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## Molecular Characterization of the Gene Encoding for the Salivaricin B Activity and its Flanking Sequences

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**Abstract:** Lactic acid bacteria (LAB) produce many different antimicrobial proteins, some of which have potential in food preservation. The molecular analysis of bacteriocins has gained much attention and has advanced rapidly in recent years, and it became routine to analyze the way in which bacteriocins are expressed and translocated. Since they are associated with foods of plant and animal origins, the shared interest is that they can be utilized as a vector to deliver the active constituents at the target in the gastrointestinal tract after digestion and be used in therapy. In this respect, the molecular pattern of the expression and translocation of salivaricin B (SalB), a bacteriocin, from *Lactobacillus salivarius* M7 was studied at the molecular level. The gene encoding SalB and the flanking sequences were obtained and sequenced. The gene encoding SalB comprised an open reading frame (ORF) of 171 bp having a 57 bp long leader sequence and a 114 bp long structural part. Ribosomal binding site (GAGG, RBS) is located at a canonical distance of 8 bp upstream from the start site.

**Key Words:** Gene, Gene structure, Salivaricin B, PCR, Inverse PCR, Degenerate primers, *Lactobacillus salivarius* M7.

### Salivarisin B Aktivitesini Kodlayan Gen ve Çevre Nükleotid Sıralarının Moleküler Karakterizasyonu

**Özet:** Laktik asit bakterileri (LAB) bazıları gıda korunmasında kullanılan birçok farklı antibakteriyel protein üretirler. Bakteriosinlerin moleküler genetik analizleri son yıllarda oldukça ilgi çekmiş ve hızlanmıştır. Buna paralel olarak bakteriosinlerin ekspresyon ve salınımları rutin olarak incelenmeye başlanmıştır. Bitki ve hayvan kökenli gıdalarla birlikte bulduklarından ortak amaç bu bakterilerin bir vektör olarak kullanılmasını ve taşıdıkları aktif içeriği gastrointestinal sistemin hedef bölgesinde saliverilmelerini sağlamak ve tedavide kullanabilmektir. Bu bakımdan bu çalışmada *Lactobacillus salivarius* M7 tarafından sentezlenen ve bir bakteriosin olan salivarisin B (SalB)'nin ifade edilmesi ve hücre dışına naklinin moleküler yapısı ortaya çıkarılarak incelenmiştir. SalB'yi kodlayan gen ve çevreleyen bölge elde edilerek nükleotid dizilimi açığa çıkarılmıştır. Buna göre SalB'yi kodlayan gen 171 baz çiftinde meydana gelmekte, 57 bazlık bir lider sıraya ve 114 bazlık bir gen yapısal bölgesine sahiptir. Başlangıç baz çiftinden 8 nukleotid uzakta ise bilinen ribosom bağlama bölgesi (RBS) yerleşmiştir.

**Anahtar Sözcükler:** Gen, Gen moleküler yapısı, Salivarisin B, PCR, Inverse PCR, Dejenere primerler, *Lactobacillus salivarius* M7.

## Introduction

Lactic acid bacteria have been used in the production of fermented foods and feeds throughout the history of humankind (1). In addition to being recognized for their health and nutritional benefits, they are now beginning to gain industrial importance in terms of the preservation of highly perishable foods of plants and animal origins. Lactic acid bacteria show a strong antagonistic activity against many food spoiling microorganisms and pathogens including listeria, clostridia, staphylococci, and streptococci. Besides the production of lactic acid as the primary metabolite, resulting in a decrease in the pH of the food to be fermented, some strains also produce inhibitory substances called bacteriocins.

