Cloning and sequencing of *sakP* encoding sakacin P, the bacteriocin produced by *Lactobacillus sake* LTH 673

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Sakacin P is a heat-stable, unmodified peptide bacteriocin produced by *Lactobacillus sake* LTH 673. The strain was isolated from fermented dry sausages and is well adapted to this habitat. The bacteriocin inhibits the growth of the opportunistic food pathogens *Enterococcus faecalis* and *Listeria monocytogenes* and therefore, it may improve the hygienic status of fermented food, i.e. meat products. Oligonucleotide probes were designed from the N-terminal amino acid sequence of sakacin P and used to identify *sakP*, the structural gene of sakacin P, on the chromosome of *L. sake* LTH 673. *SakP* was cloned into *Escherichia coli* NM554 and the nucleotide sequence of the gene and its adjacent regions were determined. Sakacin P appears to be synthesized as a prepeptide of 61 amino acids which is proteolytically processed to the mature bacteriocin consisting of 43 amino acids. Sequencing of the cloned fragment also revealed the presence of two other open reading frames *orfX* and *orfY*, which are located upstream and downstream of *sakP*, respectively, putatively encoding proteins of 52 and 98 amino acids, respectively. The functions of both ORFs remain unknown. Primer extension analysis revealed a promoter upstream of *sakP*. Two transcripts of approximately 0.35 and 1.0 kb were detected by Northern hybridization encoding either only *sakP*, or both *sakP* and *orfY*, respectively.

Keywords: *Lactobacillus sake*, bacteriocin, sakacin P, *sakP*, food hygiene

INTRODUCTION

Antimicrobial activities of lactic acid bacteria (LAB) have been investigated since 1951 (Wheater *et al.*, 1951; Vincent *et al.*, 1959; Daeschel, 1989; Hammes *et al.*, 1990). Whereas unspecific, broad-spectrum inhibition is generally attributed to organic acid production and/or to hydrogen peroxide, a narrow inhibitory spectrum, mainly including closely related species, can be linked to the synthesis and the secretion of bacteriocins. These substances are antimicrobial peptides or proteins, whose bactericidal or bacteriostatic activity usually is directed against a few bacterial species (Tagg *et al.*, 1976; Klaenhammer, 1988). However, some bacteriocins of LAB also inhibit pathogens and spoilage organisms found in foods, and thus, they may be of interest in process control and food

preservation. On the basis of the protein structure of LAB bacteriocins, several groups can be distinguished (Tichaczek *et al.*, 1993). The unmodified peptide bacteriocins share some similarities, i.e. they have a N-terminal sequence proteolytically cleaved off before or during secretion (Hastings *et al.*, 1991; Holo *et al.*, 1991). Comparing the amino acid sequences of some mature peptide bacteriocins revealed the common sequence Y-G-N-G-V-X-C in their N-terminal region, indicating that this consensus sequence may be of importance for this group of bacteriocins (Lozano *et al.*, 1992).

A number of bacteriocin genes have recently been cloned and sequenced including lactococcins (van Belkum *et al.*, 1989, 1991; Holo *et al.*, 1991), helveticin J (Joerger & Klaenhammer, 1990), lactacin F (Muriana & Klaenhammer, 1991) and leucocin A-UAL 187 (Hastings *et al.*, 1991). Some bacteriocins have been shown to be either plasmid encoded (Daeschel & Klaenhammer, 1985; Buchman *et al.*, 1988; Mørdtvedt & Nes, 1990; Muriana & Klaenhammer, 1991; Tichaczek *et al.*, 1993) or located on the chromosome (Barefoot & Klaenhammer, 1983;

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Joerger & Klaenhammer, 1986; Grinstead & Barefoot, 1992). A strain can produce more than one bacteriocin as shown for *Lactobacillus plantarum* LPCO10 (Jiménez-Díaz *et al.*, 1993) and *Lactococcus lactis* subsp. *cremoris* 9B4. From the latter strain the genes encoding three different lactococcins and their respective immunity proteins have been cloned and sequenced (van Belkum *et al.*, 1991, 1992). For the production of active lactococcin, two additional genes (*lcnC* and *lcnD*) are required, which are implicated in secretion (Stoddard *et al.*, 1992). The genes involved in the production of pediocin PA-1 are organized in an operon which includes the structural gene and additional ORFs which are required for bacteriocin biosynthesis (Marugg *et al.*, 1992).

