Characteristics of the bovicin HJ50 gene cluster in *Streptococcus bovis* HJ50

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Bovicin HJ50 is a new lantibiotic containing a disulfide bridge produced by *Streptococcus bovis* HJ50; its encoding gene bovA was reported in our previous publication. To identify other genes involved in bovicin HJ50 production, DNA fragments flanking bovA were cloned and sequenced. The bovicin HJ50 biosynthesis gene locus was encoded by a 9.9 kb region of chromosomal DNA and consisted of at least nine genes in the following order: bovA, -M, -T, -E, -F, ORF1, ORF2, bovK and bovR. A thiol–disulfide oxidoreductase gene named sdb1 was located downstream of bovR. A knockout mutant of this gene retained antimicrobial activity and the molecular mass of bovicin HJ50 in the mutant was the same as that of bovicin HJ50 in *S. bovis* HJ50, implying that sdb1 is not involved in bovicin HJ50 production. Transcriptional analyses showed that bovA, bovM and bovT constituted an operon, and the transcription start site of the bovA promoter was located at a G residue 45 bp upstream of the translation start codon for bovA, while bovE through bovR were transcribed together and the transcription start site of the bovE promoter was located at a C residue 35 bp upstream of bovE. We also demonstrated successful heterologous expression of bovicin HJ50 in *Lactococcus lactis* MG1363, which lacks thiol–disulfide oxidoreductase genes; this showed that thiol–disulfide oxidoreductase genes other than sdb1 are not essential for bovicin HJ50 biosynthesis.

INTRODUCTION

The lantibiotics represent a diverse family of bacterially produced antimicrobial peptides (de Vos *et al.*, 1995; Sahl *et al.*, 1995; van Kraaij *et al.*, 1999). They are characterized by the presence of lanthionine and β-methyllanthionine, which form intramolecular thioether rings. All lantibiotics are ribosomally synthesized as prepropeptides that are first modified by the dehydration of Ser and Thr residues. Lan or MeLan bridges are then introduced by the intramolecular addition of Cys thiols to the dehydrated amino acids (Koponen *et al.*, 2002; Kuipers *et al.*, 2006; Li *et al.*, 2006). The peptides are not fully active until the leader sequence is cleaved (Engelke *et al.*, 1992; Kuipers *et al.*, 1993). Lantibiotics are divided into two groups, type A and type B, according to their structural features (Jung, 1991; Kupke & Gotz, 1996). Type A includes lantibiotics with highly different structures, and up to three subtypes are recognized: AI lantibiotics are elongated and flexible, e.g. nisin; All peptides display an unbridged N-terminal extremity and a globular C-terminal part, e.g. lactocin 481; and the AIII group contains lactocin S as well as lantibiotics composed of two peptides (two-component bacteriocins) (Twomey *et al.*, 2002). Notably, some All peptides have the extraordinary characteristic of possessing disulfide bonds, e.g. sublancin 168 (Paik *et al.*, 1998).

Studies on lantibiotic gene clusters have proposed that the genes involved in lantibiotic production include structural genes (*lanA*), modification enzyme genes (*lanB* and *lanC*, or *lanM*), secretion ABC transporter genes (*lanT*), leader peptidase genes (*lanP*), self-protection genes (*lanFEG* and *lanI*) and regulatory genes (*lanR* and *lanK*) (Siezen *et al.*, 1996). For those lantibiotics that undergo other more unusual post-translational modifications, the genes encoding the required enzymes are usually found in the corresponding cluster, e.g. *bdhB* in the sublancin 168 gene cluster (Dorenbos *et al.*, 2002). However, not all of these genes have been detected in the gene clusters, indicating
that some of the regulatory and accessory genes may be located outside the gene cluster or that the gene functions can be provided by host-encoded proteins having similar activities (Heidrich et al., 1998). To study the function of the genes, several expression systems for production of lantibiotics have been constructed (Kuipers et al., 1992; Yuan et al., 2004).

