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Expression of bacteriocin LsbB is dependent on a transcription terminator

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ABSTRACT

The production of LsbB, leaderless class II bacteriocin, is encoded by genes (*lsbB* and *lmrB*) located on plasmid pMN5 in *Lactococcus lactis* BGMN1-5. Heterologous expression of the *lsbB* gene using the pAZIL vector (pAZIL-lsbB) in *L. lactis* subsp. *cremoris* MG7284 resulted in a significant reduction (more than 30 times) of bacteriocin LsbB expression. Subcloning and deletion experiments with plasmid pMN5 revealed that full expression of LsbB requires the presence of a complete transcription terminator located downstream of the *lsbB* gene. RNA stability analysis revealed that the presence of a transcription terminator increased the RNA stability by three times and the expression of LsbB by 30 times. The study of the influence of transcription terminator on the expression of other bacteriocin genes (*lcnB*, for lactococcin B production) indicated that this translational terminator likely functions in a *lsbB*-specific manner rather than in a general manner.

1. Introduction

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by bacteria. Bacteriocins have been the focus of considerable scientific interest for years, but they are relatively underutilized by the food, veterinary and medical industries. To fully realize the potential of bacteriocins, it is necessary to understand the biology of bacteriocins, their structure–function relationships, the regulation of gene expression, and their mode of action. The expression of genes involved in bacteriocin production is usually controlled by a quorum sensing system (Kleerebezem et al. 1997; Nes and Eijsink 1999). All quorum sensing systems utilize small, secreted signaling molecules known as autoinducers, which belong to one of the following three categories: acylatedhomoserine lactones (AHLs) used by Gram-negative bacteria, peptide signals used by Gram-positive bacteria and autoinducer-2 (AI-2) used by both types of bacteria (La Sarre and Federle 2013). In many Gram-positive bacteria, production of bacteriocins is inducible and induction requires secretion and extracellular accumulation of peptides that act as chemical messengers that trigger bacteriocin production. These inducer peptides are often referred to as autoinducers and are believed to permit a quorum sensing-based regulation of bacteriocin production. Notably, some peptides that act as autoinducers also have antimicrobial activity, and some bacteriocins act as their own autoinducers. The autoinducer-dependent induction of bacteriocin production requires histidine protein kinases and response regulator proteins for two-component signal transduction systems. The other component of this system is a cognate response regulator that promotes transcription of the regulated promoters and which is activated by the pheromone-activated kinase (Risøen et al. 2000; Eijsink et al. 2002). Class IIa bacteriocins require at least four genes including structural, immunity, ABC transporter and accessory protein genes (Fimland et al. 2005). Some members of class IIa bacteriocins (curvacin A, sakacin P, carnobacteriocin B2, enterocin A, Kleerebezem and Quadri 2001), and some class IIb bacteriocins (ABP-118, plantaricin E/F, plantaricin J/K9, Anderssen et al. 1998; Flynn et al. 2002) are transcriptionally regulated through a three-component signal transduction system. The pheromone

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peptide involved in the production of class II bacteriocins is usually a non-modified short peptide with no or very limited antimicrobial activity. In the case of nisin (class I), the bacteriocin itself acts as a pheromone, which activates a "two-component" regulatory system. In this case, nisin induces the expression of genes that are necessary for its own production. The genes and promoters involved in nisin production have been used to develop a nisin-controlled expression (NICE) system. Several *nis*-promoter vectors were constructed that are suitable for tightly controlled expression of heterologous proteins in lactococci (De Ruyter et al. 1996), lactobacilli (Pavan et al. 2000), and other Gram-positive bacteria (Eichenbaum et al. 1998), which is a great biotechnological success. Similar systems may be developed on the basis of genes and promoters involved in the production of class II bacteriocins.

Our previous studies on the expression of LsbB showed that the highest production level was obtained by the *Lactococcus lactis* BGMN1-596T strain, harboring the whole operon that is naturally involved in LsbB production in a natural organization (pMN5 plasmid, Gajic et al. 2003; Kojic et al. 2006). LsbB, a class IId bacteriocin, is a relatively hydrophilic protein synthesized without an N-terminal leader sequence or signal peptide. It is transported by LmrB, which at the same time acts as an immunity protein. Recently, it has been shown that Zn-dependent metallopeptidase is responsible for sensitivity to LsbB (Uzelac et al. 2013), representing the most likely receptor protein. Furthermore, structure–function analyses provide strong evidence that the receptor-binding part of LsbB is located in the C-terminal domain (Ovchinnikov et al. 2014). Previous experiments have shown that the strain MG7284/pAZIL-lsbB carrying *lsbB* cloned with its own promoter (*PlsbB*) expresses a lower zone of inhibition on the sensitive strain BGMN1-596 than the strain BGMN1-596T, indicating the involvement of additional sequences or genes in the increased bacteriocin activity of *L. lactis* BGMN1-596T. In the present work the regulation of LsbB expression was analyzed, indicating that the region responsible for regulation is located within 60 nucleotides immediately downstream of the *lsbB* gene on the pMN5 plasmid. Until now, the mechanism for regulation could not be confirmed. The most likely factor was thought to be the stability of messenger RNA, whether the regulation occurs at the transcriptional or translational level. It has been proven, however, that the downstream region including a transcription terminator is responsible for increased expression of LsbB bacteriocin.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

The strains, their derivatives and plasmids used in this study are listed in Table 1. *L. lactis* strains were grown in M17 medium (Merck GmbH, Darmstadt, Germany) supplemented with 0.5% glucose (GM17) at 30 °C. *Escherichia coli* strains were grown in Luria Bertani broth (LB) at 37 °C with aeration. To each medium, agar (1.5%; Torlak, Belgrade, Serbia) was added for use as a solid medium. All strains carrying constructs were stored in a growth medium containing 15% glycerol (Sigma Chemie GmbH, Deisenhofen, Germany) at −80 °C. The following antibiotic concentrations (in μg/mL) were used: erythromycin, 10 (lactococci) and 300 (*E. coli*); chloramphenicol, 10 (lactococci) and 30 (*E. coli*); and ampicillin, 100 (*E. coli*). 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) (Fermentas, Vilnius, Lithuania) was added to LB medium plates for blue/white color selection of colonies at a final concentration of 20 μg/mL (*E. coli*) and 80 μg/mL (*L. lactis* construct with *lacZ* fusion). Ortho-nitrophenyl-β-galactoside (ONPG) was used for the β-galactosidase assay (Sigma Chemical Co. St. Louis, MO).