Cloning and sequencing of bacteriocin genes and their adjacent regions allow insight into their biosynthesis, transport and regulation. This knowledge can be used to develop new protective starter organisms with the potential to improve both the hygienic status of food and their competitiveness in food fermentations (Schillinger *et al.*, 1991; Foegeding *et al.*, 1992; Vogel *et al.*, 1993). Due to the inhibitory spectrum of their bacteriocins, *L. curvatus* LTH 1174 and *L. sake* LTH 673 producing curvacin A and sakacin P, respectively (Tichaczek *et al.*, 1992), are likely candidates for application in meat fermentation. The gene encoding curvacin A has been cloned and sequenced recently (Tichaczek *et al.*, 1993).

The present work describes the cloning and nucleotide sequencing of the DNA region containing *sakP*, the gene encoding the peptide bacteriocin sakacin P. This heat-stable unmodified peptide bacteriocin is produced by *Lactobacillus sake* LTH 673 and inhibits strains of some *Lactobacillus* species as well as *Listeria monocytogenes*, *Enterococcus faecalis* and *Carnobacterium* sp. (Tichaczek *et al.*, 1992).

METHODS

Bacterial strains, plasmids and media. *Lactobacillus sake* LTH 673 was isolated from fermented sausage. *Escherichia coli* HB102 harbouring pRB473 was kindly provided by R. Brückner, Tübingen, Germany. *E. coli* NM554 and the plasmid vector pBluescript SK+ (Stratagene) were used for cloning experiments. *L. sake* LTH 673 was grown in MRS medium (de Man *et al.*, 1960) at 30 °C. *E. coli* strains were propagated at 37 °C in SOB broth with shaking (180 r.p.m.) or on SOB agar plates (Hanahan, 1983). Selective media contained ampicillin at a final concentration of 100 µg ml⁻¹ [SOB(amp)]. Bacteriocin production by *L. sake* LTH 673 was determined as described previously (Tichaczek *et al.*, 1992).

Construction and amplification of the genomic library. DNA of *L. sake* LTH 673 and *E. coli* was isolated as described before (Tichaczek *et al.*, 1993). Chromosomal DNA of *L. sake* LTH 673 was cleaved with *Bcl*I. DNA fragments of approximately 6 kb were isolated from preparative 0.8% agarose gels by the method of Dretzen *et al.* (1981). The fragments were ligated into the *Bam*HI-digested pRB473 vector with T4 DNA ligase (Boehringer). The ligation mixture was used to transform competent *E. coli* NM554 cells by the method of Hanahan (1983). The transformants were washed off the agar plates and collected by centrifugation. The cells were resuspended in a

total volume of 1 ml of SOB(amp) medium and mixed with an equal volume of sterile 60% (v/v) glycerol and stored at -85 °C.

Detection and cloning of *sakP*. For the detection of *sakP* in Southern hybridizations, DNA of *L. sake* LTH 673 was digested with various restriction enzymes and the fragments were separated in 0.8% agarose gels according to their size. *Hind*III fragments of bacteriophage λ DNA (Boehringer) were used as molecular mass markers.

A sakacin P-specific probe for DNA hybridizations was deduced from the sequence of the amino acids 11–19 of purified sakacin P which was KHSCTVDWG (Tichaczek *et al.*, 1992). The degeneration of this 26-mer oligonucleotide was 16-fold. The sequence was 5' CCC CAI TCI(C) ACI(C) GTI CAI(C) G(C)II TGT TT 3'. The probe was end-labelled with [γ -³²P]-ATP (Amersham) by using a 5' terminus labelling system (Pharmacia). Prehybridization and hybridization with the probe was performed essentially as described by Sambrook *et al.* (1989) with modifications as described by Tichaczek *et al.* (1993).

