Analysis of the sakacin P gene cluster from *Lactobacillus sake* Lb674 and its expression in sakacin-negative *Lb. sake* strains

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Sakacin P is a small, heat-stable, ribosomally synthesized peptide produced by certain strains of *Lactobacillus sake*. It inhibits the growth of several Gram-positive bacteria, including *Listeria monocytogenes*. A 7.6 kb chromosomal DNA fragment from *Lb. sake* Lb674 encompassing all genes responsible for sakacin P production and immunity was sequenced and introduced into *Lb. sake* strains Lb790 and Lb706X which are bacteriocin-negative and sensitive to sakacin P. The transformants produced sakacin P in comparable amounts to the parental strain, Lb674. The sakacin P gene cluster comprised six consecutive genes: *sppK*, *sppR*, *sppA*, *spiA*, *sppT* and *sppE*, all transcribed in the same direction. The deduced proteins SppK and SppR resembled the histidine kinase and response regulator proteins of bacterial two-component signal transducing systems of the AgrB/AgrA-type. The genes *sppA* and *spiA* encoded the sakacin P preprotein and the putative immunity protein, respectively. The predicted proteins SppT and SppE showed strong similarities to the proposed transport proteins of several other bacteriocins and to proteins implicated in the signal-sequence-independent export of *Escherichia coli* haemolysin A. Deletion and frameshift mutation analyses showed that *sppK*, *sppT* and *sppE* were essential for sakacin P production in Lb706X. The putative SpiA peptide was shown to be involved in immunity to sakacin P. Analogues of *sppR* and *spiA* were found on the chromosomes of *Lb. sake* Lb706X and Lb790, indicating the presence of an incomplete *spp* gene cluster in these strains.

Keywords: *Lactobacillus sake*, sakacin P, *spp* gene cluster, bacteriocin, two-component signal-transducing system

INTRODUCTION

Sakacin P is an anti-listerial peptide produced by *Lactobacillus sake* strains Lb674 and LTH673 (Holck *et al.*, 1994; Tichaczek *et al.*, 1994). It belongs to a group of antimicrobial proteinaceous compounds, known as bacteriocins, which are secreted by many Gram-positive bacteria. Bacteriocins usually inhibit the growth of species closely related to the producer strain but may also be active against less related bacteria. Some bacteriocins from lactic acid bacteria (LAB) are of interest to the food

industry because of their potential use in inhibiting the growth of food spoilage microorganisms and pathogens such as *Listeria monocytogenes* (Hillier & Davidson, 1991; Vandenberg, 1993).

The bacteriocins of LAB differ in structure, size and mode of action. The small, peptide-like bacteriocins are divided into two classes (Klaenhammer, 1993). Class I comprises the lantibiotics which contain the unusual amino acids lanthionine and β -methyllanthionine (Schnell *et al.*, 1988). The lantibiotic nisin produced by *Lactococcus lactis* subsp. *lactis* is used in many countries for the preservation of milk products and other foods. Class II bacteriocins comprise small, heat-stable proteins which contain no modified amino acids. Examples of class II bacteriocins are sakacins A, P and 674, which are produced by different meat strains of *Lb. sake* (Holck *et al.*, 1992, 1994; Tichaczek *et al.*, 1992), pediocin PA-1 from *Pediococcus*

Abbreviations: HPK, histidine protein kinase; LAB, lactic acid bacteria; RR, response regulator protein.

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acidilactici (Henderson *et al.*, 1992), leucocin A-UAL 187 from *Leuconostoc gelidum* (Hastings *et al.*, 1991), mesentericin Y105 from *Leuconostoc mesenteroides* Y105 (Fremaux *et al.*, 1995), carnobacteriocins B2 and BM1 from *Carnobacterium piscicola* LV17B (Quadri *et al.*, 1994), lactococcin A from *Lc. lactis* (Holo *et al.*, 1991), and plantaricin A from *Lactobacillus plantarum* (Nissen-Meyer *et al.*, 1993). Class I and class II bacteriocins are synthesized as precursor proteins. Their N-terminal leader sequence is cleaved before the mature product is released from the cell. It has emerged that sakacins P and 674 are identical (see below) and are closely related to pediocin PA-1 with regard to the mature peptides (67% amino acid identity), whereas the relation to sakacin A is less pronounced (33% amino acid identity). Sakacin P, together with pediocin PA-1, sakacin A and other bacteriocins, contains the N-terminal sequence motif YGNGV and is therefore considered to belong to a sub-family of class II bacteriocins (Klaenhammer, 1993).

Besides the bacteriocin structural genes, a number of additional genetic determinants are involved in bacteriocin production. These genes are often located in the vicinity of the bacteriocin genes and encode proteins involved in the regulation of bacteriocin production, in processing of the prepeptide, in secretion of the mature bacteriocin and in host immunity. Small genes are frequently found immediately downstream of the bacteriocin structural gene and these have in some cases been shown to encode the immunity factor (Axelsson & Holck, 1995; Quadri *et al.*, 1995; van Belkum *et al.*, 1991; van Belkum & Stiles, 1995; Venema *et al.*, 1995). In the plantaricin A system of *Lb. plantarum* C11, the reading frames *plnB*, *plnC* and *plnD* encode proteins that are homologous to members of the bacterial two-component regulatory systems (Diep *et al.*, 1994). In the vicinity of the pediocin PA-1 and lactococcin A structural genes, ORFs were identified whose predicted proteins belong to the HlyB family of ATP-dependent membrane translocators (Marugg *et al.*, 1992; Stoddard *et al.*, 1992). In the vicinity of the sakacin A structural gene, the genes of both an AgrB/AgrA-like bacterial two-component signal transducing system and of a HlyB/HlyD-like transport system were identified (Axelsson & Holck, 1995).

Recently, we reported the purification of sakacin 674 from *Lb. sake* Lb674, and the cloning and sequencing of its chromosomally located structural gene *sakR* (Holck *et al.*, 1994). Tichaczek *et al.* (1994) reported the nucleotide sequence of the sakacin P structural gene from *Lb. sake* LTH673 which is identical to sakacin 674. The strains LTH673 and Lb674 were isolated from different sources and can be distinguished from each other by their plasmid content. To avoid further confusion, the bacteriocin produced by *Lb. sake* Lb674 is henceforth referred to as 'sakacin P'. Also, to avoid confusion with the genes involved in sakacin A production (*sap* genes) by *Lb. sake* Lb706 (Axelsson & Holck, 1995), the genes associated with sakacin P production are named using the prefix '*spp*'.

Most of the bacteriocins described to date are derived

from lactobacilli that are not typically found in meat. Strains of *Lb. sake* and *Lactobacillus curvatus* are important starter cultures in meat fermentations. Because they are very competitive at lower ripening temperatures they are frequently used for the production of traditional raw fermented sausages. Lactobacilli used for raw sausage production normally do not produce bacteriocin, although such a trait could improve the microbiological safety and stability of the meat products. We are therefore interested in finding an expression system for sakacin P, which would allow improvement of established meat starter cultures, especially with respect to their inhibitory potential against *Li. monocytogenes*. In this study, we defined a DNA fragment containing all information necessary for expression of sakacin P in sakacin-negative *Lb. sake* host strains. The nucleotide sequence of this fragment was determined and mutation/deletion analyses were performed to investigate the role of the individual genes in bacteriocin production.

