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Cloning and Nucleotide Sequence of a Gene from *Lactobacillus sake* Lb706 Necessary for Sakacin A Production and Immunity

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Sakacin A is an antilisterial bacteriocin produced by *Lactobacillus sake* Lb706. In order to identify genes involved in sakacin A production and immunity, the plasmid fraction of *L. sake* Lb706 was shotgun cloned directly into a sakacin A-nonproducing and -sensitive variant, *L. sake* Lb706-B, by using the broad-host-range vector pVS2. Two clones that produced sakacin A and were immune to the bacteriocin were obtained. A DNA fragment of approximately 1.8 kb, derived from a 60-kb plasmid of strain Lb706 and present in the inserts of both clones, was necessary for restoration of sakacin A production and immunity in strain Lb706-B. The sequence of the 1.8-kb fragment from one of the clones was determined. It contained one large open reading frame, designated *sakB*, potentially encoding a protein of 430 amino acid residues. Hybridization and nucleotide sequence analyses revealed that the cloned *sakB* complemented a mutated copy of *sakB* present in strain Lb706-B. The *sakB* gene mapped 1.6 kb from the previously cloned structural gene for sakacin A (*sakA*) on the 60-kb plasmid. The putative SakB protein shared 22% amino acid sequence identity (51% similarity if conservative changes are considered) to AgrB, the deduced amino acid sequence of the *Staphylococcus aureus* gene *agrB*. The polycistronic *agr* (accessory gene regulator) locus is involved in the regulation of exoprotein synthesis in *S. aureus*. Similar to the AgrB protein, SakB had some features in common with a family of transmembrane histidine protein kinases, involved in various adaptive response systems of bacteria. This similarity included transmembrane regions in the N-terminal half of the protein and certain conserved amino acid residues.

Members of a physiologically related group of gram-positive bacteria, known as the lactic acid bacteria (LAB), are of great industrial importance, mainly in food fermentation processes (6, 9, 23). Bacteriocins of LAB have become a major area of study in the search for new and safe food preservatives. Bacteriocins are proteinaceous compounds produced by bacteria, typically inhibiting the growth of strains closely related to the producer strain (34). Certain bacteriocins produced by LAB also affect more distantly related species (17). Interestingly, many of these are potent antimicrobial agents against *Listeria monocytogenes*, a foodborne pathogen which has received a lot of attention recently. Bacteriocins and bacteriocin-producing strains are therefore considered useful for the control of *Listeria monocytogenes* in food processing. Basic knowledge about bacteriocins, such as structure, mode of action, genetic determinants, and regulation of expression, etc., is needed to exploit the potential of these antimicrobial agents in future applications. LAB bacteriocins are often heat-stable, small polypeptides with an M_r of 3,000 to 6,000, and their genes are frequently plasmid encoded. The best known is the lantibiotic nisin, which is produced by strains of *Lactococcus lactis* subsp. *lactis*. The structure of nisin was determined in 1971 (11), but some of the genetic determinants necessary for its production have been elucidated only recently (5, 8, 16). The amino acid sequences of a number of nonlantibiotic bacteriocins from LAB have now been determined, but only two, lactococcin A (produced by *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*) and pediocin PA-1 (produced by *Pediococcus acidilactici*), have been studied thoroughly with regard to the genes necessary for production and immunity (21, 33). Although the amino acid sequences of lactococcin A and pediocin PA-1 are quite different, the molecular analyses of the genes involved in production and immunity have revealed some striking similarities. First, the structural gene encodes a precursor peptide (a prebacteriocin) which is cleaved adjacent to a glycine doublet to yield the mature bacteriocin. This is true also for other LAB bacteriocins, e.g., lactacin F (24) and leucocin A-UAL 187 (12). Second, the genes necessary for bacteriocin production (and immunity in the case of lactococcin A) are clustered in an operon-like structure, and third, one of the genes in the operon encodes a protein which belongs to the HlyB family of ATP-dependent membrane translocators (3). Since the leader peptides of the prebacteriocins do not resemble normal signal sequences, these results imply that signal sequence-independent secretion systems, similar to the *Escherichia coli* hemolysin secretion system, are involved in processing and transport of lactococcin A and pediocin PA-1.

Sakacin A is an antilisterial bacteriocin produced by *Lactobacillus sake* Lb706 (31). It is a nonlantibiotic bacteriocin consisting of 41 amino acid residues with an M_r of 4,309. The amino acid sequence shows strong similarities in the N-terminal part to pediocin PA-1 and other antilisterial LAB bacteriocins (13). It has been suggested that a consensus sequence (Tyr-Gly-Asn-Gly-Val-Xaa-Cys) near the N terminus may be important for the activity of this group of

