APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 2003, p. 7194–7203 Vol. 69, No. 12
0099-2240/03/$08.00+0 DOI: 10.1128/AEM.69.12.7194–7203.2003

Functional Characterization of a Composite Bacteriocin Locus from Malt Isolate *Lactobacillus sakei* 5

Anne Vaughan,[1,2] Vincent G. H. Eijsink,[3] and Douwe van Sinderen[1,4]*

Department of Microbiology,[1] National Food Biotechnology Centre,[2] and BioScience Institute,[4] National University of Ireland, Cork, Cork, Ireland, and Department of Chemistry and Biotechnology, Agricultural University of Norway, Ås, Norway[3]

Received 27 May 2003/Accepted 18 September 2003

***Lactobacillus sakei* 5, isolated from malted barley, produces three bacteriocins. Genetic and functional analysis of the purified bacteriocins showed that this strain produces a plasmid-encoded bacteriocin that is identical to sakacin P, as well as two novel, chromosomally encoded bacteriocins, which were designated sakacin T and sakacin X. The structural genes specifying sakacin T and sakacin X are part of the *sakacin TX* locus, which consists of two adjacent but divergently oriented gene clusters. The first gene cluster includes *stxP*, *stxR*, *stxK*, and *stxT*, which, based on functional and comparative sequence analysis, are believed to encode an inducing peptide and proteins involved in regulation and secretion of these bacteriocins. The second gene cluster includes the structural and immunity genes for sakacin T, a class IIb two-peptide bacteriocin composed of $SakT_{\alpha}$ and $SakT_{\beta}$, and sakacin X, a class IIa bacteriocin. Interestingly, a so-called transport accessory protein was absent from the locus, and based on our results it appears that a dedicated accessory protein is not required for processing and transport of sakacin T and sakacin X.**

Malted cereals contain high numbers of various microorganisms representing a wide range of bacteria, yeast, and fungal species (48). Microbial spoilage may result in a variety of technological impediments in the brewing and malting processes. These include damage to raw materials, filtration problems, and deleterious effects on both the fermentation process and the final product, such as turbidity of the beer and undesirable flavors and aromas (23). Any means by which the level of this undesirable microbial contamination can be controlled would be of interest to the brewing industry.

The last decade has seen a growing interest in the application of biopreservation through the use of microorganisms and/or their metabolites to prevent food spoilage and to extend the shelf life of foods (51). Lactic acid bacteria (LAB) are of particular interest as biopreservative organisms. The preserving effects of these organisms are partially due to the production of fermentation end products but may also be due to the formation of small, heat-stable inhibitory peptides, often referred to as bacteriocins (16, 18).

Bacteriocins are ribosomally synthesized, extracellularly released, bioactive peptides or peptide complexes that have a bactericidal or bacteriostatic effect on other (usually closely related) species. Bacteriocins of LAB have previously been arranged into three classes based on their composition, size, mode of action, mechanism of export, and inhibitory spectrum (16, 33, 45). The class I bacteriocins, the so-called lantibiotics, are posttranslationally modified. The class II bacteriocins, which are nonlantibiotic bacteriocins, are further divided into three subgroups. Class IIa bacteriocins are pediocin-like bacteriocins with strong antilisterial effects and a conserved N-terminal YGNGVXC consensus motif in the mature peptide, while class IIb bacteriocins consist of two peptides, both of which are required for full antimicrobial activity. Most class IIa and IIb bacteriocins are synthesized with a double-glycine type of leader sequence (26, 28). While all bacteriocins are formed with an N-terminal leader sequence, some small, heat-stable, and nonmodified bacteriocins are translated with *sec*-dependent leaders (36, 60). Due to their similarity to the class II bacteriocins these molecules have in the past been placed in a separate subgroup, class IIc (45). There are apparently hybrid bacteriocins which display characteristics of both class IIa and IIb subgroups (12, 13), which has resulted in an alternative class II classification scheme (16), in which subclass IIc is defined as other peptide bacteriocins which do not fulfill the criteria for class IIa or IIb. Class III was defined as large protein bacteriocins. A fourth class of bacteriocins was defined by Klaenhammer (33), and this class contains bacteriocins composed of undefined mixtures of proteins, lipids, and carbohydrates. However, experimental data suggest that the complex bacteriocinogenic activities of these molecules may be artifacts caused by interactions between constituents from the cells or growth medium and that the undefined bacteriocin activities are likely to be activities of regular peptide bacteriocins, and thus recognition of this separate class may not be valid. Kemperman et al. (32) have proposed a new class, class V, of bacteriocins that consist of ribosomally synthesized, nonmodified, head-to-tail-ligated, cyclic, antibacterial peptides, such as circularin A (32) and AS-48 (39).

Recent studies have revealed that there is considerable variation in the number of bacteriocins (11) produced by a particular strain (range, one to three bacteriocins), as well as considerable flexibility in the way in which bacteriocin loci can be organized (16). However, most genetically characterized class II bacteriocin gene clusters are composed of three gene modules, a module that includes the structural and immunity genes,

* Corresponding author. Mailing address: Department of Microbiology and BioScience Institute, National University of Ireland, Cork, Western Road, Cork, Ireland. Phone: 353 21 4901365. Fax: 353 21 4903101. E-mail: d.vansinderen@ucc.ie.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source and/or reference
Strains		
L. sakei 5	Producer of sakacin T, sakacin X, and sakacin P	Malt (47, 57)
L. sakei LMG2313	Indicator strain sensitive to sakacin T, sakacin X, and sakacin P	LMG[a]
P. pentosaceus LMG2001	Indicator strain sensitive to sakacin X only	LMG
L. sakei Lb790X	Plasmid-free derivative of *L. sakei* Lb790; Bac^-	6
L. sakei Lb790(pSAK20)	*L. sakei* Lb790 containing pSAK20, host strain; Bac^- Cm^r	5
L. sakei 23K	Plasmid-free *L. sakei*	8
E. coli DH5α	ϕ80d*lacZ*ΔM15 *recA1 endA1 gyrA96 thi-1 hsdR17*(r_K^- m_K^+) *supE44 relA1 deoR* Δ(*lacZYA-argF*)*U169*	Promega
E. coli EC101	*supE thi* Δ(*lacproAB*) (F′ *traD36 proAB lacI*^{q} ZΔM15) *repA*	35
Plasmids		
pBluescript SK(+)	3-kb *E. coli* cloning vector; α*lacZ* Ap^r	Stratagene
pTOPO	3.9-kb pCR 2.1 TOPO cloning vector; Ap^r	Invitrogen
pNZ44	*E. coli-L. lactis* vector containing P44 promoter; Cm^r	42
pLPV111	4.2 kb; *E. coli-L. plantarum*/*L. sakei* shuttle vector; Em^r	6
pSAK20	11.8 kb; contains the operon *RIRorf4sapKRTE*, necessary for transcriptional activation and processing and transport of sakacin A; Cm^r	6
pLPT5	pLPV111 $sakI_T$	This study
pLPT6	pLPV111 $sakT_\beta$ $sakI_T$	This study
pLPT7	pLPV111 $sakT_\alpha$ $sakT_\beta$ $sakI_T$	This study
pLPT8	pLPV111 $sakT_\alpha$ $sakI_T$	This study
pLPX14	pLPV111 *sakX* $sakI_X$	This study
pLPXi23	pLPV111 $sakI_X$	This study
pZ235T	pNZ44 $sakI_T$	This study
pZ233X	pNZ44 $sakI_X$	This study
pSKΔTE	pSAK20 derivative with a 3.5-kb *Bam*HI deletion; $sapK^+R^+\Delta sapTE$	This study
pSK5T	pSKΔTE *stxT*	This study

[a] LMG, Laboratory of Microbial Gene Technology, Ås, Norway.

a transport gene module, and a regulatory gene module. The bacteriocin structural gene specifies a prepeptide that is processed during secretion either by dedicated transport machinery or via the *sec*-dependent pathway. In the case of two-component bacteriocins, the two structural genes are located adjacent to each other. The structural gene for the bacteriocin is cotranscribed with the corresponding immunity gene located downstream, although there are exceptions to this genetic organization (21, 22). Most bacteriocins are produced as a precursor with an N-terminal double-glycine leader peptide sequence, which is removed upon externalization of the bacteriocin by dedicated secretion machinery, which consists of an ATP-binding cassette (ABC) transporter and a so-called accessory protein (16, 25, 26). The transport machinery normally tolerates some variation in the leader peptide sequence, which allows it to transport different precursor peptides (3, 15, 16). Accessory transport proteins are postulated to facilitate membrane translocation and/or help in processing of the leader peptide, although their specific role in the translocation process is not fully understood (18).