METHODS

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids used in this study are shown in Table 1. *Lb. sake* Lb790 is a bacteriocin-negative and sakacin-P sensitive strain. *Lb. sake* Lb706X is a plasmid-free derivative of the sakacin A producer Lb706. Since the sakacin A gene cluster is located on one of the plasmids, Lb706X has lost both production of and immunity to sakacin A. The *Lactobacillus* strains used in this study were isolated from different meat sources and are not related to each other in any way other than being identified as *Lb. sake*. The *Lactobacillus* strains were cultured in MRS broth (Merck), routinely at 25 °C, but at 30 °C when used in electrotransformation experiments. *Listeria innocua* Li1 was grown in Standard-I medium (Merck) at 37 °C. *Escherichia coli* strains were propagated in LB broth (Sambrook *et al.*, 1989) at 37 °C. Solid media were prepared by adding 1.2% (w/v) agar to the broth. *E. coli* and *Lactobacillus* transformants were selected and cultured in the presence of antibiotics at the following concentrations: ampicillin, 100 µg ml⁻¹; erythromycin, 10 µg ml⁻¹ for lactic acid bacteria and 200 µg ml⁻¹ for *E. coli*.

Bacteriocin activity and immunity. Bacteriocin production and immunity were determined by using a well diffusion assay as described by Schillinger & Lücke (1989). Controls were performed with culture supernatants from bacteriocin-producing (Bac⁺) and non-producing (Bac⁻) strains, and with trypsin-digested Bac⁺ supernatants. *Li. innocua* Li1 was used as an indicator strain to represent the antagonistic activity of sakacin P against *Li. monocytogenes*. To avoid inhibition of the indicator strain by erythromycin, the antibiotic was removed from the bacteriocin-containing culture supernatants by pelleting the protein in the presence of 40% (w/v) ammonium sulfate, resuspending the pellet in H₂O and passing it through a Sephadex PD-10 column (Pharmacia). The antagonistic products in the culture supernatants of *Lb. sake* transformants were tested for heat resistance, sensitivity to trypsin and inhibitory spectra to confirm their identity with sakacin P. The immunity of *Lb. sake* transformants was tested against high purity sakacin P, which was obtained from *Lb. sake* Lb674 as described previously (Holck *et al.*, 1994).

Molecular cloning. Plasmid DNA from *E. coli* cells was isolated by the alkaline lysis method according to Sambrook *et al.* (1989) and from lactobacilli as described previously (Axelsson *et al.*,

Table 1. Bacterial strains and plasmids

Strain or plasmid	Description*	Source or reference
Strains		
<i>Lactobacillus sake</i>		
Lb674	Wild-type strain; Spp ⁺ , Imm ⁺	Holck <i>et al.</i> (1994)
Lb790	Host strain for pLPV111 and derivatives; sensitive to sakacin P, Spp ⁻ , Imm ⁻	Schillinger & Lücke (1989)
Lb706X	Host strain for pLPV111 and derivatives; sensitive to sakacin P, Spp ⁻ , Imm ⁻	Axelsson <i>et al.</i> (1993)
Lb790/111	Lb790 carrying pLPV111, indicator strain; sensitive to sakacin P, Spp ⁻ , Imm ⁻ , Em ^r	This study
<i>Listeria innocua</i> Li1	Indicator strain; sensitive to sakacin P, Spp ⁻ , Imm ⁻	This study
<i>Escherichia coli</i>		
DH5 α	Host strain for pBluescript KS ⁺ and derivatives; F ⁻ ϕ 80d <i>lacZ</i> Δ M15 <i>endA1 recA1 hsdR17 supE44 thrI-1 λ^- gyrA96 relA1 Δ(<i>lacZYA-argF</i>)U169</i>	Gibco BRL
JM105	Host strain for pLPV111 and derivatives; <i>thi rpsL endA sbcB15 hsdR4 Δ(<i>lac-proAB</i>)/F'(<i>traD36 proAB lacI^q lacZ</i>ΔM15]</i>	Pharmacia
Plasmids		
pGEM-7Zf(+)	3.0 kb cloning vector; replicates in <i>E. coli</i> , Ap ^r , <i>lacZ</i>	Promega
pBluescript KS ⁺	3.0 kb cloning vector; replicates in <i>E. coli</i> , Ap ^r , <i>lacZ</i>	Stratagene
pLPV111	4.2 kb <i>E. coli</i> / <i>Lb. plantarum</i> / <i>Lb. sake</i> shuttle vector; Em ^r , <i>lacZ</i>	Axelsson & Holck (1995)
pGEM-7Zf(+) derivative (Ap ^r)		
pBLS1	3.9 kb <i>ClaI</i> / <i>EcoRI</i> fragment from <i>Lb. sake</i> Lb674 DNA; <i>sppR⁺ A⁺ T⁺ spiA⁺</i>	Holck <i>et al.</i> (1994)
pBluescript KS ⁺ derivatives (Ap ^r)		
pBLS2	1.3 kb <i>HpaII</i> fragment from <i>Lb. sake</i> Lb674 DNA; part of <i>sppK</i> gene	This study
pBLS3	3.7 kb <i>PvuII</i> fragment from <i>Lb. sake</i> Lb674 DNA; part of <i>sppT</i> gene, <i>sppE⁺</i>	
pLPV111 derivatives (Em ^r)		
pMLS114	7.6 kb insert, combined inserts of pBLS1, pBLS2 and pBLS3; <i>sppK⁺ R⁺ A⁺ T⁺ E⁺ spiA⁺</i>	
MLS114-K	pMLS114 derivative with a frameshift mutation in <i>sppK</i> (<i>ClaI</i> site); <i>sppR⁺ A⁺ T⁺ E⁺ spiA⁺ sppK110</i>	
pMLS114-R	pMLS114 derivative with a 0.56 kb <i>NruI</i> / <i>HpaI</i> deletion in <i>sppR</i> ; <i>sppK⁺ R⁺ A⁺ T⁺ E⁺ spiA⁺ ΔsppR</i>	
pMLS114-I	pMLS114 derivative with a frameshift mutation in <i>spiA</i> (<i>BglII</i> site); <i>sppK⁺ R⁺ A⁺ T⁺ E⁺ spiA27</i>	
pMLS114-T	pMLS114 derivative with a 0.99 kb <i>SnaBI</i> / <i>EcoRV</i> deletion; <i>sppK⁺ R⁺ A⁺ E⁺ spiA⁺ ΔsppT</i>	
pMLS114-E	pMLS114 derivative with a 1.07 kb <i>ScaI</i> / <i>XhoI</i> deletion; <i>sppK⁺ R⁺ A⁺ T⁺ spiA⁺ ΔsppE</i>	
pMLS-IP	0.56 kb PCR fragment from pMLS114; <i>ermLp spiA⁺ ΔsppKRATE</i>	

* Spp, sakacin P production; Imm, immunity to sakacin P; Ap^r, ampicillin resistance; Em^r, erythromycin resistance; *ermLp*, gene under control of the *ermL* promoter.