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TABLE 1. Bacterial strains

Strain	Remarks[a]	Reference
Lactobacillus sake		
Lb706	Sak$^+$ Imm$^+$, 60- and 28-kb plasmids	Schillinger and Lücke (31)
Lb706-B	Sak$^-$ Imm$^-$, 60-kb plasmid	Schillinger and Lücke (31)
Lb706-B2	Sak$^+$ Imm$^+$, Lb706-B with plasmid pLSA2	This work
Lb706-B24	Sak$^+$ Imm$^+$, Lb706-B with plasmid pLSA24	This work
Lb706-B2401	Sak$^-$ Imm$^-$, Lb706-B with plasmid pLSA2401	This work
Lb706-B2402	Sak$^-$ Imm$^-$, Lb706-B with plasmid pLSA2402	This work
Lb706-B2403	Sak$^+$ Imm$^+$, Lb706-B with plasmid pLSA2403	This work
Lb706-B2404	Sak$^+$ Imm$^+$, Lb706-B with plasmid pLSA2404	This work
Lb706-B2405	Sak$^+$ Imm$^+$, Lb706-B with plasmid pLSA2405	This work
Lb706-S	Sak$^+$ Imm$^+$, 60-kb plasmid	This work
Lb706-X	Sak$^-$ Imm$^-$, plasmid free	This work
Lactobacillus plantarum NC8	Sak$^-$ Sak^r, efficiently transformable, plasmid free	Aukrust and Blom (2)
Lactobacillus brevis 2.14	Sak$^-$ Imm$^-$, indicator strain for sakacin A	Holck et al. (13)
Lactococcus lactis LM0230(pVS2)	Source of the vector pVS2, conferring resistance to EM and CM	von Wright et al. (38)

[a] Sak$^+$ and Sak$^-$, sakacin A producer and nonproducer, respectively; Imm$^+$ and Imm$^-$, immune and sensitive to sakacin A, respectively; Sak^r, resistance to (tolerance for) sakacin A by a mechanism that may be different from the immunity system of the producer strain.

bacteriocins (25). It has been further suggested that sakacin A production and immunity are associated with a 28-kb plasmid (31), but the structural gene (*sakA*) was shown to be located on a 60-kb plasmid (13). Similar to lactococcin A, pediocin PA-1, and other LAB bacteriocins, the structural gene encodes a precursor peptide which is cleaved adjacent to a glycine doublet to yield the active sakacin A molecule (13).

Here we report the identification and nucleotide sequence of a gene from *L. sake* Lb706 necessary for both production of and immunity to sakacin A. The putative protein encoded by this gene has some features in common with a family of transmembrane histidine protein kinases involved in adaptive response systems of bacteria. Furthermore, this study provides an example of the feasibility of using exclusively *Lactobacillus* strains as hosts in routine genetic and molecular analyses.

MATERIALS AND METHODS

Bacterial strains, plasmid vector, and growth conditions. The bacterial strains are listed in Table 1. The broad-host-range plasmid pVS2 (38), conferring erythromycin (EM) and chloramphenicol (CM) resistance, was used as cloning vector. Cloning in the single *Bcl*I site inactivates the EM resistance gene. Unless otherwise stated, *Lactobacillus* strains were grown in MRS (Difco Laboratories, Detroit, Mich.) or on MRS agar at 30°C. *Lactococcus lactis* subsp. *lactis* LM0230(pVS2) was grown at 30°C in M17 (35) supplemented with 0.5% glucose. CM (10 μg/ml) was included in the growth medium for the selection of pVS2 or constructions thereof.

Sakacin A production and immunity screening. Colonies from curing experiments were tested for sakacin A production (Sak$^+$ phenotype) by the spot test on BSM agar (36) with the sakacin A-sensitive *Lactobacillus brevis* 2.14 as an indicator. CM-resistant transformants from cloning and deletion analysis experiments were screened for sakacin A production by the same method, but CM (10 μg/ml) was included in the BSM agar and *L. sake* Lb706-B or *L. brevis* 2.14 transformed with pVS2 was used as the indicator. Sakacin A production was also confirmed by assessing the heat tolerance, trypsin sensitivity, and inhibitory spectrum of the inhibitory compounds produced by derivative strains and comparing these characteristics with those of sakacin A produced by *L. sake* Lb706 (31). Immunity to sakacin A (Imm$^+$ phenotype) was assayed by a microtiter plate method with either a pH-adjusted (pH 6.5), sterile-filtered culture broth from *L. sake* Lb706 or a concentrate after ammonium sulfate precipitation as the source of bacteriocin (13).

Plasmid curing. The MIC of novobiocin for *L. sake* Lb706 was determined to be 25 to 30 μg/ml. To induce curing of plasmids, *L. sake* Lb706 was grown for approximately 40 generations by repeated subculturing in MRS containing 20 μg of novobiocin per ml. A sample from the last culture was diluted and spread onto MRS agar. Fifty colonies were picked at random and checked for plasmid content and bacteriocin production.