2.2. Bacteriocin detection and activity assay

For detection of bacteriocin activity, an agar-well diffusion assay was performed as described previously by Lozo et al. (2004). For testing the level of LsbB production serial double dilutions of filtered supernatants (16 h old culture) of the different producers and synthetic LsbB were spotted (5 μL) on the surface of the top agar inoculated with BGMN1-596. Zones of inhibition were compared by size and intensity with zones formed by synthetic LsbB (known concentrations; ChinaPeptides Co., Ltd.; Uzelac et al. 2013) in order to quantify production by different producers.

2.3. DNA manipulations

Plasmids from lactococci were isolated by modification of the method described by O'Sullivan and Klaenhammer (1993). For plasmid isolation from *E. coli* a Thermo Scientific GeneJET Plasmid Miniprep kit was used according to the manufacturer's recommendations (Thermo Scientific, Lithuania). Digestion with restriction enzymes was conducted according to the supplier's instructions (Fermentas, Lithuania). Lactococci were transformed with plasmid constructs using a Gene Pulser Eporator (Eppendorf, Hamburg, Germany) as described previously (Holo and Nes 1989). Standard heat-shock transformation was used for plasmid transformation of *E. coli* (Hanahan 1983). The DNA fragments from agarose gels were purified using a Thermo Scientific GeneJET Gel Extraction kit as described by the manufacturer (Thermo Scientific, Lithuania). DNA was ligated with T4 DNA ligase (Agilent technologies, USA) according to the manufacturer's recommendations. Sets of specific primers used in this study are listed in Table 2. Total RNA was isolated from derivatives MG7284/pAZIL-EX and MG7284/pAZIL-lsbB by an SV Total RNA Isolation system (Promega, Madison, USA). Thermo Scientific Random hexamer, RiboLockRNAse Inhibitor and RevertAid Reverse Transcriptase were used to generate complementary DNA (cDNA) from the RNA template by RT-PCR. KapaTaq DNA Polymerase (Kapa Biosystems, Inc., Boston, MA, USA) was used to amplify DNA fragments using a GeneAmp PCR System 2700 thermal cycler (Applied Biosystems, Foster City, CA, USA). PCR products were purified with a Thermo Scientific PCR Purification Kit according to the manufacturer's protocol and sequenced by the Macrogen Sequencing Service (Macrogen, The Netherlands). The sequences were analyzed in the NCBI database using BLAST. Commercial p-GEM-T-Easy (Promega, Madison, USA) or pBluescriptT/A vectors were used for cloning of PCR products. The pBluescriptT/A vector was constructed using synthesized pBS-T/A oligonucleotide (Table 2) and *Bam*HI digested pBluescript. First, pBS-T/A oligonucleotide (designed to provide 12 amino acid in frame with *lacZ* carrying the following restriction sites *Nde*I, *Xcm*I, *Nco*I, *Sph*I, *Nco*I, *Xcm*I and *Nde*I) was self-annealed to leave *Bam*HI cohesive ends and then ligated with predigested pBluescript with *Bam*HI. The pBluescriptT/A vector was designed for efficient direct cloning of PCR products. It contains two *Xcm*I restriction sites oriented towards each other that generate 3′ thymine single nucleotide overhangs on both 3′-ends after digestion. It also contains *Nco*I and *Sph*I restriction sites for destruction of an internal fragment (*Xcm*I-*Nco*I-*Sph*I-*Nco*I-*Xcm*I) in order to prevent its re-ligation into the vector and *Bam*HI for testing and blue-white screening of cloned fragments. Efficiency of the constructed pBluescriptT/A vector to accept PCR fragments was tested in DH5α and it was found that more than 95% of the transformants contain the correct insert.

2.4. Gene knock-out

The construct pAZIL-EX was used for gene knock-out experiments. To knock out *lsbB*, pAZIL-EX was digested with the *Nco*I restriction enzyme and then blunted using Klenow enzyme

Table 1
Bacterial strains and plasmids used in this study.

