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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 IS*1163*, a recently described *L. sake* insertion element. IS*1163* 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 has now 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) exporter (11), 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 as 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-UAL 187 (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

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Reference or source
Lactobacillus sake		
Lb706	Wild-type strain, pLSA60, pLSA28; Sap^+ Imm^+	40
Lb706-B	Mutant derivative of Lb706, pLSA60, cured from pLSA28; Sap^- Imm^- *sapK406*	4
Lb706-X	Plasmid-free derivative of Lb706; Sap^- Imm^-	4
Lb790	Host strain, very sensitive to sakacin A; Sap^- Imm^-	40
Lb790(pVS2)	Lb790 transformed with pVS2, indicator strain; Sap^- Imm^- Em^r Cm^r	This study
L. plantarum NC8	Plasmid-free host strain; Sap^- Sak^r	2
Lactococcus lactis MG1363	Plasmid-free host strain; Sap^- Sak^r	13
Escherichia coli		
DH5α	Host strain for pLPV111, pGEM-7Zf(+), and derivatives; F^- Δ*lacU169*(Φ80d*lacZ*ΔM15) *recA1 end A1 hsdR17 supE44 thi-1 gyrA96 relA1* λ^-	Gibco BRL
TG1	Host strain for pVSB1, pVS2, and derivatives; Δ(*lac-proAB*) *supE thi hsd*Δ*5* F′ [*traD36 proAB*$^+$ *lac*I^q *lacZ*ΔM15]	38
Plasmids		
pLSA60	60 kb; contains genes for the Sap^+ Imm^+ phenotype in *L. sake* Lb706	4
Vectors		
pGEM-7Zf(+)	3.0-kb cloning vector; Ap^r *lacZ*	Promega
pVS2	5.0-kb broad-host-range cloning vector; Em^r Cm^r	56
pVSB1	4.0-kb *E. coli*-LAB shuttle vector; Cm^r *lacZ*	This study
pLPV111	4.2-kb *E. coli-L. plantarum-L. sake* shuttle vector; Em^r *lacZ*	This study
pVSB1 derivatives (Cm^r)		
pSAK21	10.4-kb *Sph*I fragment from pLSA60; *sapA*$^+$*K*$^+$*R*$^+$*T*$^+$*E*$^+$ *saiA*$^+$	This study
pSAK27	pSAK21 derivative with a 1.4-kb *Pac*I-*Sph*I deletion; *sapA*$^+$*K*$^+$*R*$^+$*T*$^+$*E*$^+$ *saiA*$^+$	This study
pSAK26	pSAK27 derivative with a 0.56-kb *Acy*I-*Pac*I deletion; *sapA*$^+$*K*$^+$*R*$^+$*T*$^+$*E*$^+$ *saiA*$^+$	This study
pSAK20	pSAK27 derivative with a 1.5-kb *Eco*RI-*Pac*I deletion; *sapK*$^+$*R*$^+$*T*$^+$*E*$^+$ Δ*sapA* Δ*saiA*	This study
pSAK15	pSAK21 derivative with a 1.23-kb *Bgl*II-*Sph*I deletion, *sapA*$^+$*K*$^+$*R*$^+$*T*$^+$ *saiA*$^+$ Δ*sapE*	This study
pSAK22	pSAK21 derivative with a 3.5-kb *Bam*HI deletion; *sapA*$^+$*K*$^+$*R*$^+$ *saiA*$^+$ Δ*sapTE*	This study
pSAK21A	Frameshift mutation in *Acc*III site of pSAK21; *sapA*$^+$*K*$^+$*R*$^+$*E*$^+$ *saiA*$^+$ *sapT335*	This study
pSAK21N	Frameshift mutation in *Nco*I site of pSAK21; *sapA*$^+$*K*$^+$*T*$^+$*E*$^+$ *saiA*$^+$ *sapR124*	This study
pSAK20A	pSAK20 derivative with a 0.93-kb deletion; *sapK*$^+$*R*$^+$*T*$^+$*E*$^+$ Δ*sapA* Δ*saiA* RIR	This study
pSAK20B	pSAK20 derivative with a 0.98-kb deletion; *sapK*$^+$*R*$^+$*T*$^+$*E*$^+$ Δ*sapA* Δ*saiA* ΔRIR	This study
pVS2 derivatives (Cm^r)		
pSAK30	0.49-kb *Bcl*I-*Pvu*II fragment from pSAK27; *ermCp sapA*$^+$ Δ*saiA* Δ*sapKRTE*	This study
pSAK31	0.61-kb *Bcl*I-*Acy*I fragment from pSAK27; *ermCp sapA*$^+$ *saiA*$^+$ Δ*sapKRTE*	This study
pLPV111 derivatives (Em^r)		
pSAK17B	3.0-kb *Sph*I-*Bgl*II fragment from pLSA60; *sapA*$^+$ *saiA*$^+$ Δ*sapKRTE*	This study
pSAK17C	0.78-kb PCR fragment from pLSA60; *sapA*$^+$ *saiA*$^+$ Δ*sapKRTE* ΔLIR	This study
pSAK17D	0.83-kb PCR fragment from pLSA60; *sapA*$^+$ *saiA*$^+$ Δ*sapKRTE* LIR	This study

[a] Sap, sakacin A production; Imm, immunity to sakacin A; Sak^r, resistance or tolerance to sakacin A by a mechanism that might be different from the immunity system; Ap, ampicillin; Em, erythromycin; Cm, chloramphenicol. RIR and LIR, intact RIR and LIR regions; *ermCp*, genes under control of the *ermC* promoter.

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 674, 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 *sai* 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.