Plasmid DNA isolation, transformation, and shotgun cloning. An alkaline lysis method (29) was used to enrich for plasmid DNA. The lysis step was modified by the addition of lysozyme and mutanolysin at 20 mg/ml and 50 U/ml, respectively, to the lysis buffer (solution I) followed by an incubation period (20 to 60 min, depending on the strain) at 37°C. Plasmids were purified further by CsCl-ethidium bromide buoyant density gradient centrifugation or by use of the Magic Miniprep resin and columns (Promega Corp., Madison, Wis.). *Lactobacillus* strains were transformed by electroporation according to the optimized procedure described by Aukrust and Blom (2). Briefly, the strains were grown overnight in MRS without glucose, inoculated to an optical density at 600 nm of 0.25 in MRS plus 1% glycine, and incubated at 30°C. At an optical density at 600 nm of 0.6, the cells were harvested, washed once with 1 mM $MgCl_2$ (100 mM $MgCl_2$ for *L. sake* Lb706-B) and once with 30% polyethylene glycol 1500, and resuspended in 1/100 culture volume of 30% polyethylene glycol 1500. Forty microliters of the cell suspension and 0.1 to 2.5 μg of DNA in 2 to 5 μl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) were added to a 0.2-cm cuvette for each transformation. Pulses were applied at settings of 1.5 kV, 400 Ω, and 25 μF (Gene Pulser and Pulse Controller; Bio-Rad Laboratories, Richmond, Calif.). Shotgun cloning was performed as follows. The plasmid DNA fraction of *L. sake* Lb706 was partially digested with *Sau*3AI to an average fragment size of 2 to 5 kb. This DNA (1.5 μg) was ligated to 0.8 μg of *Bcl*I-digested, dephosphorylated pVS2 in a volume of 20 μl. The ligated DNA was precipitated with ethanol, washed three times with 70% ethanol to remove salts, and resuspended in 5 μl of TE

buffer. The DNA was used to transform *L. sake* Lb706-B. CM-resistant transformants were screened for bacteriocin production by replica plating and the spot colony test with *L. sake* Lb706-B(pVS2) as the indicator strain.

General molecular cloning techniques. Standard procedures for molecular cloning were used according to the directions of Sambrook et al. (29). Enzymes (restriction enzymes, T4 DNA ligase, Klenow fragment, and calf intestine phosphatase) were used according to the manufacturer's directions (Promega). Deletions of the recombinant plasmids pLSA2 and pLSA24 were obtained by the use of available restriction enzyme sites in the inserts and in the vector. In some cases the ends were made blunt with a fill-in reaction using deoxynucleotide triphosphates and the Klenow fragment before ligation. All DNA manipulations were done in *Lactobacillus plantarum* NC8, and the desired plasmid constructions were then used to transform *L. sake* Lb706-B. In Southern blotting experiments, DNA was transferred to nylon membranes (Hybond N^+; Amersham International, Amersham, United Kingdom) by vacuum blotting according to the manufacturer's instructions (Pharmacia, Uppsala, Sweden). DNA fragments used as probes were purified from agarose gels with DEAE-nitrocellulose membranes (29) and labelled with [α-^{32}P]dCTP by the random-priming reaction (Amersham). Oligonucleotide probes were labelled with [α-^{32}P]ddATP with a 3'-end-labelling kit (Amersham).

DNA sequencing and sequence analysis. The nucleotide sequences of the *Hin*dIII-*Bgl*II fragment of pLSA2404 and parts of the corresponding region of pLSA2 containing *sakB* were obtained by the use of the dideoxy chain termination method (30) directly on denatured plasmid DNA (20). Sequencing reactions were initiated with primers derived from the known sequence of the EM resistance gene of pVS2 (pE194 [14]). Subsequent sequencing was done by a primer-walking strategy. Oligonucleotides were synthesized on an Applied Biosystems synthesizer, model 381A. Both strands of the *Hin*dIII-*Bgl*II fragment were sequenced.

Fragments encompassing the mutated *sakB* gene present in *L. sake* Lb706-B were amplified by the polymerase chain reaction. The polymerase chain reaction-amplified fragments were subsequently sequenced by the *fmol* DNA sequencing system (Promega) employing [γ-^{33}P]ATP-labelled synthetic oligonucleotide primers.

The computer analyses of sequence data were performed on an IBM personal computer employing the DNASIS sequence analysis program (Pharmacia) and the PC/Gene program package (IntelliGenetics, Inc., Mountain View, Calif.) and on a microVAX 3400 computer employing the Genetics Computer Group program package (7).

Nucleotide sequence accession number. The nucleotide sequence of the 1,810-bp fragment containing *sakB* was submitted to the EMBL Data Library and was given the accession number Z21855.

RESULTS

Shotgun cloning. The transformation of *L. sake* Lb706-B with the ligation mixture (*Sau*3AI fragments from plasmids of *L. sake* Lb706 and *Bcl*I-digested and dephosphorylated pVS2) resulted in approximately 10^3 CM-resistant transformants per μg of DNA. This was 2 orders of magnitude lower than with supercoiled vector (pVS2). Of approximately 1,000 screened transformants, 2 produced inhibitory zones with the spot colony test. Further characterization showed that the inhibitory compounds produced were heat tolerant and inactivated by trypsin and had the same inhibitory spectrum as the bacteriocin from *L. sake* Lb706, indicating that these transformants, designated *L. sake* Lb706-B2 and Lb706-B24, produced sakacin A. In addition, Lb706-B2 and Lb706-B24 were immune (Imm^+) to sakacin A produced by Lb706.

