. 5) was used as the indicator strain. Both BGSJ2-83 transformants showed the zone of inhibition on each indicator strain. However, in this case the zone of inhibition had a turbid appearance (Fig. 4B) in comparison with the zone of inhibition produced by BacSJ that has a clear appearance (Fig. 4A). Thus, plasmid pSJ2-8 contains information for the production of both bacteriocins, BacSJ and acidocin 8912.

DISCUSSION

Taking into account the importance of lactic acid bacteria (LAB), investigation of their characteristics is important from both a fundamental and applicative point of view. In the last few decades, there has been a growing interest in the natural isolates of LAB. Bacteriocins producing LAB are in the focus of research, since bacteriocins can be potential food bio-preservatives. Bacteriocin production can be considered as a part of secondary metabolism because this trait is not essential for the normal growth of the bacterial host.

The strain *Lactobacillus paracasei* subsp. *paracasei* BGSJ2-8 was isolated from home-made semi-hard cheese. Previous analyses showed that BGSJ2-8 produces the bacteriocin designated BacSJ (Lozo et al., 2007). The N-terminal extension of BacSJ contains a double-glycine consensus sequence at positions -1 and -2 relative to the cleavage site. This sequence confers a typical signal peptide for proteins processed and secreted by dedicated ABC transporters and their accessory proteins (Håvarstein et al., 1995). Therefore, the bacteriocin BacSJ is most likely synthesized as a prepeptide that is cleaved at Gly-Gly residues, followed by the release of mature BacSJ. It has been previously reported that the type of double glycine leader peptides can significantly influence the level of bacteriocin production in a heterologous host (van Belkum et al., 1997), and the highest production is often obtained when its specific ABC transporter processes the leader peptide (Horn et al., 1998; Horn et al., 1999). In this study, we found that two different bacteriocins most probably use the same ABC transporter and accessory protein. Unfortunately, this premise was not possible to prove because we could not obtain a stable construct carrying only the *acdT* and *orf1* genes using both *E. coli* and *L. paracasei* subsp. *paracasei* BGHN14 as hosts. The same results are already obtained for *L. sakei* 5 that produces two bacteriocins, sakacin T and sakacin X,

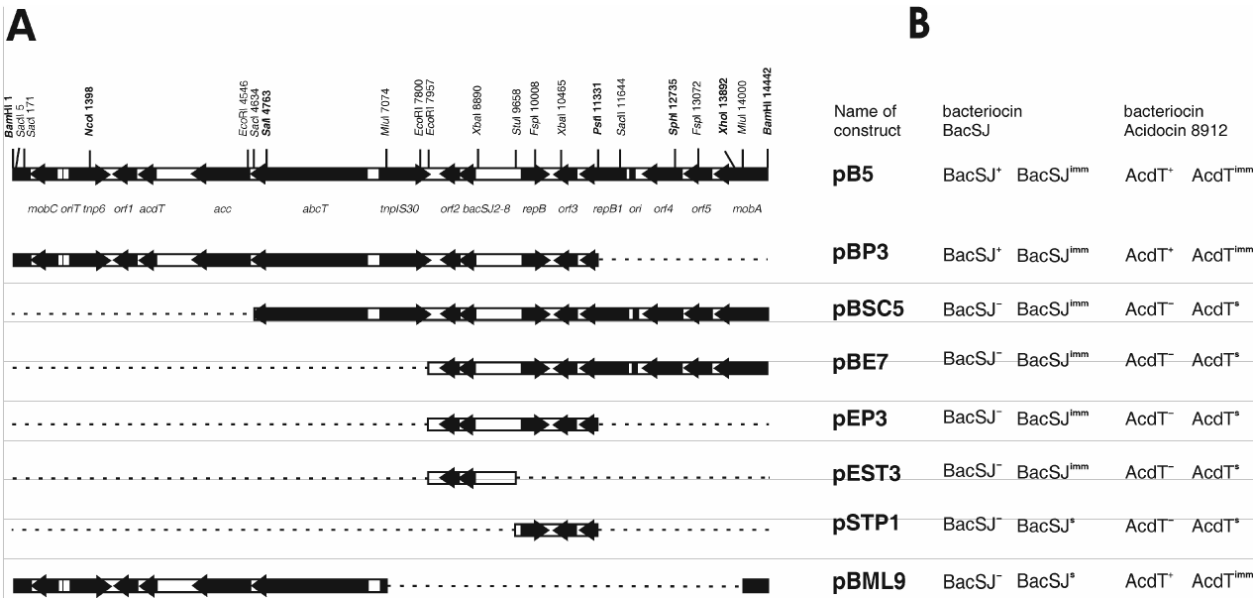


Figure 5.

