Transcriptional analysis of bacteriocin production by malt isolate
*Lactobacillus sakei* 5
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Abstract

Malt isolate *Lactobacillus sakei* 5 produces three bacteriocins, sakacin P, sakacin T and sakacin X. The structural and regulatory genes for sakacin T and sakacin X are part of the *sakacin TX* locus, which consists of two adjacent, but divergently oriented gene clusters. Primer extension transcriptional analysis pointed to the existence of three distinct promoters within the *sakacin TX* locus, indicating that the three-component regulatory system in this locus is atypical in the sense that it is divided into a pheromone-specifying operon and an operon containing the genes for the histidine protein kinase and response regulator. Quantitative real-time PCR analyses showed that a transient increase in the expression of these two regulatory operons precedes transcription of the bacteriocin genes and appearance of bacteriocins in the culture medium. The identified promoters of the *sakacin TX* locus contain putative regulatory sequences (direct repeats) at corresponding positions in front of their –10 regions, which are likely to play a role in gene regulation.

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1. Introduction

Many lactic acid bacteria (LAB) secrete anti-microbial peptides called bacteriocins, which represent ribosomally synthesized, bioactive peptides or peptide complexes that have a bactericidal or bacteriostatic effect on other (usually closely related) species [1–3]. Bacteriocins of LAB have previously been arranged into three main classes, of which Classes I and II are most prevalent, based on their composition, size, mode of action, mechanism of export and inhibitory spectrum [3–5]. Class I bacteriocins, the so-called lantibiotics, are post-translationally modified. Class II bacteriocins are small, heat-stable non-lantibiotics, which are subdivided into three subcategories [5]. Members of Class III have been defined as large protein bacteriocins.

Recent studies have revealed considerable variation in the number of different bacteriocins produced by a particular strain [6], as well as a considerable genetic flexibility in the way in which bacteriocin loci can be organised [5]. However, Class II bacteriocin gene clusters are typically built up from three gene modules: (1) a module that encompasses the structural and immunity genes, (2) a transport gene module and (3) a regulatory gene module. The latter module encodes the so-called three-component regulatory system [7], responsible for the control of bacteriocin production and consisting of a secreted bacteriocin-like, cationic peptide pheromone, a
histidine protein kinase (HPK) and a response regulator (RR). At a specific extracellular concentration the pheromone is assumed to activate the HPK through an autophosphorylation event. The phosphoryl group is then transferred to the RR, which binds to specific sequences in the promoter regions of its target genes. This binding event leads to (transient) transcriptional activation of all genes involved in bacteriocin production and secretion, and also includes the regulatory genes [5].

This regulatory circuit thus acts as a quorum-sensing device, coupling co-ordinated bacteriocin production by a particular strain to its cell density [5,8]. In several cases it has been shown that the promoters associated with the regulated transcriptional units contain a conserved pair of direct repeats to which the RR binds [9–13].

Having found growth conditions leading to loss of the bac⁺ phenotype, and studying the effect of addition of synthesised putative peptide pheromones to bac⁻ cultures, we could previously show that Lactobacillus sakei 5 possesses at least two inducible systems for regulation of bacteriocin production [14]. One of these systems regulates the production of sakacin P and has been studied extensively in other sakacin producing strains [10]. The other system regulates the production of two Class II bacteriocins so far only found in L. sakei 5, namely sakacin T and sakacin X [14]. Analyses of various open reading frames in the sakacin TX locus (Fig. 1), revealed two genes, stxK and stxR, predicted to encode a HPK and RR, respectively. In contrast to many sequenced bacteriocin loci, whose expression is regulated by three-component regulatory systems [reviewed in [5,15]], stxK and stxR are not preceded by a gene encoding a peptide pheromone. It was shown that, instead, the pheromone is encoded by a gene, stxP, located downstream of stxK and stxR [14] (Fig. 1).