Characterization of plasmids. Purification of the recombinant plasmids from the two transformants was simplified by electrotransformation of the plasmid-free *L. plantarum* NC8 with plasmid minipreparations from Lb706-B2 and Lb706-B24 with selection for CM resistance. The *L. plantarum* transformants contained only single plasmids, designated pLSA2 (from Lb706-B2) and pLSA24 (from Lb706-B24). To confirm that the plasmids contained information which restored sakacin A production and immunity and to rule out possible rearrangements during passage through another host, pLSA2 and pLSA24 prepared from *L. plantarum* NC8 were retransformed into *L. sake* Lb706-B. All transformants tested were Sak^+ and Imm^+.

pLSA2 and pLSA24, containing inserts of 4.3 and 4.7 kb, respectively, were characterized by restriction enzyme mapping. The restriction maps of the inserts were somewhat different but indicated a common internal segment of about 2 kb. Figure 1 shows the restriction map of the insert of pLSA24, the deletions that were obtained, and their effect on sakacin A production and immunity when the resulting plasmids were introduced into Lb706-B. It was concluded that the 1.8-kb *Hin*dIII-*Bgl*II fragment of pLSA24 (construction pLSA2405) was the minimal fragment required for the Sak^+ Imm^+ phenotype in Lb706-B. Similar results were obtained with pLSA2 (not shown), and sequence analysis (see below) later revealed that pLSA2 indeed contained an identical fragment.

Nucleotide sequence of the DNA fragment restoring sakacin A production and immunity. The nucleotide sequence of the 1,810-bp *Hin*dIII-*Bgl*II fragment from pLSA2404 is shown in Fig. 2. One large open reading frame (ORF) was evident, potentially encoding a protein of 430 amino acid residues with a molecular mass of 50.5 kDa. The derivatives, pLSA2401 and pLSA2402 (Fig. 1), containing deletions in the ORF, failed to restore the Sak^+ Imm^+ phenotype to strain Lb706-B. This ORF, designated *sakB*, was therefore responsible for the restoration of sakacin A production and immunity in this strain. A potential promoter region with similarity to the extended consensus gram-positive strain promoter, including the -15 (TG) and -45 (A-rich) regions (10), was located upstream of *sakB*. A putative ribosome binding site was located 2 bp upstream the start codon ATG. The third triplet of *sakB*, GTG, also a potential start codon (19), may be the actual start codon for *sakB*, considering the normal spacing of 8 to 10 bp between ribosome binding sites and start codons.

Location of *sakB* and plasmid curing. *L. sake* Lb706 contains two plasmids of approximately 60 and 28 kb (31). Results of earlier curing experiments suggested that the 28-kb plasmid was associated with sakacin A production and immunity (31). *L. sake* Lb706-B (Sak^- Imm^-), which contains only the 60-kb plasmid, was a result of these curing experiments. Preliminary hybridization experiments (not shown) revealed that (i) there was no homology between the *Hin*dIII-*Bgl*II fragment of pLSA2404 and the previously cloned 1.4-kb *Eco*RI fragment containing the structural gene for sakacin A (*sakA*) (13) and (ii) similar to the *sakA* fragment, the *Hin*dIII-*Bgl*II fragment was derived from the 60-kb plasmid and was therefore also present in strain Lb706-B. Since sakacin A production and immunity in *L. sake* Lb706-B were obtained by introducing a DNA fragment seemingly already present, a mutation in *sakB* was a more