Bovicin HJ50 is an AII lantibiotic containing two \( \beta \)-methyllyanthionines and a disulfide bond produced by *Streptococcus bovis* HJ50 (Xiao et al., 2004). It differs from other lantibiotics in that it has a disulfide bridge and reduction of the disulfide bond has no effect on its antimicrobial ability. Previously, we identified its structural gene *bovA*. To gain further insight into bovicin HJ50 production, in the present study we cloned and sequenced its biosynthesis gene cluster to determine the genetic basis for its biosynthesis. Bovicin HJ50 was also expressed in *Lactococcus lactis* MG1363, which lacks thiol–disulfide oxidoreductase genes, and the results surprisingly showed that thiol–disulfide oxidoreductases were not essential for the synthesis of a lantibiotic containing a disulfide bond; this is believed to be the first time such a result has been reported.

**METHODS**

**Bacterial strains and culture conditions.** *Streptococcus bovis* HJ50 was grown anaerobically in M17 medium with 5 g glucose l\(^{-1}\) at 37 °C. *Escherichia coli* DH5\(\alpha\), which was used as the host for DNA cloning, sequencing and constructing disruption vectors, was cultured in LB medium at 37 °C. When necessary, media were supplemented with 10 μg chloramphenicol ml\(^{-1}\), 100 μg ampicillin ml\(^{-1}\) and 100 μg erythromycin ml\(^{-1}\) for *E. coli*, and 5 μg erythromycin ml\(^{-1}\) and 5 μg chloramphenicol ml\(^{-1}\) for *S. bovis*. The indicator strain *Micrococcus flavus* NCIB 8166 was maintained in Sl medium at 30 °C.

**DNA preparation, transformation and molecular cloning techniques.** *S. bovis* HJ50 genomic and plasmid DNA were isolated as described by Lewington et al. (1987) and Takamatsu et al. (2001b), respectively. Transformation was carried out as described by Takamatsu et al. (2001b) with minor modifications. Briefly, cells were grown at 30 °C in M17 medium containing 5 g glucose l\(^{-1}\) and 40 mM DL-threonine and harvested in the mid-exponential phase (OD\(_{600}\) 0.3–0.5). Cells were subsequently treated at 50 °C for 9 min and washed with CTB (55 mM MnCl\(_2\), 15 mM CaCl\(_2\), 250 mM KCl), and 10 mM PIPES, pH 6.7. After resuspending in CTB and incubating on ice for 30 min, cells were washed twice with 0.3 M PIPES, pH 6.7. After resuspending in CTB and 10 mM PIPES, pH 6.7. After resuspending in CTB and incubating on ice for 30 min, cells were washed twice with 0.3 M sucrose and resuspended in 0.3 M sucrose containing 15 % glycerol. A 0.05 ml aliquot of cell suspension was mixed with plasmid DNA. The mixture was then placed in a pre-chilled sterile electroporation cuvette (2 mm electrode gap) and pulsed immediately with a Bio-Rad Gene Pulser (2.5 kV, 200 W, 25 mF). The mixture was then diluted with M17 broth containing 0.3 M sucrose and 10 mM MgCl\(_2\), and incubated at 30 °C for 2 h. The cells were then spread on M17 agar containing 5 g glucose l\(^{-1}\) and appropriate antibiotics, and incubated at 30 °C. The resulting clone was selected. Other molecular cloning techniques were performed according to Maniatis et al. (1982).