Strain or plasmids	Relevant characteristics	Source or reference
L. lactis subsp. *lactis*		
IL1403	Plasmid free derivative of IL596	Chopin et al. (1984)
BGMN1-596	Plasmid free derivative of *L. lactis* subsp. *lactis* BGMN1-5	Gajic et al. (1999)
BGMN1-596T	Derivative of BGMN1-596 with pMN5 plasmid	Gajic et al. (1999)
BGMN1-501	Plasmid cured derivative of *L. lactis* subsp. *lactis* BGMN1-5, LcnB producer	Kojic et al. (2006)
B464	*ptn* deletion mutant of IL1403	Diep et al. (2007)
B464/pAZIL-LcnB	Derivative of IL1403 with pAZIL-LcnB	This study
B464/pAZIL-LcnBTT	Derivative of IL1403 with pAZIL-LcnBTT	This study
L. lactis subsp. *cremoris*		
MG7284	Prt^-, Lac^-, Bac^s, MG1363 Fus^r, Spc^r	Gasson (1983)
MG7284/pAZIL-lsbB	Derivative of MG7284 with pAZIL-lsbB	Uzelac et al. (2013)
MG7284/pAZIL-EX	Derivative of MG7284 with pAZIL-EX	This study
MG7284/pNZ8150-lsbB	Derivative of MG7284 with pNZ8150-lsbB	This study
E. coli		
DH5α	*supE44 ΔlacU169 (ø80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Hanahan (1983)
EC101	JM101 containing *repA* gene of pWV01 in chromosome	Law et al. (1995)
Plasmids		
pMN5	Natural plasmid carrying lsbB operon	Kojic et al. (2006)
pAZIL	7109 bp, Em^r, shuttle cloning vector	Kojic et al. (2011)
pAZIL-EX	pAZIL carrying *Eco*RI-*Xmn*I fragment of pMN5 (2821 bp)	This study
pAZIL-bN	pAZIL-EX with *lsbB* gene knock-out in *Nco*I restriction site	This study
pAZIL-ΔNSS	pAZIL-EX with *lsbB* and *lmrB* genes deletant (*Nco*I- *Sca*I- *Sca*I fragments)	This study
pAZIL-ΔSS	pAZIL-EX with *lmrB* gene deletant of *Sca*I fragment	This study
pAZIL-ES	pAZIL carrying *Eco*RI-*Sca*I fragment of pMN5 (736 bp)	This study
pAZIL-PlsbB	pAZIL carrying *PlsbB* promoter	This study
pAZIL-PlsbB-lsbB	pAZIL carrying *lsbB* gene under *PlsbB* promoter	This study
pAZIL-PlsbB-lsbA	pAZIL carrying *lsbA* gene under *PlsbB* promoter	This study
pAZIL-PlsbB-lsbX	pAZIL carrying *lsbX* gene under *PlsbB* promoter	This study
pAZIL-PlsbB-lsbY	pAZIL carrying *lsbY* gene under *PlsbB* promoter	This study
pAZIL-PlsbB-lsbXY	pAZIL carrying *lsbXY* genes under *PlsbB* promoter	This study
pAZIL-PlsbB-lsbZ	pAZIL carrying *lsbZ* gene under *PlsbB* promoter	This study
pAZIL-lsbB	pAZIL carrying *lsbB*	This study
pAZIL-lsbBlsbX	pAZIL carrying *lsbB* and *lsbX* genes	This study
pAZIL-lsbBlsbXY	pAZIL carrying *lsbB*, *lsbX* and *lsbY* genes	This study
pAZIL-lsbBlsbX1	pAZIL carrying *lsbB* and *lsbX* genes with deletion of last 5 nucleotides of *lsbX* gene	This study
pAZIL-lsbBlsbX2	pAZIL carrying *lsbB* and *lsbX* genes with deletion of last 12 nucleotides of *lsbX* gene	This study
pAZIL-lsbBlsbX3	pAZIL carrying *lsbB* and *lsbX* genes with deletion of last 22 nucleotides of *lsbX* gene	This study
pAZIL-lsbBlsbX4	pAZIL carrying *lsbB* and *lsbX* genes with deletion of last 38 nucleotides of *lsbX* gene	This study
pAZIL-lsbBlsbXSt	pAZIL carrying *lsbB* and *lsbX* genes with inserted stop codon into *lsbX* gene	This study
pAZIL-lsbBEV	pAZIL carrying *lsbB* gene and five nucleotides downstream of *lsbB* gene	This study
pNZ8150	Cm^r, NICE expression vector	Mierau and Kleerebezem (2005)
pNZ8150-lsbB	pNZ8150 carrying *lsbB* gene	This study
pBluescript	2958 bp, Amp^r, cloning vector	Stratagene
pBluescriptT/A	2994 bp, Amp^r, PCR cloning vector	This study
pNZ8150-lacZ1	pNZ8150 carrying *lacZ1* gene	This study
pNZ8150-PlsbB-lacZ	pNZ8150 carrying *PlsbB* and *lacZ* gene	This study
p-GEM-T-Easy	3015 bp, Amp^r, PCR cloning vector	Promega
pBSTT	pBluescript carrying transcription terminator as *Hin*dIII-*Eco*RI fragment	This study
pBSLcnBTT	pBSTT carrying *lcnB* gene as *Sal*I-*Cla*I fragment	This study
pAZIL-LcnB	pAZIL vector carrying *lcnB* gene	This study
pAZIL-LcnBTT	pAZIL vector carrying *lcnB* gene and transcription terminator	This study

(insertion of four nucleotides; construct pAZIL-bN). Knocking out the *lsbB* and *lmrB* genes was performed by their deletion from pAZIL-EX by digestion with *Nco*I/*Sca*I. The obtained construct was circularized by ligase enzyme after treatment with Klenow enzyme and used for transformation of *E. coli* DH5α (construct pAZIL-ΔNSS). Knocking out *lmrB* was performed by its deletion from pAZIL-EX by digestion with the *Sca*I enzyme. The mixture was ligated and used for transformation of *E. coli* DH5α (construct pAZIL-ΔSS). Lactococci strains MG7284 and IL1403 were transformed with the constructs (pAZIL-bN, pAZIL-ΔNSS and pAZIL-ΔSS) and bacteriocin activity was tested.

2.5. Expression of target genes under the PlsbB promoter

The LsbB promoter, *PlsbB*, was amplified by PCR using primers PlsbBEcoRIF and PlsbBBamHIR (Table 2), where pAZIL-EX was used as template. The obtained fragment was cloned into the commercial pGEM-T-Easy vector, and then recloned into the pAZIL vector as a fragment *Eco*RI/*Bam*HI (the primers contain these restriction sites in order to obtain the correct orientation). The designed construct, pAZIL-PlsbB, was used for further experiments. Likewise, the genes *lsbA*, *lsbX*, *lsbY*, *lsbZ* and *lsbXY*, were amplified from pAZIL-EX using the appropriate primers (Table 2) and cloned into the commercial pGEM-T-Easy vector. The fragments were transferred into the pAZIL-PlsbB vector as *Bam*HI/*Pst*I, downstream of the *Plsb*B promoter. *L. lactis* MG7284 was transformed with each of the constructs, and the obtained derivatives were used as bacteriocin producers in the agar well diffusion assay.

2.6. Promoter cloning and LacZ fusion

For analysis of the *lsbB* promoter activity, *PlsbB* was fused to the *lacZ* gene. The *PlsbB* promoter transcriptional fusion was constructed as follows; the amplified and cloned promoter *PlsbB*

Table 2
Sequence of specific primers used in this study.