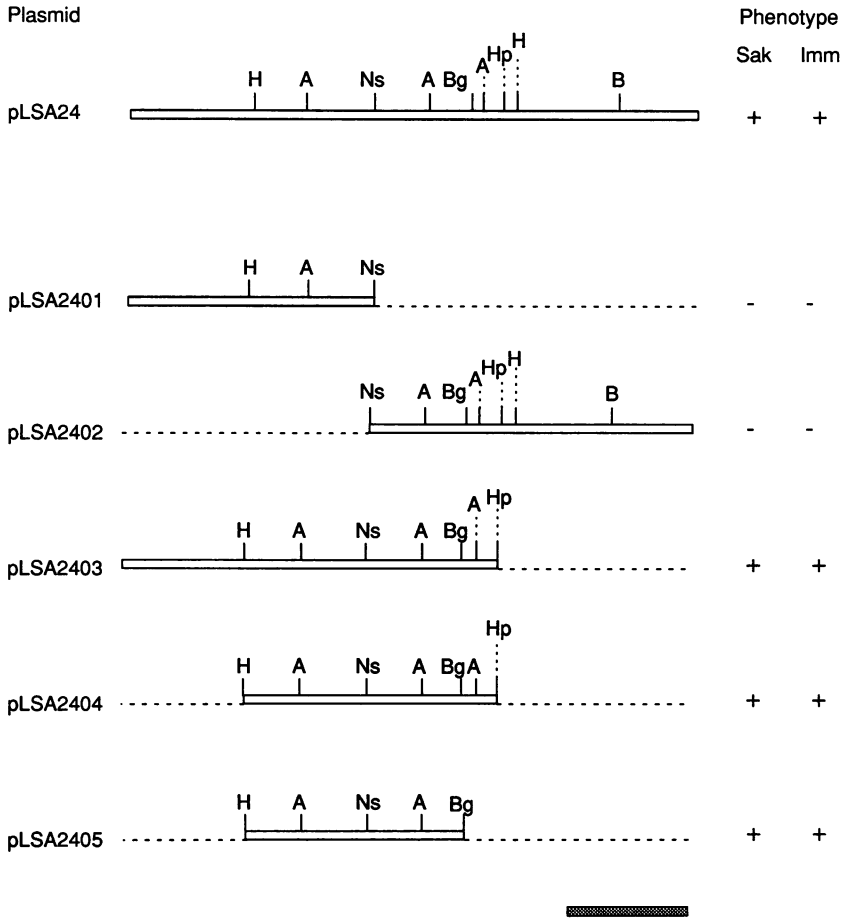

FIG. 1. Restriction maps of the insert of the recombinant plasmid pLSA24, restoring sakacin A production and immunity in *L. sake* Lb706-B, and deletion derivatives. The resulting phenotype when the plasmids were introduced in strain Lb706-B is indicated. Dashed lines indicate deleted parts. The bar represents 1.0 kb. The vector (pVS2) fragments are not shown. Abbreviations: A, *Acc*I; B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; Hp, *Hpa*I; Ns, *Nsi*I.

probable explanation for the Sak$^-$ Imm$^-$ phenotype of this strain than was the curing of the 28-kb plasmid. Thus, it seemed possible to isolate a Sak$^+$ Imm$^+$ variant of *L. sake* Lb706 that would harbor only the 60-kb plasmid. Strain Lb706 was therefore subjected to a novobiocin curing experiment.

The curing experiment resulted in a dominance (88%) of variants with no plasmids. These were all Sak$^-$. Six colonies (12%) were Sak$^+$, and they all contained only the 60-kb plasmid (results not shown). Representatives of the two groups were chosen and designated *L. sake* Lb706-X (plasmid free, Sak$^-$) and Lb706-S (60-kb plasmid, Sak$^+$). Further characterization showed that Lb706-X and Lb706-S were Imm$^-$ and Imm$^+$, respectively.

The 60-kb plasmids from *L. sake* Lb706-S and Lb706-B were compared by cleavage with restriction enzymes and subsequent hybridization with the 0.8-kb *Nsi*I-*Bgl*II fragment of pLSA2404 (*sakB* probe) and an oligonucleotide probe for *sakA* (13) (Fig. 3). The 60-kb plasmids of Lb706-S and Lb706-B were indistinguishable, suggesting that the mutation of *sakB* in Lb706-B was caused by either a point mutation or a small deletion. Furthermore, *sakB* was located in close vicinity to *sakA*, since both probes hybridized to a fragment of approximately 6 kb when the plasmid was digested with either *Nco*I or *Xba*I. *Hin*dIII and *Bgl*II sites were located between *sakA* and *sakB*. Further restriction enzyme analysis and hybridizations, together with the nucleotide sequence data, were used to create a linear restriction map of the *sakA-sakB* region of the 60-kb plasmid (Fig. 4). This analysis revealed that *sakA* and *sakB* are transcribed in opposite directions and that the distance between the genes is 1.6 kb. Note that the *Bgl*II site present in pLSA24 and derivatives was not present in the *sakA-sakB* region. Instead, a *Sau3*AI site was present at the corresponding position (not shown). The *Bgl*II site in pLSA24 was created by a chance ligation of an appropriate *Sau3*AI fragment (with a T flanking the recognition sequence GATC) to the fragment containing *sakB*.

Nucleotide sequence of the mutated *sakB*. The suspected mutation in *sakB* present in *L. sake* Lb706-B was confirmed by nucleotide sequencing. The sequence of *sakB* on the 60-kb plasmid of strain Lb706-B was identical to that of *sakB* on pLSA2404, except for a single A-T base pair deletion at position 884 (Fig. 2). A translational stop codon (TAA) six codons downstream would be the result of this frameshift mutation, presumably leading to a truncated SakB protein.

Hydropathic character and homology to transmembrane histidine protein kinases (HPKs) of the predicted SakB pro-