Southern hybridizations of the fractionated DNA and detection of clones containing the bacteriocin gene were performed as described before (Tichaczek *et al.*, 1993). Positive clones and subclones were cultured overnight in 10 ml SOB(amp) broth. Plasmids were isolated from the cultures, digested with *Hind*III/*Eco*RI and inserts were analysed by Southern hybridization. Plasmid DNA of positive clones was isolated and cleaved with *Nru*I/*Pvu*II. For the construction of subclones, a 1.0 kb *Nru*I/*Pvu*II fragment was ligated into the *Eco*RV-digested pBluescript SK+ vector and the ligation mixture was used to transform competent *E. coli* NM554 cells.

DNA sequencing and analysis. Double-stranded DNA sequencing and sequence analysis was performed as described previously (Tichaczek *et al.*, 1993). The molecular mass of the peptides was calculated with the PROSIS software (Pharmacia).

Analyses of the mRNA. mRNA was isolated from lactobacilli and *E. coli* by the method of Obst (1993). The site for transcription initiation was determined by primer extension analysis with reverse transcriptase (BRL). Two oligonucleotide primers were used which were complementary to the 5' region of the mRNA (*sakpr*1, 5' ACCGTTACCATAATATT 3'; and *sakpr*2, 5' AAACCTTTCCATTAGAA 3'). For Northern hybridizations ³²P end-labelled *sakpr*1 was used as probe. Primer extension and Northern hybridization analysis were performed as described by Sambrook *et al.* (1989).

Fig. 1. Restriction map of the DNA fragment containing *sakP* and the location of the ORFs. The ORFs are indicated by boxes, with arrowheads showing the direction of transcription. *sakpr*1 and *sakpr*2 were used for primer extension (compare Fig. 3). The arrows show the length and direction of the sequence runs obtained using these primers. Vector DNA is indicated by double lining.

RESULTS

Detection of *sakP*

The structural gene encoding sakacin P (*sakP*) was identified by Southern hybridization using a 26-mer oligonucleotide probe based on the amino acid sequence of sakacin P as determined by Tichaczek *et al.* (1992). No plasmids were detected in *L. sake* LTH 673 suggesting the chromosomal location of the hybridizing sequence. In Southern hybridizations with total DNA, a diffuse signal was obtained at the position of the chromosomal DNA in the gel. One strong signal was detected when total DNA of *L. sake* LTH 673 was subjected to single and double restriction enzyme digestions and analysed by Southern hybridization. The *sakP* gene was identified on a 5.8 kb *Bcl*I fragment and a 1.0 kb *Nru*I/*Pvu*II fragment, respectively.

Cloning and subcloning of *sakP*

*Bcl*I-generated fragments of approximately 6 kb were ligated into plasmid pRB473 and cloned in *E. coli* NM554. Positive clones were detected by colony hybridization with the 26-mer oligonucleotide probe. Plasmid DNA was isolated from the hybridizing clones, digested with *Hind*III/*Eco*RI, and shown to contain a 5.8 kb insert of *Lactobacillus* DNA reacting with the probe. One of the plasmids harbouring the 5.8 kb *Bcl*I DNA insert was designated pPT1000. The hybridizing 1.0 kb *Nru*I/*Pvu*II fragment of pPT1000 was subcloned into the *Eco*RV site of pBluescript SK+ *E. coli* NM554, resulting in pPT1100.

Sequencing of the cloned fragments

Sequencing of the 1.0 kb insert of pPT1100 and parts of the insert of pPT1000 revealed the presence of three ORFs that could encode polypeptides of 52, 98 and 61 amino acids referred to as *orfX*, *orfY* and *orfZ*, respectively. The restriction map of the cloned fragment and the location of the ORFs are depicted in Fig. 1. Translation of *orfZ* resulted in a sequence, part of which was identical to the amino acid sequence determined by Edman degradation of purified sakacin P (Tichaczek *et al.*, 1992). Thus, *orfZ* is the structural gene encoding sakacin P and will subsequently be referred to as *sakP*. The nucleotide sequence of *sakP* and its adjacent regions is shown in Fig. 2. The *sakP* primary product has a deduced M_r of 6380, and the M_r values of the peptides putatively encoded by *orfX* and *orfY* were calculated to be 6000 and 11500, respectively.