In view of the growing consumer demand for natural food additives, most applied research has been focused on the possibility of using either these bacteria or the products of them, bacteriocins, as natural food additives. Recently, *Lactobacillus salivarius* M7 was isolated from the dental plaque of rodents (2), its physiology has been studied (3), and the amino acid sequence of its logarithmic phase product salivaricin B (SalB), a bacteriocin, was partially identified (4). In order to obtain the whole sequence information of the gene encoding the SalB activity from *L. salivarius* M7, this report describes the isolation of chromosomal DNA, the amplification of the sequence where the related gene lies by Inverse PCR, and sequencing the fragment obtained by Inverse PCR with the technique called cycle sequencing (5).

## Materials and Methods

### Cell Growth

*Lactobacillus salivarius* M7 were propagated in MRS (de Man Rogosa Sharpe) broth (Difco Laboratories, Detroit, Michigan) at 37°C overnight. *Escherichia coli* was propagated in LB (Luria Bertani) broth (Difco) at 37°C overnight as well.

### DNA Preparation

The genomic DNA was isolated by using the technique of Posno et al. (1991). *Lactobacillus salivarius* M7 was grown overnight in MRS medium containing 40 mM DL threonine at 37°C. Cells were pelleted. The pellet was resuspended in 1 ml, 20 mM maleate buffer and centrifuged again. Supernatant was discarded and the pellet was freeze-dried at -20°C for 1 h. The pellet was resuspended in 0.6 M lactose/20 mM maleate buffer containing 2 mg lysosyme/ml and incubated in a waterbath for 1 h at 37°C. The cells were centrifuged at 12,000 rpm for 10 min. The pellet was resuspended in 400 µl 120 mM Tris-HCl (pH 8.0). Addition of 88 µl of 0.5 M EDTA was followed by 110 µl 5% sarcosyl. The mixture was incubated at 37°C for 10 min. After 200 µl 5 M NaCl was added. Equal amounts of phenol:chloroform (150 µl) were added. The mixture was shaken smoothly on a rotary shaker for 5 min followed by incubation on ice for 5 min. Two volumes of absolute alcohol and 200 µl 3 M NaCl were added and centrifuged

(Hettich) at 12,000 rpm for 5 min. The pellet was washed with 70% EtOH and vacuum dried for 10 min. The DNA was dissolved in 450 µl 0.1X SSC. 8 µl RNase (10 mg/ml) was added to the mixture and incubated at 65°C for 20 min. The addition of 52 µl 5 M NaCl was followed by phenol-chloroform extraction. Final precipitation was carried out with 2 volumes of EtOH and 3 M NaCl at -20°C at maximal speed. The pellet was washed with 70% EtOH and vacuum dried (Gallenkamp). The DNA was dissolved in 100 µl Milli-Q water.

### Restriction Enzyme Digestion

Genomic DNA was digested with *Ase* I, *Bgl* II, *Bam* HI, *Eco* RI (Gibco BRL), *Hin* d III, and *Sau* 3A1 (New England, Biolabs). Each reaction mixture was incubated overnight at 37°C. The reaction mixture contained 10 µg genomic DNA, 10 µl of reaction buffer, 5 µl (10 U/µl) of enzyme, and 60 µl Milli-Q water (7).

### Ligation

A total of 20 µl of digestion product, 10 µl buffer for T<sub>4</sub> DNA ligase (Boehringer, Mannheim), and 4 µl T<sub>4</sub> DNA ligase was added to each tube containing the reaction mixture and the tubes were incubated at 14°C overnight (7).