MATERIALS AND 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 plasmid vectors, pVSB1 and pLPV111, were constructed in connection with this work. pVSB1 is a derivative of the broad-host-range vector pVS2 (56), in which the erythromycin resistance gene (*ermC*) has been 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 *Escherichia 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, pVSB1, and derivatives, since *E. coli* DH5α does not support proper replication of these plasmids. pVSB1 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; erythromycin, 10 μg/ml (LAB) and 200 μg/ml (*E. coli*); and chloramphenicol, 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 (Em^r Cm^r) in all experiments. Antibiotics were added as needed depend-

ing on the plasmid(s) introduced. The negative controls were *L. sake* Lb706-X carrying pVS2 and the indicator strain itself. Immunity to sakacin A was tested by using a microtiter plate dilution assay as described previously (4).

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 H_2O 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 directed by the manufacturers (Promega; Boehringer Mannheim, Mannheim, Germany). DNA fragments were isolated from agarose gels by using low-melting-point agarose (SeaPlaque agarose; FMC Bioproducts, Rockland, Maine). The agarose slice containing the fragment was melted at 65°C. The DNA was then extracted by a phenol-freeze method (6) and subsequently purified by using a Magic Clean-up system (Promega). Deletions of plasmids were obtained by the use of available restriction sites. In some cases, the ends were made blunt with a fill-in reaction using deoxynucleotide triphosphates (dNTPs) and the Klenow fragment before ligation. Certain specific deletions were made by a PCR technique (see below and Results). Frameshift mutations were introduced by linearizing plasmids at appropriate restriction sites (which generate protruding ends), flushing the ends with the Klenow fragment plus dNTP, recircularizing the plasmids, and subsequently introducing them into suitable host strains. The desired mutations were confirmed by the absence of the restriction site used and by determining the nucleotide sequence. In general, all manipulations were done in *E. coli*, and the desired constructions were then transferred to various hosts, in most cases to *L. sake* Lb790 or Lb706-X.

Southern and Northern (RNA) analyses. In Southern blotting experiments, DNA was transferred to nylon membranes (Hybond N+; Amersham International, Amersham, United Kingdom) by vacuum blotting as instructed by the manufacturer (Pharmacia, Uppsala, Sweden). DNA fragments used as probes were labelled with [α-^{32}P]dCTP by the random priming reaction (Amersham). Oligonucleotides were labelled with [α-^{32}P]ddATP with a 3′-end-labelling kit (Amersham). Hybridization was performed according to standard procedures (38). For Northern analysis, RNA was prepared from *Lactobacillus* strains as described by Igo and Losick (23), digested with RQ1 RNase-free DNase (Promega) for 2 min at 37°C, and separated in 1% agarose gels containing formaldehyde (38). The RNA was blotted onto nitrocellulose filters (Schleicher & Schuell, Dassel, Germany) and hybridized with labelled probes as above. When necessary, labelled DNA was removed from the filters by prewetting them in 2× SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0]) before incubation in 0.1% sodium dodecyl sulfate for 7 min at 95°C.

Amplification (PCR) reactions. DNA was amplified in 50-μl reactions, using a Perkin-Elmer Cetus Thermal Cycler (Perkin-Elmer Corp., Norwalk, Conn.). The mixtures contained 1 μM each primer, 200 μM each dNTP, 1 ng of template DNA, 2.5 mM $MgCl_2$, 0.8 U of *Taq* DNA polymerase (Promega), and 1× reaction buffer (Promega). Amplification proceeded through 30 cycles after a 3-min hot start (95°C) with a program including denaturation (94°C, 1 min), primer annealing (47°C, 1.5 min), and polymerization (72°C, 2.5 min).

DNA sequencing and sequence analysis. A total of 8,668 bp containing the sakacin A gene cluster was sequenced by the dideoxy-chain termination method (39) directly on denatured plasmid DNA (29). Both strands were sequenced completely. Parts of the sequence reported here have been published previously (4, 20). These fragments and accompanying regions within a 4.6-kb *Pac*I-*Cla*I fragment were sequenced by a primer walking strategy using newly synthesized oligonucleotide primers (Applied Biosystems model 381A synthesizer). The remaining 4.1-kb *Cla*I-*Sph*I fragment was first cloned in pLPV111, then subcloned as a *Cla*I-*Acy*I fragment into the *Cla*I site of pGEM-7Zf(+), and subsequently sequenced by using an Erase-a-Base system (Promega).