**Cloning and sequencing of genes involved in bovicin HJ50 synthesis.** To obtain flanking regions of *bovA*, a nested PCR method (Xiao et al., 2004) with slight modifications was employed. Genomic DNA was digested with different restriction enzymes and ligated to pBluescript II SK digested with the corresponding enzymes. The resulting ligation mixtures were used as PCR templates with a set of primers specific for the cloned gene and plasmid pBluescript II SK, respectively. To obtain more specific fragments, the resulting PCR products were used as PCR templates with another set of inward primers specific for the cloned gene and plasmid pBluescript II SK. PCR was performed under the following conditions: 94 °C for 2 min, followed by 30 cycles of 94 °C for 1 min, 54 °C for 1 min, 72 °C for 5 min, and 72 °C for 10 min. The longest fragment of the resulting PCR products was isolated, ligated to the pMD18-T (TaKaRa) vector, and sequenced. LA Taq (TaKaRa) was used for its high specificity and ability to amplify long fragments. Flanking regions of the newly cloned DNA fragments were also amplified and sequenced using the same method until the cloned DNA fragment did not have any relation to bovicin HJ50 biosynthesis. Both strands were sequenced twice. DNA sequences were edited using the EditSeq program (DNASTAR), and sequence similarity searches were performed using the BLASTX or BLASTP algorithms on the NCBI website (www.ncbi.nlm.nih.gov/BLASTX).

**Construction of gene-disruption mutants.** To disrupt *sdb1*, corresponding fragments of the *sdb1* gene were amplified with primers DX1/DX2 and DX3/DX4, and then subcloned into the temperature-sensitive vector pSET15s (Takamatsu et al., 2001a), resulting in the vector pDX. An erythromycin-resistant cassette from pLEM415 (Fons et al., 1997) amplified by PCR was digested with BamHI and subcloned into plasmid pDX, resulting in pDDEX. The vector was electrooporated into *S. bovis* and incubated at a permissive temperature (37 °C) overnight. The resulting resistant clone was selected and treated with a high temperature (37 °C) for 9 min at 37 °C in the presence of 5 μg erythromycin ml\(^{-1}\) to force double-crossover recombination. Successful recombinant clones were screened by PCR with primers specific for the resistance cassette and primers located in the flanking regions of the targeted gene. All primers used in constructing the disruption vectors are listed in Table 1. The PCR screen was conducted twice.

**Northern blot analysis and primer extension mapping.** Total RNA from *S. bovis* was isolated from 10 ml of culture at the mid-exponential phase. Each sample was transferred without delay to a centrifuge tube containing crushed ice to stop cell growth, and the cells were precipitated by centrifugation at 20000 g for 10 min at 4 °C. The cells were treated with lysozyme (10 mg ml\(^{-1}\)) at 37 °C for 10 min and the RNA was extracted using an Invitrogen TRIzol kit. RNA was quantified by measuring the absorbance at 260 nm.

For Northern blot analysis, samples (20 μg per lane) were separated on a 1.5 % agarose gel containing 18 % formaldehyde and transferred to a Hybond-N\(^{-}\) nylon membrane. The DNA probe for each gene was generated by PCR. The amplified DNA fragment was labelled with [\(\alpha\)-\(\beta\)]dCTP under the following conditions. The 20 μl reaction mixture contained 1× standard PCR buffer; 2 ng DNA template; 40 μM (each) dGTP, dATP and dTTP; 2.5 μM (each) ‘cold’ and ‘hot’ dCTP; and 1 μL DNA polymerase. The reaction was performed in an automatic thermocycler for 30 cycles at 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min. After PCR, the labelled probe was separated from the free isotope by ethanol precipitation and the radioactivity of the probe was measured with a scintillation counter. Other molecular biology techniques were performed as described by Maniatis et al. (1982) or according to the manufacturer’s instructions. Northern blots were repeated twice.