### Inverse PCR

Based on the already known DNA sequence, a set of four non-degenerate perfectly matching primers were synthesized and Inverse PCR amplifications were carried out with divergent primers (Bioscience). Several primer sets were used for Inverse PCR amplifications. The primer OC95R, 5'-GGATGTACAATCGCTAATAC-3' was used in combination with OC96F, 5'-TCAAAAGATTAAGACATTCAAATC-3'. The primer, OC95R, was also used in combination with OC94F, 5'-TAGAGGAAGGAAGAGATATG-3' as well as OC97R, 5'-CCATCTAACCAGATATTTCCA-3'. The reaction mixture was prepared to a final volume of 50 µl including 5 µl of 10X reaction buffer, 20 mM of each dNTP, 2 mM MgCl<sub>2</sub>, 2 U Taq polymerase (Perkin Elmer, USA), 1 µl of each primer (50 nmol) and 50 ng of template DNA. The reaction conditions were as follows: 6 min of hot start at 95°C, 30 seconds of melting at 95°C, 50 seconds of annealing at 49°C, and 3 min of extension at 72°C. The reaction was performed for 35 cycles. Inverse PCR products were purified using a clean kit (Qiagen) after agarose gel electrophoresis. Amounts of 200-500 nM purified cycle sequencing amplification products were loaded on the sequencer and used in sequencing (5) (ABI Prism 310, Applied Biosystems, Foster City, USA).

## Results

Amplification product of a 550 bp sequence (Figure 1) generated from ligated *Ase* I cut sites was purified, sequenced, and showed to contain the open reading frame (ORF) coding for the *sa/B* gene. However, in the ORF of the *sa/B* gene there was an insertion of almost 100bp (Figure 2). One last perfectly matching primer OC98F (a 21-mer) was synthesized and used

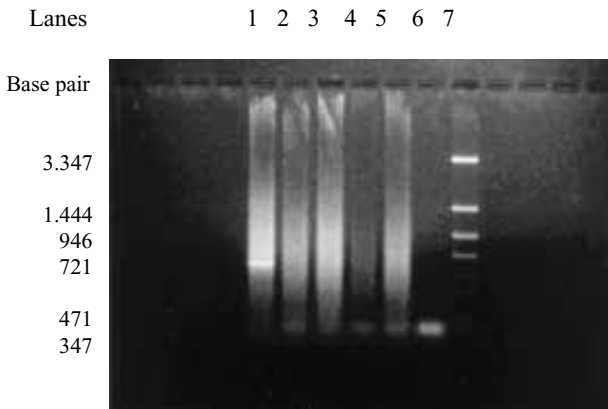


Figure 1. *Ase* I digested fragment of 550 bp obtained by Inverse PCR amplifications is in lane 1; marker DNA pEMBL-8 is in lane 7.

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AGATTAGAAG AAATAGTCAA AAAGAGCAGA TGAAGTGAAT AATTATAAGT
          ───────────────────────────────────▶
                    OC98F

GTAAATTATA GCTATAATAA ATTTTAAAAA TTATGAGGTG TTGAAGTTAT
GAATAATAAT TTTATACAAG TTGATAAGAA AGAATTGGCA CATATAATTG

                                                H I I
GTGGAAGAAA TTCTTATGAG TTATATAGAT AGCGGACAGT TTGGGTATG
   G G R N S Y E L Y R S G   Q F G L   L Y
ATTATATATTGA TAGGGGACAG TTTGGTTATG ATATAGGATG TACAATCGCT
   G T W M L   Y R M           N T K
AATACCAAAT TTTTCAAAG ATTAAGACAT TCAAATCAA ATATTTGTAG
   F F K R L R H S Q N I C
TAAACAGAA  ATGTAACCAA CTAGGTATAA AACATTTTAT GCTAAATTGA
CGGAATGATA ATACAATAAA AGTGTAAGCA TATGTTTCAC AAGTGTTTTA
GAGAGGATGT GTTCTATGTG ◀TATGGAATAT CTGGTTAGAT GCGGATTAA
                    ◀──────────────────────────────────
                             OC97R