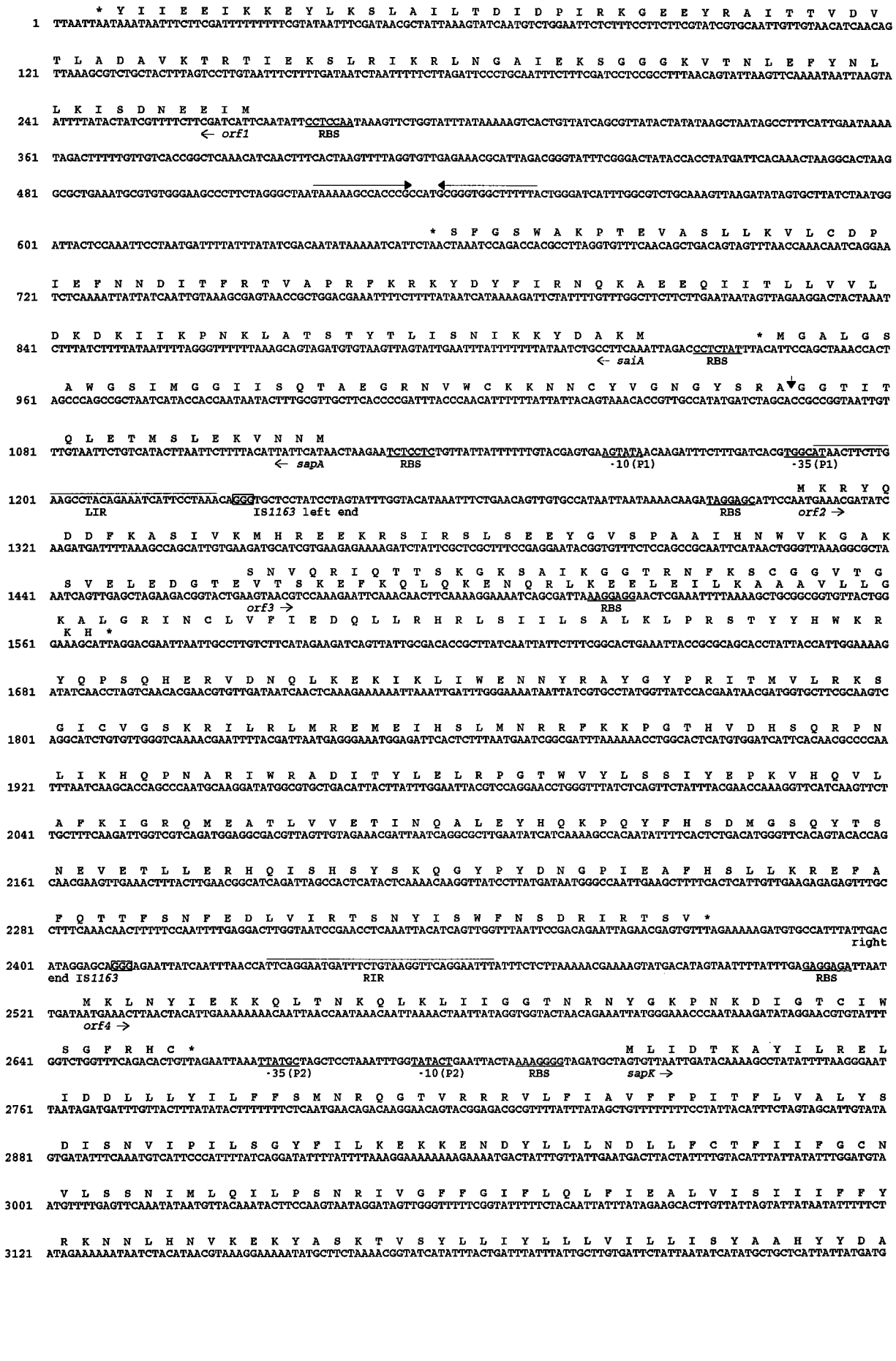
The computer analyses of sequence data were conducted with the DNASIS sequence analysis program (Pharmacia), the PC/Gene program package (IntelliGenetics, Inc., Mountain View, Calif.), and the Genetics Computer Group sequence analysis software package (version 7.2) (14). Databases used in homology searches were GenBank (release 82.0), EMBL (release 39), SWISSPROT (release 29.0), and NBRF-Protein (release 41.0).

Nucleotide accession number. The nucleotide sequence presented in this report was submitted to the EMBL Data Library and was given accession number Z46867.

RESULTS

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 LA100), 5′-TT(T/C)CC(T/C)GAACCACTCATGCC (T/C)ACAATAGT-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 *Sph*I fragment from pLSA60 was found suitable, 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 pVSB1, giving rise to pSAK21. When pSAK21 was transferred to *L. sake* Lb706-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 *Pac*I-*Sph*I fragment, present on plasmid pSAK27, was sequenced in its entirety.

DNA sequence analysis. The complete DNA sequence of the 8,668-bp *Pac*I-*Sph*I 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 (*orf1*) predicts a 87-aa peptide. The remaining seven ORFs are encoded by the opposite strand. The previously reported *sapK* (previously *sakB*) 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, *orf2*, *orf3*, and *orf4*, are positioned upstream of *sapK*. For reasons that will be discussed later, *orf3* is displayed without a proper start codon and an RBS within the coding region. Immediately downstream of *sapK* follows an ORF, designated *sapR*, probably starting with a TTG codon and encoding a protein of 247 aa. *sapR* is followed by *sapT*, starting with an ATG codon and potentially encoding a protein of 719 aa. Finally, an ORF, designated *sapE*, follows immediately downstream of *sapT*. *sapE* starts with an ATG codon and potentially encodes a


orf1
RBS
saiA
RBS
sapA
RBS
-10(P1)
-35(P1)
LIR
IS1163 left end
RBS
orf2
orf3
RBS
right
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RIR
RBS
orf4
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-10(P2)
RBS
sapK

