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

Petra S. Tichaczek, Rudi F. Vogel† and Walter P. Hammes

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 secretion of bacteriocins. These substances are attributed to organic acid production and/or to hydrogen unspecific, broad-spectrum inhibition is generally attributed to organic acid production and/or to hydrogen unspecific, broad-spectrum inhibition is generally attributed to organic acid production and/or to hydrogen unspecific, broad-spectrum inhibition is generally attributed to organic acid production and/or to hydrogen unspecific, broad-spectrum inhibition is generally attributed to organic acid production and/or to hydrogen unspecific, broad-spectrum inhibition is generally attributed to organic acid production and/or to hydrogen

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

Present address: Lehrstuhl für Technische Mikrobiologie, Technische Universität München, 85350 Freising, Germany.

The GenBank accession number for the sequence reported in this paper is X75081.

† Present address: Lehrstuhl für Technische Mikrobiologie, Technische Universität München, 85350 Freising, Germany.
Joerger & Klaenhammer, 1986; Grinstead & Barfoot, 1992). A strain can produce more than one bacteriocin as shown for Lacticobacillus plantarum LPCO10 (Jiménez-Díaz et al., 1993) and Lactococcus lactis subsp. cremoris 9B4. From the latter strain the genes encoding three different lactococci and their respective immunity proteins have been cloned and sequenced (van Belkum et al., 1991, 1992). For the production of active lactococci, two additional genes (lcnC and lend) 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., 1992). A strain can produce more than one bacteriocin as potential to improve both the hygienic status of food and 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., 1992). For the production of active lactococcins and their respective immunity proteins have shown for Lactobacillus sake LTH 673 was digested with various restriction enzymes and the fragments were separated in 0.8 % agarose gels according to their size. HindIII 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 KHSC4TDWG (Tichaczek et al., 1992). The degeneration of this 26-mer oligonucleotide was 16-fold. The sequence was 5’ CCC C1T (C) A1(C) G1(C) C1G (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 HindIII/EcoRI and inserts were analysed by Southern hybridization. Plasmid DNA of positive clones was isolated and cleaved with NraI/PraII. For the construction of subclones, a 10 kb NraI/PraII fragment was ligated into the EcoRV-digested Bluescript SK+ vector and the ligation mixture was used to transform competent E. coli NM554 cells.

**METHODS**

**Bacterial strains, plasmids and media.** Lacticobacillus sake LTH 673 was isolated from fermented sausage. Escherichia coli HB102 harbouring pBR427 was kindly provided by R. Brückner, Tübingen, Germany. E. coli NM554 and the plasmid vector Bluescript 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 containing 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 BglII. 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 BamHI-digested pBR427 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. HindIII 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 KHSC4TDWG (Tichaczek et al., 1992). The degeneration of this 26-mer oligonucleotide was 16-fold. The sequence was 5’ CCC C1T (C) A1(C) G1(C) C1G (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).

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**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 Prosite 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 (sakpr1, 5’ ACCGTACCATAATATT 3’; and sakpr2, 5’ AAACCTTTGCATTAGAA 3’). For Northern hybridizations the 32P end-labelled sakpr1 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. sakpr1 and sakpr2 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.
Cloning of sakP from Lactobacillus sake LTH673

**Fig. 2.** Nucleotide sequence of sakP and its adjacent regions. The boxed areas indicate sakP, orfX and orfY. The shaded box covers the mature sakacin P. The sequences responsible for transcription initiation and the putative ribosome binding sites are boxed and marked 1, -10, -35 and rbs, respectively. The stop codons are marked with asterisks. Putative terminator sites are indicated by arrows. The AGO values of the terminator structures observed downstream of orfX, sakP and orfY were -21.1, -18.1 and -18.6 kcal mol⁻¹, respectively.
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 BclI fragment and a 1.0 kb NraI/PvuII fragment, respectively.

Cloning and subcloning of sakP

BclI-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 HindIII/EcoRI, 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 BclI DNA insert was designated pPT1000. The hybridizing 1.0 kb NraI/PvuII fragment of pPT1000 was subcloned into the EcoRV 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 Mr of 6380, and the Mr 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 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)
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Curvacin A (1)/Sakacin A (2)  
Sakacin P (3)  
Pediocin PA-1 (4)  
Mesentericin Y 105 (5)  
Leucocin A (6)  

| Curvacin A (1)/Sakacin A (2) |  
|-----------------------------|---|
| 1 | ARSYGNGVYCNKCCWNRGEATQISOIGMSGWSGLAGM  
| 10 |  
| 20 | KYYGNVHCEKCSSTWGVTAIGN1GNAAANWATGNGAWK  
| 30 |  
| 40 |  

| Pediocin PA-1 (4) |  
|-----------------|---|
| 1 | KYYGNVHTCKSGCVSNVGEA---ASAGIHLANGGNGFW  
| 10 |  
| 20 |  
| 30 |  

| Mesentericin Y 105 (5) |  
|---------------------|---|
| 1 | KYYGNVHCTKSNGCVSNVEA---ASAGIHLANGGNGFW  
| 10 |  
| 20 |  
| 30 |  

| Leucocin A (6) |  
|---------------|---|
| 1 | KYYGNVHTKSNGCVSNVEA---FSAGVHLANGGNGFW  

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)  
Pediocin PA-1 (2)  
Curvacin A(3)/sakacin A (4)  
Lactococcin A (5)  
Leucocin A-UAL 187 (6)  
Lactacin F (7)  
Neutral protease*(7)  
Cell wall protein (8)  

| Sakacin P (1) |  
|---------------|---|
| 1 | MEEFLSLKVEAITGGLKY  
| 10 |  
| 20 | +---+---+---+---+  
| 30 |  

| Pediocin PA-1 (2) |  
|-----------------|---|
| 1 | MKKIELSLKVEAITGGLKY  
| 10 |  
| 20 | +---+---+---+---+  
| 30 |  

| Curvacin A(3)/sakacin A (4) |  
|-----------------------------|---|
| 1 | MNNKELSMTETQITIGGAR  
| 10 |  
| 20 | +---+---+---+---+  
| 30 |  

| Lactococcin A (5) |  
|------------------|---|
| 1 | MNQKLFNIVDSDEELSEAGGKL  
| 10 |  
| 20 | +---+---+---+---+  
| 30 |  

| Leucocin A-UAL 187 (6) |  
|------------------------|---|
| 1 | MNKNTPSYEQDNLSEALQVGGKLY  
| 10 |  
| 20 | +---+---+---+---+  
| 30 |  

| Lactacin F (7) |  
|---------------|---|
| 1 | MNKFNLYSLKDAIVVGGGRRN  
| 10 |  
| 20 | +---+---+---+---+  
| 30 |  

| Neutral protease*(7) |  
|---------------------|---|
| 1 | MNKRA11GIAQFLGGLLAAPNGASLG  
| 10 |  
| 20 | +---+---+---+---+  
| 30 |  

| Cell wall protein (8) |  
|----------------------|---|
| 1 | MKNKVNLSALVLTPAFA1AEE  
| 10 |  
| 20 |  

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 GGA 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), Hollo 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 orfY 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; Hollo 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 of orfY.

ACKNOWLEDGEMENTS

This work was supported by the Bundesministerium für Forschung und Technologie grant 0319280B and the Commission of the European Communities in the BRIDGE T-project on Lactic Acid Bacteria Contract BIOT CT91 0263 (SSMA).

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L. Tschaczek, R. F. Vogel and W. P. Hammes


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Received 7 June 1993; revised 3 September 1993; accepted 14 September 1993.