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; Vandenbergh, 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*
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), cananobacteriocins 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 L. 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 (Merek), routinely at 25 °C, but at 30 °C when used in electrotransformation experiments. Listeria innocua L1 was grown in Standard-I medium (Merek) 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 Schilling & Lücke (1989). Controls were performed with culture supernatants from bacteriocin-producing (Bac⁺) and non-producing (Bac⁻) strains, and with tryspin-digested Bac⁺ supernatants. L. innocua L1 was used as an indicator strain to represent the antagonistic activity of sakacin P against L. 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, resuspend 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

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description*</th>
<th>Source or reference</th>
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<tr>
<td><strong>Strains</strong></td>
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<tr>
<td><em>Lactobacillus sake</em></td>
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<tr>
<td>Lb674</td>
<td>Wild-type strain; Spp*, Imm*</td>
<td>Holck et al. (1994)</td>
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<tr>
<td>Lb790</td>
<td>Host strain for pLPV111 and derivatives; sensitive to sakacin P, Spp*, Imm*</td>
<td>Schillinger &amp; Lücke (1989)</td>
</tr>
<tr>
<td>Lb706X</td>
<td>Host strain for pLPV111 and derivatives; sensitive to sakacin P, Spp*, Imm*</td>
<td>Axelsson et al. (1993)</td>
</tr>
<tr>
<td>Lb790/111</td>
<td>Lb790 carrying pLPV111, indicator strain; sensitive to sakacin P, Spp*, Imm*, Em*</td>
<td>This study</td>
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<tr>
<td><strong>Listeria innocua Lil</strong></td>
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<td>DH5a</td>
<td>Host strain for pBluescript KS+ and derivatives; F- ΔlacZΔM15 endA1 recA1Δ hisD17 supE44 thr-1 Δ gyrA96 relA1 Δ(lacZYA-argF)U169</td>
<td>Gibco BRL</td>
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<tr>
<td>JM105</td>
<td>Host strain for pLPV111 and derivatives; thi rpsL endA ΔshvB15 hisR4 Δ(lac-pro-AB)/F’tralD36 pro-AB lac*ΔlacZΔM15</td>
<td>Pharmacia</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pGEM-7Zf(+)</td>
<td>3.0 kb cloning vector; replicates in <em>E. coli</em>, Ap*, lacZ</td>
<td>Promega</td>
</tr>
<tr>
<td>pBluescript KS+</td>
<td>3.0 kb cloning vector; replicates in <em>E. coli</em>, Ap*, lacZ</td>
<td>Stratagene</td>
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<tr>
<td>pLPV111</td>
<td>4.2 kb <em>E. coli/Lb. plantarum/Lb. sake</em> shuttle vector; Em*, lacZ</td>
<td>Axelsson &amp; Holck (1995)</td>
</tr>
<tr>
<td>pGEM-7Zf(+) derivative (Ap*)</td>
<td>3.9 kb ClaI/<em>EcoRI</em> fragment from <em>Lb. sake</em> Lb674 DNA; sppR* A* T* spiA*</td>
<td>Holck et al. (1994)</td>
</tr>
<tr>
<td>pBLS1</td>
<td>3.0 kb EcoRI fragment from <em>Lb. sake</em> Lb674 DNA; part of sppK gene, sppE+</td>
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<tr>
<td>pBLS2</td>
<td>1.3 kb HpaII fragment from <em>Lb. sake</em> Lb674 DNA; part of sppK gene</td>
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<tr>
<td>pBLS3</td>
<td>3.7 kb PstI fragment from <em>Lb. sake</em> Lb674 DNA; part of sppT gene, sppE+</td>
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<tr>
<td>pLPV111 derivatives (Em*)</td>
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<tr>
<td>pMLS114</td>
<td>7.6 kb insert, combined inserts of pBLS1, pBLS2 and pBLS3; sppK<em>R</em> ΔTP* spiA*</td>
<td>This study</td>
</tr>
<tr>
<td>pMLS114-K</td>
<td>pMLS114 derivative with a frameshift mutation in sppK (ClaI site); sppK<em>R</em> ΔTP* spiA* sppK110</td>
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<tr>
<td>pMLS114-R</td>
<td>pMLS114 derivative with a 0.56 kb NruI/HpaI deletion in sppR; sppK<em>R</em> ΔTP* spiA* ΔsppR</td>
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<tr>
<td>pMLS114-I</td>
<td>pMLS114 derivative with a frameshift mutation in spiA (BglII site); sppK<em>R</em> ΔTP* spiA* ΔsppT</td>
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<tr>
<td>pMLS114-T</td>
<td>pMLS114 derivative with a 0.99 kb SmaI/EcoRV deletion; sppK<em>R</em> ΔTP* spiA* ΔsppT</td>
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<tr>
<td>pMLS114-E</td>
<td>pMLS114 derivative with a 1.07 kb ScaI/XhoI deletion; sppK<em>R</em> ΔTP* spiA* ΔsppE</td>
<td></td>
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<tr>
<td>pMLS-IP</td>
<td>0.56 kb PCR fragment from pMLS114; ermLp spiA* ΔsppK ΔATE</td>
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*Spp, sakacin P production; Imm, immunity to sakacin P; Ap*, ampicillin resistance; Em*, erythromycin resistance; ermLp, gene under control of the ermL promoter.