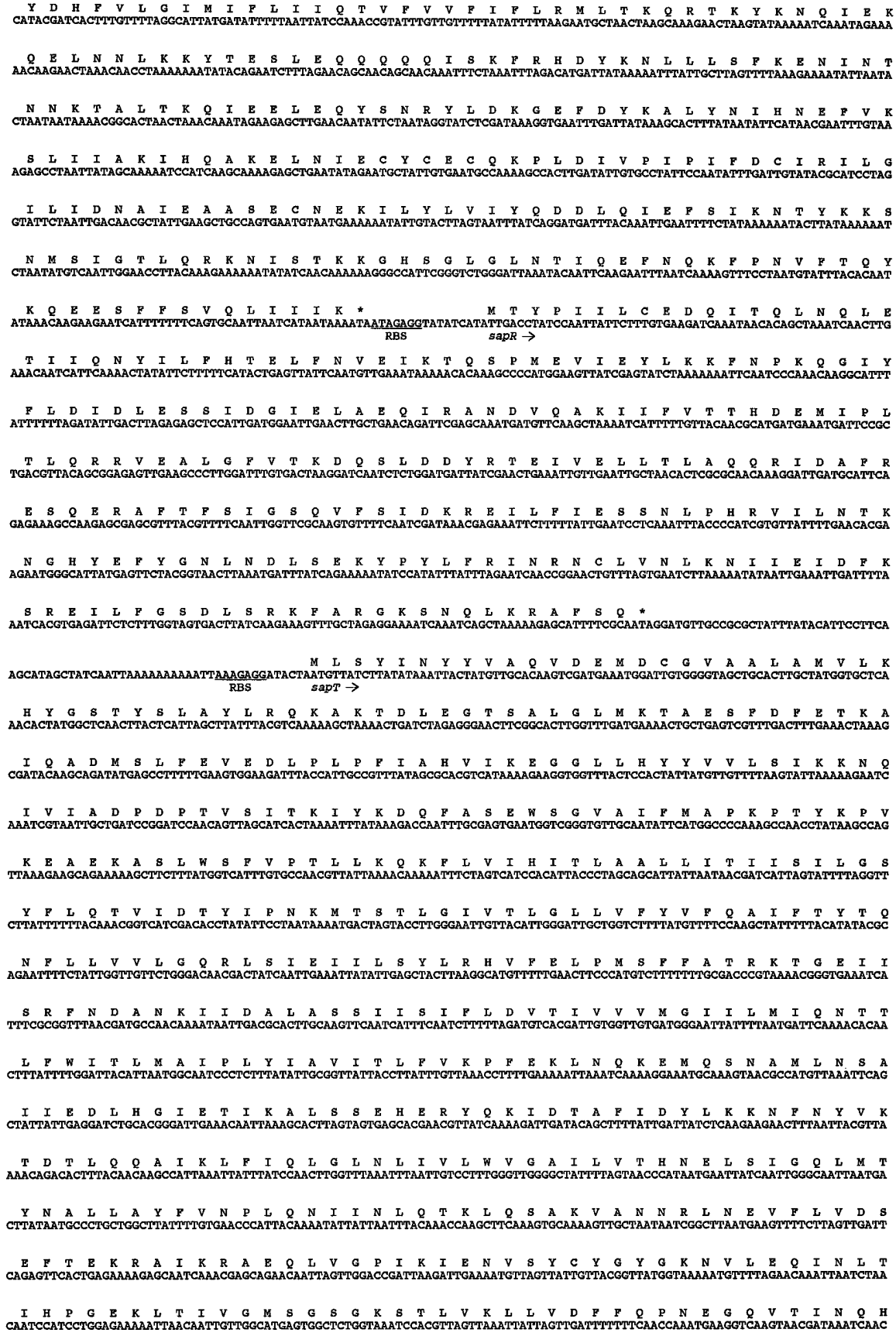

FIG. 1—*Continued*

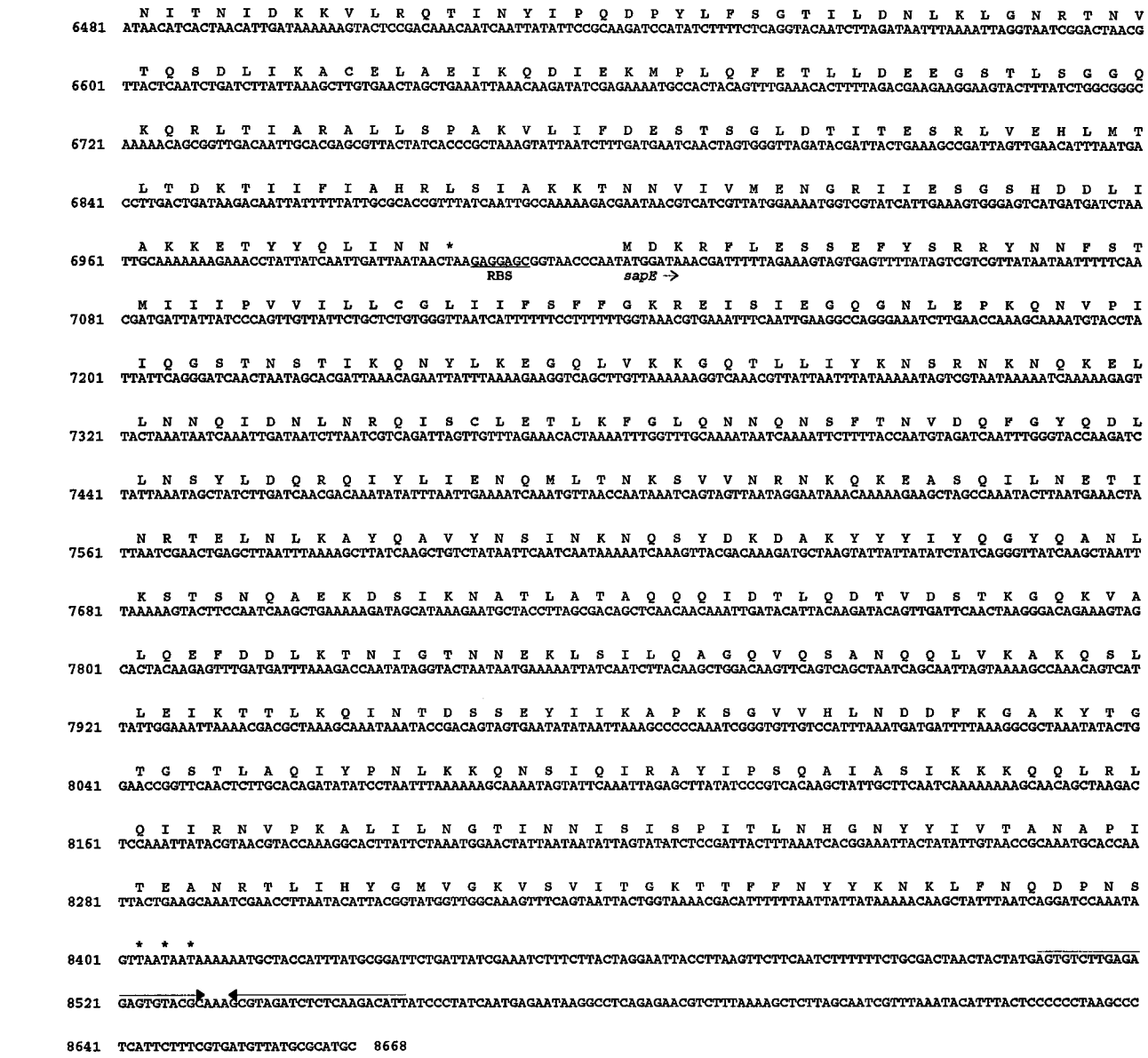

FIG. 1. Nucleotide sequence of the 8,668-bp fragment encompassing the sakacin A gene cluster and deduced amino acid sequences (shown above the nucleotide sequence) of 10 ORFs. Translational termination codons are labeled by asterisks. Predicted −10 and −35 promoter sites of two promoters (P1 and P2) as well as putative RBSs are underlined. Inverted repeat regions LIR and RIR are overlined. The sakacin A processing site (20) is indicated by a downward-directed arrow. Horizontal arrows indicate regions of dyad symmetry. The duplicated GGG sequences at the ends of IS*1163* are boxed. The sequences from coordinates 883 to 1161 and 2247 to 4056 have been reported previously (4, 20) with accession numbers Z14233 and Z21855, respectively.