1993). Chromosomal DNA of *Lb. sake* Lb674 was isolated by the following method. The cells of a 150 ml culture (OD₆₀₀ 1.0) were harvested and lysed in 15 ml lysozyme solution (10 mg lysozyme ml⁻¹ in 10 mM Tris/HCl, 0.3 M sucrose, pH 8.0) at 37 °C for 1 h. The cells were collected by centrifugation and resuspended in 10 ml NAE buffer (0.15 M NaCl, 0.1 M EDTA, pH 8.0). Two millilitres of a proteinase K (Boehringer) solution [1 mg (ml NAE)⁻¹] and 1 ml 20% (w/v) sodium dodecyl sulfate

were added and the suspension was incubated for 3–4 h at 37 °C, followed by an additional incubation step for 20 min at 60 °C. The DNA was extracted with phenol/chloroform/isoamyl alcohol (24:24:1), treated with RNase and re-extracted before precipitation with ethanol.

The basic cloning techniques of Sambrook *et al.* (1989) were used. Restriction enzymes, T4 DNA ligase and calf intestinal

alkaline phosphatase (from Amersham, Pharmacia and Boehringer, respectively) were used according to the suppliers' recommendations. Amplification (PCR) reactions were performed as described by Axelsson & Holck (1995). DNA fragments were isolated from agarose gels using the USBio-Clean-MP kit (Amersham). Deletions/inactivations of genes were achieved by the use of available restriction sites. Frameshift mutations were introduced by linearizing plasmids at appropriate restriction sites; the ends were made blunt with a fill-in reaction using deoxynucleotide triphosphates and the Klenow fragment before ligation, recircularizing the plasmids and subsequently introducing them into the recipient strains. The desired mutations were confirmed by the absence of the restriction site used and by determining the nucleotide sequence. All manipulations were done in *E. coli* and the desired constructions were then transferred to the *Lb. sake* hosts. Transformation of *Lb. sake* was done by electroporation according to the protocol of Aukrust & Blom (1992).

Plasmid constructions. Derivatives of the plasmid pMLS114, carrying the *spp* gene cluster were made as follows. A frameshift mutation was introduced in the *ClaI* site of *sppK* using an intermediate pLPV111 construct containing the joint pBLS2 and pBLS1 fragments. The single *ClaI* site was made blunt and the plasmid religated. Then the purified *NdeI*-*XhoI* fragment derived from pMLS114 was added to form pMLS114-K. A variant of pMLS114 with a deletion in *sppR* (positions 1983–2540), was obtained by ligating the purified 0.63 kb *HpaI*-*BglII* fragment (positions 2540–3169) to the *NruI*/*BglII*-digested pMLS114. To introduce a frameshift mutation in *spiA*, the single *BglII* site in pMLS114 was made blunt and the plasmid religated. This resulted in pMLS114-I. A deletion mutation in *sppT* was achieved by cutting pMLS114 with *SnaBI* and *EcoRV* and religating the resulting 10.8 kb plasmid to the new construct pMLS114-T. A deletion mutation in *sppE* was created by digesting pMLS114 first with *XhoI*; the *XhoI* ends were made blunt and the linearized plasmid was subsequently cut with *NdeI*. A purified 2.1 kb *NdeI*-*ScaI* fragment from pMLS114 was then ligated to the 8.6 kb *NdeI*-*XhoI* fragment resulting in a pMLS114 derivative, pMLS114-E, with a deletion of the 3'-end of *sppE*. The construct pMLS-IP was created using a *spiA* fragment which was amplified from pMLS114 by PCR (positions 3066–3629); the *ermL* promoter, derived from the *Lactobacillus reuteri* erythromycin resistance gene (Axelsson *et al.*, 1988), was cloned on a 0.4 kb fragment upstream of the *spiA* gene and the combined fragments were introduced into pLPV111. The *ermL* promoter is known to be utilized, since this gene is present in pLPV111 conferring erythromycin resistance to *Lb. sake*.

Southern and Northern analysis. Chromosomal DNA of *Lb. sake* Lb674 was digested with several restriction enzymes. The resulting fragments were separated on a 1% (w/v) agarose gel and blotted onto a nylon membrane (Boehringer). Synthetic oligonucleotides used as hybridization probes were labelled with digoxigenin-dUTP from a DIG Oligonucleotide 3'-End Labeling kit (Boehringer). DNA fragments were labelled by random primed incorporation of digoxigenin-labelled dUTP from a DIG DNA Labeling and Detection kit (Boehringer) and hybridization was performed as recommended by the supplier. No restriction digests were performed in the case of hybridization to *Lb. sake* Lb706X and Lb790 DNA. For the plasmid-free strain Lb706X, total DNA was used; for Lb790, the DNA preparation was enriched for either chromosomal DNA (by 'spooling', i.e. winding DNA threads onto the tip of a Pasteur pipette) or plasmid DNA (by a normal plasmid mini-preparation). Total RNA was isolated from *Lb. sake* Lb674 and Lb790 according to the procedure described by van der Vossen

et al. (1987). The RNA (10 µg per lane) was separated on an 1% agarose gel in the presence of 5.5% (v/v) formaldehyde and blotted onto a nylon membrane. Synthetic oligonucleotides and random-labelled DNA fragments were used as hybridization probes. When synthetic oligonucleotides were used, hybridizations were performed at 39 °C and the last washing step at 52 °C.

DNA sequencing. DNA was sequenced according to the dideoxy chain-termination method of Sanger *et al.* (1977). The nucleotide sequence of the double-stranded DNA was determined bidirectionally using the Sequenase version 2.0 DNA Sequencing kit (Amersham) in combination with [³⁵S]dATPαS (1.5 × 10⁸ Ci mmol⁻¹; 5.55 × 10¹³ Bq mmol⁻¹; DuPont) and appropriate synthetic oligonucleotide primers. PCR fragments amplified from *Lb. sake* Lb706X and Lb790 chromosomes were sequenced using the Sequenase PCR Product Sequencing kit (Amersham). For databank searches, the EMBL and SwissPIR databases were used. Sequence analysis and alignments were performed with the HUSAR program package (release 3.0, EMBL).

RESULTS

Cloning of the sakacin P gene cluster

As reported previously, the sakacin P (sakacin 674) structural gene, *sppA*, was located on a 3.9 kb *ClaI*-*EcoRI* chromosomal DNA fragment of *Lb. sake* Lb674 (Holck *et al.*, 1994). To find out whether, in addition to *sppA*, other ORFs possibly involved in bacteriocin production were present on this fragment, its remaining nucleotide sequence was determined. Computer analysis revealed four additional ORFs which were transcribed in the same direction as *sppA* (pBLS1 in Fig. 1). Two of them, *sppK* and *sppR*, were located upstream, and two, *spiA* and *sppT*, were located downstream of *sppA*. Database searches revealed that the proteins encoded by these ORFs showed homology to proteins involved in the production of other LAB bacteriocins (see below).