Another set of genes that is responsible for the control of the production of many, but not all, bacteriocins is the so-called three-component regulatory system (44), which consists of a secreted bacteriocin-like peptide pheromone, a histidine protein kinase, and a response regulator. This three-component regulatory system acts as a quorum-sensing device, coupling coordinated bacteriocin production by a strain to its cell density (16, 34).

In this paper, we describe functional characterization of a locus involved in the production of a novel class IIb bacteriocin, sakacin T, and a novel class IIa bacteriocin, sakacin X, produced by *Lactobacillus sakei* 5. Isolation of this strain from malt and purification of the bacteriocins that it produces have been described previously (47, 57).

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *Lactobacillus* and *Pediococcus* strains were cultivated in MRS broth (Oxoid, Basingstoke, England) at 30°C for approximately 16 h prior to experimental use. To obtain nonproducing (Bac^-) derivatives, the bacteriocin-producing (Bac^+) strain *L. sakei* 5 was cultivated in wort, which was made by using a standard mashing procedure (Analytica-EBC 4.5.1; relative density, 1.061). *Escherichia coli* strains were grown at 37°C in Luria-Bertani broth (49) with vigorous agitation. Agar media were prepared by adding 1.5% (wt/vol) granulated agar (Difco) to liquid broth media; overlay agars were prepared by adding 0.7% (wt/vol) granulated agar to the liquid broth media. The antibiotics used in the selective media were added at the following concentrations: ampicillin, 100 μg ml^{-1}; chloramphenicol, 10 μg ml^{-1}; and erythromycin, 200 μg ml^{-1} (*E. coli*) or 10 μg ml^{-1} (*Lactobacillus*). All chemical reagents were obtained from Sigma, St. Louis, Mo.

Bacteriocin activity and immunity assays. Bacteriocin assays were performed by using either bacterial colonies, cell-free supernatant (CFS), or samples obtained at various stages of the bacteriocin purification procedure as described previously (57). Bacteriocin activity was quantified by critical dilution by using the direct, spot-on-lawn assay (40). The number of activity units per milliliter was determined by determining the inverse of the last dilution at which growth inhibition was still detectable. *L. sakei* LMG2313 was used as the indicator strain unless otherwise specified.

Bacteriocin purification and characterization of the purified peptides. Bacteriocins were purified chromatographically and subjected to N-terminal amino acid sequencing and mass spectrometry as described previously (57).

General molecular cloning techniques. Plasmid DNA was isolated from *E. coli* strains with a JetQuick plasmid miniprep spin kit (Genomed, Löhne, Germany) and from *Lactobacillus* strains by the method of Birnboim and Doly (9). *E. coli* and *Lactobacillus* strains were transformed by electroporation by using the methods of Sambrook et al. (49) and Aukrust and Blom (4), respectively. All electroporations were carried out with a Gene-Pulser apparatus (Bio-Rad). Restric-

tion enzymes, T4 DNA ligase, and shrimp alkaline phosphatase were used as directed by the manufacturer (Roche Diagnostics, Mannheim, Germany). PCR amplification was performed by standard procedures by using the Expand High Fidelity or Long Template PCR system (Roche Diagnostics) and an Omnigene thermocycler (Hybaid, Ashford, United Kingdom). Primer synthesis and sequencing were performed by MWG Biotech (Ebersberg, Germany). DNA fragments were isolated and purified from agarose gels by using a QIAEX II agarose gel extraction kit (Qiagen, Crawley, United Kingdom). PCR products were purified by using the Concert PCR rapid purification system (Gibco/BRL). Southern blot hybridization onto Hybond-N+ nylon membranes (Amersham, Uppsala, Sweden) was performed by standard methods (49). An enhanced chemiluminesence kit (ECL; Amersham) was used to label the PCR-generated probes.

DNA sequencing. Total DNA of *L. sakei* 5 was obtained by the alkaline lysis method of Anderson and McKay (2) and was used as a DNA template for PCRs and DNA sequencing. Total DNA of *L. sakei* 5 was digested with *Cla*I, *Eco*RI, *Eco*RV, *Hin*cII, and *Hin*dIII, and the DNA fragments obtained from each of the digests were ligated to dephosphorylated pBluescript II SK+ (Stratagene, La Jolla, Calif.) digested with *Cla*I, *Eco*RI, *Eco*RV, *Hin*cII, and *Hin*dIII, respectively. Each of the ligation reaction mixtures, representing a restriction fragment library of total DNA of *L. sakei* 5, was then used as a template to amplify DNA segments near the bacteriocin structural gene by using degenerate primers that were designed on the basis of the amino acid sequences of $SakT_{\beta}$ and SakX (57). Two primers, T1D (5′-AARACNAAYTGGGGNTCNGTNGT-3′) and T2 (5′-ATNGCRTCYTGNCCNGCNCC-3′), were designed based on the $SakT_{\beta}$ amino acid sequence, and two other primers, Bac-X1 (5′-GGNGGNAARTAYTAYG GNAAYGG-3′) and Bac-X2 (5′-TTCCANCCNGCNGCNCCNCC-3′), were designed based on the sakacin X N-terminal sequence. Specific PCR products were obtained from the ligation reaction templates when a primer designed from the vector sequence was used in combination with one of the degenerate primers. Purified PCR products were cloned into the vector pTOPO (Invitrogen). DNA sequencing was performed by MWG Biotech with the universal forward and reverse primers. Based on the sequence information obtained from these DNA fragments, new sequence-specific primers were synthesized, and the procedure described above was repeated until the complete sequence of a 17-kb chromosomal fragment of *L. sakei* 5 encompassing the bacteriocin locus was determined. The integrity of this sequence was verified by resequencing the same region with overlapping PCR products, which had been generated by using chromosomal DNA as the template. Assembly and analysis of the DNA sequences were performed by using the DNASTAR software package. Database searches were performed by using the BLAST program (1) with the latest release of the nonredundant databases of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

Plasmid construction and analysis. The DNA inserts in the pLPV111 derivatives pLPT5, pLPT6, and pLPT7 (Table 1) were generated by using the high-fidelity PCR system (Roche Diagnostics) and primers with introduced *Pst*I and *Xba*I restriction sites which were ligated into the *Pst*I and *Xba*I sites of pNZ44. The insert and the P44 promoter were excised from the plasmids by restriction with *Bgl*I and *Xho*I, which are compatible for ligation into the *Bam*HI and *Xho*I sites of pLPV111. Plasmid pLPT5 contains the proposed immunity gene, $sakI_T$, which is assumed to confer resistance to sakacin T. Plasmid pLPT6 contains only one of the proposed structural genes of sakacin T, *sakT*, upstream of the proposed immunity gene, while pLPT7 harbors both proposed structural genes for sakacin T activity upstream of the proposed immunity gene. The SOEing technique (29) was used to generate a PCR product encompassing the $sakT_{\alpha}$ structural gene upstream of the $sakI_T$ gene, which was subsequently cloned into pLPV111 to generate pLPT8. A similar strategy was used for the genes thought to encode sakacin X (*sakX*) and its immunity protein ($sakI_X$). Two PCR fragments with incorporated restriction sites were obtained; one of these fragments contained *sakX* and $sakI_X$, and the other contained only $sakI_X$. These two fragments were cloned into the *Bam*HI and *Xho*I sites of pLPV111 to generate pLPX14 and pLPXi23, respectively. Two other constructs were made; these constructs, pZ235T and pZ233X, contained the putative sakacin T and sakacin X immunity genes, $sakI_T$ and $sakI_X$, respectively. The latter genes were amplified with incorporated restriction sites by PCR and cloned into the *Pst*I and *Xba*I sites of pNZ44.

Three derivatives of pSAK20, which contains the genes necessary for activation of transcription of the sakacin A structural gene, as well as the genes encoding the proteins needed for export and processing of presakacin A (6), were constructed. This plasmid has been successfully used for heterologous expression of other class II bacteriocins (5). All manipulations with pSAK20 were performed by using plasmids isolated from *E. coli* EC101. Restriction of pSAK20 with *Bam*HI and subsequent self-ligation allowed construction of pSKΔTE, containing a 3.5-kb deletion of the *sapT* and *sapE* transport and accessory protein genes. A PCR fragment containing the *stxT* gene with flanking *Bam*HI restriction sites was cloned into pSKΔTE to obtain plasmid pSK5T.