TTAGAGGAAG GAAGAGATAT GTAATCATTC CAATTATTTT TGCAATATTT
CTTCCCCAAT
  
```

Figure 2. 550 bp long product of cycle sequencing and four primers used in Inverse PCR amplifications. Partial amino acid sequence of salivaricin B is given under the nucleotide sequence.

with (OC97R) in forward PCR amplifications. The PCR product was purified and directly sequenced. A fragment of 500 bp was obtained (Figure 3). This fragment contained the ORF1 coding for *Sa/B* precursor protein and an additional ORF further downstream. The analysis of the nucleotide sequence revealed that the actual size of the peptide is 38 amino acids. This was contrary to the previous findings of Bart ten Brink, (1995). The ORF1 was found to start with the ATG codon at position 86. The coding sequence of *sa/B* comprised 171 nt and 57 of which code for the leader peptide of 19 amino acid. The actual peptide did not contain the leader peptide. The leader peptide ends with a double glycine motif at positions -2 and -1, which are preceded by two isoleucine residues at positions -4 and -3. A ribosome binding site, RBS (GAGG), locates at a canonical distance of 8 nucleotides from the +1 position. Immediately downstream of the ORF1 the second ORF is located. The second ORF also starts with an RBS followed by another start codon.

## Discussion

Nucleotide sequence analysis revealed that salivaricin B was synthesized as a precursor protein of 57 amino acids. It has a leader peptide of 19 amino acids. The lack of 19 amino acid leader sequence from the mature protein suggests that on its way to the cell surface, just before release, the leader peptide cleaves itself off the rest of the protein. The leader peptide contains the canonical double glycine motif for signal sequence independent (sec-independent) translation and translocation of the bacteriocin. Preceding the double glycine leader sequence at positions -3 and -4, there are two isoleucine residues.

Thus, the presence of a strongly conserved cleavage site with a double glycine motif indicates that salivaricin B produced by *L. salivarius* M7 belongs to the class II LAB bacteriocins as well (8). A comparison of amino acid sequences of the other bacteriocins confirmed this notion. As is known, the class II bacteriocins contain positively charged residues (Lys or Arg) at positions +1 and +2. The amino acid sequence of salivaricin B conforms to these patterns only for the N-terminal part of its sequence. Although the amino acid sequences at positions 4 to 11 of salivaricin B do not show much similarity to the consensus sequence (Tyr-Gly-Asn-Gly-Val-Xaa-Cys) in the N-terminal region of the anti-listerial bacteriocins, such as acidocin A (9), carnobacteriocin BM1 (10), curvacin A (11), it has a strong antilisterial activity to all listeria species tested (data not included). Thus, it is conceivable that salivaricin B belongs to the class II LAB bacteriocins, though may not follow the same structural fashion found in the anti-listeria family. Comparisons of the primary structure of the purified peptide with the *sa/B* structural gene indicates that salivaricin B is synthesized as a precursor protein. As is known, precursor proteins are translated and processed by three apparently distinct mechanisms: formation of dehydroresidues, thioester cross bridging, and proteolytic cleavage. The first two features are mainly utilized by the lantibiotics, a group of bacteriocins that contain unusually formed amino acids, whereas the last feature is utilized by bacteriocins of class II. The sequence of the

AGATTAGAAGAAATAGTCAAAAAGAGCAGATGAAGTGAATAATTATAA

OC98 SAF

GTGTAAATTATAGCTATAATAAATTTTTAAAATTATGAGGTGTTGAAGT

RBS

TATGAATAATAATTTTATACAAGTTGATAAGAAAGAATTGGCACATATA

Start N\* N\* N\* F\* I\* Q\* V\* D\* K\* K\* E\* L\* A\* H\* I\*

ATTGGTGAAGAAATTCTTATGATTATATAGATAGCGGACAGTTTGGTT

I\* G\* G\* R\* N\* S\* Y\* D\* Y\* I\* D\* S\* G\* Q\* F\* G\*

Maturation site

ATGATATAGGATGTACAATCGCTAATACCAAATTTTCAAAGATTAAG

Y\* D\* I\* G\* C\* T\* I\* A\* N\* T\* K\* F\* F\* K\* R\* L\*

ACATTCAAATCAAATATTTGTAGTTAAACAGAAATGTAACCAACTAGG