Primers	Sequence of primers	Templates
PlsbBBamHIR	5′-CGTAGGATCCTTTTCATAGG-3′	pAZIL-EX
PlsbBEcoRIF	5′-CTCCAAGAATTCCTAAAAAAATAGG-3′	pAZIL-EX
LsbAPstIR	5′-CAATAGCTGCAGTTTTTATTATG-3′	pAZIL-EX
LsbABamHIF	5′-GAATTAAATAATGGATCCAATAATGAG-3′	pAZIL-EX
LsbXPstIR	5′-TTTGTACTGCAGTGTTTGCC-3′	pAZIL-EX
LsbXBamHIF	5′-CCATGGGATCCTGGAAAAGC-3′	pAZIL-EX
LsbYPstIR	5′-GTATCTGCAGTTGGAACTTGATGG-3′	pAZIL-EX
LsbYBamHIF	5′-CAATTGGATCCTATTGATTAGTC-3′	pAZIL-EX
LsbZBamHIF	5′-CGTAGGATCCGTTTTCATAGG-3′	pAZIL-EX
RT1	5′-TCTATGCTCCAAAAAGCGCT-3′	cDNK MG7284/pAZIL-EX, cDNK BGMN1-596T
RT2	5′-GGATATTTAAAATGATAATTGTAGG-3′	cDNK MG7284/pAZIL-EX, cDNK BGMN1-596T
RT3	5′-TGACTAATCAATATGTTCC-3′	cDNK MG7284/pAZIL-EX, cDNK BGMN1-596T
RT4	5′-TGTATCTTGATAGAAAGGAAG-3′	cDNK MG7284/pAZIL-EX, cDNK BGMN1-596T
rpoBsalcod F	5′-TGTATCTTGATAGAAAGGAAG-3′	cDNK MG7284/pAZIL-lsbB, cDNK MG7284/pAZIL-lsbB cDNK MG7284/pAZIL-ES
rpoBsalcod R	5′-TGTATCTTGATAGAAAGGAAG-3′	cDNK MG7284/pAZIL-lsbB cDNK MG7284/pAZIL-ES
RlsbBA30STOP	5′-TGATATCTTATTATTTTCCACGTTCCCATGG-3′	cDNK MG7284/pAZIL-lsbB cDNK MG7284/pAZIL-ES
LsbX1R	5′-GTTGTCGACTAATCAATATGTTCC-3′	pAZIL-EX
LsbX2R	5′-CAATATGTTCCAATTGATTATAATG-3′	pAZIL-EX
LsbX3R	5′-CCAATTGATTATAATGTACC-3′	pAZIL-EX
LsbX4R	5′-CCAAGATATGCATAATAAAAACTGC-3′	pAZIL-EX
LsbXStR	5′-GTTATTGACTAATCAATATGTTTCAATTG-3′	pAZIL-EX
LcnBF	5′-GAAAACTTATTTCAATTAC-3′	BGMN501
LcnBR	5′-ATCGATTAGTGGAATGTTTTTCCCCATCC-3′	BGMN501
pBS-T/A	5′-GATCCATATGTCCCATGGCATGCCATGGGACATATG-3′	For self annealing

Introduced restriction sites are indicated by underlined sequences

was transferred from pGEM-T-Easy (pGEM-T-PlsbB) as an *Eco*RI-*Bam*HI fragment to pNZ8150-lacZ1 (Vukotic et al. 2015). The ligation mixture was used to transform *E. coli* EC101. The construct, designated pNZ8150-PlsbB-lacZ1, was used for further experiments. *L. lactis* MG7284 was transformed with construct pNZ8150-PlsbB-lacZ, resulting in the strain MG7284/pNZ8150-PlsbB-lacZ1, which was used for additional transformation with constructs pAZIL-PlsbB-lsbA, pAZIL-PlsbB-lsbX, pAZIL-PlsbB-lsbY, pAZIL-PlsbB-lsbZ, pAZIL-PlsbB-lsbXY and pAZIL-bN. The resulting transformants were screened on solid GM17 medium supplemented with 80 μg/mL X-gal.

2.7. Enzymatic activity-β-galactosidase assay

β-galactosidase activity was determined essentially as described by Miller (1972) with the following modification. The resulting derivatives initially screened on GM17 agar plates supplemented with X-gal and controls (MG7284/pNZ8150-PlsbB-lacZ1 and MG7284/pNZ8150-lacZ1) were analyzed. Enzyme activity was quantified in *L. lactis* in the exponential phase (OD_{600} = 1) of growth. One milliliter of cultures was harvested, and the pellet was resuspended in 500 μL of PP buffer with lysozyme (4 mg/mL). The reaction mixture was incubated for 30 min in a water bath at 37 °C. Bacterial cells were harvested by centrifugation at 5000 × *g* and resuspended in 500 μL of Z buffer (60 mM $Na_2HPO_4{\cdot}7H_2O$, 40 mM $NaH_2PO_4{\cdot}H_2O$, 10 mM KCl, 1 mM $MgSO_4{\cdot}7H_2O$, 50 mM β-mercaptoethanol). Twenty microliters of chloroform and 20 μL of 0.1% sodium dodecyl sulfate (SDS) were added to the mixture and it was vortexed for 20 s. After 5 min of incubation at room temperature, 200 μL of ONPG (4 mg/mL) was added. After the appearance of yellow color the reaction was stopped by the adding of 250 μL of 1 M sodium carbonate. After centrifugation, A_{420} value was measured for the supernatant. β-galactosidase activity in Miller units was calculated as $(1000{\cdot}A_{420})/(t{\cdot}v{\cdot}OD_{600})$ where "*t*" is time in minutes, "*v*" is the volume of culture used in the assay in milliliters and OD_{600} is the optical density of the culture at 600 nm.

2.8. RT-PCR

Total mRNA was isolated from lactococcal derivatives MG7284/pAZIL-lsbB and MG7284/pAZIL-ES. For DNAse treatment a DNA-free™ Kit (Ambion, Life technologies, USA) was used. The quantity and purity of mRNA were checked on a 1.2% formaldehyde-agarose gel (FA gel). First strand cDNA synthesis with reverse transcriptase was carried out with Thermo Scientific RevertAid Reverse Transcriptase. The mRNA (2.5 μg) was reverse transcribed with 50 ng of Thermo Scientific Random hexamers. The obtained cDNA was subsequently amplified by PCR using appropriate pairs of primers (Table 2). The size of the obtained PCR products was checked on a 1% agarose gel.