For primer extension analysis, 20 μg total RNA and 30 pmol [\(\alpha\)-\(\beta\)]dATP-labelled primer were mixed in a volume of 12 μl, denatured at 70 °C for 10 min, and then quickly chilled on ice. Then 4 μl 5× first-strand buffer ( Gibco-BRL), 2 μl 0.1 M DTT, 1 μl 10 mM dNTP and 1 μl Superscript RT II (200 U; Gibco-BRL) were added and incubated at 70 °C for 2 min. The reaction was terminated by adding 2 μl stop buffer and 10 μl 5 % sucrose followed by incubation at 65 °C for 5 min. 2 μl of the reaction mixture was size fractionated on a 20 % polyacrylamide gel containing 8 M urea and UV-stained. The primer extension products were visualized on an UV box and the lengths of the bands were measured by imaging analysis software.
added to the RNA/primer mixture and incubated at 42 °C for 1 h. The reaction was terminated by incubation at 70 °C for 15 min. RNase reaction mix (50 μl of 100 μg salmon sperm DNA ml−1 and 20 μg RNase A ml−1) was added, and the mixture was incubated at 37 °C for 15 min. The primer extension products were extracted with phenol/chloroform and precipitated with ice-cold ethanol. DNA sequencing reactions were simultaneously performed with the same primers using the fmol DNA cycle sequencing system (Promega) according to the manufacturer’s instructions. The products of primer extension and DNA-sequencing reaction were respectively resuspended in the same sequencing stop solution and heated for 2 min at 70 °C, then analysed on a 6 % acrylamide sequencing gel. Radioactive DNA fragments on the gels were visualized on X-ray film. Primer extension mapping was performed twice.

Heterologous expression of the bovicin HJ50 biosynthetic gene cluster. A 7.1 kb fragment containing bovA–ORF2 and a 9.9 kb DNA fragment containing bovA–bovR were amplified with primers BGC1/BGC2 and BGC3/BGC4, respectively. The corresponding fragments were digested and subcloned into pMG36c (van de Guchte et al., 1989) at the SacI/Sall site, resulting in vectors pBGC1 and pBGC3 (see Fig. 5), respectively. The plasmids were electroporated into L. lactis MG1363 and the resulting clones were selected.

Analysis of bovicin HJ50 production in S. bovis mutant strains and L. lactis recombinant strains. In order to analyse bovicin HJ50 production, strains were cultured at 37 °C or 30 °C for 12 h. Cells were removed by centrifugation at 4 °C, and culture supernatants were retained for the following analyses. Antimicrobial activity analyses were measured by agar well diffusion assay with plates seeded with indicator strain M. flavus NCIB 8166 as described by Cintas et al. (1995). The wells were filled with 20 μl culture supernatant, and the plates were incubated at 30 °C overnight prior to examining inhibition zones. Bovicin HJ50 proteins were purified and quantified by the Pierce BCA protein assay kit (product no. 23225). One microgram of purified bovicin HJ50 from S. bovis HJ50 and L. lactis MG1363/pBGC1 was used to measure activity against M. flavus NCIB 8166. The culture supernatant was also used to measure activity against other bacteria to determine the antimicrobial spectrum of active protein produced by the L. lactis recombinant. In addition, bovicin HJ50 was analysed by MALDI-TOF MS as described previously (Xiao et al., 2004). Both the antimicrobial activity test and MALDI-TOF MS were repeated twice.

## RESULTS

### Cloning and sequencing of putative genes involved in bovicin HJ50 production

DNA fragments flanking bovA were cloned by nested PCR, and sequence analysis showed that the 14 kb fragment comprised 12 coding sequences (Fig. 1). The first two ORFs downstream of bovA were termed bovM and bovT, respectively. The bovM gene encoded an 837-residue protein which showed similarity to a series of putative modification enzymes involved in the post-translational modification of lacticin 481-type lantibiotics, e.g. 46 % identity to the lantibiotic mersacidin modifying enzyme found in Streptococcus suis (Chen et al., 2007). The putative bovT product consisted of 311 amino acids and showed similarity to the MrST protein of Bacillus sp. HIL-Y85/54728 (32 % identity) (Altena et al., 2000), suggesting that the putative bovT translational product may be involved in the secretion of bovicin HJ50.