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 sequence thus indicates the presence of two divergently transcribed operons: one encompassing *sapA* and *saiA*, and the other encompassing *sapKRTE*. 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 (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 between positions 1230 and 2409 was completely identical to IS*1163*, a newly described 1,180-bp insertion element from *L. sake* (43). IS*1163* 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 IS*1163*. IS*1163* 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 IS*1163* sequence. An RBS and a translational frameshift window (5′-TTTAAAA-3′) are present in the cod-

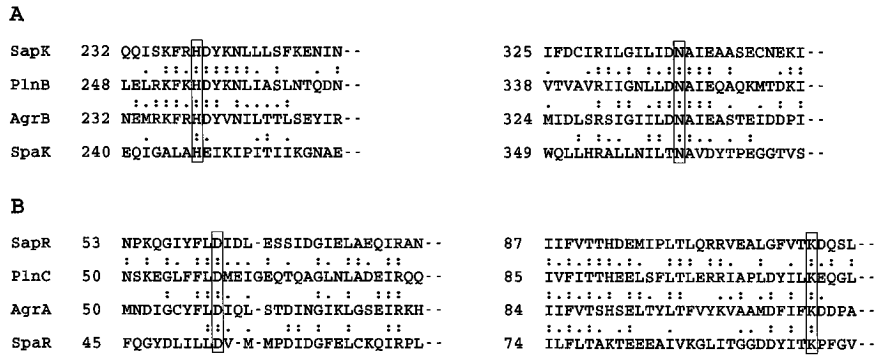

FIG. 2. Protein homology in conserved regions of SapK and SapR to analogous proteins in the plantaricin A (PlnB/PlnC [10]), *agr* (AgrB/AgrA [28]), and subtilin (SpaK/SpaR [27]) systems, respectively. Double and single dots indicate identical and similar amino acid residues, respectively. (A) Homology 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).

ing region of *orf3*. This is typical for members of the IS*3* family and probably plays a role in a translational frameshift, creating a fusion protein coded by both *orf2* and *orf3* (43). Hybridization experiments with chromosomal DNA from the plasmid-free strain *L. sake* Lb706-X and an IS*1163*-specific probe suggested that IS*1163* is present in at least two copies in the Lb706 chromosome (not shown).

Nearly 100% DNA homology was found with the published 836-bp DNA sequence encompassing the structural gene (*curA*) for curvacin A, a bacteriocin produced by *Lactobacillus curvatus* LTH1174 and shown to be identical with sakacin A (49). The region from positions 3 to 836 in the *curA* sequence is equivalent to positions 2551 to 532 (Fig. 1, complementary strand) in the sequence reported here, but the homology is interrupted by IS*1163*, not present in the *curA* sequence. The duplicated sequence GGG present outside the ends of IS*1163* is present only once in the *curA* sequence. Furthermore, the *curA* sequence is missing two bases compared with the sequence in Fig. 1 at positions 815 and 829. This leads to a different interpretation of the ORF downstream of the bacteriocin structural gene, resulting in no amino acid homology between the putative *orfB* product (*curA* sequence) and the putative SaiA peptide.

Significant DNA sequence similarity, approximately 60%, was also noted between the region encompassing *sapT* and genes likely to encode ABC exporters such as *pedD* (32), *lcnC* (45), and *comA* (22) (see below).

Protein homology. The homology of sakacin A to other members of the pediocin family has been established (20, 25). The bacteriocin most similar to sakacin A (besides the 100% identical curvacin A) is the recently reported carnobacteriocin BM1 produced by *Carnobacterium piscicola* LV17B (34). There are 13 amino acid differences between the mature 41-aa sakacin A and the 43-aa carnobacteriocin BM1. As reported previously, SapK (previously designated SakB) shows similarity to the histidine protein kinase (HPK) family, in particular to AgrB, a *Staphylococcus aureus* protein (28, 44). Similarly, the 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

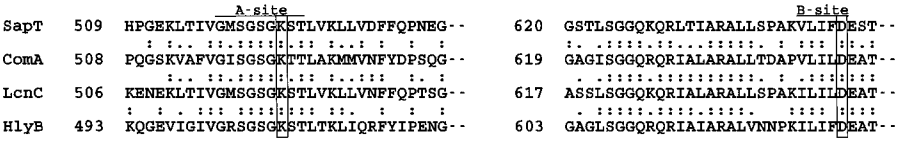

FIG. 3. Protein homology of SapT to members of the ABC exporter family in regions corresponding to the A and B sites, constituting the ATP-binding motif (11). Double and single dots indicate identical and similar amino acid residues, respectively. The highly conserved lysine (A-site) and aspartate (B-site) residues are boxed.

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, which is suggested to be a transmembrane region (not shown).

The only significant similarity of the putative SaiA peptide to other proteins was to the deduced amino acid sequence of *ORF*-α2, an ORF located downstream the structural gene for carnobacteriocin BM1 (34). The amino acid identity between the 88-aa *ORF*-α2 product and the 90-aa SaiA was 49%.