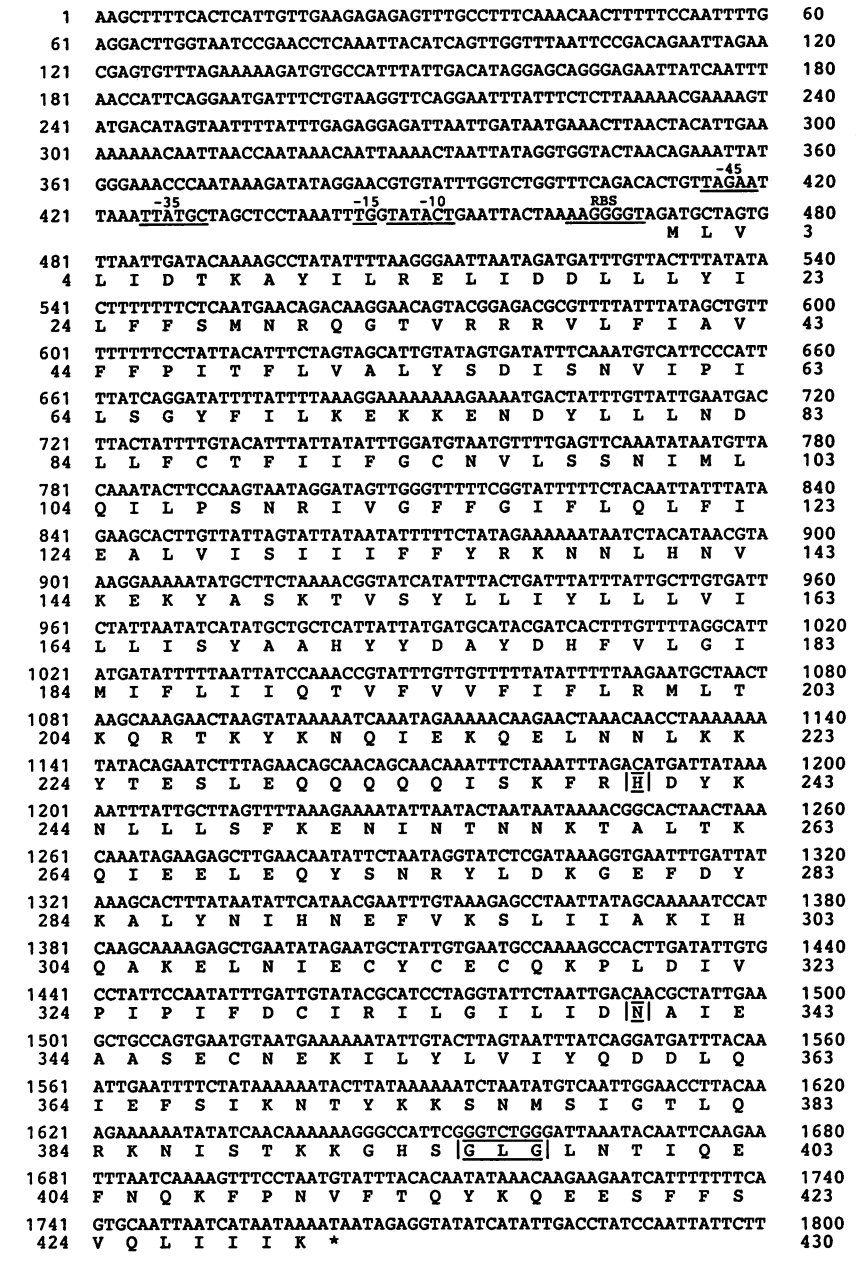

FIG. 2. Nucleotide sequence of the 1,810-bp *Hin*dIII-*Bgl*II fragment from pLSA2404. The predicted amino acid sequence of the large ORF (*sakB*) is shown below the nucleotide sequence. The putative −45, −35, −15, and −10 promoter regions as well as the putative ribosome binding site (RBS) are underlined. Conserved amino acid residues of the HPK family are boxed (32).

tein. A hydropathic plot revealed that the putative SakB protein was essentially divided into two regions, a highly hydrophobic N-terminal half and a more hydrophilic C-terminal half (results not shown). This interpretation was further supported by a buried-helix parameter plot according to the method of Rao and Argos (27) (Fig. 5). The hydrophobic, N-terminal part of the protein appeared to contain five transmembrane regions.

The SakB amino acid sequence was compared with other amino acid sequences in the SwissProt (release 24.0) and NBRF (release 35.0) data bases. The highest similarity score was obtained with the deduced amino acid sequence of a gene from *Staphylococcus aureus*, *agrB* (18, 37) or *agr*-ORF2 (32). The putative AgrB protein, consisting of 423 amino acid residues, had 22% amino acid identity with SakB. When conservative amino acid changes were included, the similarity was increased to 51%. A buried-helix parameter plot of the AgrB protein by using the same computer modelling as for SakB revealed a strikingly similar pattern (not shown). AgrB has been suggested to be a member of a family of transmembrane HPKs, which serve as sensor proteins in responses of bacteria to various external stimuli (32). The SakB protein also shares some distinctive features of this family, with its transmembrane regions in the N-ter-

