The Genes Involved in Production of and Immunity to Sakacin A, a Bacteriocin from Lactobacillus sake Lb706

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Sakacin A is a small, heat-stable, antilisterial bacteriocin produced by Lactobacillus sake Lb706. The nucleotide sequence of a 8,668-bp fragment, shown to contain all information necessary for sakacin A production and immunity, was determined. The sequence revealed the presence of two divergently transcribed operons. The first encompassed the structural gene sapA (previously designated sakA) and saiA, which encoded a putative peptide of 90 amino acid residues. The second encompassed sapK (previously designated sakB), sapR, sapT, and sapE. sapK and sapR presumably encoded a histidine kinase and a response regulator with marked similarities to the AgrB/AgrA type of two-component signal-transducing systems. The putative SapT and SapE proteins shared similarity with the Escherichia coli hemolysin A-like signal sequence-independent transport systems. SapT was the HlyB analog with homology to bacterial ATP-binding cassette exporters implicated in bacteriocin transport. Frameshift mutations and deletion analyses showed that sapK and sapR were necessary for both production and immunity, whereas sapT and sapE were necessary for production but not for immunity. The putative SaiA peptide was shown to be involved in the immunity to sakacin A. The region between the operons contained IS1163, a recently described L. sake insertion element. IS1163 did not appear to be involved in expression of the sap genes. Northern (RNA) blot analysis revealed that the putative SapK/SapR system probably acts as a transcriptional activator on both operons. A 35-bp sequence, present upstream of the putative sapA promoter, and a similar sequence (30 of 35 nucleotides identical) upstream of sapK were shown to be necessary for proper expression and could thus be possible targets for transcriptional activation.

Lactic acid bacteria (LAB), a physiologically related group of gram-positive bacteria, produce a variety of compounds with antimicrobial activity (9, 31). Some of these are proteins or peptides and are termed bacteriocins. According to a classical definition (46), bacteriocins are proteinaceous compounds that are bacteriocidal to strains closely related to the producer strain. However, it now has become evident that many bacteriocins from LAB and other gram-positive bacteria have a somewhat broader spectrum of activity, affecting also more distantly related species (24, 25). Bacteriocins with activity toward the food-borne pathogen Listeria monocytogenes are of interest for potential applications to enhance food safety. To exploit these potentials, further biochemical and genetic characterization of LAB bacteriocins is needed.

Bacteriocins from LAB are currently divided into four major classes (25). The most well-known LAB bacteriocin is perhaps nisin. Nisin belongs to the class I bacteriocins, characterized by containing unusual amino acids such as lanthionine and usually termed lantibiotics (41). However, the most common bacteriocins produced by the LAB belong to class II. These peptides are characterized as being small (<10 kDa), mostly hydrophobic, heat stable, and nonmodified, with similar modes of action, i.e., activity on the cell membrane. The leader sequences of the precursors of class II bacteriocins have similarities, in particular a double-glycine motif at positions −1 and −2 before the processing site.

The class II bacteriocins best characterized at the genetic level are lactococcin A, produced by Lactococcus lactis (45, 51), lactacin F, produced by Lactobacillus johnsonii (26), and the identical bacteriocins pediocin PA-1 and pediocin AcH, produced by Pediococcus acidilactici (7, 32, 35). The production of lactococcin A and pediocin PA-1 (pediocin AcH) is dependent on the expression of a gene encoding an ATP-dependent membrane translocator, also termed ABC (ATP-binding cassette) transporter (15), belonging to the HlyB family. Although it has not been proven for any other class II bacteriocin, these dedicated transport systems may be a general feature, since the leader peptides of the bacteriocin precursors are similar and distinct from classical signal sequences (25). The genes necessary for production and immunity are commonly organized in operon-like structures.