To find out more about the regulation of the production of sakacin T and X, we have analysed promoters in the sakacin TX gene cluster. In addition, we have employed quantitative PCR to study the expression of operons in the sakacin TX locus during normal growth of a bacteriocin-producing culture.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Lactobacillus sakei 5 [16] and Pediococcus pentosaceus LMG2001 (LMG collection Ås, Norway) were cultivated in MRS broth (Oxoid) at 30 °C for approximately 16 h prior to experimental use. MRS medium was removed and cells washed twice and resuspended in quarter strength Ringers solution, to remove bacteriocin-inducing factors, prior to all inoculations of L. sakei 5. Agar media were prepared by adding 1.5 % (w/v) granulated agar (Difco) to corresponding liquid broth media; overlay agars were prepared by the addition of 0.7 % (w/v) granulated agar to the liquid broth media.

2.2. Bacteriocin activity assay

Lactobacillus sakei 5 produces three bacteriocins, sakacin P, sakacin T and sakacin X. It has been shown in a previous study that the production of sakacin T and sakacin X is linked, whereas sakacin P production is regulated independently from sakacin T and sakacin X production [14]. For this reason, P. pentosaceus LMG2001 was used as the indicator strain for measuring sakacin T and X, because it is sensitive only to

Fig. 1. Schematic representation of nine genes involved in the production of sakacin T and sakacin X [14]. Arrows indicate ORFs and the proposed direction of transcription with the gene name positioned underneath: stxP, peptide pheromone; stxR, response regulator; stxK, histidine kinase; stxT, transporter; sakT₆, sakT₇, sakX, bacteriocins; sakI₆, sakI₇, immunity. P indicates the three promoters identified by primer extension analysis in this study. A putative terminator structure is indicated by the lollipop symbol. Primers used in RT-PCR analysis are indicated by solid arrowheads. The dashed lines indicate the transcripts detected by RT-PCR.
sakacin X and not to sakacin P. Bacteriocin assays were performed using cell-free supernatant (CFS) obtained at various stages of culture growth. Bacteriocin activity was quantified by critical dilution using the direct, spot-on-lawn assay [17]. Activity units (AU) per millilitre were determined as the inverse of the last dilution at which growth inhibition was still detectable.

2.3. Influence of the inducing peptide IP-TX on bacteriocin production

In order to assay the effect of the inducing peptide IP-TX on sakacin T and X production, 400 ng ml⁻¹ of the chemically synthesised peptide, enough to induce production [14], was added to a growing culture of L. sakei 5, after 4 h of growth corresponding to early log phase (OD₆₀₀ nm 0.35).

2.4. RNA extraction and cDNA synthesis

Total RNA was isolated from L. sakei 5 using the RNeasy Mini Kit (Qiagen). The procedure was modified to accommodate physical lysis by means of a bead beater before isolating the RNA. The RNA was prepared at different growth time points of L. sakei 5 and quantified spectrophotometrically at 260 and 280 nm. This RNA was then used as a template to generate first strand cDNA in reaction mixtures containing 1 μg of RNA, 600 ng of random hexamer primer (Promega), 10 mM (each) deoxynucleotide triphosphates, 100 mM DTT, 50 U of Expand reverse transcriptase and 5× reverse transcriptase buffer (Roche) as described previously [18]. Reaction volumes were adjusted to 20 μl with HPLC grade water and incubated at 30 °C for 10 min and 42 °C for 45 min, and terminated by heating to 93 °C for 2 min.

The cDNA generated was used as a template for RT-PCR reactions to determine the number of transcriptional units within the sakacin TX locus.

In order to accurately determine and compare transcript levels of any differentially regulated gene, it is necessary to normalise cDNA levels from the individual time points. Consequently, using primers and parameters previously described [19], the constitutively expressed 16S rRNA region was standardised for each time point by adjusting the volume of cDNA added prior to PCR until the crossing point values were identical for all samples. Subsequently, using these standardised volumes of cDNA, the relative expression of the target genes was determined as outlined below.