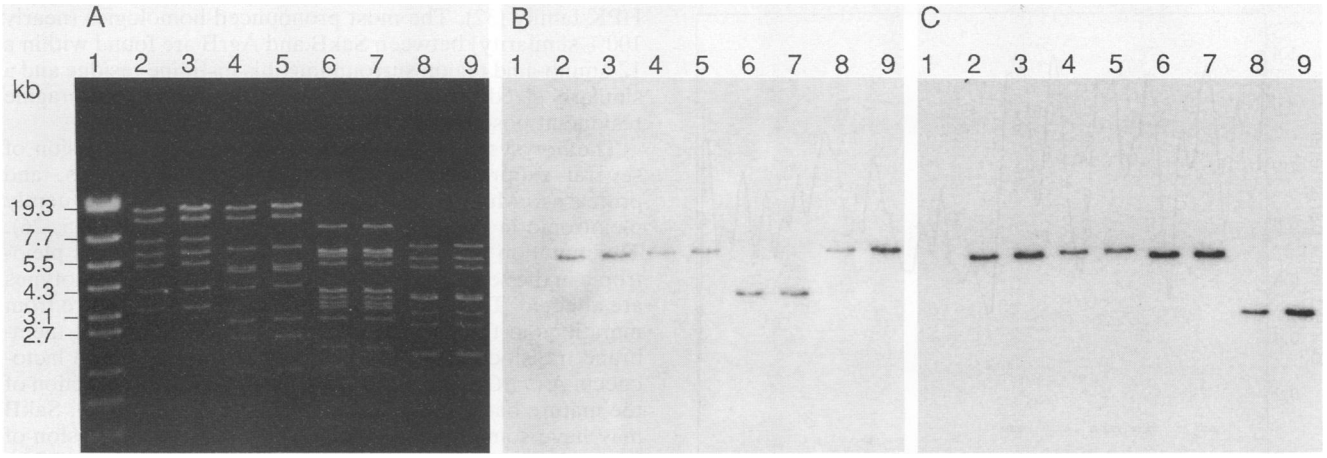

FIG. 3. Restriction enzyme analysis of the 60-kb plasmid from *L. sake* Lb706-S (Sak$^+$ Imm$^+$) and *L. sake* Lb706-B (Sak$^-$ Imm$^-$) and subsequent hybridization with a *sakA* probe and a *sakB* probe. (A) Agarose gel; (B) corresponding blot and hybridization with *sakA* probe; (C) corresponding blot hybridized with *sakB* probe. Lanes 2, 4, 6, and 8, Lb706-S 60-kb plasmid; lanes 3, 5, 7, and 9, Lb706-B 60-kb plasmid. Enzymes: *Nco*I (lanes 2 and 3), *Xba*I (lanes 4 and 5), *Bgl*II (lanes 6 and 7), and *Hin*dIII (lanes 8 and 9). Lane 1 is molecular weight marker IV (Boehringer, Mannheim, Germany).

minal half and certain conserved amino acid residues regarded as important for function (Fig. 2). Although the homology between SakB and AgrB was fairly evenly distributed along the entire sequences, the similarity was higher around these conserved amino acid residues.

DISCUSSION

This report describes the identification and characterization of a gene (*sakB*) necessary for sakacin A production and immunity in *L. sake* Lb706. The Sak$^+$ Imm$^+$ phenotype is associated with the 60-kb plasmid present in this strain, since both *sakB* and the structural gene *sakA* are located on this plasmid. Contrary to previous assumptions, the 28-kb plasmid seems to be irrelevant in this regard, since Sak$^+$ Imm$^+$ variants and transformants without this plasmid or any part of it could be obtained. This plasmid appears to be easily cured, as shown by our curing experiment in which all colonies tested had lost it. In retrospect, it is perhaps not surprising that strain Lb706-B is a mutant in addition to being cured of the 28-kb plasmid, since the previous curing experiment involved a rather mutagenic compound (acriflavine) and the selection was made for loss of the Sak$^+$ phenotype (31). Novobiocin, used in this study, inhibits DNA gyrase (22) and is therefore not as mutagenic as acriflavine and other DNA-intercalating compounds. It has been used successfully as a curing agent for several bacteria (22), including lactobacilli (28).

No recombination was observed between the pLSA plasmids containing *sakB* and the 60-kb plasmid present in *L. sake* Lb706-B. The reasons for this apparent lack of recombination, despite the obvious sequence homology, are unknown.

Two genes necessary for sakacin A production have now been identified, the structural gene *sakA* (13) and *sakB*. A recombinant plasmid containing the entire region depicted in Fig. 4 has been used to transform the plasmid-free variant *L. sake* Lb706-X, but the transformants remained Sak$^-$ Imm$^-$ (results not shown). Genes other than *sakA* and *sakB* are apparently needed for expression. In view of the similarities between sakacin A and pediocin PA-1, both in the mature bacteriocin and in the leader peptide (13, 21), this is not surprising. It is likely that the processing and transport system of sakacin A is similar to that of pediocin PA-1 and therefore involves an ATP-dependent membrane translocator. The SakB protein does not belong to this family of proteins but has some other role (see below). Two completely different approaches have been used to identify *sakA* (oligonucleotide probes derived from the amino acid sequence) and *sakB* (shotgun cloning and assay for activity). It is significant that the two genes were located close to each other on the 60-kb plasmid (Fig. 4). This may indicate that all

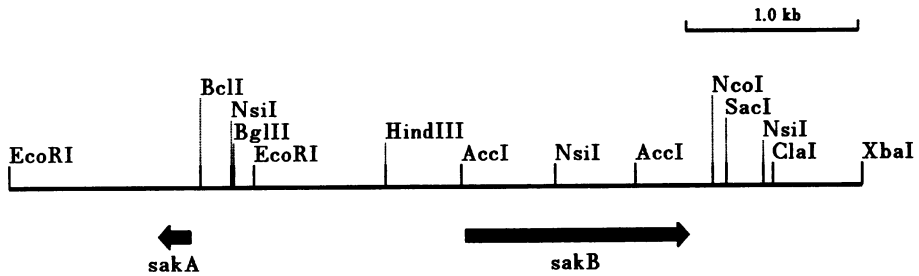

FIG. 4. Linear restriction map of the 5-kb *sakA-sakB* region of the 60-kb plasmid from *L. sake* Lb706. The transcription directions of the *sakA* and *sakB* genes are indicated by arrows.