Since the ORFs *sppK* and *sppT* were not completely contained on the 3.9 kb *ClaI*-*EcoRI* insert, Southern hybridization experiments were performed with *Lb. sake* Lb674 DNA. For cloning of the remaining part of *sppK* a synthetic oligonucleotide FR2 (5'-CTGTTGCACTATT GTTC-3') complementary to part of the *sppK* sequence was used as a probe. The probe identified a 1.3 kb *HpaII* fragment which was ligated into the *ClaI* site of pBlue-script KS⁺, resulting in pBLS2 (Fig. 1). A 3.7 kb *PvuII* fragment hybridizing to the labelled 0.9 kb *SpeI*-*EcoRI* fragment of the pBLS1 insert (Fig. 1) was isolated and cloned into the *EcoRV* site of pBlue-script KS⁺. The resulting plasmid was termed pBLS3 (Fig. 1). Positive *E. coli* transformants containing the plasmids pBLS2 and pBLS3 were identified by colony hybridization.

DNA sequence analysis

The DNA sequence of the inserts from pBLS1, pBLS2 and pBLS3 was determined. The combined nucleotide sequence comprises 7597 bp and encompasses the six ORFs *sppK*, *sppR*, *sppA*, *spiA*, *sppT* and *sppE*, the small

Fig. 1. Schematic overview of the subclones derived from the sakacin P gene cluster. The 7.6 kb insert in pMLS114 was combined from the inserts of pBLS1, pBLS2 and pBLS3. The location and orientation of the ORFs in pMLS114 are indicated by arrows. Deletion (Δ) and frameshift mutation (*) derivatives of pMLS114 are shown below. The arrowhead with the designation *ermLp* indicates the direction of the *ermL* promoter present in this construction. The thin line in pMLS-IP represents the *ermL* promoter sequence (Axelsson *et al.*, 1988). Spp and Imm denote sakacin P production and immunity to sakacin P, respectively, of *Lb. sake* Lb706X carrying the pMLS114 derivatives. Restriction sites: B, *Bgl*II; C, *Cl*aI; E, *Eco*RI; EV, *Eco*RV; H, *Hind*III; Ha, *Hpa*I; Hp, *Hpa*II; K, *Kpn*I; N, *Nru*I; Nd, *Nde*I; P, *Pvu*II; S, *Spe*I; Sc, *Sc*aI; Sn, *Sna*BI; X, *Xho*I. On the insert of pMLS114 not all of the *Hpa*I and *Hpa*II restriction sites are indicated. The dotted line represents part of the vector's multiple cloning site.

ORF1 upstream of *sppK* (Fig. 2), and an incomplete reading frame downstream of *sppE* which we refer to as ORF2 (not shown in Fig. 2). Potential ribosome binding sites (RBS) were identified upstream of all ORFs. ORF1 potentially encodes a protein of 36 aa. The *sppK* gene encodes a putative protein of 448 aa. Immediately downstream of *sppK*, *sppR* starts with the less frequently used leucine initiation codon (TTG) and could encode a protein of 248 aa. Downstream of *sppR*, a sequence element is located representing a possible *rho*-independent transcription termination signal with a calculated ΔG of -18.4 kcal mol^{-1} (-77.0 kJ mol^{-1}) (Tinoco *et al.*, 1973). The

structural gene of sakacin P, *sppA*, starts 147 bp downstream of *sppR*. It is followed by *spiA* encoding a potential protein of 98 aa. Downstream of *spiA* a possible *rho*-independent transcription terminator with a calculated ΔG of -19.4 kcal mol^{-1} (-81.2 kJ mol^{-1}) was found. The *sppT* gene starts 350 bp downstream of *spiA* and could encode a protein of 718 aa. The reading frame *sppE* is located immediately downstream of *sppT* and predicts a protein of 458 aa. No obvious transcription termination signal was detected in the region downstream of *sppE*. Potential promoter sequences are located upstream of ORF1, *sppA*, *sppT* and ORF2 (Fig. 2). Thus, three major