R\* H\* S\* N\* Q\* N\* I\* C\* S\* Stop codon

TATAAAACATTTTATGCTAAATTGATATTTGAATACGGAATGATAATAC

AATAAAAGTGTAAGCATATGTTTCACAAGTGTTTTAGAGAGGATGTGT

2.Ribosome Binding site

TCTATGTGCTGAAAAAATTATGGAATATCTGGTTAGATGGCGGATTAAT

Start

OC97 SAR

TAGAGGAAGGAAGAGATATGTAATCATTCCAATTATTTTTGCAATATTTT

TTCCCAAT

Figure 3. The complete nucleotide sequence of the 498 bp fragment carrying the gene coding for salivaricin B and an additional ORF which is thought to be the gene encoding for the immunity protein. The letters starred are the residues found in the original sequence of salivaricin B.

maturation of class II bacteriocins or SalB is not currently known; however, accumulated data suggest that the processing of SalB follows the following path: enzymatic modification, proteolytic processing, and exportation. The *sa/B* gene started with the initiation ATG codon at position 99. The putative ribosome binding (RBS) site, GGAGG, is present at canonical distance (8 bp upstream) from the ATG codon at nucleotide position 85. The *salB* gene encodes a 57-amino-acid precursor that undergoes proteolytic cleavage of a 19-amino-acid leader peptide sequence. This leader peptide of *sa/B* also has a high degree of homology to those of other class II LAB bacteriocins (Figure 4). The Gly-Gly motif, which is typical among the class II LAB bacteriocin precursors previously described, is found at the -1 and -2 positions before the processing site. Also preceding the double glycine motif are the two isoleucine residues at positions -3 and -4, and are accepted as the feature of class II LAB bacteriocins. These similarities found among the leader peptides suggest that a common processing and/or secretion mechanism for the class II LAB bacteriocins could exist. Analysis of the nucleotide sequence revealed the presence of a second ORF, ORF2, immediately downstream from the ORF1 for *salB*. For other well-characterized bacteriocins, it has been demonstrated that genes involved in immunity functions are closely associated with the bacteriocin structural gene. Therefore, it is highly likely that the ORF2 encodes a protein involved in immunity to salivaricin B. To clarify the function of this ORF2-encoded protein, further investigation is obviously required.

As mentioned above, the strong conservation of the cleavage site of the leaders strongly suggests a common processing mechanism for these peptides (12). Another line of evidence suggests that ABC-transporters are required for extracellular activation of class II bacteriocins (13). Today it is well established that the secretion of double-glycine leader containing bacteriocins is mediated by a dedicated transmembrane translocator belonging to the ATP-binding cassette (ABC) transporter superfamily (14).

The hydropathy plot of salivaricin B suggests that it is mostly a hydrophilic protein with 18 amino acids containing uncharged polar R-groups (15). Thus, outside of the cell, depending on the presence of both positively and negatively charged residues, salivaricin B forms an amphiphilic transmembrane helix with a hydrophobic inner core in the cell membrane of the target bacterium. This view seems appropriate with the general structures of bacteriocins, as they induce membrane spanning pore formation complexes to disturb the proton motive force and the ion-exchange mechanisms of susceptible cells.

**MNNFIQVDKKELAHIGG**

The leader sequence      Glycine doublet

Figure 4. The complete sequence of the mature protein salivaricin B From *Lactobacillus salivarius* M7.

**RNSYDYIDSGQFGYGIGCTIANTKFFKRLRHSNQICS**



Cycle sequencing of the fragments from the chromosomal DNA coding for salivaricin B activity revealed a sequence of 550 nt. In the sequence, there were two consecutive ORFs. The first one was of the salivaricin B peptide. The second ORF was thought to be the immunity gene. The nucleotide sequence of the sal-B gene is composed of 171 nt. A 57 nt long leader sequence codes for the 19 amino acid leader peptide. A glycine doublets are located in the -1 and -2 positions.

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