Induction factor synthesis and induction assays. The proposed induction factor for sakacin T and sakacin X production (designated IP-TX) and the sakacin P-inducing peptide (IP-673) (10) were synthesized at the Facility for Molecular Biology at the University of Newcastle Upon Tyne (Newcastle Upon Tyne, United Kingdom) and were purified to >97% homogeneity by reverse-phase high-performance liquid chromatography with an acetonitrile-H_2O gradient. The molecular weights of the high-performance liquid chromatography-purified peptides were verified by laser desorption mass spectrometry. To assay biological activity, 1-mg ml^{-1} stock solutions of both IP-TX and IP-673 were prepared in sterile distilled water. To generate a Bac^- derivative of *L. sakei* 5, an overnight culture of this strain was inoculated (1%) into wort and subcultured up to five times at 37°C until the culture medium became turbid. Individual colonies, as well as CFS, were assayed for loss of inhibitory activity against *L. sakei* LMG2313 (which is sensitive to all three bacteriocins produced by *L. sakei* 5) and *Pediococcus pentosaceus* LMG2001 (which is sensitive only to sakacin X). The *L. sakei* LMG2313 derivatives pZ235T and pZ233X, which contained the putative sakacin T and sakacin X immunity genes $sakI_T$ and $sakI_X$, respectively, were also used as indicator cultures. The resulting completely Bac^- culture was inoculated (1%) into wort containing the inducing peptide IP-TX (this study) or IP-673 (10) at a concentration of 400 ng ml^{-1} and incubated at 37°C for 16 h. The cultures were then tested for bacteriocin production.

Nucleotide sequence accession number. The sequence determined in this study has been deposited in the GenBank database under accession number AY206863.

RESULTS

Sequence analysis of the *sakacin TX* locus. *L. sakei* 5 produces three bacteriocins, sakacin P, sakacin T, and sakacin X (previously referred to as sakacin 5P, sakacin 5T, and sakacin 5X), which were purified and subjected to N-terminal amino acid sequencing previously (57). Degenerate primers were designed on the basis of the amino acid sequences obtained for one component of sakacin T, $SakT_{\beta}$ (see below), and sakacin X. Two PCR products that were approximately 130 bp long were generated, and these products were shown to contain coding regions that were consistent with the derived amino acid sequences (data not shown). Additional rounds of anchored PCR were performed until the complete nucleotide sequence of a 17,243-bp region, referred to as the *sakacin TX* locus, was determined by several rounds of ligation-anchored PCR. Sequence analysis revealed the presence of 14 complete putative open reading frames (ORFs) and eight incomplete ORFs arranged in divergent operons (Fig. 1) (the ORFs were designated ORFs 1 to 22, but some of them were renamed if functions could be assigned to the encoded products). The ORFs were identified based on the criterion that an ORF consists of least 25 codons preceded by a potential ribosome binding site at an appropriate distance from one of the commonly used initiation codons (24). A nine-gene cluster in the middle of the locus seems to contain all the genes needed for sakacin T and sakacin X production, regulation, and transport (see below).

Analysis of the protein products encoded by the *sakacin TX* locus. The protein products encoded by ORFs 6, 10, 11, 13, and 17′ all resemble class II bacteriocin precursors containing an N-terminal extension or leader sequence. The leader sequences of these ORFs conform closely to the consensus leader sequences proposed by Nes et al. (45). Two important features of these leaders are the presence of two glycine residues in the C terminus at positions −2 and −1 relative to the processing site and the remarkable degree of similarity in the

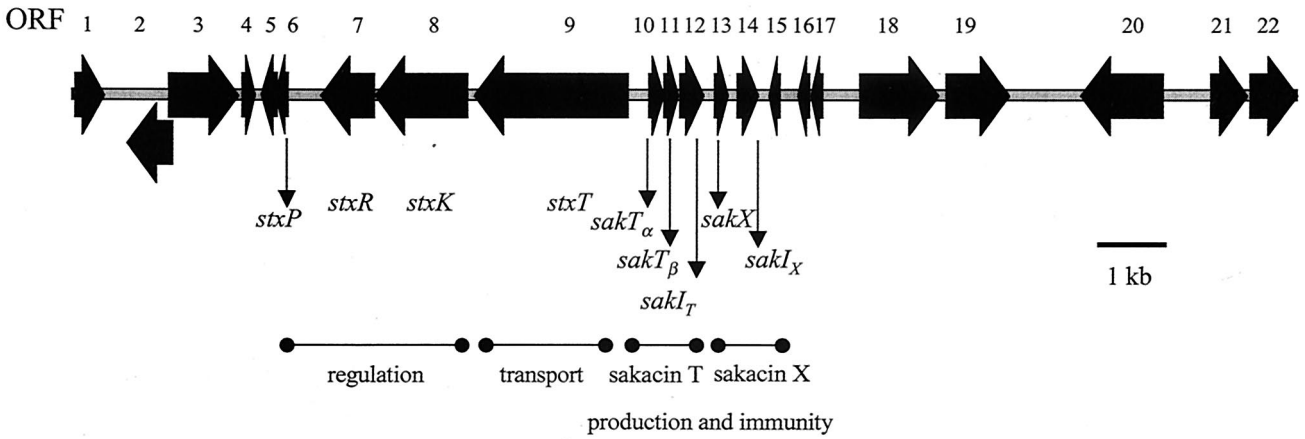

FIG. 1. Schematic representation of the *sakacin TX* locus sequenced to date, obtained by using a combination of ligation-anchored PCR and the primer walking strategy. A total of 22 potential ORFs are shown. The arrows indicate ORFs and the proposed directions of transcription. ORFs with deduced functions in production and secretion of active sakacin T and sakacin X are indicated by *stx* or *sak*. Sections of the *stx* locus assumed to be dedicated to production, immunity, regulation, and transport are indicated.

hydropathic profiles. Defined distances separate conserved hydrophobic and hydrophilic residues between the conserved residues (Table 2).

Based on the amino acid sequencing results (57), the gene encoding sakacin X activity corresponds to ORF 13 and thus was designated *sakX*. The N-terminal sequence obtained for sakacin T corresponds to ORF 11, and this gene was designated *sakT*$_\beta$. The product of *sakT*$_\beta$ was found to constitute part of a two-component bacteriocin (see below). The second part of this bacteriocin was shown to be encoded by ORF 10, which is located immediately upstream of *sakT*$_\beta$ and was therefore designated *sakT*$_\alpha$. The products of ORF 12 and ORF 14, which were designated *sakI*$_T$ and *sakI*$_X$, confer immunity to the antimicrobial activities of sakacin T and sakacin X, respectively (see below), and these genes are located immediately downstream of the corresponding bacteriocin structural genes.

The proteins encoded by ORF 6 (designated *stxP*), ORF 7 (*stxR*), and ORF 8 (*stxK*) exhibited significant sequence similarity to a small, cationic pheromone with a double-glycine leader and to response regulator and histidine protein kinase proteins, respectively. These three associated genes therefore seem to represent a so-called three-component regulatory system, similar to those involved in the control of several class II bacteriocins (34, 44).

The deduced protein product of ORF 9, designated *stxT*, exhibits highly significant similarity to ABC transporter proteins. It has been well established that secretion of bacteriocin peptides possessing a double-glycine leader is mediated by a dedicated membrane-associated translocator belonging to the HlyB ABC transporter superfamily (20, 25, 45). In addition, a so-called accessory protein has been implicated to play an essential, but still unidentified, role in this transport process. The genes encoding the dedicated bacteriocin ABC-type transporter and the accessory protein are usually located adjacent to each other as part of a bacteriocin production gene cluster. However, no complete homologue of an accessory-encoding gene was identified in the *sakacin TX* locus.