The bovE and bovF products consisted of 292 and 302 amino acids, respectively. They showed high sequence similarities with a series of ABC-transporter proteins. The BovE protein contained an ABC-transporter signature motif and was shown to be an ATPase. The BovF protein showed 42 % overall identity to MutF in mutacin II biosynthesis (Qi et al., 1999) and the bovF product possessed the consensus amino acid sequence of the ATP-binding domain. ORF1 and ORF2 encoded 240 and 238 amino acids, respectively, located downstream of bovF. ORF1 showed similarities only to several ABC-transporter proteins in the database, e.g. 34 % identity to the putative

### Table 1. Primers and plasmids used in this study

<table>
<thead>
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<th>Primer/Plasmid</th>
<th>Sequence/Characteristics</th>
<th>Use/Source or reference</th>
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<td>BGC2</td>
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<td>PEM2</td>
<td>TTAACAAAGGCATTCCACTAC</td>
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<td>Em`</td>
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<td>This study</td>
</tr>
<tr>
<td>pBGC3</td>
<td>Cm`, pMG36c derivative, expression of bovA-R</td>
<td>This study</td>
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### Analysis of bovicin HJ50 production in S. bovis mutant strains and L. lactis recombinant strains

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ABC transporter in *Clostridium perfringens* SM101 (Myers *et al.*, 2006). ORF2 showed 22% identity to the ABC transporter protein found in *Silicibacter pomeroyi* DSS-3 (Buchan *et al.*, 2004), and 25% identity to SpaE encoded on the subtilin locus of *Bacillus subtilis* A1/3 (Chung & Hansen, 1992). These results suggested that bovE, bovF, ORF1 and ORF2 might form an ABC-transporter-like complex which contributes to self-protection of *S. bovis* HJ50 from bovicin HJ50.

The bovK and bovR products consisted of 503 and 198 amino acids, respectively. The BovK protein showed 30% identity to SalK, and BovR showed 39% identity to SalR in the salivaricin biosynthesis locus (Upton *et al.*, 2001). The bovR gene is presumably translated from TTG. The ORF downstream of bovR encoded a 161 amino acid protein. It contained a thioredoxin domain and was named sdb1 (for *Streptococcus* disulfide bond). In the region upstream of bovA and downstream of sdb1, the genes appeared to be unrelated to the biosynthesis of bovicin HJ50.

**Transcriptional analysis of the bovicin HJ50 gene cluster**

To determine how many transcription units constituted the bovicin HJ50 biosynthetic locus, Northern blot analyses were performed with 32P-labelled DNA probes (200–300 bp) specific to the genes in the cluster (Fig. 2). Hybridization with the bovA probe detected two putative transcripts, of approximately 0.25 and 3.8 kb. The 0.25 kb RNA was the most abundant transcript and hybridized only with the bovA probe. This result suggested that the majority of the transcripts terminated at the end of the bovA gene. In contrast to the 0.25 kb RNA, the 3.8 kb transcript hybridized with every probe of bovA, bovM and bovT. The size of the transcript agreed with the length of the DNA from bovA to the end of bovT, indicating that this transcript arose from the bovAMT operon. Probes of bovE and bovR, conversely, hybridized with transcripts of 5.3 kb, indicating that the bovE through bovR genes are in the same operon. Taken together, these data indicate that there are three transcription units in the bovicin HJ50 biosynthetic cluster: bovA, bovAMT and bovE–bovR.

To locate the promoter of the transcriptional operon, 5' end-labelled primers complementary to the bovA and bovE genes, respectively, were used. Primer extension mapping detected a single transcript, which was initiated at a G residue 45 bp upstream of the translation start codon for bovA (Fig. 3a). Analysis of the bovA promoter region revealed two inverted repeats, IR I and IR II, and three 6 bp direct repeats (Fig. 3b). A putative −10 region with the sequence TAT**ACT** was found, but no obvious −35 region. Primer extension mapping showed that the transcription start site of bovE corresponded to a C located 35 bp upstream of the ATG codon, while no obvious −10 or −35 regions or RBS were found (Fig. 3c, d).