Analyses of the mRNA

The site for transcription initiation was determined by primer extension as shown in Fig. 3. Additional signals of mRNAs with a smaller size were observed probably resulting from partially degraded mRNA. The guanine residue 365 was identified as the 5' terminus of the mRNA and corresponding -10 and -35 regions were detected upstream of guanine residue 365. A putative ribosome

Fig. 3. Determination of the transcription initiation site in *sakP*. The result is depicted by sequence gel runs and a corresponding primer extension experiment with reverse transcriptase using two different oligonucleotides (*sakpr1* and *sakpr2*) directed against *sakP* mRNA. The sequence runs are marked A, C, G and T for the dideoxy nucleotides used in the Sanger reaction. The primer extension lanes with mRNA from *L. sake* LTH 673 and *E. coli* NM554(pPT1000) are marked L and E, respectively. Both signals marked by arrowheads indicate that guanine 365 is the starting point of transcription. The probable -10 region is boxed.

binding site was present 10 bp upstream of the ATG specifying the N-terminal methionine of pre-sakacin P (Fig. 2). In Northern hybridizations with RNA isolated from *L. sake* LTH 673, two signals were obtained corresponding to the mRNAs of 0.35 and 1.0 kb in length, respectively (results not shown). With RNA isolated from *E. coli* NM554 harbouring pPT1000 the same primer extension signal was obtained which was much less intense with the same amount of RNA applied (Fig. 3). This indicates weak transcription of *sakP* in the *E. coli* transformant. *E. coli* NM554 containing pPT1000 was not able to inhibit the indicator organism.

Similarity to other bacteriocins

Comparison with a DNA sequence databank (HUSAR) revealed similarity to pediocin PA-1 (Marugg *et al.*, 1992)

	1	10	20	30	40
Curvacin A (1)/ sakacin A (2)	ARSYGNVYCNKKCWNRGEATQSIIGGMISGWASGLAGM				
Sakacin P (3)	KYYGNGVHCCKHSCVDWGTAGNIGNNAAANWATGGNAGWNK				
Pediocin PA-1 (4)	KYYGNGVTCGKHSCVDWGTATTCIINNGAMAWATGGHQQGNHCK				
Mesentericin Y 105 (5)	KYYGNGVHCTKSGCSVNWGEA----ASAGIHLRANGGNGFW				
Leucocin A (6)	KYYGNGVHCTKSGCSVNWGEA----FSAGVHRLANGGNGFW				

Fig. 4. Similarity of the mature sakacin P to other bacteriocins. The presence of identical and related amino acids is indicated by * and :, respectively. (1), Tichaczek *et al.* (1993); (2), Holck *et al.* (1992); (3), this work; (4), Marugg *et al.* (1992); (5), Héchard *et al.* (1992); (6), Hastings *et al.* (1991).

Sakacin P (1)	MEKFIELSLKEVTAITGG↓KY
Pediocin PA-1 (2)	MKKIEKLTEKEMANIIGG↓KY
Curvacin A(3)/sakacin A (4)	MNNVKELSMTELQTITGG↓AR
Lactococcin A (5)	MKNQLNFNIVSDEELSEANGG↓KL
Leucocin A-UAL 187 (6)	MMNMKPTESYEQLDNSALEQVVG↓KY
Lactacin F (7)	MKNFNLYLSHKDLAVVVG↓RN
Neutral protease*(7)	MNKRAMLGAIGLAFLGLAAPIGASA↓KG
Cell wall protein (8)	MKKVVNSVLASALALTVPMAFA↓AE

Fig. 5. Comparison of the leader sequences of small unmodified peptide bacteriocins. The cleavage site between pre-sequence and mature bacteriocin is indicated by an arrow. Two typical signal sequences from *Bacillus* species are added to demonstrate their dissimilarity to the bacteriocin leader sequences. Positively and negatively charged amino acids are indicated by + and -, respectively. The bacteriocin leader sequences share a GG consensus sequence at the cleavage site and absence of a hydrophobic core present in the signal peptides. (1), This work; (2), Marugg *et al.* (1992); (3) Tichaczek *et al.* (1993); (4), Holck *et al.* (1992); (5), Holo *et al.* (1991); (6), Hastings *et al.* (1991); (7), Muriana & Klaenhammer (1991); (8), Simonen & Palva (1993). The neutral protease (*) cleavage site was suggested, not experimentally proved.