It has been found previously that many LAB contain remnants of bacteriocin gene clusters on both the chromosome and resident plasmids (16, 30). It is therefore not surprising that the *sakacin TX* locus harbors genes whose deduced proteins are related to bacteriocin production and immunity but which do not seem to be involved in the production of or immunity to sakacin T and sakacin X. ORF 16 displays 63% identity to *brcI*, the immunity gene for brochocin C. ORF 17, which is located immediately upstream of ORF 16, does not strictly conform to the ORF definition described above in that it is not preceded by a ribosome binding site. A putative leader peptide-encoding

TABLE 2. Prepeptides of the sakacin TX locus containing double-glycine leader sequences

Protein	Amino acid sequence of prepeptide[a] (↓ cleavage site)	Prepeptide size (amino acids)	Mature peptide size (amino acids)
ORF 6 (IP-TX)	MTNRKTLPKEELKKIKGG ↓ TPGGFDIISGGPHVAQDVLNAIKDFFK	45	27
ORF 10 (SakT$_\alpha$)	MKNVQSLSKEELVLVVGG ↓ YTAKQCLQAIGSWGIAGTGAGAAGGPAGAFVGAHVGVIAGSAVCIGGFLGQ	69	51
ORF 11 (SakT$_\beta$)	MKTANIKLLTNQEMIEIFGG ↓ KTNWGSVVGSCVAGGLVGALGGTPISIGAGCLVGAGQDWISQK	63	43
ORF 13 (SakX)	MEAIKKLDLQAMKGIVGG ↓ KYYGNGLSCNKSGCSVDWSKAISIIGNNAVANLTTGGAAGWKS	61	43
ORF 17′	MRKFQKLNEQEMKRLMGG[b] ↓ SSKDCLKDIGKGIGAGTVAGAAGGGCLTGAIGSIWDQW	56	38
Consensus[c]	-XO-OOX--X-GG ↓		

[a] X, hydrophobic residue; O, hydrophilic residue. The arrow indicates the conserved cleavage site.
[b] Frameshift occurs at this site.
[c] Suggested consensus sequence (45).

sequence designated ORF 17′ precedes ORF 17, but at the DNA sequence specifying the double-glycine residues an apparent frameshift has taken place. The 5′ part of ORF 17 is homologous *brcA*, while the 3′ part exhibits homology to *brcB*; *brcA* and *brcB* are the two genes encoding the two peptides of brochocin C (41). Furthermore, ORF 5 and ORF 15 appear to represent truncated bacteriocin-related genes, since they encode proteins that are between 45 and 50% identical to the first 45 amino acids (in the case of ORF 15) and 74 amino acids (in the case of ORF 5) to BrcD, the transport accessory protein for brochocin C. The protein products of the remaining ORFs identified in the *sakacin TX* locus exhibit significant similarity to various transposases and hypothetical proteins with unknown functions produced by different species of LAB (Table 3).

Immunity genes. The assumption that $sakI_T$ and $sakI_X$ encode immunity proteins was based on the fact that in many bacteriocin-encoding gene clusters the immunity gene is located immediately downstream of the structural gene(s) and the fact that such immunity-conferring proteins are hydrophobic (45). Expression clone pZ235T, containing only $sakI_T$, was constructed to confirm the role of this gene in immunity to sakacin T. This plasmid was introduced into *L. sakei* LMG2313, which is sensitive to sakacin T, and this strain was subsequently used as an indicator in the deferred antagonism assay. *L. sakei* LMG2313 harboring pZ235T was shown to be immune to sakacin T produced by clone pLPT7 (see below), demonstrating that the insert on the plasmid, $sakI_T$, is capable of conferring immunity to a previously sensitive strain.

A similar construct, pZ233X containing $sakI_X$, was made in the same manner. *L. sakei* LMG2313 is sensitive to sakacin X, and so this strain was used as an indicator. *L. sakei* LMG2313 containing pZ233X was shown to be immune to sakacin X produced by a colony of the pLPX14 clone, although it was not completely immune to purified sakacin X at concentrations greater than 800 activity units ml^{-1}. Thus, the insert on pZ233X is capable of conferring sakacin X immunity to a previously sensitive strain.

To investigate whether the immunity genes in the *sakacin TX* locus conveyed cross-immunity to sakacin T and sakacin X, the *L. sakei* LMG2313 derivatives harboring pZ235T and pZ233X were used as indicators. The construct that included the sakacin T immunity gene did not provide immunity to sakacin X, and likewise, $sakI_X$ did not confer immunity to sakacin T to the indicator strain harboring it. Plasmid pNZ44 without an insert was used as a control in both the sakacin T and sakacin X immunity experiments and did not confer immunity.

Bacteriocin structural genes. A system for heterologous expression of class II bacteriocins has been constructed previously, and this system is based on introducing two plasmids into a bacteriocin-negative *L. sakei* strain (6). The first plasmid (pSAK20) contains the genes necessary for export and processing of several bacteriocin precursors, while the second plasmid (a pLPV111 derivative) supplies the structural and immunity genes for the bacteriocins (5). To investigate whether both $sakT_\alpha$ and $sakT_\beta$ are essential for sakacin T production, $sakT_\alpha$ and $sakT_\beta$ were cloned individually and in combination upstream of an intact $sakI_T$ immunity gene, under control of the constitutive lactococcal promoter P44, in pLPV111 in order to produce plasmids pLPT6, pLPT7, and pLPT8 (see Materials and Methods) (Table 1). These three plasmids were introduced into *L. sakei* Lb790X or a derivative of this strain harboring plasmid pSAK20. Only *L. sakei* Lb790X transformants containing both pLPT7 and pSAK20 were capable of producing antimicrobial activity. *L. sakei* Lb790X clones containing either pLPT6 or pLPT8 along with pSAK20 were assayed for inhibitory activity by using the deferred antagonism assay with the indicator *L. sakei* LMG2313. No zones of inhibition were observed surrounding the colonies of either the pLPT6 or pLPT8 transformants (Fig. 2). However, when colonies of each transformant type were grown adjacent to each other, a clear zone of inhibition was observed between the colonies (Fig. 3). These results clearly show that sakacin T requires two peptides for full activity and that this bacteriocin therefore is a member of the class IIb bacteriocins.

To establish heterologous expression of sakacin X, *sakX* was cloned upstream of the $sakI_X$ immunity gene under control of the P44 promoter in the vector pLPV111. The resulting construct, designated pLPX14, was introduced into *L. sakei* strain Lb790 containing pSAK20 as described above. The resulting strain was shown to exhibit bacteriocin activity (Fig. 2), which was purified as described previously (57) (see Materials and Methods). The constructs pLPT5 and pLPXi23, which contained the immunity genes for sakacin T and sakacin X, respectively, without the structural genes under investigation, did not exhibit any inhibitory activity when they were transformed into *L. sakei* Lb790(pSAK20).

Sakacin P genes are plasmid encoded, while the *sakacin TX* locus is chromosomally encoded. In addition to sakacin X and sakacin T, a bacteriocin identical to sakacin P was purified from *L. sakei* 5 (57). Several genes in the assumed *sakacin P* locus were PCR amplified and sequenced, which showed that the gene organization and sequence of this locus are identical to the gene organization and sequence described previously (10, 30, 55; results not shown). Total or plasmid DNA preparations isolated from *L. sakei* 5 were digested with a range of restriction enzymes and used in Southern hybridization experiments performed with the PCR products corresponding to the structural genes of sakacin P, sakacin X, and sakacin T. Hybridization signals were observed with a number of distinct bands of various sizes obtained from total DNA, whereas no hybridization signals were obtained when plasmid DNA was probed with the PCR-amplified sakacin T and sakacin X fragments. However, positive signals were detected when the plasmid DNA was probed with the PCR fragment corresponding to the DNA region encoding sakacin P (data not shown). These results show that the sakacin T and sakacin X structural genes are chromosomally encoded, whereas the sakacin P structural gene is plasmid encoded.