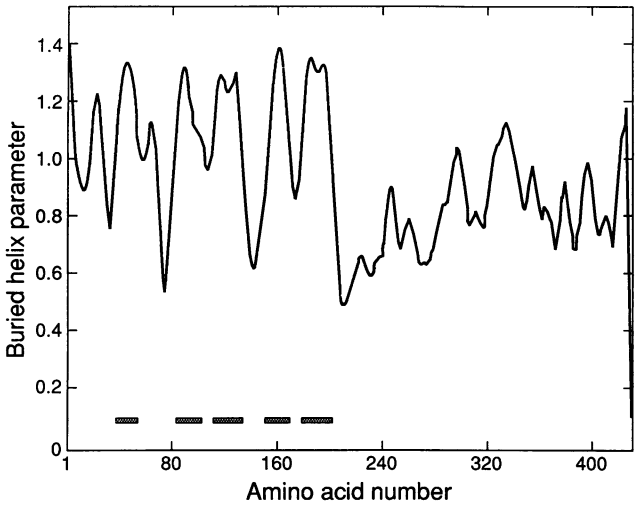

FIG. 5. Buried-helix parameter plot of the putative SakB protein according to the method of Rao and Argos (27). Potential transmembrane regions are indicated by horizontal bars.

the genes necessary for sakacin A production and immunity are clustered in a way which resembles the pediocin PA-1 (*ped*) and lactococcin A (*lcn*) gene clusters (21, 33). The organization for the *sak* genes appears to be more complex, however, since *sakA* and *sakB* are transcribed in opposite directions. In addition to *sakB*, the nucleotide sequence of the 1,810-bp fragment revealed a small ORF, potentially encoding a peptide of 45 amino acid residues (not shown in Fig. 2) and located upstream of the putative promoter and *sakB*. The role, if any, of this ORF in the expression of sakacin A is unknown.

No significant sequence homology was found between the putative SakB protein and proteins encoded by the *ped* and *lcn* gene clusters, which are involved in the expression of bacteriocins (21, 33). A structural resemblance was evident in that SakB, PedD, LcnC, and LcnD all had hydrophobic N-terminal regions, suggesting that they were membrane associated. A significant sequence homology was observed, however, between SakB and AgrB, an *S. aureus* protein. The *agr* (accessory gene regulator) locus consists of four ORFs, in which *agrA* and *agrB* are believed to be the genes of greatest importance (18). This is based on the finding that the corresponding proteins AgrA and AgrB share some distinctive features with the so-called two-component signal transducing systems. These systems are present in many bacteria and mediate responses to environmental stimuli through kinase reactions (32). The first component is an HPK, which often is membrane located and serves as a sensor protein. An internal histidine residue is phosphorylated in response to some external signal. The phosphoryl group is then transferred to a cytoplasmatic protein, the response regulator. The level of phosphorylation of the response regulator protein determines the activity, which often is an activation of transcription of the appropriate target genes. In this context, AgrB is the sensor protein and AgrA is the response regulator component (18, 32). Similar to AgrB, SakB appears to be a member of the HPK family, showing the same distinctive features. Among these are certain conserved amino acid residues (32). The histidine residue at position 240 (Fig. 2) corresponds to the amino acid likely to be phosphorylated during signal transduction in the HPK family (32). The most pronounced homologies (nearly 100% similarity) between SakB and AgrB are found within a 12-amino-acid region surrounding this histidine residue and a similarly sized region surrounding the conserved asparagine residue at position 340 (Fig. 2).

The *agr* system in *S. aureus* regulates the expression of several exoproteins, e.g., hemolysins, enterotoxins, and proteases. Mutations in the *agr* locus are consequently pleiotropic in that many target genes are affected (15, 26). The mutation in *sakB* present in *L. sake* Lb706-B is pleiotropic in the sense that both the Sak and the Imm phenotypes are affected. This mutation is clearly different in nature from mutations in the genes encoding the ATP-dependent membrane translocators in the pediocin PA-1 (*pedD*) and lactococcin A (*lcnC*) systems, which affect only the production of the mature bacteriocin and not the immunity (21, 33). SakB may have some overall regulatory role in the expression of both sakacin A production and immunity. Preliminary DNA sequencing data from the region downstream of *sakB* on the 60-kb plasmid indicate the presence of a gene with similarity to *agrA*. A classical two-component signal transducing regulatory system may thus be operational in connection with sakacin A production and immunity in *L. sake* Lb706.

With the recent advances in the techniques for introducing DNA into *Lactobacillus* strains, mainly through electrotransformation (electroporation) (1, 2, 4, 39), these bacteria may be used as hosts in cloning work and subsequent molecular analysis. One of the aims of this study was to evaluate this possibility. The reasons for this were (i) to avoid problems with expression of certain traits in other commonly used cloning hosts and (ii) that future genetic manipulations of strains that are aimed for use in food may require that the DNA has been passed only through generally recognized as safe organisms, e.g., lactobacilli. The sakacin A system was suitable for this evaluation, since the production of this bacteriocin was associated with plasmids (13, 31). In addition, we had access to a sakacin A-nonproducing and -sensitive variant (*L. sake* Lb706-B), and the conditions for electrotransformation had been optimized to reach appreciable levels of transformation efficiency (2). The results reported here illustrate the feasibility of using *Lactobacillus* strains as hosts in cloning work and genetic analyses.

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