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
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 pLV111 construct containing the joint pBLS2 and pBLS1 fragments. The single ClaI site was made blunt and the plasmid religated. The desired mutations were confirmed by the absence of the respective recombinant plasmid bands in agarose gels 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).

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, sppA 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 site, Southern hybridization experiments were performed with Lb. sake Lb674 DNA. For cloning of the remaining part of sppK a synthetic oligonucleotide FR2 (5'-CTGTTTGACTATT GTTC-3') complementary to part of the sppK sequence was used as a probe. The probe identified a 1.3 kb HpaI fragment which was ligated into the ClaI site of pBlueScript KS+, resulting in pBLS2 (Fig. 1). A 3.7 kb PvuI fragment hybridizing to the labelled 0.9 kb Spel–EcoRI fragment of the pBLS1 insert (Fig. 1) was isolated and cloned into the EcoRV site of pBluescript 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, sppA, 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, BglII; C, ClaI; E, EcoRI; EV, EcoRV; H, HindIII; Ha, Hpal; Hp, HpalII; K, KpnI; N, NruI; Nd, Ndel; P, PvuII; S, SpeI; Sc, ScaI; Sn, SnaBI; X, XhoI. On the insert of pMLS114 not all of the Hpal and Hpall 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 −19·4 kcal mol⁻¹ (−81·2 kJ mol⁻¹) was found. The sppT gene starts 350 bp downstream of sppA 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 promotor sequences are located upstream of ORF1, sppA, sppT and ORF2 (Fig. 2). Thus, three major
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, sppA, 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.

**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 & Kofoid, 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%), which 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 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.

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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 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 gelidum 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; 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 (Felmllee 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 (Hävarstein 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; Felmllee 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'-TCCACCCGTAATTTGCTG-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 sppA 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, sppA, 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
and were sensitive to sakacin P, indicating that \textit{sppK} and \textit{sppT} were necessary. A deletion mutation in \textit{sppE} (pMLS114-E) resulted in Lb706X transformants exhibiting a Spp\textsuperscript{+}, Imm\textsuperscript{-} 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 \textit{sppR} (pMLS114-R) and \textit{spiA} (pMLS114-I) had no effect; the transformants were in these cases not distinguishable from \textit{Lb. sake} Lb706X carrying pMLS114. However, cells of Lb706X transformed with pMLS-IP were immune to sakacin P, showing the involvement of \textit{spiA} in immunity.

**Presence of \textit{sppR} and \textit{spiA} analogues in \textit{Lb. sake} Lb706X and Lb790 chromosomes**

The results obtained with the derivatives pMLS114-R and pMLS114-I prompted us to investigate if the strains \textit{Lb. sake} Lb706X and Lb790 contained genes that could complement these mutations. This was first done with hybridization and gene-specific probes. A \textit{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 \textit{spiA}-specific probe. Hybridization to DNA from \textit{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 \textit{sppR}- and \textit{spiA}-specific probes were used in PCR reactions with Lb706X and Lb790 chromosomal DNA as templates. With both \textit{sppR} and \textit{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 \textit{sppR} which were 100\% identical between each other at the amino acid level and 97\% identical to \textit{sppR} at the DNA level. The \textit{spiA} analogues on the chromosome of Lb706X and Lb790 were 100\% identical to \textit{spiA} at the DNA level.

**DISCUSSION**

The sakacin P gene cluster from \textit{Lb. sake} Lb674 was cloned, sequenced and expressed in the bacteriocin-negative hosts \textit{Lb. sake} Lb790 and Lb706X. By mutation analyses it was shown that \textit{sppK}, \textit{sppT} and \textit{sppE} were essential for sakacin P production and immunity in Lb706X. We also showed that \textit{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 \textit{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 \textit{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;
Stock et al., 1989). A mutation in $sppK$ abolished both production and immunity, while immunity could be obtained by placing $spi.A$ under the control of a constitutive, heterologous promotor. 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 $Lb. sake$ and $Lb. sake$ strains (Axelsson et al., 1993; van der Meet 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 Lb706X). Assuming a classical route for signal transduction involving a HPK and RR, it was found that a copy of $sppR$ for sakacin P production could not be shown in the $Lb. sake$ 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 transcriptional level since $spi.A$ under control of a heterologous promotor 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 haemolsyn A. In the haemolsyn translocation model, HlyD together with HlyB forms a specific secretory complex which is responsible for the recognition and export of haemolsyn 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 haemolsyn 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 haemolsyn 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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