Bacteriocin production is inducible. Spontaneous loss of bacteriocin production was not observed when standard cultures of *L. sakei* 5 were diluted in MRS medium, as observed previously for many class II bacteriocin-producing bacteria in which bacteriocin production is regulated by a so-called three-component regulatory system (44). However, loss of production was observed when *L. sakei* 5 was grown in wort at temperatures above 35°C. Different indicators were used to determine the identities of the bacteriocins which were produced during isolation and induction of the Bac^- strains. Sakacin X inhibits *P. pentosaceus* LMG2001, and it was shown by

TABLE 3. Similarities of the proteins encoded by the identified ORFs of the *sakacin TX* gene cluster to their homologues[a]

ORF (gene)	Size of product (amino acids)	Homologue		
		Identity	Homology	Reference or accession no.
ORF 1	148	Transposase	100% identity to TraISLp1 of *Lactobacillus plantarum*	AF459445[b]
			Conserved domain Tra8 of IS*30* family involved in DNA replication, recombination, and repair	COG2826[b]
ORF 2	220	Tyrosyl-tRNA synthetase	69% identity to *TyrS*-1 of *Enterococcus faecalis*	14
			Conserved domain TyrS involved in translation, ribosomal structure, and biogenesis	COG0162[b]
ORF 3	332	Transposase	40% identity to *BH3986* encoding transposase (24) of *Bacillus halodurans*	52
			Conserved domain transposase_11 necessary for efficient DNA transposition	pfam01609[b]
ORF 4	34	Transposase (fragment)	100% identity to IS*1163* of *L. sakei* L45	50
ORF 5	88	Transport accessory protein (fragment)	45% identity to *brcD* of *Brochothrix campestris*	41
ORF 6 (*stxP*)	45	Inducing peptide	38% identity to carnobacteriocin A precursor of *Carnobacterium piscicola* LV17A	60
ORF 7 (*stxR*)	252	Response regulator	36% identity to *entR* of *Enterococcus faecium*	46
			Conserved domain LytT, response regulator involved in transcription and signal transduction	COG3279[b]
ORF 8 (*stxK*)	437	Histidine kinase	28% identity to *entK* of *Enterococcus faecium*	46
			Conserved domain of signal transduction protein	COG2972[b]
ORF 9 (*stxT*)	723	ABC transporter	53% identity to *entT* of *Enterococcus faecium*	46
			Conserved domain SunT, ABC-type bacteriocin exporters containing an N-terminal double-glycine peptidase domain	COG2274[b]
ORF 10 ($sakT_{\alpha}$)	69	Bacteriocin	57% identity to *brcA* of *Brochothrix campestris*	41
ORF 11 ($sakT_{\beta}$)	63	Bacteriocin	52% identity to *brcB* of *Brochothrix campestris*	41
ORF 12 ($sakI_T$)	112	Bacteriocin immunity protein	No homologue	
ORF 13 (*sakX*)	61	Bacteriocin	71% identity to *pisA* of *Carnobacterium piscicola* JG126	31
ORF 14 ($sakI_X$)	97	Bacteriocin immunity protein	59% identity to *pisI* of *Carnobacterium piscicola* JG126	31
ORF 15	45	Transport accessory protein (fragment)	50% identity to *brcD* of *Brochothrix campestris*	41
ORF 16	52	Bacteriocin immunity protein	61% identity to *brcI* of *Brochothrix campestris*	41
ORF 17	56	Bacteriocin	69% identity to *brcA* of *Brochothrix campestris*	41
			47% identity to *brcB* of *Brochothrix campestris*	
ORF 18	370	Transposase	68% identity to *Efae1527* of *Enterococcus faecium*	NZAAAK01000222[b]
			Conserved domain transposase and inactivated derivatives	COG3547[b]
ORF 19	301	Transposase	45% identity to *TNP*-encoding IS*1070* of *Leuconostoc lactis*	58
			Conserved domain Tra8 of IS*30* family involved in DNA replication, recombination, and repair	COG2826[b]
ORF 20	354	Transposon	25% identity to SMU207 of *Streptococcus mutans* UA159	NC004350[b]
			Conserved domain Rep_trans, replication initiation factor	pfam02486[b]
ORF 21	165	Transcriptional regulator	34% identity to SAG1991 of *Streptococcus agalactiae*	54
			Conserved domain HTH_XRE, helix-turn-helix xenobiotic response element family of transcriptional regulators	cd00093[b]
ORF 22	219	Hypothetical protein	65% identity to *Efae1295* of *Enterococcus faecium*	NZAAAK01000203[b]
			Conserved domain XerD, site-specific recombinase	COG4974[b]

[a] Only the best matches are shown.
[b] Accession number of sequence deposited directly in the National Center for Biotechnology Information database.

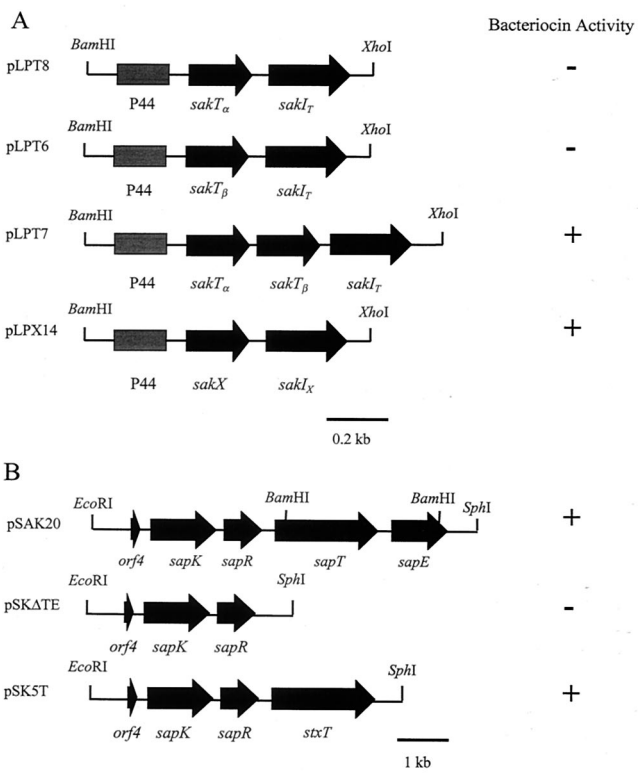

FIG. 2. (A) PCR-derived inserts in pLPV111 derivatives with bacteriocin structural and immunity genes. The plasmid designations are indicated on the left, and the phenotypes when the plasmids are introduced (in conjunction with pSAK20) into *L. sakei* Lb790 are indicated on the right. (B) Inserts in pSAK20, containing the genes necessary for processing and transport of the bacteriocins used, and pSAK20 derivatives used in this study. The phenotypes when these constructs were introduced into *L. sakei* Lb790 or *L. sakei* 23K harboring the pLPV111 derivatives with bacteriocin structural and immunity genes are indicated.

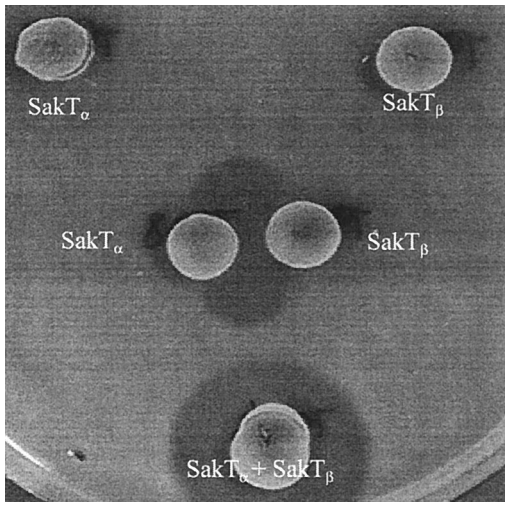

FIG. 3. Bacteriocin activity and complementation of the SakT$_\alpha$ and SakT$_\beta$ peptides. *L. sakei* Lb790(pSAK20) containing pLPT8 (SakT$_\alpha$) and *L. sakei* Lb790(pSAK20) containing pLPT6 (SakT$_\beta$) were grown and overlaid with the sensitive indicator strain *L. sakei* LMG2313. Cultures were grown adjacent to each other and as a mixed culture (SakT$_\alpha$ + SakT$_\beta$).

using this indicator that isolates of *L. sakei* 5 could be obtained which did not exhibit sakacin X production but which still produced sakacin P. Total bacteriocin production was abolished when *L. sakei* 5 was grown in wort at an elevated temperature, 37°C. Once *L. sakei* 5 had lost the ability to produce bacteriocin (Bac$^-$), subcultures of it continued to be Bac$^-$ when they were grown in wort. Addition of sterile CFS derived from a Bac$^+$ culture of *L. sakei* 5 induced stable bacteriocin production in wort at 37°C, whereas CFS derived from a Bac$^-$ culture did not. To determine whether the product of *stxP* was the induction factor for sakacin T and sakacin X production, chemically synthesized mature peptide IP-TX was used to induce bacteriocin production in a Bac$^-$ culture of *L. sakei* 5 grown in wort. Addition of IP-TX at a concentration of 400 ng ml^{-1} restored sakacin T and sakacin X production. To demonstrate that sakacin T production and sakacin X production were switched on simultaneously upon addition of IP-TX, the *L. sakei* LMG2313 derivatives harboring pZ235T and pZ233X with immunity gene inserts (see above) were used as indicators. Both indicator strains were inhibited when IP-TX was added to the wort. To investigate whether the sakacin P-inducing peptide (10) could induce sakacin X production, chemically synthesized mature peptide IP-673 was used to induce bacteriocin production in a Bac$^-$ culture of *L. sakei* 5. Sakacin P was produced, which inhibited *L. sakei* LMG2313 but not *P. pentosaceus* LMG2001, which indicated that sakacin X was not produced. When inoculated into wort without IP-TX, the Bac$^-$ culture failed to produce bacteriocin and served as a negative control. In all cases, whether bacteriocin production was switched on or off following growth in wort, subsequent subculturing in MRS broth resulted in bacteriocin-producing cultures.