2.9. Measurements of mRNA degradation

Actinomycin D was used as a transcription inhibitor to enable the measurement of the stability of mRNA in *L. lactis* subsp. *cremoris* derivatives MG7284/pAZIL-lsbB and MG784/pAZIL-ES. When used, the actinomycin D (Serva, GmbH, Deisenhofen, Germany) concentration in cultures was 2 μg/mL. Cultures were grown to mid-log phase (OD_{600} = 0.5) before actinomycin D was added, and reactions were stopped after 10 and 30 min mRNA was isolated as described above, and samples were diluted in nuclease-free water to a standard concentration of total RNA (1 μg/mL).

Fig. 1. Inhibition plate assay comparing LsbB activity of LsbB producers (1) *L. lactis* subsp. *cremoris* MG7284 pAZIL-lsbB with wild type strain *L. lactis* subsp. *lactis* BGMN1-596T (2). *L. lactis* subsp. *lactis* BGMN1-596 was used as an indicator strain. Plates were incubated overnight at 30 °C for development of inhibition zones.

2.10. qRT-PCR

mRNA purified from derivatives MG7284/pAZIL-ES and MG7284/pAZIL-lsbB was quantified with real-time quantitative PCR (qRT-PCR) using a SYBR Green ER kit (Life technologies, Carlsbad, CA) in a thermocycler (7500 Real Time PCR System) using pairs of primers (rpoBsalcodF and rpoBsalcodR; RLsbBA30STOP and RT4) to amplify the genes of interest, *rpoB* and *lsbB* (Table 2). qRT-PCRs were carried out according to the manufacturers protocol using 50 ng total RNA per 20 μL of reaction mixture unless otherwise specified. The absence of contaminating DNA was confirmed by performing PCR in the absence of reverse transcriptase (efficiencies ranged from 99.0%).

3. Results and discussion

Synthesis of LsbB bacteriocin is plasmid encoded. Previously, it was reported that a derivative *L. lactis* subsp. *lactis* BGMN1-596T contains plasmid pMN5 carrying the structural bacteriocin gene *lsbB* (Gajic et al. 2003; Kojic et al. 2006). The LmrB protein, located upstream of the *lsbB* gene, transcribed in the opposite orientation, is involved in bacteriocin transport and immunity. Non-lantibiotics, the genetic determinants of which have been proposed to confer immunity, are frequently found in the bacteriocin operons, often located downstream of the bacteriocin structural genes (Klaenhammer 1993). In previous experiments it was observed that derivative MG7284/pAZIL-lsbB expresses a significantly lower zone of inhibition than the strain BGMN1-596T (Fig. 1; Uzelac et al. 2013). Considering that the construct pAZIL-lsbB carries *lsbB* cloned with promoter *PlsbB*, we analyzed the influence on LsbB production of *lsbB* surrounding genes and sequences located on plasmid pMN5. The reason for a larger zone of inhibition for the strain carrying pMN5 could be due to another bacteriocin production that is plasmid encoded. To test this possibility the following experiments were completed: (a) deletion of different parts of pMN5 and knock-out of ORFs, and (b) cloning and expression of each ORF present on pMN5 in the pAZIL vector alone. Because plasmid pMN5 is a natural lactococcal plasmid, which is unsuitable for manipulation and gene knock-out experiments, we prepared construct pAZIL-EX (*Eco*RI-*Xmn*I, 2821 bp) which showed that it contains all of the required genes to regulate LsbB bacteriocin expression; expressing the same size of inhibition zone as the strain carrying pMN5. *L. lactis* subsp. *cremoris* MG7284 is a naturally resistant strain to LsbB and was successfully transformed with the construct pAZIL-EX, giving the derivative MG7284/pAZIL-EX which produces the same size of inhibition zone as strain BGMN1-596T (7 mm) on sensitive strain BGMN1-596. This construct was used for further gene knock-out experiments. Gene knock-out of *lsbB*, *lmrB* and *lmrB*/*lsbB*, resulted in the constructs pAZIL-bN (with its *Nco*I restriction site blunted by incorporating four nucleotides by Klenow enzyme, inducing changes to amino acids after 25 in LsbB), pAZIL-ΔSS and pAZIL-ΔNSS, respectively (for details see Section 2 and Fig. 2). Three lactococcal strains, MG7284, IL1403 and BGMN1-596 were transformed with all constructs and the obtained transformants were used as bacteriocin producers in an agar well diffusion assay. The results show that the *lsbB* gene knock-out carriers MG7284/pAZIL-bN and IL1403/pAZIL-bN, as with *lsbB*/*lmrB* deletants MG7284/pAZIL-ΔNSS and IL1403/pAZIL-ΔNSS, have no bacteriocin activity against the sensitive strain BGMN1-596. In both constructs *lsbB* is knocked-out, and the absence of this gene leads to the absence of antimicrobial activity. The transformant MG7284/pAZIL-ΔSS with knocked-out *lmrB* gene showed bacteriocin activity as the primary strain BGMN1-596T. Transformants of IL1403 and BGMN1-596 were not obtained, as we expected, with constructs lacking the *lmrB* gene because IL1403 and BGMN1-596 do not possess the *lmrB* or *lmrP* genes which code for proteins necessary for immunity to LsbB bacteriocin, unlike MG7284, which expresses LmrP (Bolotin et al. 2001). These results strongly indicate that strain BGMN1-596T (a carrier of plasmid pMN5) produces only LsbB bacteriocin, and the different sizes of antimicrobial zones between derivatives MG7284/pAZIL-lsbB and BGMN1-596T is due to other factors. In addition a new construct was made, pAZIL-ES (containing only 736 bp *Eco*RI-*Sca*I fragment of pMN5), and the bacteriocin activity of obtained MG7284 transformants (MG7284/pAZIL-ES) were tested. Because the constructs MG7284/pAZIL-ES showed the same antimicrobial activity as MG7284/pAZIL-EX and MN1-596T it was concluded that for full expression of *lsbB* it is a sufficient *Eco*RI-*Sca*I sequence. Four potential genes downstream of the *lsbB* gene on the *Eco*RI-*Sca*I fragment were defined (*lsbA*, *lsbX*, *lsbY*, *lsbZ*; Fig. 2) as potential candidates for regulators responsible for increased activity. Because bioinformatics analysis showed that none of the defined genes have their own promoter, all of them were cloned downstream of promoter *PlsbB*. *PlsbB* promoter was first cloned in the pAZIL vector as an *Eco*RI-*Bam*HI fragment by the incorporation of restriction sites into primers for amplification, and after that additionally amplified fragments carrying *lsbA*, *lsbX*, *lsbY* or *lsbZ* individually or combined into a group of two (*lsbX*-*lsbY*) were cloned downstream of the *PlsbB* promoter as *Bam*HI-*Pst*I fragments. The resulting constructs were transferred into *L. lactis* MG7284 and the bacteriocin production of the transformants was tested. None of the obtained transformants showed bacteriocin activity no matter what construct was used for transformation, demonstrating that not one of the cloned genes (*lsbA*, *lsbX*, *lsbY*, *lsbZ* or *lsbX*-*lsbY*) encodes for bacteriocin or that the greater inhibition zone is not a result of the synergistic effect of LsbB and another bacteriocin. In addition the best bacteriocin candidate, LsbA was chemically synthesized (ChinaPeptides Co., Ltd.), but it did not show any antibacterial activity against BGMN1-596.