Sakacin A, produced by Lactobacillus sake Lb706 (20, 40), belongs to a subgroup of the class II bacteriocins. The bacteriocins in this subgroup, sometimes referred to as the pediocin family or class IIa (25), are characterized by being active against Listeria species and sharing a consensus sequence (-Tyr-Gly-Asn-Gly-Val-Xaa-Cys-) (Xaa means any amino acid residue) near the N terminus (25). Other members in this group are pediocin PA-1 (pediocin AcH), leucocin A-UAL187 (17), mesentericin Y105 (19), sakacin P (sakacin 674) (21, 48), carnobacteriocins B2 and BM1 (34), and curvacin A (48), the latter shown to be identical to sakacin A when the structural gene (curA) was cloned and sequenced (49).

Sakacin A production and immunity are associated with a 60-kb plasmid present in L. sake Lb706. So far, two genes involved in sakacin A production and immunity have been identified: the structural gene, sakA (20), and sakB, which encodes a putative histidine kinase with homology to sensor proteins in bacterial two-component signal-transducing systems (4). The two genes are located close to each other on the 60-kb plasmid and are transcribed in opposite directions (4).

This report describes the cloning, nucleotide sequence, and analysis of a 8.7-kb fragment from the 60-kb plasmid of L. sake Lb706, containing all information necessary for sakacin A production and immunity. The analysis revealed that sakacin A production is dependent on a putative dedicated transport...
system of the HlyB/HlyD type. Furthermore, a presumed two-component signal-transducing system was found to be involved in expressing the genes for both production and immunity. There are now several genes published with the prefix sak, in addition to sakA and sakB mentioned above, such as sakP and sakR, the structural genes for the identical bacteriocins sakacin P and sakacin K, respectively (21, 50). These are separate from sakacin A, and to avoid further confusion, we now introduce the prefix sap for genes involved in sakacin A production and sui for genes associated with sakacin A immunity. Thus, the structural gene is now designated sapA (previously sakA). The gene encoding the presumed histidine kinase is designated sapK (previously sakB), the K suffix referring to the putative kinase function.

MATHEMATICAL METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this work are listed in Table 1; phenotypes used in the text are defined in the footnote to Table 1. Two new plasmids, pVS1 and pSV2, and a new shuttle vector, pLPV111, were constructed in connection with this work. pVS1 is a derivative of the broad-host-range vector pVS2 (56), in which the lacZ polylinker region of pGEM-7Zf(+) was replaced by a 0.63-kb fragment from pGEM-7Zf(+) containing the lacZ polylinker region. pLPV111 is essentially a pGEM-7Zf(+) derivative in which the ampicillin resistance gene (bla) has been replaced by a fragment containing the erythromycin resistance gene from the Lactobacillus reuteri plasmid pLUL631 (5) and a replication region derived from the Lactobacillus plantarum plasmid p256 (8). Both of these vectors replicate in E. coli in addition to LAB and contain the lacZ polylinker region of pGEM-7Zf(+) which enables blue/white selection on plates containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and IPTG (isopropyl-β-D-thiogalactopyranoside). E. coli TG1 was used as the host for pVS2, pVS1, and derivatives, since E. coli DH5α does not support proper replication of these plasmids. pVS1 and pLPV111 (and derivatives) are compatible when present in the same strain.

Unless otherwise stated, Lactobacillus strains were grown in MRS broth (Difco Laboratories, Detroit, Mich.) without agitation at 30°C. Lactococcus strains were grown in M17 medium (47) supplemented with 0.5% glucose (GM17) at 30°C. E. coli strains were grown in brain heart infusion (BHI) broth (Difco) with vigorous agitation at 37°C. Agar plates were made by adding 1.5% (wt/vol) agar to broth media. Selective antibiotic concentrations were as follows: ampicillin, 100 μg/ml; chloramphenicol, 10 μg/ml (LAB) and 200 μg/ml (E. coli); and erythromycin, 10 μg/ml (LAB) and 30 μg/ml (E. coli). When selecting for blue/white colonies of E. coli transformants, X-Gal and IPTG (each at a final concentration of 40 μg/ml) were included in the BHI agar.