although the accessory protein was not detected in this strain (Vaughan et al., 2003). The accessory gene is usually located immediately downstream of the corresponding ABC transporter gene and cotranscribed with it, which makes the genetic organization of these two genes relatively conserved (Diep et al., 1996; Nes et al., 1996). The ABC transporter gene in pSJ2-8 is homologous to the same gene from the plasmid pLA103 of *L. acidophilus* TK8912 (Kanatani et al., 1995) (Table 2). In contrast, the accessory protein encoded by pSJ2-8 is shorter than that in pLA103. Nevertheless, the functionality of the truncated form of the accessory protein encoded by pSJ2-8 is maintained.

Most bacteriocin loci of Gram-positive bacteria contain the corresponding immunity gene that usually resides directly downstream of the bacteriocin structural gene and is cotranscribed with it. These proteins are usually 50 to 150 amino acid residues long and they usually do not share sequence similarities (Ennahar et al., 2000; Nes et al., 1996). Cloning experiments revealed that *orf2*, located next to the *bacSJ2-8* gene, encodes the cognate immunity protein of the BacSJ. Furthermore, the construct pEST3 that contains only the *orf2* and *bacSJ2-8* genes (Fig. 5) confers the immunity of transformants harboring it to BacSJ, and therefore *orf2* was annotated as *bacSJ2-8i*.

Genes for bacteriocin production are often located on mobile elements like transposons or transferable plasmids (Kojić et al., 2006). Therefore, these genes may be frequently transferred to other bacterial lineages, and are thereby prone to different genetic rearrangements. The homology detected between the plasmids pLA103 in *L. acidophilus* TK8912 and pSJ2-8 in *L. paracasei* subsp. *paracasei* BGSJ2-8 suggests horizontal gene transfer (HGT) between bacteria of the same or different species. Furthermore, the presence of a mobilization protein and transposable elements in the plasmid pSJ2-8 indicates that they may contribute to the transfer and mobilization of other DNA elements among lactobacilli. Interestingly, two bacteriocins clusters (BacSJ and acidocin 8912) are interrupted and flanked with transposase genes. The functionality of transposase and other genes on pSJ2-8, namely *repB, repB1, ori*, *mobA, mobC* and *oriT*, will be subject of further investigation.

In the last decade, several LAB genomes have been partially or completely sequenced. Comparative genomics, together with the conventional methods for the molecular characterization of new bacteriocins will enable a more rapid elucidation of the genes responsible for bacteriocin synthesis, maturation and export. This approach will also reveal whether bacteriocin genes belong to the group of genes that are frequently involved in HGT. However, further efforts should be made towards the elucidation of the mechanisms of transfer and mobilization of bacteriocin gene clusters among LAB, especially those from different ecological niches. There are already many examples of an independent characterization of the same bacteriocin that is synthesized by the bacteria belonging to the same or different species (Holck et al., 1992; Marugg et al., 1992; Motlagh et al., 1992).

In conclusion, the strain *L. paracasei* subsp. *paracasei* BGSJ2-8 was isolated from home-made semi-hard cheese manufactured in a village household. BGSJ2-8 produces two bacteriocins, designated BacSJ and acidocin 8912. To our knowledge, BacSJ is the first completely characterized bacteriocin produced by the *L. paracasei* subsp. *paracasei* species. The genes' encoding for bacteriocin BacSJ and acidocin 8912 production and immunity are located on the 14.4 kb large plasmid pSJ2-8. The molecular characterization of the plasmid pSJ2-8 was performed using the new shuttle cloning vector pA13.

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