**Gene disruption to demonstrate the involvement of the cloned genes in bovicin HJ50 production**

To further demonstrate that the cloned gene cluster was involved in bovicin HJ50 biosynthesis, we knocked out the sdb1 gene and designated the clone *S. bovis* DX. In *S. bovis* DX, a DNA fragment of approximately 450 bp in sdb1
was deleted and a 1.2 kb erythromycin-resistance cassette was inserted in the corresponding position. Antimicrobial activity assays showed that the \textit{S. bovis} DX product retained activity, but it was slightly lower than that of the \textit{S. bovis} HJ50 and \textit{S. bovis} HJ50/pDDEX proteins (Fig. 4a). Purified protein was subsequently quantified; the amount of bovicin HJ50 in \textit{S. bovis} DX was approximately 30% lower than that in \textit{S. bovis} HJ50 and \textit{S. bovis} HJ50/pDDEX. The protein was then subjected to MALDI-TOF MS analysis (Fig. 4b). This showed that the molecular mass was 3428.5 Da, which was very close to the result for bovicin HJ50 in \textit{S. bovis} HJ50 (3428.3 Da), suggesting that \textit{sdb1} is probably not involved in bovicin HJ50 production.

**Heterologous expression of the bovicin HJ50 biosynthetic gene cluster**

In order to determine whether the genes identified were sufficient for bovicin HJ50 production, \textit{L. lactis} MG1363 was transformed with pBGC1 and pBGC3. In \textit{L. lactis} MG1363/pBGC1, \textit{bovAMTEF}, ORF1 and ORF2 were expressed from the P32 promoter (van der Vossen et al., 1987), whereas in \textit{L. lactis} MG1363/pBGC3, \textit{bovA} through...
Fig. 4. Characterization of bovicin HJ50 produced by *S. bovis* ΔDX. (a) Antimicrobial activity. Culture supernatants (20 µl) of *S. bovis* HJ50, *S. bovis* HJ50/pDDEX and *S. bovis* ΔDX were used for an agar diffusion assay. Inhibitory activity against *M. flavus* NCIB 8166 was slightly decreased in *S. bovis* ΔDX (3) compared to *S. bovis* HJ50 (1) and *S. bovis* HJ50/pDDEX (2). (b) MALDI-TOF MS of bovicin HJ50 purified from *S. bovis* ΔDX.

Fig. 5. Maps of pBGC1 and pBGC3. *cat*, cassette containing the chloramphenicol acetyltransferase gene responsible for chloramphenicol resistance.
bovR were expressed from bovA’s promoter (Fig. 5). No active bovicin HJ50 could be detected in recombinant L. lactis MG1363/pBGC3, but the clone was no longer sensitive to bovicin HJ50 (Fig. 6e). L. lactis MG1363/pBGC1 was not sensitive to bovicin HJ50 and the culture supernatant of this clone showed antimicrobial activity against M. flavus NCIB 8166; however, the production level of bovicin HJ50 was much lower than that in wild-type S. bovis HJ50 (Fig. 6). The cell-free culture supernatant was active against Lactobacillus curvatus LTH1174, Bacillus subtilis AS1.1087, Bacillus megaterium AS1.941, Leuconostoc dextranicum 181 and Leuconostoc mesenteroides AS1.2, but showed no activity against Listeria monocytogenes. No difference in antimicrobial spectrum was found between the heterologously expressed bovicin HJ50 and that produced by wild-type S. bovis HJ50. MALDI-TOF-MS analysis of the heterologously expressed peptide gave a molecular mass of 3431.0 Da (Fig. 7). This peak apparently corresponds to bovicin HJ50 having no disulfide bridge, which should have a molecular mass of 3430.5 Da. Antimicrobial activity assays with 1 μg samples of wild-type bovicin HJ50 and mutant bovicin HJ50 produced by L. lactis MG1363/pBGC1 showed that bovicin HJ50 and mutant bovicin HJ50 (without disulfide bridges) demonstrated the same activity against M. flavus NCIB 8166 (Fig. 6b). Thus, thiol–disulfide oxidoreductase may not be essential for production of active bovicin HJ50.