Sakacin A production and immunity. Colonies from transformation experiments were tested for sakacin A production by a deferred assay as described previously (4, 48), with some modifications. A modified MRS (B-MRS) agar was used. This medium has a pH of 6.5 and is identical to MRS except for its low glucose (0.2% [wt/vol]) and high potassium phosphate (100 mM) content. When Lactococcus or E. coli strains were tested for bacteriocin production, BHI agar was used as the basal medium. The plates were incubated anaerobically (BBL anaerobic system; Becton Dickinson and Co., Cockeysville, Md.) both before and after overlay with the indicator strain, which was L. sake Lb790 transformed with pVS2 (Emr, Cm) in all experiments. Antibiotics were added as needed depend-
Plasmid DNA isolation and transformation. Plasmids from *E. coli* were prepared by using a Magic Miniprep kit (Promega Corp., Madison, Wis.). For *E. coli* TG1, a phenol-chloroform extraction step had to be included before the Magic column in order to inactivate residual endonuclease activity. *E. coli* colonies were screened for plasmid content by picking a visible amount of cells followed by a microscale version (15-µl volumes) of the Magic Miniprep procedure, excluding the column purification. Instead, the neutralizing step was followed by an ethanol precipitation and finally the DNA was resuspended in a small volume of H₂O and electrophoresed in agarose gels. Plasmids from *Lactobacillus* strains were purified by an alkaline lysis method as described previously (4). Transformation of the different strains with plasmid DNA was done by electroporation using the Gene Pulser and Pulse Controller unit (Bio-Rad Laboratories, Richmond, Calif.) and previously published protocols (2, 16, 57).

General molecular cloning techniques. Standard procedures for molecular cloning were as described by Sambrook et al. (38). Enzymes (restriction enzymes, T₄ DNA ligase, Klenow fragment, and calf intestine phosphatase) were used as described by Sambrook et al. (38). Enzymes (restriction enzymes, ligase, and polymerase) were used as described by Sambrook et al. (38). Enzymes (restriction enzymes, ligase, and polymerase) were used as described by Sambrook et al. (38).

Cloning of the sakacin A gene cluster. To map the location of a gene putatively encoding an ABC exporter associated with sakacin A production, advantage was taken of a highly conserved (nearly 100% identical) region in LcnC and PedD, the putative ABC exporters of lactococcin A (45) and pediocin PA-1 (32), respectively. The region corresponds to the A site in the ATP-binding motif (11). A 29-mer degenerate probe (termed LAI100), 5'-TT/T(C/T)CC(T/C)GAACCACTGAGGCT(T/C)CAATTAGT-3', complementary to the lcnC and pedD sequences, was used in hybridization experiments with pLSA60. A significant signal was obtained (not shown), and the location was mapped to a position 2.0 to 2.5 kb downstream *sapK* (4). Since *sapA* and *sapK* are transcribed in opposite directions (4), this was an indication that the fragment from pLSA60 to be cloned in order to obtain the entire machinery for sakacin A production should contain a large piece of DNA downstream of *sapK* rather than downstream of *sapA*. An approximately 10.4-kb *SplI* fragment from pLSA60 was found sufficient, encompassing 2.6 kb downstream of *sapA* and 4.7 kb downstream of *sapK* in addition to the two genes and the region in between. This fragment was cloned in pVS81, giving rise to pSAK21. When pSAK21 was transferred to *L. sake* LB760-X (Sap+ Imm+), all transformants tested became Sap+ and Imm-. Heterologous expression of both sakacin A production and immunity was obtained in the indicator strain *L. sake* LB790 by introducing pSAK21, indicating that the 10.4-kb fragment contained all information needed for sakacin A production and immunity. No expression was detected when pSAK21 was present in *E. coli* TG1, *Lactococcus lactis* MG1363, or *L. plantarum* NC8. A simple deletion experiment was performed to narrow the fragment somewhat. It was possible to remove 1.7 kb at the *sapA* side of the fragment without affecting sakacin A production or immunity. The remaining 8.7 kb *PacI-SplI* fragment, present on plasmid pSAK27, was sequenced in its entirety.