1	COGGCTCTTTGACCATAAGCACCTAGGTCGATTAAATATGTTCTTCAGATTTATTGAGA	2221	K I A P L D Y I L K D Q T M A E I K R Q GAAAAATAGCGCTTTAGATTACATTTCTCAAGGACCAAACTATGCGTGAATCAAAAGGC
61	TGAATTTTACCAAGGCCAACACTATAAATAATTTGCGAGGCGGTGACTAACACTTCA		L I D D L L L A E K Q N E A A A Y H R E
121	GGATTCTGGCAGTTTCAAGTGTGAGATAGACCTGACTCAAAACATCGGTAATGTCGAGA	2281	AATTGATTGATGATCTATTGTTAGCTGAGAAGCAAAAGAGGCGCAGCGATACCGAG
181	AGCGCGTTTGATTTATCTGAGTAGGCCATTAGGCTGTTGTAAGGTGTATGAATGTTTCC		N L F S Y K I G P R F F S L P L K E V V
241	TTAGCAATTTCTTGATTAGTCATAATGAAAGCCTCCACAAAAGTATTATCAACATCTG	2341	AAAATTTATTAGTTATAAATAGGTCCTCGCTTTTCTCAATTACCAATTAAGGAAGTTG
301	TTCTAGTCTCTTAGTAAATTTGTCACTTCAATAGAGTCTTAAACGTTAATCGGAAAA	2401	Y L Y T E K E N P G H I N L L A V T R K TTTATTATATCTGAAAAAGAAAAATCGGGTCATATTATTGTTAGCGGTACCAAGAA
361	AAACTAAGCTTAATATTAATAAATAGATTCGCTTGTGAATTATGTATAATTGATTAGA	2461	V T F P G N L N A L E A Q Y P M L F R C AGGTTACTTTTCCAGGAAATTTAAATGCGCTGGAAGCCCAATATCCAATGCTCTTTCGGT
421	CTAAGAATAGGAGAAAGTATGATGATTTAAAAAATTTTCAAAAAAGAAATGCAAAA	2521	D K S Y L V N L S N I A N Y D S K T R S GTGATAAAGGTACTTAGTTAACCTATCTAATATTGCGCAATTATGACAGTAAACACGGA
481	INGGMAGNSSSNFIHKIKQIF AATAAACGGTGTATGGCAGGAATAGTCTCAATTTTATTCATAGATTAAACAAATTTT	2581	L K F V D G S E A K V S F R K S R E L V GTTTAAATTTGTAGATGCGAGTGAAGCAAAAGTCTCGTCCGGAATCAGGGGAACCTAG
541	THR * M L Y T D V S V S L TACCCATCGTTAAGATAGGTTTGGTGAGCATGTTATATACGGATGATCGGTTCCCT	2641	A K L K Q M M * TGGCAAAATTAACAAATGATGTAGCGCATATTAAACGTTAAACGATAAAGTTGAACGT
601	M Q N F V A I L L I F L L Y R Y I Q R K AATGCAAAATTTGTGCTATCTTATTTTCTTATATATAGATATATTCAAGAAA	2701	TAATATTTTITTTTGGCGAGAAATGGTAAATGAAGCATAGTCTTGAAGTATTATAG
661	I T F K R I I L D I L I A I I F S I L Y GATAACATTTAAACGGATCATATTAGATATTTTAAATAGCGATTATTTTCAATATTATA	2761	CTGGCTGGCGTAAGATATGCTTTATAAAATAATATATAGGATATGATCTAATGGAAAA
721	L F I S D A S L L L V M V L M R L G W H F TCGTATTATTCAGATGCGCTACTTGTATGTAATGTAATTAATGCGATTAGGTTGCAATT	2821	F I E L S L K E V T A I T G G K Y Y G N GTTTATTGAATTCCTTTAAAGAAAGTAACAGCAATTGACAGGTGGAAATATTATGGTAA
781	H Q Q K E N K I K T T D T A N L I L I I TCATCAACAAAGAAATTAAGATAAAGACGATGATCAGCTAATTTAATCTAATATTAT	2881	G V H C G K H S C T V D W G T A I G N I CGGTGTACACTGTGGAAAACTTCATGTACCGTAGACTGGGGAACGACTATTGGAATAT
841	V I Q L L L L V A V G T I I S Q P T I S I CGTGATCCAGTTATTTAGTTTGGGTTGGGACTATTATTAGTCAGTTTACCATATCGAT	2941	G N N A A A N W A T G G N A G W N K * CGGAAATATGCGAGCTGCAACTGGGCGCAGCGGAAACGCTGCGTGAATAAATAA
901	I K S D F S Q I L N N S A T D I T L L L TATCAAAAGTATTTTTCAGCCAAATATATTGAACAATAGTGCACAGATATAACTTTATT	3001	AGCAAACTTAATTTAGATTTAAGGACTCTCTATCATTTTGTATGGGATTTTTTAGCTTAT
961	G I F F A V L F D G L F F I L L K N K R AGGTATTTTCTTGGCTGTTTATTGACGGCTTGTCTTTATATATTATGAAGATAAAGCG	3061	M K I L K W Y S G G AGTAACAGAATAAAATAGAGCTTTCAGATTATGAAAAATATTGAAGTGTATTTCAGGTG
1021	T E L Q H L N Q E I I E F S L E K Q Y P GACTGAATTCACCAATTTAAATCAAGAAATCATTTGAATTTTGGTTAGAAAAAATATTT	3121	K D R G E R A N D I I G Q L L L D L N H TAAGGATCGCGGAGAACGGGCAATGATATTATAGGCCAATTTGCTGATAGCTGAACCA
1081	I F I F I L F I V I E I I L A V G N L Q TATATTATATTATTTTATTTATAGTAATAGAAATTTTATAGCAGTTTGGGAATCTTCA	3181	D P K N E H L E A I L I N Y Q N E I K R TGATCCAAAAATGAACATTTAGAAGCAATTAATAAATATTACAGTAAGAAATTAAGAG
1141	G V T A T I L L T I I I F C V L I G M AGGAGTAACAGCACAGATATTATTAACCATTTATCATTTTGTGCTCTTATCGGGAT	3241	K E S S V P F I L S R M N I S I A N T I GAAAGAAAGTTCGGTGCCATTTATTTAGTGGGATGAATATATCAATAGCCAATACAT
1201	T F W Q V M L F L K A Y S I R Q E A N D GACTTTTGGCAAGTATGCTTTTGTGAAGCTTATTCGATTTCGCCAAGAACCAATGA	3301	R R D R L I L T D F Q E D K L K L L T A CAGAAGAGATAGGCTCATTTTAAACCGATTTTCAAGAGATAAATGAAATTTGTTAACTGC
1261	Q L V R N Q Q L Q D Y L V N I E Q Q Y T CCAATTGGCTCCGAATCAAACTTCAAGATTATCTAGTCAATATCGAACAGCAGTACAC	3361	L S N I R Y G Y * GTTGCTAATATAAGATATGGCTATTAGCAGCTTAATTCGTAGCACACTATTCTAAGAA
1321	E L R R F K H D Y Q N I L L S L E S F A CGAATTACGGGATTTAAGCATGATTATCAAAACATCTTATATCGTTGGAGAGTTTTCG	3421	TACGCTAGAACGTATTATAGGAATTTTCAATATTAAATTTGGTTGATAGTGAACCTGATGA
1381	E K G D Q Q Q F K A Y Y Q E L L A Q R P CGAAAGGGGCAATCAAACTTAAAGCGTATTACCAAGAAATTATTAGCAACCAAGCGGC	3481	GATATTCTATTATTAATAGTGTATATAGTACGATCGTTATGCTGGTTATGAATACAT
1441	I Q S E I Q G A V I A Q L D Y L K N D P AATTCAAAAGTGAATCCAAAGGGCAGTCAITGCACAACTGCACTAGTGAATAATGATCC	3541	TGTAATGAAAGATTAAAGCTTTAATCTATACAAATTATACGTTAGTCTTTTATTATTATA
1501	I R G L V I O K F L A A K Q A G V T L K TATTCGAGGATTAGTCATTCAAAAGTTTGGCAGCCAAACAGGCTGGTGTACTTTAAA	3601	GGAATAGCCATAATATAACAGCTTAGATTTTAAATGCGAGCATTAAAGCTTAATTTGTATA
1561	P E M T E P I E L A T A N L L T V I R I ATTGAAATGACCGAACCAATGGAATTAGCAACCGCTAATCTATTAAACGTTATTTCGAT	3661	ACGTAAAGCTTAATGATATATCATCTGTTTCAAAATAGTGTATGACATAAATTAAGTAAT
1621	I G I L L D N A I E Q A V Q E T D Q L V TATCGGTATTTATTAGCAATGCGATTGAACAAGCGGTTCAAGAAACGATCAATGTGT	3721	M I D R K I Y I P Q V D E M AACTTACCGAGCTCTTATGATGATAGATGTAATTTATATACCAAGTAGATGAATG
1681	S C A F L Q S D G L I E I T I E N T A S GAGTTGTGCTTTCTTACAATCTGATGGTTTAAATCGAAATTACGATTGAAAAATACGGCCAG	3781	D C G V A A L A M I L K H Y G S S A S L GATTGTGCGTAGCAGCTTTAGCGATGATCTTAAACATTATGTTGTTCTTCACTGCTGCTG
1741	Q V K N L Q A F S E L G Y S T K G A G R TCAAGTTAAGATCTCCAGCATTTTTCAGAGTTAGGCTATTCAACGAAAGGCGCTGTCG	3841	A Y L R N E A K T D L L E A G T A C C A C G G C G T T G L V GCTTATTATCGTAATGAGGCTAAAACTGATTAGAGGGAACCAACGGCGCTTGGCTAGTG
1801	G G G A C T G G T T A G C T A A T G C A G G A T T G A T T G C C A A C A A C C A A T T T A T C T T A G A	3901	K T A E N L G F E T K A I Q A D M S L F AAGACAGCTGAGATTTAGGTTTTCGAAACGAAAGCGGATTCAGCTGATATGAGTTTGT
1861	T Q I E N R K L R Q T L M I T E E T * L AACACAGATTGAAAAAGTATGACAGACATTGATGATTACGAGGAACTTAAT	3961	E V A D L P F P F F I A H V L K N G E L L GAAGTCGAGATTACCATTTTCTTTTATGTCACATGTTTCTAATAAATGCGCGCTATTA
1921	Y P V Y L L E D D L Q Q Q A I Y Q Q I I TGATCCCGTTTATTATTAGAGGATTTTACAGCAACAGCGATTATTCAGCAAAATTA	4021	H Y Y V V L G I K K D A I I I A D P D P CATTTATTATGTTGTATAGGATAAAAAAGATGCGCAATTAATATGCGCGATCTGATCCA
1981	A N T I M I N E F A M T L T C A A S D T TCGGAATACGATTATGATTAAAGAAATTTGCAATGACTTTAAATCGCGCTCCAGTGATA	4081	T V G I A K M T Q S Q F K S E W T G V A ACAGTGGGCTGCTTAAGATGACGCAATCAAGCTTTAAATCGGAATTAATTAATTAATTA
2041	E T L L A A I K D Q Q R G L F F L D M E CTGAGACATTGTTGGCGCAATTAAGGATCAGCAACAGAGGTTTATCTTTTGGATATGG	4141	L F I A P K P S Y Q P V K Q Q T K A S L TTATTATTGCTCCAAACCGAGCTATCAACAGTTAAGCAGCAACAAAGCGAGTTTA
2101	I E D N R Q A G L E V A T K I R Q M M P AAATTGAGGATAACCGCAAGCGGTTTAGAAGTGGCACTAAGATTCCGCGAGATGATGC	4201	F S F V P T L M Q Q K R L V I N I T L A TTTTATTGTTCCAACTTGTAGTCAGCAAAAGCGATTGGTAATTAATTAATTAATTA
2161	F A Q I V F I T T H E E L T L L T L E R CGTTTGGCGAAATGTTCTTATTACCAACCAAGAGGAAGTGCATTTAATCGTTAGAAC	4261	A L L I T I I S I L G S Y F L Q T V I D CGGCTACTATAACCAATTATTAGTATCTTAGGATCTTTTCTTACAGACTGCTCATGAT
		4321	T Y I P N N M Q S T L A I V A I G L I I ACCTATATCCCAATAACATGACAGCAAGCTGGCGGATTTGTCATTTGTTTAAATTATC