**DISCUSSION**

We have cloned a 14 kb gene cluster proposed to be involved in bovicin HJ50 production. DNA sequence analysis shows that the gene cluster contains nine ORFs in the following order: bovA, -M, -T, -E, -F, ORF1, ORF2, bovK and bovR. A thiol–disulfide oxidoreductase gene named sdb1 is located downstream of bovR. Our previous results showed that inhibitory activity of bovicin HJ50 was neither decreased nor increased when the peptide was reduced by DTT (Xiao et al., 2004). To determine whether sdb1 is involved in bovicin HJ50 biosynthesis, this gene was knocked out. The molecular mass of bovicin HJ50 produced by the mutant was not changed. Bovicin HJ50 was quantified and we found that the amounts of bovicin HJ50 in S. bovis HJ50 and S. bovis HJ50/pDDEX were the same, but the amounts of bovicin HJ50 in S. bovis ΔDX were approximately 30 % lower than in S. bovis HJ50 and S. bovis HJ50/pDDEX. These results showed that sdb1 might not be required for bovicin HJ50 biosynthesis, directly, but did have an effect on efficiency of bovicin HJ50 biosynthesis.

We demonstrated that there were three transcripts in the bovicin HJ50 gene cluster: bovA, bovAMT and bovE–bovR. The bovA and bovAMT transcripts were transcribed from the same promoter. As there was a stem–loop structure between bovA and bovM, the presence of the bovAMT transcript could be a result of readthrough from the bovA promoter, which was also reported in the mutacin II gene locus (Qi et al., 1999). The bovE–bovR genes were transcribed as an operon (Fig. 2) and the genes were either overlapping or adjoining; they were likely to be cotranscribed. Further, primer extension mapping showed that there was no promoter upstream of bovK (data not shown). In most lantibiotic gene clusters, the ABC transporter lanFEG confers immunity to the host, while in S. bovis HJ50 only bovE and bovF were found. A gene equivalent to lanG, which is typically involved in export of lantibiotics, was missing in the bovicin HJ50 gene cluster. L. lactis MG1363/pBGC1, in which bovA through ORF2 were expressed, showed resistance to bovicin HJ50. Although it is possible that a lanG-like gene is not located in the vicinity of the bovicin HJ50 gene cluster, we presume that ORF1 and ORF2 take over this function.

The transcription start site of bovA was located by primer extension mapping as a G residue 45 bp upstream of the translation start codon. Analysis of the bovA promoter region revealed two inverted repeats (IR I and IR II) and three 6 bp direct repeats (DRI, DRII and DRIII) (Fig. 3b). IR I overlaps the transcription start site, whereas IR II is located upstream of the −10 region. Similar structures are

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**Fig. 6.** Antimicrobial activity of heterologously expressed bovicin HJ50. (a) Inhibition zones of culture supernatants from S. bovis HJ50 (1), L. lactis MG1363/pBGC1 (2) and L. lactis MG1363 (3), tested against M. flavus NCIB 8166. (b) Inhibition zones of 1 μg bovicin HJ50 from S. bovis HJ50 (left) and L. lactis MG1363/pBGC1 (right), tested against M. flavus NCIB 8166. (c) Inhibition zone of bovicin HJ50 tested against L. lactis MG1363. (d) Inhibition zone of bovicin HJ50 tested against L. lactis MG1363/pBGC1. (e) Inhibition zone of bovicin HJ50 tested against L. lactis MG1363/pBGC3.
also present in the epidermin biosynthetic operon, in which IR II serves as the binding site for the regulatory protein EpiQ (Peschel et al., 1993), and in the promoter region of mutA (Qi et al., 1999). DRI to DRIII are located upstream of bovA. Similar structures were reported in the subtilin promoter (Kleerebezem et al., 2004). The transcription start site of bovE was a C residue 35 bp upstream of the ATG codon, whereas no obvious −10 or −35 regions or ribosome-binding site were found. Two inverted repeats were found upstream of the translation start site (Fig. 3d). Lack of possible RBS and obvious −35 and −10 regions could be the result of a different regulation system compared with the bovA promoter. L. lactis MG1363/pBGC3 was not sensitive to bovicin HJ50; however we did not find any bovA transcripts. The results show that bovE could be transcribed but bovA was not transcribed. Thus, although lacking an obvious RBS or promoter region, the bovE promoter might be regulated through a different pathway. The lack of consensus in the bovA and bovE promoter regions also indicated a different regulatory pathway between the promoters. The presence of the two-component regulatory system (bovK and bovR) indicated that bovicin HJ50 production could be regulated in a manner similar to that of the subtilin and nisin loci (Klein et al., 1993; Kuipers et al., 1995). In the case of nisin, the expression of the biosynthetic genes is controlled by an autoregulatory circuit via the histidine kinase NisK and the response regulator NisR (Kuipers et al., 1995). Such an autoregulatory circuit is involved in the expression of the biosynthetic genes of other lantibiotics, such as the type A lantibiotics subtilin (Klein et al., 1993) and streptococcin A-FF22 (McLaughlin et al., 1999) and the type B lantibiotic mersacidin (Altena et al., 2000). Bovicin HJ50 production in S. bovis HJ50 was shown to be inducible by the addition of purified bovicin HJ50 (data not shown).