Transport of sakacin X and sakacin T does not appear to require an accessory protein. To establish the role of the putative transport protein StxT and the lack of a gene encoding an accessory protein in the native *L. sakei* 5 bacteriocin locus, two derivatives of pSAK20 were constructed. Deletion of the *sapT* and *sapE* transport and accessory protein genes from pSAK20 resulted in construction of pSKΔTE. The *stxT* gene, encoding the putative native transport protein, was cloned into pSKΔTE to obtain plasmid pSK5T. Each transport construct (pSAK20, pSKΔTE, or pSK5T) was transformed into host strain *L. sakei* Lb790X. Then one of the structural gene constructs, pLPT7 (sakacin T) or pLPX14 (sakacin X), was introduced into the resulting transformants. *L. sakei* Lb790X clones containing either pLPT7 or pLPX14 in conjunction with one of the transport constructs (pSAK20, pSKΔTE, or pSK5T) were assayed for inhibitory activity against the indicator strain *L. sakei* LMG2313. The results of this series of experiments for both sakacin T and sakacin X were the same and are discussed collectively below. Bacteriocin production was observed for the pSAK20 and pSK5T transformants. No zones of inhibition were detected surrounding the colonies of the pSKΔTE transformants. However, it is known that while the host strain *L. sakei* Lb790X does not produce bacteriocin, it does contain homologues of the *spp* gene cluster (30, 43), and this could have affected the results obtained with the transport gene con-

structs by gene complementation. The bacteriocin tests were therefore repeated with a different host strain, *L. sakei* 23K, which does not appear to contain *spp* gene homologues (L. Axelsson, personal communication). Bacteriocin production by the pSAK20 and pSK5T transformants was observed. However, no inhibitory activity was observed for pSKΔTE (Fig. 2).

DISCUSSION

In this paper we describe genetic characterization of a bacteriocin cluster of *L. sakei* 5, which was originally isolated from malt. The genetic analysis focused on a 17-kb chromosomal fragment, which was shown to contain the genetic information responsible for production of sakacin X and the two-component bacteriocin sakacin T. This strain also produces sakacin P (57), which was found to be plasmid encoded by Southern blot experiments. The ability to produce several different bacteriocins is expected to endow the producer with a wider inhibitory capacity and therefore an enhanced ability to compete with other bacteria in the same environment. Alternatively, these bacteriocins may act synergistically with each other or with other antimicrobial peptides in a particular environment, as was shown recently (37).

In many cases, bacteriocins produced by an LAB inhibit species that are closely related to the producing strain (38). This is particularly relevant in the brewing industry, as LAB account for a large percentage of spoilage bacteria in the brewing environment (7, 27). In a previous study, sakacin X was shown to inhibit a range of beer spoilage LAB (57), and this was the driving force for characterizing the genetic information of this and other bacteriocins produced by *L. sakei* 5. This strain was shown to produce a single peptide bacteriocin, sakacin X, as well as sakacin T, which requires the complementary activity of two small, unmodified hydrophobic peptides. This finding identifies sakacin T as a member of the class IIb bacteriocins. Both peptides are required for full activity as neither component exhibited bacteriocin activity on its own when it was produced in a heterologous host. However, one component of sakacin T, $SakT_{\beta}$, exhibits a low but detectable level of activity at high concentrations, as observed during purification of this peptide (57).

The organization of the (putative) genes required for sakacin T and sakacin X production and regulation is similar to that of other class II bacteriocin gene clusters (45). The adjacent genetic elements $sakT_{\alpha}$, $sakT_{\beta}$, and $sakI_T$ were identified as the structural and immunity genes for sakacin T. The neighboring genes *sakX* and $sakI_X$ were demonstrated to be the structural and immunity genes, respectively, for sakacin X.

Sakacin T and sakacin X seem to be controlled by a three-component regulatory system consisting of IP-TX, StxK, and StxR (10, 44). In a situation similar to that observed for ABP-118 production by *Lactobacillus salivarius* UCC118 (19), *L. sakei* 5 does not lose the Bac^+ phenotype upon extreme dilution of the culture, a strategy used successfully for obtaining Bac^- derivatives of other bacteriocin systems (10). Obtaining a Bac^- derivative of *L. sakei* 5 was further complicated by the ability of the organism to produce multiple bacteriocins and by the presence of more than one regulatory system controlling the production of sakacin P on the one hand and sakacin T and sakacin X on the other hand. Production of the latter two bacteriocins was specifically induced by addition of chemically synthesized IP-TX to the medium, while supplementation of wort with synthetic peptide IP-673 exclusively induced sakacin P production.

It is not unusual for LAB to produce more than one bacteriocin and to contain a diverse range of bacteriocin-related genes that are dispersed over the chromosome and plasmids (15, 16, 30, 43). For example, the sakacin P gene cluster ends with an incomplete gene, *orfX*, that is likely to encode a bacteriocin (10, 17). ORF 3 of the *abp-118* locus (19) encodes a peptide that is almost identical to presalivaricin B, a bacteriocin produced by *L. salivarius* M6 (53). Four genes in the *sakacin TX* locus exhibit homology to genes required for brochocin C production. A leader peptide sequence precedes ORF 17, but at the sequence specifying the predicted double-glycine motif an apparent frameshift seems to have taken place. This suggests that these genes are the remains of a gene cluster encoding a brochocin C-like bacteriocin. The presence of what seems to be a putative intact immunity gene, ORF 16, might provide *L. sakei* 5 protection against a brochocin C-type bacteriocin produced by competing strains in the same environment.

The sequenced chromosomal fragment flanking the bacteriocin gene cluster in *L. sakei* 5 contains several genes whose products have not been characterized and have unknown functions. The protein products of these genes display significant similarity to hypothetical proteins found in other LAB, which resemble transposases or IS elements. The presence of these proteins, the finding that sakacin P production is plasmid encoded rather than chromosomally encoded (10, 43), and the rearrangement of the *brc*-type genes are interesting and indicate that bacteriocin gene clusters are highly mobile and subject to strong evolutionary pressures.

There is a notable absence of an accessory transport gene in the *sakacin TX* locus. In the present study, we obtained evidence which suggests that an accessory protein is not required for processing or transport of sakacin T and sakacin X, although we cannot completely rule out the possibility that this function is provided by the host strains used in this study. It may be that there is some form of complementation by an unknown chromosomal gene fulfilling the role of an accessory protein. While the specific role of accessory transport proteins in bacteriocin processing is still not fully understood (18), it has been demonstrated for several bacteriocins that such proteins are needed for production (6, 56, 59). Sakacin T and sakacin X, while different from previously described bacteriocins, nevertheless exhibit homology to other bacteriocins and seem to share many characteristics with other standard class II bacteriocins, and so the absence of an accessory transport gene in the locus is unexpected. This adds to the genetic complexity of bacteriocin production in LAB mentioned above.

The antimicrobial properties of the multiple bacteriocins produced by malt isolate *L. sakei* 5 may be used to enhance the microbiological stability of the brewing process and its product, beer. In addition to the sakacin T and sakacin X bacteriocin structural genes, *L. sakei* 5 appears to contain genetic information encoding additional bacteriocin-related peptides, and while some proteins may be inactive, the presence of at least four immunity proteins, which provide immunity to sakacin P, sakacin T, sakacin X, and the brochocin C-like bacteriocin,

should give this bacterium a competitive advantage in its environment. Since *L. sakei* 5 was isolated from malt and since it also inhibits LAB that cause problems for the brewing industry, this bacteriocin-producing strain might be a suitable candidate for use in the industry.