Fig. 2. For legend see opposite.

Fig. 2. Nucleotide sequence of the sakacin P gene cluster and the predicted amino acid sequences of the ORFs ORF1, *sppK*, *sppR*, *sppA*, *spiA*, *sppT* and *sppE*. Putative –35 and –10 promoter regions and possible ribosome-binding sites (RBS) are underlined. The divergent arrows above the DNA sequence indicate putative transcription terminators. Translation termination codons are marked by asterisks. The transcription start of the *sppA* gene determined by Tichaczek *et al.* (1994) is underlined and marked with (+1). The vertical arrow marks the processing site of SppA. The parts of this sequence previously published by Holck *et al.* (1994) and Tichaczek *et al.* (1994) comprise positions 2792–3131 and 2384–3737, respectively.

transcripts are predicted from the sequence, the first encompassing ORF1/*sppK*/*sppR*, the second *sppA*/*spiA*, and the third, at least *sppT* and *sppE*.

Homologies of the *sppK* and *sppR* gene products

The putative proteins encoded by *sppK* and *sppR* are homologous to several proteins of two-component signal transducing systems (Parkinson & Kofoed, 1992; Stock *et al.*, 1989). These systems in general consist of a histidine protein kinase (HPK) and a response regulator protein (RR). SppK shows strongest similarity to PlnB (59%), the suggested HPK of the plantaricin A operon from *Lb. plantarum* C11 (Diep *et al.*, 1994). SppR shows strong similarity to PlnD (68%) and PlnC (65%), the proposed RR proteins of the plantaricin A operon. SppK and SppR are also homologous to the proposed two-component regulatory system of the sakacin A gene cluster, SapK (54% similarity) and SapR (50% similarity) (Axelsson & Holck, 1995). SppK/SppR also revealed similarity to the

AgrB/AgrA system from *Staphylococcus aureus* which is involved in the post-exponential expression of proteins (AgrB 54 %, AgrA 50 %) (Kornblum *et al.*, 1990; Peng *et al.*, 1988). SppK and SppR displayed all the general features of HPKs and RRs as described by Stock *et al.* (1989), such as conserved amino acid residues, transmembrane regions, etc. (not shown).

Homologies of the *sppA* and *spiA* gene products

Homology of sakacin P to other class II bacteriocins has been discussed elsewhere (Holck *et al.*, 1994; Tichaczek *et al.*, 1994). The reading frame *spiA* is identical to the proposed ORFY protein from the sakacin P site of *Lb. sake* LTH673, a strain which was isolated and investigated by Tichaczek *et al.* (1994). The DNA sequence of the sakacin P site published by Tichaczek *et al.* (1994) comprises positions 2384–3737 of our sequence. Except for an additional T residue in position 2426 and a missing T residue in position 2471 it is identical to the sequence shown in Fig. 2. In addition, the putative *spiA* product shows 61 % similarity to the predicted protein encoded by ORF- β 3 of the carnobacteriocin B2 site from *C. piscicola* LV17B (Quadri *et al.*, 1994).

Homologies of the *sppT* and *sppE* gene products

The deduced SppT protein is homologous to proteins of the HlyB family of ATP-dependent translocators, which are involved in the signal-sequence independent transport of proteins across the bacterial membrane. Homologies exist to SapT of *Lb. sake* Lb706 (79 % identity), to ComA of *Streptococcus pneumoniae* (75 %), to LcaC of *Leuconostoc gelidium* UAL187 (58 %), to MesD of *Leuconostoc mesenteroides* Y105 (58 %), to LcnC of *Lc. lactis* subsp. *lactis* biovar. *diacetylactis* WM4 (55 %), and to PedD (54 %) of *P. acidilactici* PAC-1.0 (Axelsson & Holck, 1995; Fremaux *et al.*, 1995; Hui & Morrison, 1991; Marugg *et al.*, 1992; Stoddard *et al.*, 1992; van Belkum & Stiles, 1995). These proteins are the predicted translocators of sakacin A, leucocin A, mesentericin Y105, lactococcin A and pediocin PA-1, respectively. In *S. pneumoniae*, ComA was proposed to be responsible for the secretion of a competence factor which coordinates the induction of genetic competence among the cells. Significant homology of SppT was also observed to HlyB (27 % identity), a protein which is essential for haemolysin A secretion in *E. coli* (Felmlee *et al.*, 1985). With regard to other members of this family of transporters, SppT was predicted to contain several membrane-spanning domains within the N-terminal half and about 200 conserved aa residues within the C-terminal part (not shown). The former was also homologous to the suggested proteolytic domain of bacteriocin ABC (ATP-binding cassette) exporters responsible for cleavage of the bacteriocin leader peptide (Håvarstein *et al.*, 1995). The latter contained the conserved ATP-binding site, invariably found in the superfamily of ABC exporters (Fath & Kolter, 1993).