2.5. Quantitation of gene expression by real-time PCR

To prepare quantitation standards for LightCycler (LC)-PCR, an approximate 120 bp region from stxP, stxK or sakTₚ, representing the three putative transcriptional units (Fig. 1), was amplified using primers designed from the L. sakei 5 sakacin TX locus [14]. Each PCR product generated was purified using the Concert PCR rapid purification system (Gibco/BRL) and was subsequently quantified with a GeneQuant® spectrophotometer (Pharmacia). The copy number for each target was calculated, and appropriate dilutions ranging from 1⁰¹ to 1⁰⁸ copies of the standards were prepared in elution buffer (Qiagen), aliquoted and stored at 4 °C.

The Faststart SYBR Green master mix kit (Roche) was used for all LC-PCR reactions. Using the standards generated above, conditions were optimised for the real-time PCR as recommended by the manufacturer. This included titrating MgCl₂ and primer concentrations as well as optimising annealing temperatures and extension times. The final optimised conditions used in all subsequent analyses were: 2 μl of either the standard dilutions or standardised dilutions of each RT reaction mixture was used as a template, 2 mM MgCl₂, 2 μl of the Faststart SYBR Green master mix (Roche) with 0.5 μM of each relevant primer, in a total reaction volume of 20 μl. The PCR parameters for amplification included a hotstart (95 °C for 8 min) followed by 40 cycles of denaturation (95 °C for 5 s), annealing (48 °C for 5 s), and extension (72 °C for 8 s) and finally the melting curve profile (65–95 °C at 0.1 °C/s). All LC assay data analysis was carried out using the fit points option of the Roche LC software (version 3.53). The standard dilutions were used to generate crossing point values, which served to quantify the transcript levels of each gene being investigated. By fitting the crossing point values of the known standards to those from dilutions of the unknown real-time reaction mixtures, the number of gene transcripts for each target was calculated. Each experiment was performed in triplicate and the standard deviations calculated to ensure statistical integrity.

2.6. Primer extension analysis

Total RNA was isolated from L. sakei 5 as described above. Primer extension was performed by annealing 10 pmol 5′-end γ³²P-labelled synthetic 18-mer oligonucleotides, designed from the L. sakei 5 sakacin TX locus [14], to 50 μg of RNA as described by Pujic et al. [20]. Primers were designed from regions where promoters were likely to occur within the sakacin TX locus, i.e., between the adjacent, divergent gene clusters and upstream of the gene encoding the inducing peptide. Sequence ladders were produced using the same primers as the primer extension product with the aid of the Sequenase sequencing kit (version 2.0) (Amersham).
3. Results

3.1. Bacteriocin production

When grown in MRS medium, *L. sakei* 5 produces all its three identified bacteriocins without the need for addition of external peptide pheromone. Maximum sakacin X activity as measured in cell-free supernatants (CFS) of *L. sakei* 5 was observed 10 h after inoculating a culture in fresh medium, which corresponds to the late exponential phase (OD<sub>600 nm</sub> 3.15). Addition of the peptide pheromone encoded by *stxP* (IP-TX) during early log phase resulted in an earlier appearance of sakacin X activity, with maximum levels being reached at 8 h (OD<sub>600 nm</sub> 2.45), 2 h earlier than the non-induced culture (results not shown).

3.2. Real-time PCR analysis of bacteriocin gene transcription

PCR products, using cDNA samples from different time points as templates, were amplified with primers based on the three genes selected for investigation, *stxP*, *stxK* and *sakT<sub>b</sub>*. These genes were selected to represent the three putative transcriptional units, based on the proposed direction of transcription and their arrangement within the *sakacin TX* locus (Fig. 1). These transcriptional units were confirmed by RT-PCR analysis (Fig. 1). The purified PCR products were used as standards, and dilutions of each standard were used to generate a standard curve. The sensitivity of the LightCycler (LC)-PCR was estimated to be 10 copies of a given target, which was the cut-off point for accurate detection of DNA. Dilutions corresponding to copy number ranging between 10<sup>4</sup> and 10<sup>8</sup> were used in all LC-PCR reactions as standards.