No homologies to known sequences were found for the putative *orf1* and *orf4* products when the peptides were used in standard homology searches. However, it was noted that both contained a glycine doublet at positions 21 and 22, indicating possible similarities with bacteriocin leader peptides. The first 22 aa of the peptides were compared with some of the known leaders of the double-glycine type (18). Both of these peptides shared some signatures with bacteriocin leader sequences (not shown). In particular, the similarity of the putative leader of the *orf4* peptide to the plantaricin A leader sequence was highly significant (45% amino acid identity, 68% similarity).

Mutations and deletions in the sakacin A gene cluster. The 8,668-bp *Pac*I-*Sph*I fragment is presented as a restriction map including the features described above (Fig. 4). In order to assign some role in sakacin A production and/or immunity to the different genes, a scheme of deletions and introduction of frameshift mutations was used. In these experiments, *L. sake* Lb790 was used instead of Lb706-X as the host strain for the various plasmid constructions since it is generally an easier strain to work with (e.g., no slime production, higher transformation efficiencies, and higher sensitivity to sakacin A). However, plasmids pSAK27, pSAK26, pSAK22, pSAK21N, pSAK21A, and pSAK15 were also introduced into *L. sake* Lb706-X, confirming the results obtained for Lb790. In some cases, two plasmids were introduced, each containing different parts of the gene cluster. Deletion of *orf1* (plasmid pSAK26) did not alter the Sap^+ Imm^+ phenotype, indicating that this gene is unnecessary. Further deletion at this end, including the removal of *sapA* and *saiA* (pSAK20), abolished both sakacin A production and immunity. As previously reported, a mutation in *sapK* resulted in a Sap^- Imm^- phenotype (4). A mutation in *sapR* (pSAK21N) also abolished both production and immunity. However, a mutation in *sapT* (pSAK21A), a deletion of the 3′ end of *sapE* (pSAK15), or deletion of both *sapT* and *sapE* (pSAK22) only abolished sakacin A production, but the derivative strains were still immune to the bacteriocin. A construction containing only *sapA* and *saiA* (pSAK17B) resulted in a Sap^- Imm^- phenotype. The result obtained with pSAK22 (Imm^+ phenotype) was contradictory to a previously reported result with a similar plasmid construction (4). Upon reinvestigation, this construction gave an Imm^+ phenotype, supporting the result obtained with pSAK22. The initial result remains unexplained.

Immunity could be expressed without the need for *sapK* and *sapR* if the *sapA-saiA* region was cloned behind another promoter. This was done in plasmid pSAK31, in which the −35 region of P1 is missing, and thus *sapA* and *saiA* are under control of the *ermC* promoter present in pVS2. This promoter is known to be utilized since pVS2 confers erythromycin resistance in *L. sake*. In a similar cloning experiment, pSAK30 was constructed. pSAK30 contains an intact *sapA* gene and a deleted *saiA* gene in such a way that theoretically the last 12 aa of the 90-aa SaiA peptide are missing. The only difference between pSAK31 and pSAK30 is the deletion in the *saiA* gene. The fact that pSAK31 conferred immunity to sakacin A and pSAK30 did not strongly suggests a role of the SaiA peptide in the immunity to sakacin A.

The two operons could be separated and present on two plasmids. pSAK20 (*sapKRTE*) 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 IS*1163* 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 *sapKRTE* operon, but detailed analysis was hindered by the difficulties in detecting it. If *sapKRTE* is transcribed as one polycistronic 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 *Acy*I site is located at position 564; Fig. 1) downstream of *saiA*. 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