DNA sequence analysis. The complete DNA sequence of the 8.688-bp *PacI-SplI* fragment is shown in Fig. 1. Ten open reading frames (ORFs) preceded by putative ribosome binding sites (RBSs) were identified. ORFs previously (4, 20) and later (see below) shown to be directly involved in sakacin A production and immunity were designated by the prefixes *sap* and *sai*, respectively. The structural gene for sakacin A, now designated *sapA*, is located at positions 1119 to 943. Immediately downstream *sapA* is an ORF designated *saiA*. *saiA* starts with a TTG codon and potentially encodes for a 90-amino-acid (aa) peptide. A region of dyad symmetry (calculated free energy, −20.4 kcal [1 cal = 4.184 J/mol] representing a putative rho-independent terminator structure is located about 100 bp downstream *saiA*. Further downstream, another ORF (*orfI*) predicts a 87-aa peptide. The remaining seven ORFs are encoded by the opposite strand. The previously reported *sakF* (previously *sakK*) gene is located at positions 2724 to 4007. Because of a more appropriate distance to the putative RBS, the GTG codon is presumed to be the start codon of *sapK* rather than ATG located at position 2718 (4). Three ORFs, *orfI*, *orfIII*, and *orfIV*, are positioned upstream of *sapK*. For reasons that will be discussed later, *orfIII* is displayed without a proper start codon and an RBS within the coding region. Immediately downstream *sapK* follows an ORF, designated *saiB*, probably starting with a TGA codon and encoding a protein of 247 aa. *sapR* is followed by *saiT*, starting with an ATG codon and potentially encoding a protein of 719 aa. Finally, an ORF, designated *sapE*, follows immediately downstream of *saiT*. *sapE* starts with an ATG codon and potentially encodes a
protein of 461 aa. Three stop codons (TAA) in a row mark the end of sapE. A stem-and-loop structure (calculated free energy, \(-21.5\) kcal/mol), possibly a transcriptional terminator, is located approximately 100 bp downstream of sapE. Putative promoter sequences were found upstream of sapA (designated P1) and, as previously suggested (4), upstream of sapK (P2). No other obvious promoters conforming to the gram-positive consensus sequence (15) could be detected. The sequences thus indicate the presence of two divergently transcribed operons: one encompassing sapA and sapK, and the other encompassing sapRTKE. The region between sapA and sapK contains, besides the ORFs, two 35-bp stretches that are inverted repeats of each other. They were designated LIR and RIR (left inverted repeat, positions 1224 to 1190) and RIR (right inverted repeat, positions 2432 to 2466). LIR and RIR have 30 of 35 nucleotides identical.

**DNA homology.** A homology search revealed that the nucleotide sequence of positions 1230 to 2409 is completely identical to IS1163, a new insertion element from *L. sake* (43). IS1163 contains inverted repeat sequences at the left and right ends (43), but note that the regions designated LIR and RIR (Fig. 1) are located outside the ends and are not part of IS1163. IS1163 reportedly creates a 3-bp duplication of the target sequence upon insertion (43). This is in accordance with the sequence reported here, in which a GGG sequence is found preceding and following the IS1163 sequence.
putative SakR protein has homology to AgrA (35% identity, 46% similarity), a member of the response regulator (RR) family (28, 44). AgrA and AgrB have been suggested to constitute a two-component signal-transducing system in S. aureus (28). SapK and SapR also have homology to PlnB, PlnC, and PlnD, the suggested HPK/RR system involved in plantaricin A production (10). This homology is similar to the homology to the Agr proteins; i.e., 40 to 50% similarity if conservative changes are allowed for. As shown in Fig. 2, there is considerably higher local amino acid sequence similarity between these proteins in the vicinity of some of the suggested conserved amino acids in the HPK and RR families (44). In these regions, homology is also found to otherwise distantly related HPK/RR proteins, such as SpaK and SpaR (27).