The second possible explanation for the defined genes' influence on the bacteriocin activity of LsbB is if they were part of two-component bacteriocins (LsbB representing one component, which alone has bacteriocinogenic activity). It is known that components of two (or more) component bacteriocins can sometimes have bacteriocin activity alone, while together having or increasing activity (Oppegard et al. 2007; Cooper et al. 2008; Hu et al. 2010; Coelho et al. 2014). In order to test this hypothesis all of the cloned genes were co-expressed in-trans with the gene *lsbB*. For that purpose *lsbB*, with its own promoter was transferred as a *Sac*I-*Pst*I fragment

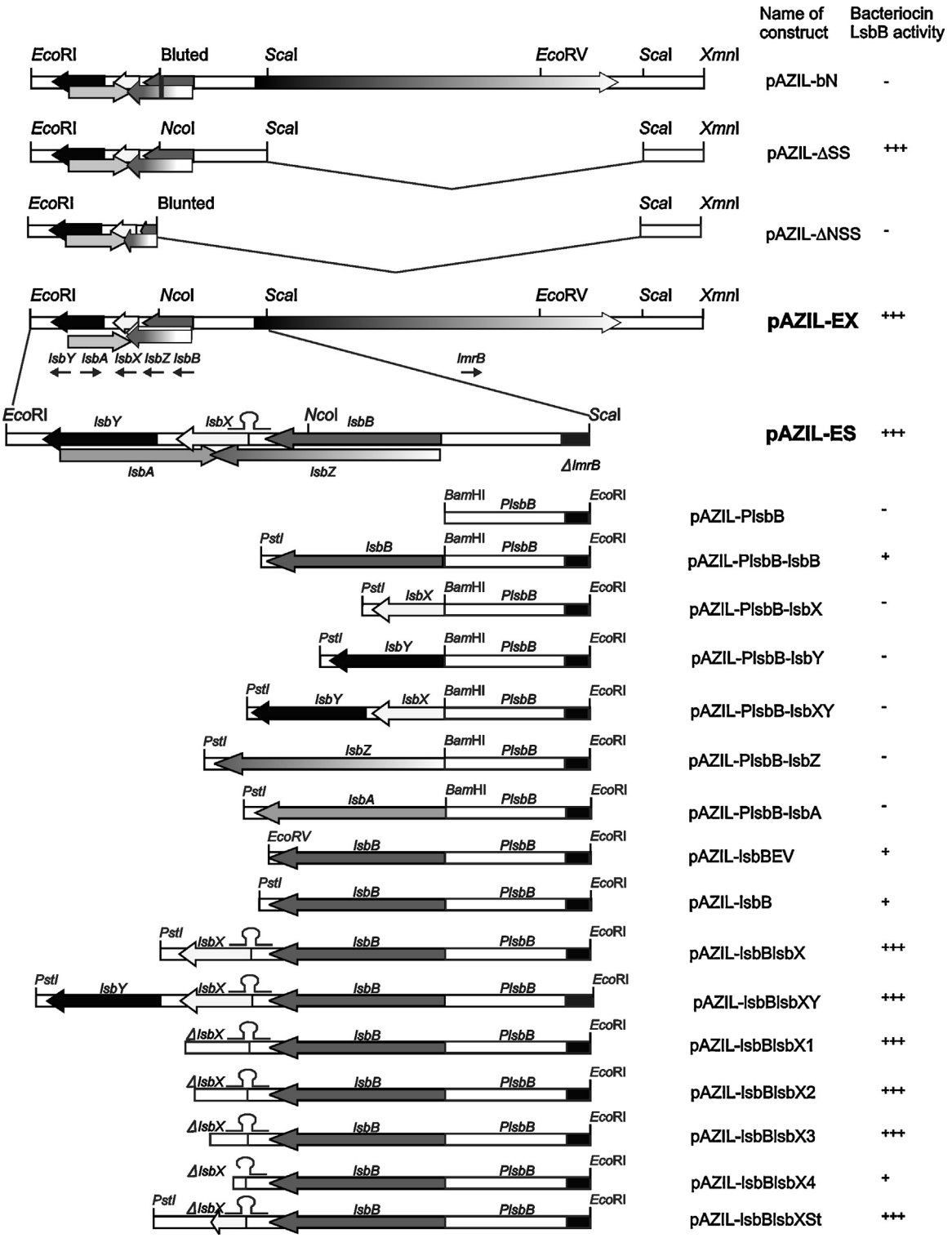

Fig. 2. Linear gene map of the *Eco*RI-*Xmn*I region of pMN5 plasmid carrying the *lsbB* operon and the scheme of construction knock-out mutants, deletants and clones used for analysis of influence of different genes/regions on the expression of the *lsbB* gene. Relevant restriction sites are indicated. The size and orientation of predicted ORFs are indicated by arrows.

from pAZIL (erythromycin resistance) to the pNZ8150 vector (chloramphenicol resistance), giving the pNZ8150-lsbB construct. Strain MG7284 was first transformed with pNZ8150-lsbB and after that additionally transformed with pAZIL constructs carrying the other genes, *lsbA*, *lsbX*, *lsbY*, *lsbZ* or *lsbX-lsbY*, under *PlsbB*. Again not one pAZIL construct increased production of LsbB in-trans compared to the control carrying only pNZ8150-lsbB. From these results we conclude that the other genes present on plasmid pMN5 are not part of a two-component bacteriocin and that they are not involved in LsbB overexpression in the strain BGMN1-596T.