We demonstrated the heterologous production of bovicin HJ50 in L. lactis MG1363 by the expression of bovAMTEF-ORF1-ORF2 from the P32 promoter. However, in L. lactis MG1363/pBGC3, which expressed bovA through bovR from the bovA promoter, no active bovicin HJ50 was detected. Northern blots revealed no transcripts of bovA (data not shown), but the clone was not sensitive to bovicin HJ50. These results indicate that the bovA and bovE promoters are regulated by a different pathway. It seems that the bovKR two-component system does not function efficiently in L. lactis MG1363. The reason for this is presently unknown. One possibility is that there may be other unknown factors necessary for expression of the bovicin HJ50 genes in S. bovis that were not transferred into L. lactis MG1363. Another possible reason for inefficient signal transduction is cross-talk between two different two-component systems in the host. Different two-component regulatory systems that exhibit extensive similarity in protein structure can each affect the signal transduction of the other (Fisher et al., 1995; Wright et al., 1993). For example, the kinase sensor protein VanS of the vancomycin-resistance regulon was shown to activate PhoB (regulator protein of phosphate synthesis) in E. coli (Fisher et al., 1995). As the BoVK protein showed similarities to several sensor protein kinases in the L. lactis genome, it is therefore possible that some gene competition exists among different two-component systems in L. lactis MG1363, which impedes full expression of the bovicin HJ50 genes.

To date only two lantibiotics containing a disulfide bond have been described. Disulfide bridges were reported to be essential for the inhibitory activity of sublancin 168 (Dorenbos et al., 2002). In B. subtilis 168, there are four thiol–disulfide oxireductases, BdbA, BdbB, BdbC, and BdbD. BdbB and BdbC are involved in the production of active sublancin 168, whereas BdbA is not required (Dorenbos et al., 2002; Kouwen et al., 2007). In S. bovis HJ50, only one thiol–disulfide oxireductase gene was found. Our results showed that the inhibitory activity of DTT-reduced bovicin HJ50 neither decreased nor increased (Xiao et al., 2004). Gene disruption analysis showed that the thiol–disulfide oxireductase encoded by sdb1 was not involved in bovicin HJ50 biosynthesis. Heterologous expression of the bovicin HJ50 gene cluster resulted in a protein with a molecular mass of 3431.0 Da, slightly larger than that of bovicin HJ50 (3428.3 Da); this result indicated that the protein was the bovicin HJ50 protein without a disulfide bridge. However we did not find any difference in antimicrobial spectrum between the active proteins from wild-type S. bovis HJ50 and S. lactis MG1363/pBGC1. Furthermore, purified bovicin HJ50 and mutant bovicin HJ50 showed the same antimicrobial activity. These observations show that thiol–disulfide oxireductase is not essential for bovicin HJ50 biosynthesis. The role of the disulfide bridge in bovicin HJ50 biosynthesis is still unknown. Further elucidation is progressing in our laboratory.

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