ACKNOWLEDGMENTS

This work was supported by the Department of Agriculture and Food (contracts 97/R&D/C/131 and 00/R&D/C/53), by Heineken Ireland Ltd. through a Heineken Ireland fellowship to A. Vaughan, and by Enterprise Ireland through the International Collaboration Programme (RE:IC/1999/065).

Wort used in this study was kindly provided by Declan Goode of the Department of Food Science, Technology and Nutrition, National University of Ireland, Cork. We are grateful to Lars Axelsson, MATFORSK, Norwegian Food Research Institute, Ås, Norway, for providing results before publication and for providing *L. sakei* Lb790 and plasmids pLPV111 and pSAK20. We thank Monique Zagorec, Unité FLEC, INRA, Domaine di Vilvert, Jouy-en-Josas, France, for providing *L. sakei* 23K.

REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
2. **Anderson, D. G., and L. L. McKay.** 1983. Simple and rapid methods for isolating large plasmid DNA from lactic streptococci. Appl. Environ. Microbiol. **46:**549–552.
3. **Anderssen, E. L., D. B. Diep, I. F. Nes, V. G. H. Eijsink, and J. Nissen-Meyer.** 1998. Antagonistic activity of *Lactobacillus plantarum* C11: two new two-peptide bacteriocins, plantaricins EF and JK, and the induction factor plantaricin A. Appl. Environ. Microbiol. **64:**2269–2272.
4. **Aukrust, T., and H. Blom.** 1992. Transformation of *Lactobacillus* strains used in meat and vegetable fermentations. Food Res. Int. **25:**253–261.
5. **Axelsson, L., T. Katla, M. Bjswsl]ornslett, V. G. H. Eijsink, and A. Holck.** 1998. A system for heterologous expression of bacteriocins in *Lactobacillus sake.* FEMS Microbiol. Lett. **168:**137–143.
6. **Axelsson, L., and A. Holck.** 1995. The genes involved in production of and immunity to sakacin A, a bacteriocin from *Lactobacillus sake* Lb706. J. Bacteriol. **177:**2125–2137.
7. **Back, W.** 1994. Secondary contamination in the filling area. Brauwelt Int. **4:**326–328.
8. **Berthier, F., M. Zagorec, M. C. Champomier-Vergès, S. D. Erlich, and F. Morel-Deville.** 1996. Efficient transformation of *Lactobacillus sake* by electroporation. Microbiology **142:**1273–1279.
9. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
10. **Brurberg, M. B., I. F. Nes, and V. G. H. Eijsink.** 1997. Pheromone-induced production of antimicrobial peptides in *Lactobacillus.* Mol. Microbiol. **26:**347–360.
11. **Casaus, P., T. Nilsen, L. M. Cintas, I. F. Nes, P. E. Hernández, and H. Holo.** 1997. Enterocin B, a new bacteriocin from *Enterococcus faecium* T136 which can act synergistically with enterocin A. Microbiology **143:**2287–2294.
12. **Cintas, L. M., P. Casaus, C. Heranz, L. S. Håvarstein, H. Holo, P. E. Hernández, and I. F. Nes.** 2000. Biochemical and genetic evidence that *Enterococcus faecium* L50 produces enterocins L50A and L50B, the *sec*-dependent enterocin P, and a novel bacteriocin secreted without an N-terminal extension termed enterocin Q. J. Bacteriol. **182:**6806–6814.
13. **Cintas, L. M., P. Casaus, L. S. Håvarstein, P. E. Hernández, and I. F. Nes.** 1997. Biochemical and genetic characterization of enterocin P, a novel *sec*-dependent bacteriocin from *Enterococcus faecium* P13 with a broad antimicrobial spectrum. Appl. Environ. Microbiol. **63:**4321–4330.
14. **Connil, N., Y. Le Breton, X. Dousset, Y. Auffray, A. Rince, and H. Prevost.** 2002. Identification of the *Enterococcus faecalis* tyrosine decarboxylase operon involved in tyramine production. Appl. Environ. Microbiol. **68:**3537–35544.
15. **Diep, D. B., L. S. Håvarstein, and I. F. Nes.** 1996. Characterization of the locus responsible for the bacteriocin production in *Lactobacillus plantarum* C11. J. Bacteriol. **178:**4472–4483.
16. **Eijsink, V. G. H., L. Axelsson, D. B. Diep, L. S. Håvarstein, H. Holo, and I. F. Nes.** 2002. Production of class II bacteriocins by lactic acid bacteria; an example of biological warfare and communication. Antonie Leeuwenhoek **81:**639–654.
17. **Eijsink, V. G. H., M. Skeie, P. H. Middelhoven, M. B. Brurberg, and I. F. Nes.** 1998. Comparative studies of class IIa bacteriocins of lactic acid bacteria. Appl. Environ. Microbiol. **64:**3275–3281.
18. **Ennahar, S., T. Sashihara, K. Sonomoto, and A. Ishizaki.** 2000. Class IIa bacteriocins: biosynthesis, structure and activity. FEMS Microbiol. Rev. **24:**85–106.
19. **Flynn, S., D. van Sinderen, G. M. Thornton, H. Holo, I. F. Nes, and J. K. Collins.** 2002. Characterization of the genetic locus responsible for the production of ABP-118, a novel bacteriocin produced by the probiotic bacterium *Lactobacillus salivarius* subsp. *salivarius* UCC118. Microbiology **148:**973–984.
20. **Franke, C. M., J. Tiemersma, G. Venema, and J. Kok.** 1999. Membrane topology of the lactococcal bacteriocin ATP-binding cassette transporter protein LcnC. J. Biol. Chem. **274:**8484–8490.
21. **Franz, C. M. A. P., M. J. van Belkum, R. W. Worobo, J. C. Vederas, and M. E. Stiles.** 2000. Characterization of the genetic locus responsible for production and immunity of carnobacteriocin A: the immunity gene confers cross-protection to enterocin B. Microbiology **146:**621–631.
22. **Franz, C. M. A. P., R. W. Worobo, L. E. N. Quadri, U. Schillinger, W. H. Holzapfel, J. C. Vederas, and M. E. Stiles.** 1999. Atypical genetic locus associated with constitutive production of enterocin B by *Enterococcus faecium* BFE900. Appl. Environ. Microbiol. **65:**2170–2178.
23. **Haikara, A., and A. Laitila.** 1995. Influence of lactic acid starter culture on the quality of malt and beer, p. 249–256. *In* Proceedings of the 25th Congress of European Brewing Convention, Brussels. IRL Press, Oxford, United Kingdom.
24. **Harley, C. B., and R. P. Reynolds.** 1987. Analysis of *E. coli* promoter sequences. Nucleic Acid Res. **15:**2343–2361.
25. **Håvarstein, L. S., D. B. Diep, and I. F. Nes.** 1995. A family of bacteriocin ABC transporters carry out proteolytic processing of their substrates concomitant with export. Mol. Microbiol. **16:**229–240.
26. **Håvarstein, L. S., H. Holo, and I. F. Nes.** 1994. The leader peptide of colicin V shares consensus sequences with leader peptides that are common amongst peptide bacteriocins produced by Gram-positive bacteria. Microbiology **140:**2383–2389.
27. **Hollerová, I., and P. Kubizniaková.** 2001. Monitoring Gram positive bacterial contamination in Czech breweries. J. Inst. Brew. **107:**355–358.
28. **Holo, H., Ø. Nilssen, and I. F. Nes.** 1991. Lactococcin A, a new bacteriocin from *Lactococcus lactis* subsp. *cremoris*: isolation and characterization of the protein and its gene. J. Bacteriol. **173:**3879–3887.
29. **Horton, R. M., Z. L. Cai, S. N. Ho, and L. R. Pease.** 1990. Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. BioTechniques **8:**528–535.
30. **Hühne, K., A. Holck, L. Axelsson, and L. Kroeckel.** 1996. Analysis of the sakacin P gene cluster from *Lactobacillus sake* Lb674 and its expression in sakacin-negative *L. sake* strains. Microbiology **142:**1437–1448.
31. **Jack, R. W., J. Wan, J. Gordon, K. Harmark, B. E. Davidson, A. J. Hillier, R. E. Wettenhall, M. W. Hickey, and M. J. Coventry.** 1996. Characterization of the chemical and antimicrobial properties of piscicolin 126, a bacteriocin produced by *Carnobacterium piscicola* JG126. Appl. Environ. Microbiol. **62:**2897–2903.
32. **Kemperman, R., A. Kuipers, H. Karsens, A. Nauta, O. Kuipers, and J. Kok.** 2003. Identification and characterization of two novel clostridial bacteriocins, circularin A and closticin 574. Appl. Environ. Microbiol. **69:**1589–1597.
33. **Klaenhammer, T. R.** 1993. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol. Rev. **12:**39–85.
34. **Kleerebezem, M., L. E. Quadri, O. P. Kuipers, and W. M. de Vos.** 1997. Quorum sensing by peptide pheromones and two-component signal-transduction systems in Gram-positive bacteria. Mol. Microbiol. **24:**895–904.
35. **Law, J., G. Buist, A. Haandrikman, J. Kok, G. Venema, and K. Leenhouts.** 1995. A system to generate chromosomal mutations in *Lactococcus lactis*, which allows fast analysis of targeted genes. J. Bacteriol. **177:**7011–7018.
36. **Leer, R. L., J. M. B. M. van der Vossen, M. van Giezen, J. M. van Noort, and P. H. Pouwels.** 1995. Genetic analysis of acidocin B, a novel bacteriocin produced by *Lactobacillus acidophilus.* Microbiology **141:**1629–1635.
37. **Lüders, T., G. A. Birkemo, G. Fimland, J. Nissen-Meyer, and I. F. Nes.** 2003. Strong synergy between a eukaryotic antimicrobial peptide and bacteriocins from lactic acid bacteria. Appl. Environ. Microbiol. **69:**1797–1799.
38. **Marciset, O., M. C. Jeronimus-Stratingh, B. Mollet, and B. Poolman.** 1997. Thermophilin 13, a nontypical antilisterial poration complex bacteriocin, that functions without a receptor. J. Biol. Chem. **272:**14277–14284.
39. **Marinez-Bueno, M., M. Maquedam A. Galvez, B. Samyn, J. van Beeumen, J. Coyette, and E. Valdivia.** 1994. Determination of the gene sequence and the molecular structure of the enterococcal peptide antibiotic AS-48. J. Bacteriol. **176:**6334–6339.
40. **Mayr-Harding, A., A. J. Hedges, and R. C. W. Buckley.** 1972. Methods for studying bacteriocins. Academic Press, New York, N.Y.
41. **McCormick, J. K., A. Poon, M. Sailer, Y. Gao, K. L. Roy, L. M. McMullen, J. C. Vederas, M. E. Stiles, and M. J. van Belkum.** 1998. Genetic characterization and heterologous expression of brochocin C, an antibotulinal, two-peptide bacteriocin produced by *Brochothrix campestris* ATCC 43754. Appl. Environ. Microbiol. **64:**4757–4766.
42. **McGrath, S., G. F. Fitzgerald, and D. van Sinderen.** 2001. Improvement and optimization of two engineered phage resistance mechanisms in *Lactococcus lactis.* Appl. Environ. Microbiol. **67:**608–616.
43. **Møretrø, T.** 2000. Optimization of production of the bacteriocin sakacin P by