In parallel, the level of *PlsbB* promoter transcription was measured using the newly constructed promoter fusion vector pNZ8150-lacZ1 (Vukotic et al. 2015) in order to test the in-trans influence of the *labA*, *lsbX*, *lsbY*, *lsbXY* and *lsbZ* genes

on *PlsbB* promoter activity. The initial derivative carrying the *PlsbB* promoter fusion MG7284/pNZ8150-PlsbB-lacZ1 was used for further transformation with constructs pAZIL-PlsbB-lsbA, pAZIL-PlsbB-lsbX, pAZIL-PlsbB-lsbY, pAZIL-PlsbB-lsbXY, pAZIL-bN and pAZIL-PlsbB-lsbZ. The obtained Cm^R, Em^R transformants were used for measurements of β-galactosidase activity, where it was shown that none of the derivatives have a significant influence on β-galactosidase activity compared to the control derivative MG7284/pNZ8150-PlsbB-lacZ1. The difference in activity between the control strain and derivatives did not have statistical significance ($P < 0.5$). From the results of promoter activity it is possible to conclude that increased LsbB bacteriocin production in BGMN1-596T is not the result of increased promoter activity or transcription governed by any of tested genes; none of genes are transcription regulators, pheromones or quorum-sensing peptides.

Because none of the analyzed elements were able to increase expression of *lsbB* in-trans, further experiments focused on testing the in-cis influence of the various elements. The cis-acting elements that could stabilize mRNA from degradation are stable stem-loops at the 5′ end of the transcript and terminators or REP sequences at their 3′ end (Grunberg-Manago 1999).

Considering that construct pAZIL-ES (*Eco*RI-*Sca*I, 736 bp) that provides full expression of LsbB bacteriocin as BGMN1-596T possesses the same 5′ region as pAZIL-lsbB, it was concluded that increased activity could be attributed to the 3′ region of the clone. To test this assumption, deletion of the 3′ region of the construct was performed, and further constructs were made: pAZIL-lsbBlsbX and pAZIL-lsbBlsbXY, which were shorter than pAZIL-ES. After testing the activity of the constructs in strain MG7284 it was realized that both express full bacteriocin activity, showing that the region responsible for enhanced expression of LsbB is located within gene *lsbX*. In order to test for possible involvement of *lsbX* in-cis or as a protein, additional constructs were prepared and expressed in MG7284: pAZIL-lsbBlsbX (with full *lsbX*), pAZIL-lsbBlsbX1 (with deletion of the last five nucleotides of *lsbX*), pAZIL-lsbBlsbX2 (with deletion of the last 12 nucleotides of *lsbX*), pAZIL-lsbBlsbX3 (with deletion of the last 22 nucleotides of *lsbX*), pAZIL-lsbBlsbX4 (with deletion of the last 38 nucleotides of *lsbX*) and pAZIL-lsbBlsbXSt (containing full length *lsbX* with stop codon inserted in the middle). In an agar well diffusion assay, transformants carrying construct pAZIL-lsbBlsbX4 showed smaller zones of inhibition like those carrying pAZIL-lsbB, while all others expressed the same inhibition zone as BGMN1-596T, MG7284/pAZIL-EX and MG7284/pAZIL-ES. From the results of bacteriocin activity tests we can conclude that regulation of LsbB expression is maintained by RNA structure; no one ORF is involved. Computer analysis of the sequence of 53 nucleotides downstream of *lsbB* (using the shortest construct that gave full bacteriocin activity, pAZIL-lsbBlsbX3) (Macke et al. 2001) showed the presence of a transcription terminator (CTATTAAGGAAAAACTGCAACAATAGTTGCAGTTTTTATTATGCATA), which finishes five nucleotides before the end of clone pAZIL-lsbBlsbX3. The presence of this transcription terminator, we can assume, has an influence on the stability of mRNA, which is important for full expression of *lsbB* in *L. lactis* BGMN1-596T. As RNA stability is an important component of gene expression, we measured the mRNA decay patterns for genes encoding RNA polymerase subunit *rpoB* and structural bacteriocin gene *lsbB*. The results showed greater mRNA stability in derivative MG7284/pAZIL-ES, which contains the transcription terminator, than in MG7284/pAZIL-lsbB without the transcription terminator region. We also compared the secondary structures of these two transcripts, which differ by only 21 nucleotides, using ViennaRNA Web Services (rna.tbi.univie.ac.at) and found that the secondary RNA structure of the transcript that contains the transcription terminator has a free energy value of −46.20 kcal/mol, in contrast to −36.30 kcal/mol for the shorter one. Data related to the regulation