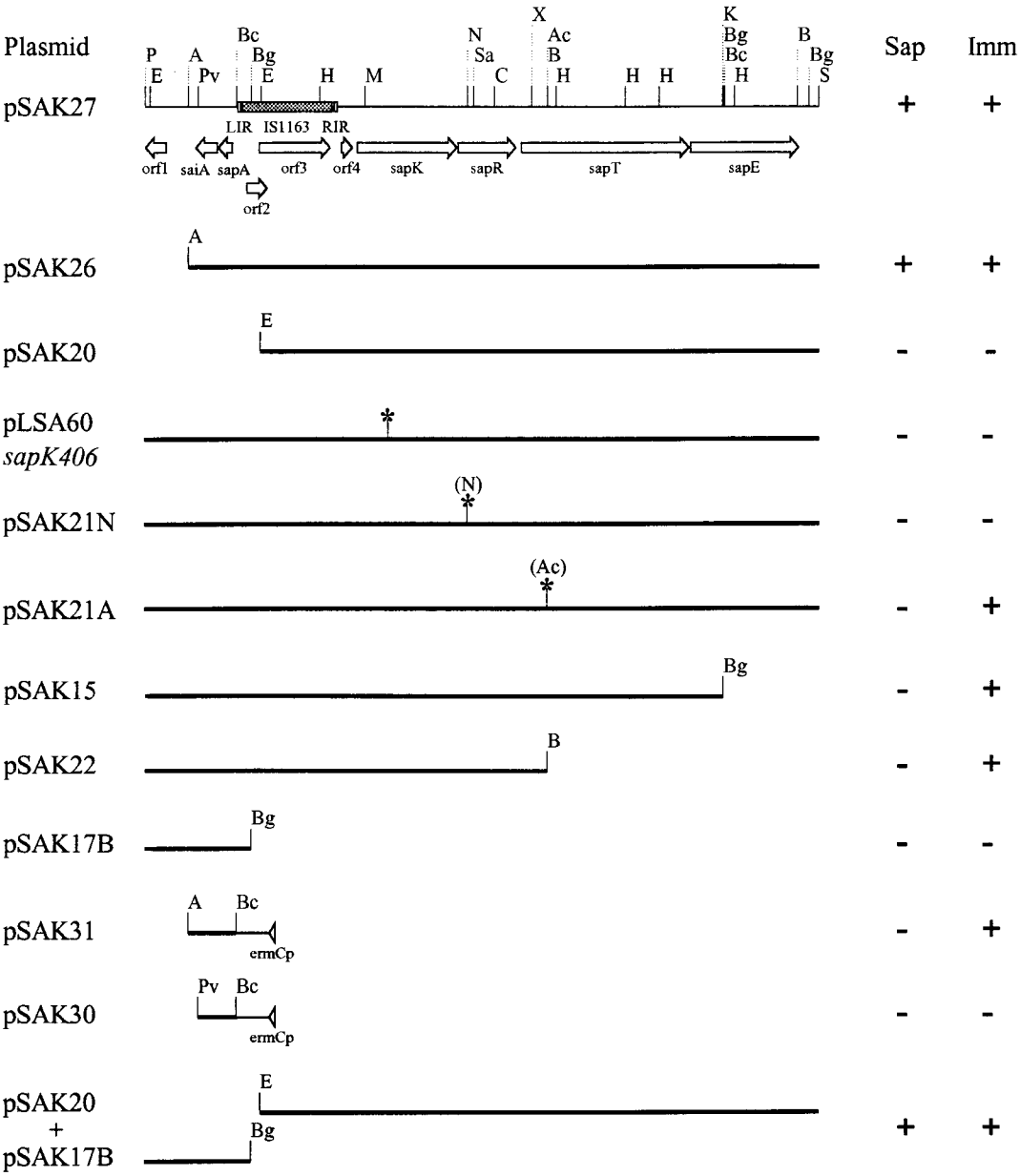

FIG. 4. Linear restriction map of the 8,668-bp sequenced region encompassing the sakacin A gene cluster and structures of derived deleted and frameshift-mutated constructs. The organization and direction of the 10 ORFs are indicated by arrows. Shaded box indicate IS*1163*. The small open boxes outside IS*1163* represent the inverted repeat regions LIR and RIR. Below the restriction map are the structures of the different deletion and frameshift-mutated derivatives. pLSA60*sapK406* refers to the *sapK* mutation in pLSA60 (4). The restriction enzyme sites used in the construction of these derivatives are indicated. Restriction sites in brackets indicate the sites used for creating frameshift mutations (see Materials and Methods). *, frameshift mutation. The plasmid names are shown on the left, and the phenotypes when the plasmids were introduced into *L. sake* Lb790 are shown on the right. The arrowhead with the designation ermCp indicates the direction of the *ermC* promoter present in these constructions. Thin lines in pSAK31 and pSAK30 represent pVS2 sequences. Enzyme abbreviations: A, *Acy*I; Ac, *Acc*III; B, *Bam*HI; Bc, *Bcl*I; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; M, *Mlu*I; N, *Nco*I; P, *Pac*I; Pv, *Pvu*II; S, *Sph*I; Sa, *Sac*I; X, *Xba*I.