The oligonucleotide probe LA100 was directed against a conserved region in the nucleotide sequence encoding part of the ATP-binding cassette of ABC exporters (see above). Not surprising, the nucleotide sequence where the probe was hybridizing was indeed part of gene encoding a protein (SapT) with homology to bacterial ABC exporters (Fig. 3). The highest score was obtained with ComA (57% amino acid identity, 75% similarity including conservative changes), an ATP-dependent membrane translocator required for competence in Streptococcus pneumoniae (22). High scores were also obtained with LcnC (56% identity, 73% similarity) and PedD (55% identity, 73% similarity), which are required for lactococcin A and pediocin PA-1 production, respectively (32, 45). Homology was also found between SapT and HlyB (28% identity, 48% similarity), a transporter required for hemolysin A secretion in E. coli and referred to as a prototype bacterial ABC exporter (11). The similarity between these proteins is highly significant around two conserved regions in the C-terminal part, the A and B sites, which together constitutes the ATP-binding motif (11). The conserved lysine (A-site) and aspartate (B-site) residues (Fig. 3) are thought to be close in space in the folded protein and interact directly with the ATP molecule (11). Similar to ComA, PedD, LcnC, and HlyB (11, 22, 32, 45), SapT is

FIG. 2. Protein homology in conserved regions of SapK and SapR to analogous proteins in the plantaricin A (PlnB/PlnC [10]), agr (AgrB/AgrA [28]), and subtilin (SpaK/SpaR [27]) systems, respectively. Double and single dots indicate identical and similar amino acid residues, respectively. (A) Homology around the conserved histidine and asparagine residues (boxed) present in the C-terminal half of proteins in the HPK family (44). (B) Homology around the conserved aspartic acid and lysine residues (boxed) present in the N-terminal half of proteins in the RR family (44).
likely to contain several transmembrane regions in the N-terminal part (not shown).

The putative SapE protein has homology to LcnD, which is regarded as an accessory factor to the ABC exporter (LcnC) in lactococcin A transport (11, 45). The overall amino acid identity between SapE and LcnD is 33%. If conservative changes are considered, the similarity reaches 50%. A weak but significant similarity was also noted to HlyD, the accessory factor for HlyB (11) (22% identity, 38% similarity). A structural similarity between SapE, LcnD, and HlyD was evident when the hydrophobicity characteristics were compared. All of these proteins are essentially hydrophilic except for a marked hydrophobic stretch of approximately 20 aa near the N terminus, proteins are essentially hydrophilic except for a marked hydrophobic stretch of approximately 20 aa near the N terminus.

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The two operons could be separated and present on two plasmids. pSAK20 (sapKRT) could thus complement pSAK17B (sapa-saiA), giving a Sap⁺ Imm⁺ phenotype, while either plasmid alone resulted in a Sap⁻ Imm⁻ strain. This experiment also showed that an intact IS1163 sequence was not necessary for the Sak⁺ Imm⁺ phenotype.

Role of the inverted repeats LIR and RIR. The possibility of using two complementing plasmids to obtain sakacin A production and immunity was exploited to investigate the significance of the inverted repeats LIR and RIR (Fig. 1). This was done by constructing plasmid derivatives of pSAK17B and pSAK20 with specific deletions in these regions, using a PCR technique (Fig. 5). The results of the experiments when these derivatives were introduced into strains containing a complementing plasmid are shown in Table 2. The LIR region upstream sapa and saiA appeared essential for expression of both these genes, and the RIR region was essential for production but not for immunity.