The *sppE* product is homologous to SapE of the sakacin A gene cluster (57 % identity), to LcaD of the leucocin A

site (33 %), to MesE of the mesentericin Y105 locus (32 %), to ComB (30 %), to LcnD (29 %), and to HlyD (25 %). These proteins are viewed as accessory proteins for the ABC transporters (Axelsson & Holck, 1995; Blight & Holland, 1990; Fath & Kolter, 1993; Felmlee *et al.*, 1985; Fremaux *et al.*, 1995; Hui *et al.*, 1995; Mackman *et al.*, 1986; van Belkum & Stiles, 1995). A common feature of SppE and other HlyD analogues is that they are largely hydrophilic except for an approximately 20 aa hydrophobic region close to the N-terminus (not shown).

Northern analysis

Total RNA of *Lb. sake* Lb674 was hybridized to probes specific for the predicted transcription units. RNA of strain Lb790 was used as a negative control. A 2.3 kb signal was detected with a DNA fragment specific for *sppK* and *sppR* (Fig. 3b). This mRNA size corresponds well to the expected ORF1/*sppK*/*sppR* transcript. A synthetic oligonucleotide M3R12 (5'-TCCACCTGTAA-TTGCTG-3') specific for *sppA* detected two mRNA signals of 0.9 kb and 0.4 kb (Fig. 3a). This suggests that *sppA* is transcribed both in a transcription unit with *spiA* and in a mRNA encompassing only the *sppA* cistron. However, no obvious transcriptional termination structure was found in the corresponding DNA region downstream of *sppA*. Similar results were obtained by Tichaczek *et al.* (1994) using mRNA from their strain *Lb. sake* LTH673. The weaker hybridization signals also present in the control lanes corresponded to the 23S and 16S rRNAs (Fig. 3). When random-labelled pBLS3 specific for *sppT*/*sppE* was used as a probe, a faint signal was detected at 6.9 kb (not shown). It is at present uncertain whether this represents a read-through transcript starting from the promoter in front of ORF1 or a transcript starting in front of *sppT* and extending beyond *sppE* and ORF2. Difficulties in detecting transcripts involved in bacteriocin systems have been encountered previously (Axelsson & Holck, 1995).

Deletions/mutations in the *spp* gene cluster and expression in sakacin-negative *Lb. sake* strains

The inserts of pBLS1, pBLS2 and pBLS3 were cloned as a continuous sequence of 7.6 kb into the vector pLPV111, resulting in pMLS114 (Fig. 1). The sakacin-P-sensitive (Imm⁻) and bacteriocin-deficient (Bac⁻) hosts *Lb. sake* Lb790 and Lb706X transformed with pMLS114 corresponded to the parental strain Lb674 with respect to the extent of bacteriocin production and immunity. Thus, pMLS114 contained all information necessary to confer the ability of sakacin P production and immunity. To establish whether *sppK*, *sppR*, *spiA*, *sppT* and *sppE* are essential for bacteriocin production, frameshift or deletion mutations were introduced in the respective genes on pMLS114. Sakacin P production and immunity were tested in *Lb. sake* Lb706X (Fig. 1) and Lb790 (not shown in Fig. 1) after transformation with the pMLS114 derivatives. Transformants carrying the derivative with a frameshift in *sppK* (pMLS114-K) and a deletion in *sppT* (pMLS114-T), respectively, did not produce bacteriocin

Fig. 3. Northern blot analysis of total RNA from *Lb. sake* Lb674 (Bac⁺) and from *Lb. sake* Lb790 (Bac⁻). Before blotting the RNA onto a nylon filter the 23S and 16S rRNA was visualized by UV-shadowing. Lanes: 1, UV-shadowing photograph of the agarose gel showing the 16S and 23S rRNA used as size markers (Brosius *et al.*, 1968, 1980); 2, hybridization of RNA (a) to the *sppA*-specific oligonucleotide probe M3R12 and (b) to a randomly labelled 1.8 kb *Clal-SpeI* DNA fragment specific for *sppK* and *sppR*.

and were sensitive to sakacin P, indicating that *sppK* and *sppT* were necessary. A deletion mutation in *sppE* (pMLS114-E) resulted in Lb706X transformants exhibiting a Spp⁻, Imm⁻ phenotype. However, the Lb790 transformants carrying pMLS114-E were able to produce bacteriocin and also were immune to sakacin P. Somewhat surprisingly, the mutations in *sppR* (pMLS114-R) and *spiA* (pMLS114-I) had no effect; the transformants were in these cases not distinguishable from *Lb. sake* Lb706X carrying pMLS114. However, cells of Lb706X transformed with pMLS-IP were immune to sakacin P, showing the involvement of *spiA* in immunity.

Presence of *sppR* and *spiA* analogues in *Lb. sake* Lb706X and Lb790 chromosomes

The results obtained with the derivatives pMLS114-R and pMLS114-I prompted us to investigate if the strains *Lb. sake* Lb706X and Lb790 contained genes that could complement these mutations. This was first done with hybridization and gene-specific probes. A *sppR*-specific probe was made by PCR using pMLS114 as a template. The primers were chosen to amplify a fragment corresponding to position 1940–2615 in the reported sequence (Fig. 2). In an analogous manner, a PCR fragment corresponding to position 3072–3623 was used as a *spiA*-specific probe. Hybridization to DNA from *Lb. sake* Lb706X and Lb790 with both probes resulted in strong signals from the chromosomal band from both strains (not shown). Next, the same primers used to make the *sppR*- and *spiA*-specific probes were used in PCR reactions with Lb706X and Lb790 chromosomal DNA as templates. With both *sppR* and *spiA* specific primers, PCR fragments were obtained that were of the same size as when using pMLS114 as a template. Sequencing of

these fragments, corresponding to 70–80% of the genes, revealed that Lb706X and Lb790 have chromosomal analogues of *sppR* which were 100% identical between each other at the amino acid level and 97% identical to *sppR* at the DNA level. The *spiA* analogues on the chromosome of Lb706X and Lb790 were 100% identical to *spiA* at the DNA level.

DISCUSSION

The sakacin P gene cluster from *Lb. sake* Lb674 was cloned, sequenced and expressed in the bacteriocin-negative hosts *Lb. sake* Lb790 and Lb706X. By mutation analyses it was shown that *sppK*, *sppT* and *sppE* were essential for sakacin P production and immunity in Lb706X. We also showed that *spiA* under the control of a promoter that functions in LAB conferred immunity. These results, together with the homologies of SppK/SppR and SppT/SppE to the proteins of bacterial two-component regulatory systems and signal-sequence-independent transport systems, respectively, indicate that sakacin P production in *Lb. sake* Lb674 is controlled by a two-component regulatory system and that its secretion is dependent on a dedicated transport system.