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

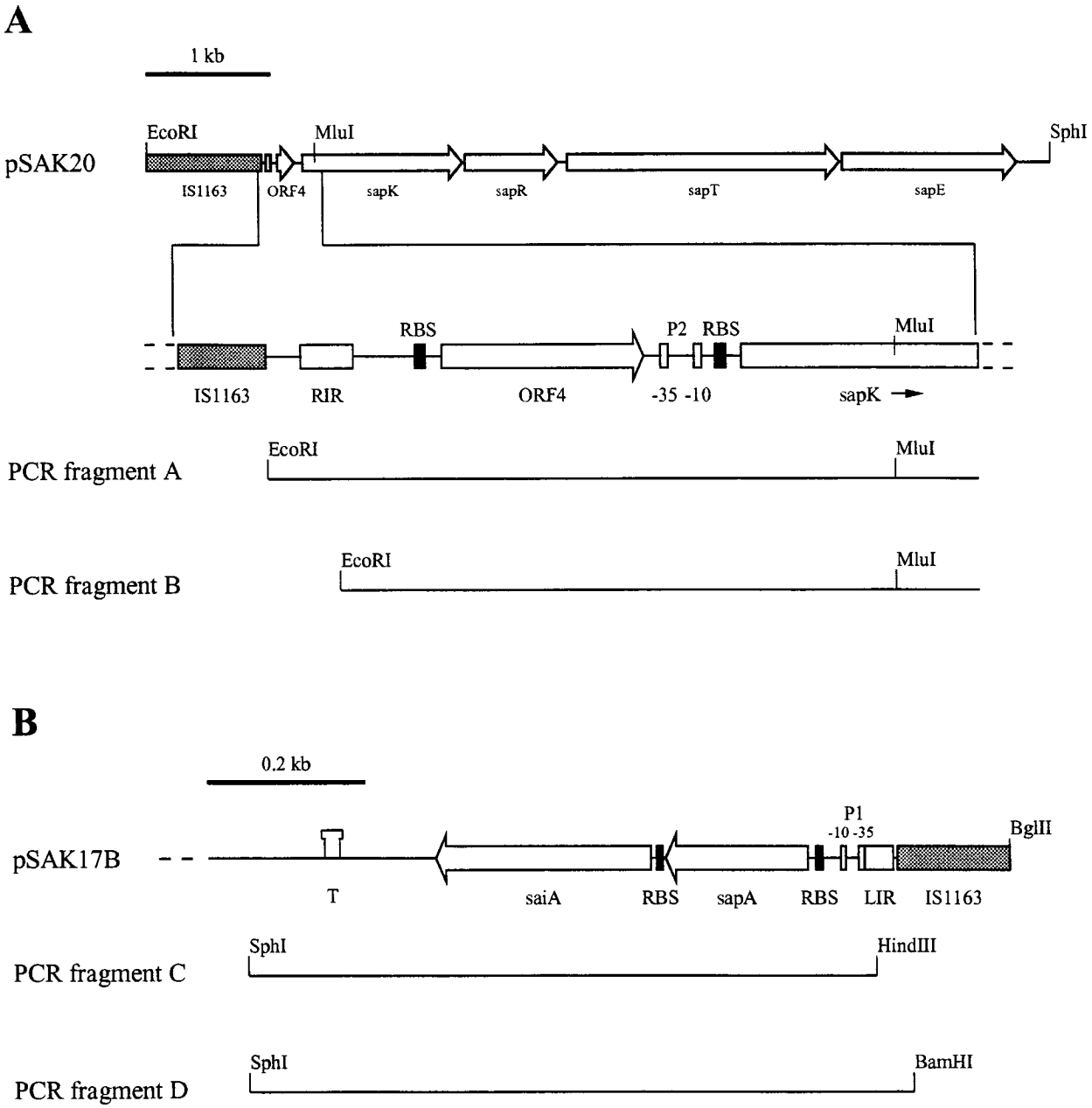

FIG. 5. Construction of deletion derivatives of pSAK20 (A) and pSAK17B (B) by PCR amplification. Primers were designed so as to introduce suitable restriction sites at the ends of the fragments. The enlarged fragment of pSAK20 represents 0.53 kb. PCR fragments A and B were subsequently digested with *Eco*RI plus *Mlu*I and cloned in *Eco*RI-*Mlu*I-digested pSAK20, thus replacing the original 1.35-kb *Eco*RI-*Mlu*I fragment and creating pSAK20A and pSAK20B. PCR fragments C and D were digested with *Sph*I plus *Hin*dIII and *Sph*I plus *Bam*HI, respectively, and cloned in pLPV111, 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.

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 *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 *S. aureus* (28). The *agr* locus consists of two divergently transcribed operons: one encompassing *agrDCBA* and

TABLE 2. Phenotype of *L. sake* Lb790 containing different pSAK20 and pSAK17 derivatives

Plasmid(s)[a]	Phenotype	
	Sap	Imm
pSAK17B (LIR)	−	−
pSAK20 (RIR)	−	−
pSAK17B (LIR) + pSAK20 (RIR)	+	+
pSAK17B (LIR) + pSAK20A (RIR)	+	+
pSAK17B (LIR) + pSAK20B (ΔRIR)	−	+
pSAK17C (ΔLIR) + pSAK20 (RIR)	−	−
pSAK17D (LIR) + pSAK20 (RIR)	+	+

[a] 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-lysin 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

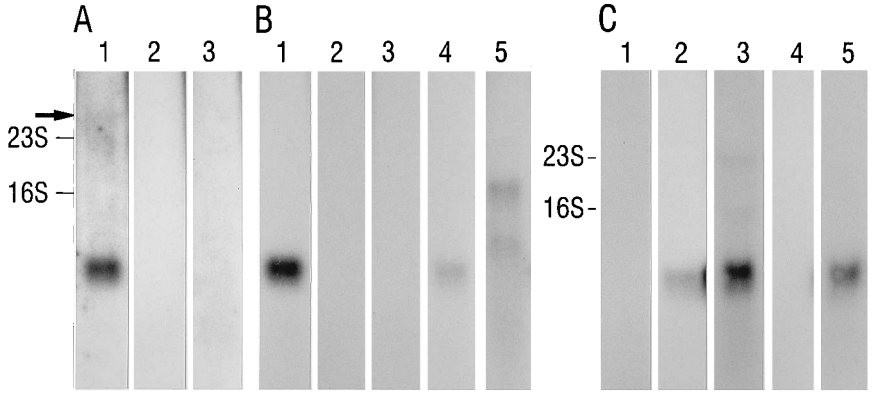

FIG. 6. Northern blot analysis of RNA prepared from *L. sake* strains. (A) *saiA sapA orf4 sapKRTE* probe. Lane 1, Lb706; lane 2, Lb706-X; lane 3, Lb706-B. (B) *saiA sapA* probe. Lanes 1 to 3, same as in panel A; lane 4, Lb706-X(pSAK27); lane 5, Lb706-X(pSAK26). (C) *saiA sapA* probe. Lane 1, Lb790; lane 2, Lb790(pSAK21); lane 3, Lb790(pSAK17D, pSAK20); lane 4, Lb790(pSAK17C, pSAK20); lane 5, Lb790(pSAK17B, pSAK20B). 23S and 16S indicate the migration of these rRNA molecules (approximate sizes, 2,900 and 1,540 nucleotides, respectively). The arrow indicates the presence of a weak signal at an estimated size of 4,000 to 5,000 nucleotides in lane 1 of panel A (see text).

SapK/SapR system, thus resembling the situation with P1 and LIR. The weak, constitutive transcription mentioned above could be mediated by P2. This model is analogous to the *agrDCBA* operon, which also has a weak and a strong promoter, the latter being activated when the system is triggered (28). Glucose concentration and pH have been shown to affect the *agr* system in *S. aureus* (36, 37), but nothing is known regarding the signal that turns on the sakacin A system. Apparently, the signal is present from the beginning in standard media since sakacin A is produced throughout growth in liquid culture (20).

Small ORFs, encoding peptides of 50 to 150 aa, are often found downstream the structural genes of class II bacteriocins (7, 12, 17, 32, 34, 45, 49, 50, 52–54). These peptides have repeatedly been suggested to be immunity factors, needed for self-protection against the bacteriocin. However, only in a few cases have these genes been clearly correlated with the immunity phenotype. The best studied is perhaps the lactococcin A immunity factor, which is encoded by *lciA* located downstream of the bacteriocin gene *lcnA*. The 98-aa LciA 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 *saiA* in the immunity to the bacteriocin. The SaiA peptide does not display any significant sequence similarity with LciA. However, both SaiA 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 *saiA* abolish both production and immunity. These somewhat surprising results indicate that production requires expression of *saiA* in addition to *sapA*, and immunity requires expression of *sapA* in addition to *saiA*. 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 LafA and LafX 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 IS*1163*, located between the operons, does not seem to be involved in the expression of the *sap* genes. The presence of IS*1163* 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 IS*1163*. Although limited, the sequence information available upstream the curvacin A structural gene also indicates that the gene cluster normally is devoid of IS*1163*.

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/RR 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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