Northern blot analysis. A Northern blot of RNA prepared from the wild-type strain (Lb706) when hybridized with a probe encompassing the insert in pSAK26 (Fig. 4), i.e., the whole gene cluster, is shown in Fig. 6A, lane 1. In comparison with the plasmid-free derivative (Lb706-X; lane 3), in which no transcripts were detected, Lb706 contained two transcripts interpreted as specific for the probe. The small transcript (estimated size, 700 nucleotides) was clearly visible, whereas the large transcript (estimated size, 4,000 to 5,000 nucleotides) appeared as a faint, smeared band. No transcripts could be detected from the sapK mutant Lb706-B (lane 2). Figure 6B, lanes 1 to 3, shows the same blot, but in this case the probe was specific for the sapa-saiA region. This finding indicates that the small transcript represents the sapa-saiA operon, confirming the sequence data which suggested the size of the transcript to be 630 to 640 nucleotides if the promoter (P1) and the terminator downstream of saiA were utilized in vivo. The large transcript probably represent the sapKRT operon, but detailed analysis was hindered by the difficulties in detecting it. If sapKRT is transcribed as one polycistrionic messenger, it should be at least 5,700 nucleotides long. This transcript was even more difficult to detect when strains with the different plasmid constructions were analyzed. Similar problems have been encountered in the lactocin S system, in which no transcription of clearly necessary genes could be detected. Also in this case, transcription of the structural gene was clearly visible (42). Figure 6B further shows a clear difference between the signals obtained from strains containing pSAK27 and pSAK26, both conferring a Sap⁺ Imm⁺ phenotype. The deletion made to construct pSAK26 (Fig. 4) also deleted the putative terminator (the acrI site is located at position 564; Fig. 1) downstream of sai4. The RNA analysis thus indicated that this terminator is in fact used in vivo. Figure 6C shows that the same sapa-saiA transcript was present in derivatives of strain Lb790. The deletion of LIR clearly turned off transcription (Fig. 6C, lane 4), whereas the deletion of RIR did not (lane 5). The Sap⁺ phenotype in the latter case (Table 2) can therefore
not be caused by an effect of transcription of the sapA-saiA operon (see below).

**DISCUSSION**

A 8,668-bp fragment from the *L. sake* Lb706 plasmid pLSA60, containing the genes necessary for sakacin A production and immunity, has been cloned and sequenced. These genes are organized differently from other LAB bacteriocin gene clusters described so far in that two divergently transcribed operons are involved. However, the individual components have many similarities with other known systems. Thus, a dedicated, signal sequence-independent transport system with similarities to the HlyB/HlyD secretion family (11) seems to be involved in the secretion of sakacin A, as well as for lactococcin A (45) and pediocin PA-1 (pediocin AcH) (7, 32). The putative SapT and SapE proteins are the HlyB and HlyD analogs, respectively. In the work described here, a probe (LA100) was designed specifically to locate the gene for the ABC exporter, i.e., *sapT*. Recently, the LA100 probe has also been used for mapping the location of an ABC exporter gene...
associated with the production of sakacin 674 (3). This strategy could thus be useful as a complement to the identification of the structural gene in the genetic characterization of new LAB bacteriocins. HlyB and HlyD are believed to form a complex consisting of dimers of the proteins, which recognizes the substrate (hemolysin A) and facilitates its transport at the expense of ATP. In the case of hemolysin A secretion in \textit{E. coli}, the transport is completed by TolC, located in the outer membrane (11). For gram-positive bacteria, such as LAB, which lack an outer membrane, the HlyB and HlyD analogs are sufficient for secretion. At some stage, the leader sequence of the sakacin A precursor (the prebacteriocin) has to be cleaved off. It can be anticipated that this is done in connection with the SapT/SapE transport process, but the role of the individual proteins is unclear.

SapK and SapR show the features of a HPK and RR, respectively. It is therefore very likely that these proteins constitute a classical bacterial two-component signal-transducing system, mediating a response to an environmental signal (44). The most similar counterpart to SapK/SapR is the AgrB/AgrA system in \textit{S. aureus} (28). The \textit{agr} locus consists of two divergently transcribed operons: one encompassing \textit{agrDCBA} and

\textbf{A}

\textbf{FIG. 5.} Construction of deletion derivatives of pSAK20 (A) and pSAK17B (B) by PCR amplification. Primers were designed so as to introduce suitable restriction sites at the ends of the fragments. The enlarged fragment of pSAK20 represents 0.53 kb. PCR fragments A and B were subsequently digested with EcoRI and MluI and cloned in EcoRI-MluI-digested pSAK20, thus replacing the original 1.35-kb EcoRI-MluI fragment and creating pSAK20A and pSAK20B. PCR fragments C and D were digested with SphI plus HindIII and SphI plus BamHI, respectively, and cloned in pPV111, thus creating pSAK17C and pSAK17D. The identities of these PCR-derived constructions were confirmed by sequencing appropriate parts of the plasmids. Putative terminator (T), RBS, and -35 and -10 regions of promoters P1 and P2 are indicated.
TABLE 2. Phenotype of *L. sake* Lb790 containing different pSAK20 and pSAK17 derivatives