Lactobacillus sakei cultural conditions and genetics. Ph.D. thesis. Agricultural University of Norway, Ås, Norway.
44. **Nes, I. F., and V. G. H. Eijsink.** 1999. Regulation of group II peptide bacteriocin synthesis by quorum-sensing mechanisms, p. 175–192. *In* G. M. Dunny and S. C. Winans (ed.), Cell-cell signaling in bacteria. American Society for Microbiology, Washington, D.C.
45. **Nes, I. F., D. B. Diep, L. S. Håvarstein, M. B. Brurberg, V. G. H. Eijsink, and H. Holo.** 1996. Biosynthesis of bacteriocins in lactic acid bacteria. Antonie Leewenhoek **70:**113–128.
46. **O'Keeffe, T., C. Hill, and R. P. Ross.** 1999. Characterization and heterologous expression of the genes encoding enterocin A production, immunity, and regulation in *Enterococcus faecalis* DPC1146. Appl. Environ. Microbiol. **65:**1506–1515.
47. **O'Mahony, A. T., F. O'Sullivan, Y. Walsh, A. Vaughan, M. Maher, G. F. Fitzgerald, and D. van Sinderen.** 2000. Characterization of antimicrobial producing lactic acid bacteria from malted barley. J. Inst. Brew. **106:**403–410.
48. **O'Sullivan, T. F., Y. Walsh, A. O'Mahony, G. F. Fitzgerald, and D. van Sinderen.** 1999. A comparative study of malthouse and brewhouse microflora. J. Inst. Brew. **105:**55–61.
49. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
50. **Skaugen, M.** 1994. Transposition in *Lactobacillus sake* and its abolition of lactocin S production by insertion of IS1163, a new member of the IS3 family. Appl. Environ. Microbiol. **60:**2818–2825.
51. **Stiles, M. E.** 1996. Biopreservation by lactic acid bacteria. Antonie Leeuwenhoek **70:**331–345.
52. **Takami, H., K. Nakasone, Y. Takaki, G. Maeno, R. Sasaki, N. Masui, F. Fuji, C. Hirama, Y. Nakamura, N. Ogasawara, S. Kuhara, and K. Horikoshi.** 2000. Complete genome sequence of the alkaliphilic bacterium *Bacillus halodurans* and genomic sequence comparison with *Bacillus subtilis.* Nucleic Acids Res. **28:**4317–4331.
53. **ten Brink, B., M. Minekus, J. M. van der Vossen, R. J. Leer, and J. H. Huis in't Veld.** 1994. Antimicrobial activity of lactobacilli: preliminary characterization and optimisation of production of acidocin B, a novel bacteriocin produced by *Lactobacillus acidophilus* M46. J. Appl. Bacteriol. **77:**140148.
54. **Tettelin, H., V. Masignani, M. J. Cieslewicz, J. A. Eisen, S. Peterson, M. R. Wessels, I. T. Paulsen, K. E. Nelson, I. Margarit, T. D. Read, L. C. Madoff, A. M. Wolf, M. J. Beanan, L. M. Brinkac, S. C. Daugherty, R. T. DeBoy, A. S. Durkin, J. F. Kolonay, R. Madupu, M. R. Lewis, D. Radune, N. B. Fedorova, D. Scanlan, H. Khouri, S. Mulligan, H. A. Carty, R. T. Cline, S. E. Van Aken, J. Gill, M. Scarselli, M. Mora, E. T. Iacobini, C. Brettoni, G. Galli, M. Mariani, F. Vegni, D. Maione, D. Rinaudo, R. Rappuoli, J. L. Telford, D. L. Kasper, G. Grandi, and C. M. Fraser.** 2002. Complete genome sequence and comparative genomic analysis of an emerging human pathogen, serotype V *Streptococcus agalactiae.* Proc. Natl. Acad. Sci. **99:**12391–12396.
55. **Tichaczek, P. S., J. Nissen-Meyer, I. F. Nes, R. F. Vogel, and W. P. Hammes.** 1992. Characterization of the bacteriocins curvacin A from *Lactobacillus curvatus* LTH1174 and sakacin P from *Lactobacillus sake* LTH673. Syst. Appl. Microbiol. **15:**460–468.
56. **Van Belkum, M. J., and M. E. Stiles.** 1995. Molecular characterization of genes involved in the production of the bacteriocin leucocin A from *Leuconostoc gelidum*. Appl. Environ. Microbiol. **61:**3573–3579.
57. **Vaughan, A., V. G. H. Eijsink, T. F. O'Sullivan, K. O'Hanlon, and D. van Sinderen.** 2001. An analysis of bacteriocins produced by lactic acid bacteria isolated from malted barley. J. Appl. Microbiol. **91:**131–138.
58. **Vaughan, E. E., and W. M. de Vos.** 1995. Identification and characterization of the insertion element IS070 from *Leuconostoc lactis* NZ6009. Gene **155:** 95–100.
59. **Venema, K., J. Kok, J. D. Marugg, M. Y. Toonen, A. M. Ledeboer, G. Venema, and M. L. Chikindas.** 1995. Functional analysis of the pediocin operon of *Pediococcus acidilactici* PAC1.0: PedB is the immunity protein and PedD is the precursor processing enzyme. Mol. Microbiol. **17:**515–522.
60. **Worobo, R. W., T. Henkel, M. Sailer, K. L. Roy, J. C. Vederas, and M. E. Stiles.** 1994. Characteristics and genetic determinant of a hydrophobic peptide bacteriocin, carnobacteriocin A, produced by *Carnobacterium piscicola* LV17A. Microbiology **140:**517–526.