In typical bacterial two-component regulatory systems, a transmembrane HPK is responsible for reception and transduction of an environmental signal to a cytoplasmic RR protein, which mediates a response within the cell. Homology comparisons revealed that SppK/SppR belong to the AgrB/AgrA subfamily of the HPK/RR systems. The *agr* (accessory gene regulator) locus regulates the expression of several virulence determinants and other exoproteins during post-exponential growth (Kornblum *et al.*, 1990; Peng *et al.*, 1988). AgrA functions as a transcriptional regulator (Parkinson & Kofoid, 1992;

Fig. 4. Sequence alignment of promoter regions within the *spp* gene cluster. Identical nucleotides are indicated by asterisks, the suggested -10 region is boxed and inverted repeats are indicated by arrows. ORF2 is the reading frame downstream of *sppE* not completely contained on the 7.6 kb insert of pMLS114 (not shown in Fig. 2). Numbers in parentheses denote the start positions of the respective sequences in Fig. 2

Stock *et al.*, 1989). A mutation in *sppK* abolished both production and immunity, while immunity could be obtained by placing *spiA* under the control of a constitutive, heterologous promoter. These results suggest that the Spp signal transducing system acts at the transcriptional level, similar to AgrB/AgrA. Since sakacin P is produced constitutively by *Lb. sake* Lb674 in MRS medium (data not shown), nothing is known about the nature of the signal that triggers its production.

Both HPK and RR proteins are also essential for the biosynthesis of several other bacteriocins as shown by mutational analyses of the respective genes in bacteriocin-producing strains (Axelsson & Holck, 1995; Klein *et al.*, 1993; van der Meer *et al.*, 1993). Although the necessity of SppR for sakacin P production could not be shown in the *Lb. sake* Lb706X and Lb790 genetic background, SppR is probably essential for sakacin P production in other hosts and in the donor strain *Lb. sake* Lb674. The explanation for the apparent non-necessity of *sppR* in Lb706X probably lies in the fact that a chromosomal analogue is present in this strain (and in Lb790). Assuming a classical route for signal transduction involving a HPK and a RR, the chromosomally encoded RR is only activated in the presence of an intact SppK. Otherwise it would also be able to complement the *sppK* mutation. It is worthy of note that such complementation was not seen in the sakacin A system using the same host strain (Axelsson & Holck, 1995), indicating that the sakacin A and P systems cannot complement each other, despite a high level of amino acid sequence similarity in the HPK and RR proteins.

The Imm⁺ phenotype of pMLS-IP transformants strongly suggests that *spiA* is the immunity gene in the sakacin P system. This result and results obtained with pMLS114-I (mutated *spiA*) at first appear to be contradictory. However, using the same approach as described above for the RR, it was found that a copy of *spiA* is present in the chromosome of both *Lb. sake* Lb706X and Lb790. This gene is normally not expressed in these strains since they are Imm⁻. However, assuming a transcriptional activation event involving SppK/SppR, it may be expressed when the *spp* gene cluster is introduced, thus complementing the mutation in *spiA*. Our results therefore indicate the presence of an incomplete sakacin P system (at least *sppR* and *spiA* analogues) on the chromosome of two separate *Lb. sake* strains. We do not know the relative organization of the *sppR* and *spiA* analogues on the chromosomes of

Lb706X and Lb790 since no restriction enzyme mapping was done. The significance of these findings needs to be studied further. It can also be deduced that the gene products from *sppT* and *sppE* are necessary not only for production (obvious from the homology with known processing and transport systems, see below), but also for immunity in the normal case (Fig. 1). However, similar to the situation described above for SppK, the involvement of SppT and SppE in immunity must be on the transcription level since *spiA* under control of a heterologous promoter confers immunity.

SppT belongs to the HlyB family of signal-sequence-independent translocator proteins (Blight & Holland, 1990; Mackman *et al.*, 1986). This family also includes SapT, LcaC, LcnC and PedD, which are essential for the production of sakacin A, leucocin A, lactococcin A and pediocin PA-1, respectively. All proteins exported by the HlyB-like transporters lack a classical signal sequence which is required for export in the general secretory pathway. ComA, which is most similar to SppT, is also a member of this protein family. However, in *S. pneumoniae* ComA is not involved in bacteriocin export but is assumed to be responsible for the secretion of the competence factor (Chandler & Morrison, 1987).

SppE is a HlyD-like protein which is required for sakacin P production and immunity in *Lb. sake* Lb706X but not in *Lb. sake* Lb790. In *E. coli*, HlyD is necessary for the translocation of haemolysin A. In the haemolysin translocation model, HlyD together with HlyB forms a specific secretory complex which is responsible for the recognition and export of haemolysin across the cytoplasmic membrane. Because of the homology between SppE and HlyD we assume that SppT/SppE form the secretory unit for sakacin P export. Requirement of HlyD analogues for bacteriocin production was established for several other bacteriocin systems (Axelsson & Holck, 1995; Marugg *et al.*, 1992; Stoddard *et al.*, 1992; van Belkum & Stiles, 1995). In the haemolysin model, HlyD spans the periplasm and is anchored in the inner membrane by its hydrophobic N-terminal region. It is assumed to connect the *E. coli* inner and outer membranes and facilitate the export of haemolysin through both membranes. Because secreted proteins in Gram-positive bacteria have only one membrane to traverse, the role of SppE in our system is not clear (Fath & Kolter, 1993). Why *sppE* was not essential in *Lb. sake* Lb790 is unknown. A labelled *sppE*-specific PCR fragment derived from pMLS114 could not detect a

homologous gene in Lb790. This could mean that Lb790 in contrast to *Lb. sake* Lb706X is able to supply an accessory protein from another transport system to complement the destroyed *sppE* gene.

Certain sequence similarities upstream of the suggested promoter regions were noted in front of ORF1, *sppA*, *sppT* and ORF2 (Fig. 4). These may be of significance in transcriptional control of the system. Such regions were also found in the sakacin A system and shown to be necessary for proper expression of the *sap* genes (Axelsson & Holck, 1995). The 18 N-terminal residues of the putative peptides encoded by ORF1 and ORF2 exhibit significant similarities with leader sequences of class II bacteriocins, including a potential double-glycine cleavage site. These features, together with the DNA sequence homologies upstream of the promoter regions, may indicate that ORF1 and ORF2 have a function in the sakacin P system. The putative mature ORF1 peptide was not homologous to any known peptide in the databases searched but a function in the sakacin P system cannot be excluded since the gene was not inactivated in this study.

In conclusion, the gene cluster involved in sakacin P production and immunity shows a high degree of similarity to other bacteriocin gene clusters from lactic acid bacteria. In particular, it is similar to the sakacin A gene cluster, although the organization of the genes is somewhat different (Axelsson & Holck, 1995). Both encode a putative two-component signal transducing system, which seems to act at the transcriptional level in the expression of the genes encoding actual functional proteins, i.e. structural, immunity and transport genes. However, further studies are necessary to determine the precise mechanisms of regulation. The identification of the signal that induces the HPK/RR system should provide a key for a better understanding of this regulation. Our finding that analogues of some of the genes in the *spp* cluster can be found on the chromosome of other, bacteriocin-negative *Lb. sake* strains is interesting and raises questions regarding the origin and evolution of these systems.

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