<table>
<thead>
<tr>
<th>Plasmid(s)*</th>
<th>Phenotype</th>
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<tr>
<td>pSAK17B (LIR)</td>
<td>SapImm</td>
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<tr>
<td>pSAK20 (RIR)</td>
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<td>pSAK17B (LIR) + pSAK20 (RIR)</td>
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<tr>
<td>pSAK17B (LIR) + pSAK20A (RIR)</td>
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<tr>
<td>pSAK17B (LIR) + pSAK20B (ΔRIR)</td>
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<tr>
<td>pSAK17C (ΔLIR) + pSAK20 (RIR)</td>
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<tr>
<td>pSAK17D (LIR) + pSAK20 (RIR)</td>
<td>SapImm</td>
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* LIR and RIR, intact LIR and RIR regions; ΔLIR and ΔRIR, deleted (truncated) LIR and RIR regions.

The other encompassing the *hld* gene, encoding the small toxic δ-lysine peptide. The only apparent function of the products of *agrDCBA* is the activation of transcription from their own promoters as well as from the divergent promoter upstream of *hld* (55). The small *hld* transcript (RNAIII, 517 nucleotides) appears to be the specific effector molecule which, together with other signals, regulates the expression of various exoproteins in *S. aureus* (55). The organization of the *sap* genes shows striking similarities to the organization of the *agr* locus in that it contains one large operon (*sapKRTE*), including genes for the signal-transducing system, and one divergently transcribed small operon (*sapA-saiA*), encoding a toxic peptide. By analogy alone, this would suggest that the SapK/SapR system activates transcription of both *sapKRTE* and *sapA-saiA*. The data presented support this suggestion. First, Northern blot analysis indicated that two major transcripts were produced in the original sakacin A producer strain Lb706: one, encompassing *sapA* and *saiA*, strongly expressed and one, probably encompassing *sapKRTE*, weakly expressed. A mutation in *sapK*, as in strain Lb706-B, turned off transcription of both operons. This was most evident with regard to the *sapA-saiA* transcript, since the large transcript was difficult to detect. However, even a substantial overexposure of the autoradiograph shown in Fig. 6 could not reveal the presence of any transcription. The same result was obtained when the RNA was analyzed from a strain containing the *sapR* mutation (pSAK21N) (not shown). Second, the immunity could be expressed independently of *sapKR*, but only when the *sapA-saiA* region was under the control of a heterologous promoter (Fig. 4). The most probable explanation for this finding is that the SapK/SapR system acts at the transcriptional level as an activator. A possible role of the inverted repeat regions LIR and RIR in this regard was investigated. A deletion of most of the LIR region turned off transcription of *sapA-saiA* (Fig. 6C). Tichaczek et al. (49) showed that transcription of the *curA* gene was initiated at the G corresponding to position 1153 (complementary strand) 34 bp upstream of *sapA* (Fig. 1). Since the *curA* sequence is 100% identical in this region to the sequence reported here, it is likely that *sapA-saiA* is initiated at the same position. This means that the promoter P1 most probably is utilized in vivo and that LIR is located at positions −71 to −37 relative to the initiation site. A −45 region has been implicated as being important for gram-positive promoters (15). The construction pSAK17C (truncated LIR) still contains a putative −45 region (up to −50). Thus, the lack of transcription of *sapA-saiA* when LIR is truncated is not likely to be caused by a removal of promoter sequences. Our interpretation of these results is that LIR may be a target region for the SapK/SapR-mediated activation of transcription. The fact that a similar region (RIR) is found upstream the other operon and shown to affect expression (Table 2) strengthens this hypothesis. The deletion of RIR did not affect the transcription of the *sapA-saiA* operon and, therefore, not the immunity. This finding can be explained if it is assumed that a weak constitutive transcription of *sapKR* (and *sapTE*) is functional even in a nonactivated state. This would be necessary for a signal-transducing system; otherwise, no proteins would be available for receiving the environmental signal that triggers the system. In a strain containing pSAK20B (truncated RIR), a small amount of SapK/SapR may be present, and since the environmental signal is present (see below), the system can activate the *sapA-saiA* operon on pSAK17B. The amount of transport/processing proteins (SapT and SapE) required for measurable amounts of sakacin A may not be enough in such a strain, hence the Sap− phenotype. The region between RIR and orf4 does not contain sequences that conform as well as P1 and P2 do with a consensus gram-positive promoter, considering also typical signatures outside the −10 and −35 regions (15). However, it is very likely that a promoter exists; a possible candidate is located at positions 2472 to 2499 (TTAAAA−16 nucleotides−AGTAAT; Fig. 1). Transcription from this putative promoter would presumably be dependent on an intact RIR region and activation by the
puter analysis suggests that the putative mature bacteriocins first 22 aa of the putative peptides are similar to bacteriocin certain- ly warrants further research.