and leucocin A-UAL 187 (Hastings *et al.*, 1991). As depicted in Fig. 4, further similarities to curvacin A (Tichaczek *et al.*, 1992, 1993) and mesentericin Y 105 (Héchard *et al.*, 1992) were detected. The comparison of the amino acid sequences of the mature peptides is depicted in Fig. 4, the amino acid sequences of N-terminal leader sequences common for peptide bacteriocins are shown in Fig. 5. No sequence homology to other proteins was found for the putative *orfX* and *orfY* products.

DISCUSSION

The comparison of the amino acid sequence deduced from *sakP* with the amino acid sequence of sakacin P determined by Edman degradation of the purified bacteriocin, revealed that sakacin P is synthesized as a prepeptide. The location of the putative AGGAG ribosome binding site 10 bp upstream of the ATG at position 429 indicates

that the methionine specified by this codon probably is the N-terminal end of pre-sakacin P (Thomas *et al.*, 1982). Thus, *sakP* encodes a 61 amino acid prepeptide. The N-terminal leader peptide appears to be proteolytically cleaved off before or at secretion, because the biologically active substance does not contain this region. This leader sequence does not share any homology with conserved regions common in signal peptides (Simonen & Palva, 1993). It lacks the typical hydrophobic α -helix core region as it contains some charged amino acids which are also found in the leader sequences of other bacteriocins (Marugg *et al.*, 1992). Furthermore, the presence of two glycine residues adjacent to the cleavage site in the C terminus of the leader sequence is common in prepeptides of unmodified small bacteriocins (Marugg *et al.*, 1992). This type of cleavage site is not observed in typical signal peptides for exported proteins (Simonen & Palva, 1993; Pugsley, 1993). The C terminus of the leader sequence with the Gly-Gly residues is inconsistent with the (-3, -1) rule of von Heijne (1988). A novel excretion mechanism for the bacteriocins, as has been described for lactococcins (Stoddard *et al.*, 1992), may account for these differences from the expected structure of signal peptides (Gierasch, 1989). In contrast, the leader sequence might function as a chaperone maintaining a specific, inactive conformation of the bacteriocin (Tichaczek *et al.*, 1993). Upstream of *sakP* a second ORF, *orfX*, is located which could encode a protein of 52 amino acids. The function of this putative protein remains unknown and it shares no sequence homology with any known protein. Sequence analysis of this region revealed the presence of a putative ribosome binding site 11 bp upstream of the putative initiating methionine codon. Downstream of *orfX*, a terminator sequence was detected suggesting that *orfX* forms a mRNA independent of *sakP* transcription.

Downstream of *sakP* another ORF, *orfY*, was identified which could encode a protein of 98 amino acids. *orfY* is probably transcribed together with *sakP* from the same transcription initiation site. Transcription of *sakP* together with *orfX* upstream of *sakP* is unlikely, due to a strong terminator structure between *orfX* and *sakP*. An operon consisting of a bacteriocin structural gene closely followed by an ORF of 90–110 amino acid residues seems to be a general feature for this type of bacteriocin (Hastings *et al.*, 1991). These ORFs could encode immunity proteins (Hastings *et al.*, 1991; Holo *et al.*, 1991). The presence of immunity genes was demonstrated by van Belkum *et al.* (1992). These (putative) immunity proteins do not share amino acid sequence homology.

Nevertheless, the length of *orfY* and its organization in one operon with *sakP* suggest a function of its product as an immunity protein. *sakP* was also transcribed as a single transcript. This may be due to a weak terminator structure between *sakP* and *orfY* in addition to the terminator downstream of *orfY*. Studies on the expression of *sakP* and immunity against sakacin P may reveal and confirm the role *orfY*.

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