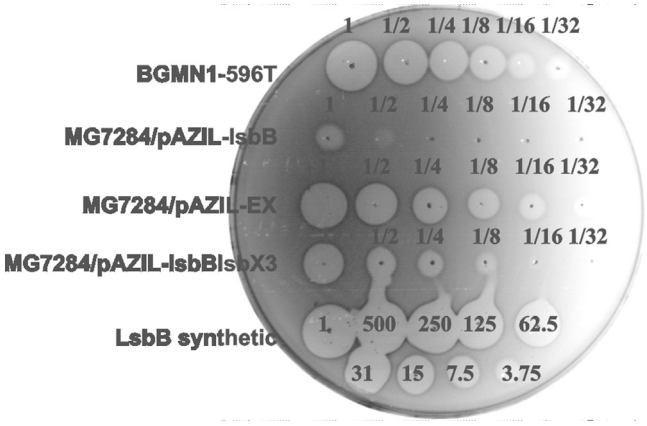

Fig. 3. Agar plate inhibition assay for measuring the level of LsbB expression by the WT strain *L. lactis* subsp. *lactis* BGMN1-596T (BGMN1-596T) and different clones in *L. lactis* subsp. *cremoris* MG7284: pAZIL-lsbB (MG7284/pAZIL-lsbB), pAZIL-EX (MG7284/pAZIL-EX) and pAZIL-lsbBlsbX3 (MG7284/pAZIL-lsbBlsbX3). Known concentrations of synthetic LsbB, as standards, were used for quantification of LsbB production (LsbB synthetic). Numbers on Petri dishes indicate dilution ratio (for LsbB producers) and concentrations of LsbB (from 1 mg to 3.75 μg; for synthetic LsbB). Solidified GM17 top agar containing *L. lactis* subsp. *lactis* BGMN1-596 as an indicator strain were spotted with 5 μL of filtered supernatants or synthetic LsbB. Plates were incubated overnight at 30 °C for development of inhibition zones.

of gene expression by transcription terminators are limited (Klug 1993; Ciampi 2006; Peters et al. 2009; Caballero et al. 2012; Czyz et al. 2014; Shashni et al. 2014) and none are related to lactic acid bacteria. It is interesting that the presence of a complete transcription terminator sequence in the transcript increased RNA stability by three times, while double dilution bacteriocin assay resulted in the expression of LsbB being increased by 30 times (Fig. 3). In addition, comparing the expression of LsbB of two constructs carrying the *lsbB* gene, one containing half of the transcription terminator sequence (pAZIL-lsbB) and the other with only the first four nucleotides (pAZIL-lsbBEV) and we conclude that because we were not able to detect any differences in production of LsbB between them, and together with results obtained by other deletion experiments, for full expression of LsbB the presence of a complete transcription terminator sequence is necessary. It may be that other factors are involved in this enhanced expression of LsbB, but this will be a subject for further investigation.

From our current results we are able to conclude that the expression of bacteriocin LsbB in *L. lactis* subsp. *lactis* BGMN1-5 is driven by a sequence located immediately downstream of the *lsbB* gene carrying only a transcription terminator (transcription terminator sequence start one nucleotide after stop codon of gene *lsbB*). This has for the first time demonstrated that a transcription terminator sequence can strongly influence bacteriocin expression. A similar increment (more than 10 times) of gene expression dependent on the transcription terminator was obtained for the *gluQ-rs* gene in *Shigella flexneri* (Caballero et al. 2012). The effect of the *lsbB* transcription terminator sequence on other genes of interest was tested in order to determine whether this sequence could be used to enhance their expression. For that experiment the *lcnB* gene (for expression of lactococcin B, another class II bactriocin) was cloned immediately upstream of the transcription terminator in the pAZIL vector. First, the transcription terminator was cloned as a *Hin*dIII-*Eco*RI fragment from construct pAZIL-lsbBlsbX3 into pBluescript predigested with the same enzymes giving the construct pBSTT. After that the amplified *lcnB* gene with its own promoter using primers LcnBF and LcnBR (Table 2) was re-cloned from pBluescriptT/A into pBSTT as the *Sal*I-*Cla*I fragment giving the pBSLcnBTT construct. Finally, two constructs were obtained: pAZIL-LcnB carrying the *lcnB* gene with its own promoter and pAZIL-LcnBTT carrying

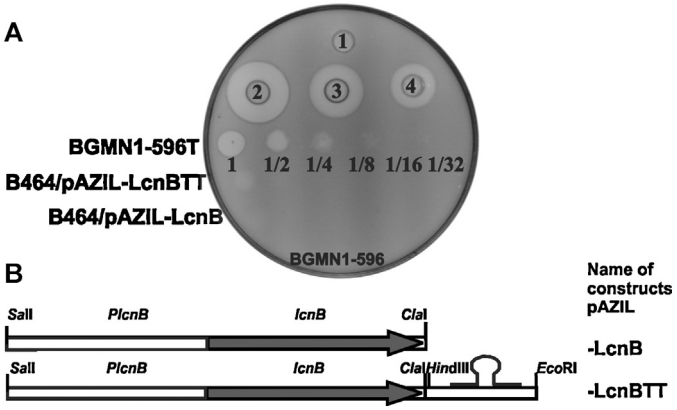

Fig. 4. (A) Agar plate inhibition assay for measuring the level of LcnB expression by different clones in *L. lactis* subsp. *lactis* B464: pAZIL-lcnB (B464/pAZIL-lcnB), and pAZIL-lcnBTT (B464/pAZIL-lcnBTT) and (B) schematic presentation of constructs. Numbers on Petri dish indicate dilution ratio (forLsbB and LcnB producers). Numbers on the Petri dishes indicate the dilution ratio (forLsbB and LcnB producers). Solidified GM17 top agar containing *L. lactis* subsp. *lactis* BGMN1-596 as indicator strain was spotted with 5 μL of filtered supernatants of producers. Plates were incubated overnight at 30 °C for development of inhibition zones. 1. *L. lactis* subsp. *lactis* BGMN1-596; 2. *L. lactis* subsp. *lactis* BGMN1-596 T; 3. *L. lactis* subsp. *lactis* B464/pAZIL-lcnBTT; 4. *L. lactis* subsp. *lactis* B464/pAZIL-lcnB. Relevant restriction sites are indicated. The size and orientation of predicted ORFs are indicated by arrows.

the *lcnB* gene with its own promoter and transcriptional terminator located immediately downstream. Bacteriocin activity of transformants of B464 (*L. lactis* subsp. *lactis* IL1403 mutant resistant to LcnB; Diep et al. 2007) carrying one of these two constructs was compared. It was observed that the bacteriocin activity of transformants carrying the transcription terminator was higher (at least two times, Fig. 4), but not as high as for LsbB. This result demonstrates the importance of this transcription terminator in bacteriocin expression and that it can act also on heterologous genes, but not as strongly as for lsbB classifying it as being more gene specific.

4. Conflict of interest

The authors declare no conflict of interest.

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