the preliminary genetic analysis indicates a similar organization of Lb706 might represent a special case. Recently, another sakacin A peptide has been purified, and its cellular location has been determined (33). The analysis of the sakacin A gene cluster clearly points to an involvement of expression of saaA in the immunity to the bacteriocin. The SaA peptide does not display any significant sequence similarity with LciA. However, both SaA and LciA are largely hydrophilic, lysine-rich peptides with similar calculated isoelectric points, 10.3 and 10.2, respectively. Nisin immunity is correlated with the NisI peptide encoded within the nis operon. However, full immunity also requires expression of the structural gene nisA (30). A similar and perhaps even more complicated situation might be the case in the sakacin A system. Preliminary results show that specific mutations in either sapA or saaA abolish both production and immunity. These somewhat surprising results indicate that production requires expression of saaA in addition to sapA, and immunity requires expression of sapA in addition to saaA. This complex interaction between the expression of these genes and their products certainly warrants further research.

orf1 and orf4 may be structural genes for bacteriocins, as the first 22 aa of the putative peptides are similar to bacteriocin leader peptides of the double-glycine type. Furthermore, computer analysis suggests that the putative mature bacteriocins (excluding the leader sequences) have isoelectric points of 9.8 and 9.6, respectively, which is similar to typical class II bacteriocins. orf1 could be deleted without affecting the Sap+ Imm– phenotype. However, we cannot exclude the involvement of orf4 in sakacin A production at this stage. orf4 is located between RIR and sapK and may be expressed in the normal case (intact RIR) but nonexpressed in strains with pSAK20B (truncated RIR), thereby affecting sakacin A production. The expression of orf1 and/or orf4 could possibly also expand the activity spectrum of sakacin A, similar to the complementary action of the LaA and LaX peptides in the lactacin F system (1). The possibility was not tested in this study, since the Sap+ phenotype was defined as activity against only one indicator strain, L. sake LB790(pVS2). Further investigations are needed to clarify the role of these ORFs.

The insertion element IS1163, located between the operons, does not seem to be involved in the expression of the sap genes. The presence of IS1163 in the sakacin A gene cluster in strain LB706 might represent a special case. Recently, another sakacin A producer has been identified in our strain collection. A preliminary genetic analysis indicates a similar organization of the sap genes but without IS1163. Although limited, the sequence information available upstream the curvacin A structural gene also indicates that the gene cluster normally is devoid of ISI63.

Heterologous expression of sakacin A production was investigated in only a few strains. We obtained expression of both production and immunity in another L. sake strain (LB790) but not in E. coli, Lactococcus lactis, or L. plantarum. The test for production by E. coli TG1(pSAK21) and Lactococcus lactis MG1363(pSAK21) was done on BHI agar plates because of poor growth on B-MRS agar. The choice of medium is not likely to play a role here, since both L. sake LB706 and LB790(pSAK21) do show distinct inhibition zones also with a BHI agar-based bacteriocin test, although growth is poor (3). Pediocin PA-1 (pediocin AcH) can be produced by E. coli (7, 32), but this system does not involve any proteins of the HPK/RK type. One therefore could imagine that the lack of expression in heterologous species is because the signal-transducing system is not functioning properly. Experiments are now in progress in order to circumvent the signal-transducing system and possibly obtain heterologous expression in several LAB.

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