Quantitative real-time PCR assays were performed to quantitate the transcription levels of *stxP*, *stxK* and *sakT<sub>b</sub>*. The gene encoding the inducing peptide for sakacin T and sakacin X, *stxP*, was transcribed at the highest level. Two hours after inoculation (OD<sub>600 nm</sub> 0.15), the transcript’s level was determined to be 4.36 × 10<sup>3</sup> ± 4.44 × 10<sup>3</sup>. There was a noticeable 14-fold increase in the copy number of the *stxP*-containing transcript at 7 h followed by a rapid decline in copy number (Fig. 2). While the *stxK*-encompassing transcript was produced at a lower (17-fold) level the changes in transcriptional levels of this gene followed the same pattern as *stxP* with a dramatic 21-fold increase in transcription at 7 h (Fig. 2). The initial measurement of the bacteriocin structural gene, *sakT<sub>b</sub>*, copy number at 2 h was 4.4 × 10<sup>3</sup> ± 3.51 × 10<sup>3</sup>. At 6 h a steady increase was observed, until 9 h to 4.57 × 10<sup>5</sup> ± 8.82 × 10<sup>4</sup> when the transcription level remained relatively constant (Fig. 2). A detectable increase in bacteriocin activity was observed after 8 h and this elevated level reached its maximum at 10 h, followed by a decrease at 11 h, after which the level remained relatively constant.

3.3. Primer extension analysis

The transcriptional start site for each of the three presumed transcriptional units involved in sakacin T and X production [14] was investigated by primer extension analysis (Fig. 3). This analysis permitted identification of transcriptional start points for all three putative promoters, which in each case is preceded by a plausible –10 promoter sequence (Fig. 4). Clear –35 consensus promoter sequences could not be identified. Interestingly, the primer extension analysis confirmed the suggestion that *stxP* is transcribed from a different

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**Fig. 2.** Transcription of the bacteriocin structural gene, *sakT<sub>b</sub>* - , *stxP* - - - and *stxK* - - as measured by the copy number detected by LC-PCR, and comparison with bacteriocin activity (AU/ml - - - ) in the culture medium.
promoter to that from which the genes encoding the cognate histidine kinase and response regulator are transcribed although additional stxP transcription may occur as a result of transcriptional readthrough from the stxTKR promoter located upstream.

When these promoter sequences were aligned (Fig. 4), all were seen to contain two or three direct repeats located upstream of the assumed –10 sites. Each repeat consists of 11 bp with the consensus sequence 5'-ATTTCAGGAC/TA-3' derived from the alignment of all copies of the repeat sequence present. The left (L) and right (R) direct repeats are spaced by an AT-rich stretch of 10 bp (Fig. 4). There are 21 nucleotides separating the beginning of the first repeat and the second, which approximates to two turns of the DNA helix (Fig. 4). Similar conserved sequence elements have been observed when analysing putative promoter regions in front of bacteriocin-related genes in other lactic acid bacteria [9,10]. It is noteworthy that the stxP and stxT promoters contain what seems to be an additional copy of this direct repeat sequence. These less conserved third copies of the repeat are located 22 bp downstream of the right repeat, partly overlapping the –10 promoter elements. The adenine at the beginning of these additional repeats is located 32 bp upstream of the first nucleotide of the right repeat (Fig. 4), which is approximately three turns of the DNA helix.
4. Discussion

It was previously reported that the structural and regulatory genes for sakacin T and sakacin X are part of the sakacin TX locus, which consists of two adjacent, but divergently oriented gene clusters (Fig. 1). Sakacin T and sakacin X production are simultaneously induced by the product of the stxP gene, IP-TX [14].

Through the analysis of a representative gene from each of the three operons in the sakacin TX gene cluster, we have shown in the present study that the three operons are differentially regulated with respect to strength and timing. All three genes were transcribed constitutively, but at different levels, during early log phase, with stxP-encompassing transcripts being the most abundant. The other operon involved in regulation, monitored by stxK, was expressed at lower levels, but its expression followed the temporal pattern as stxP. Since regulation of bacteriocin production relies on both stxK and stxP, it is not surprising that the expression of these genes shows the same temporal regulation. What is surprising, however, is that this does not appear to be achieved by having the genes in one (three-component regulatory) operon, as is normally observed [5,7], but by the coordinated action of two promoters.

Expression of the two regulatory genes monitored in this study showed a remarkably sharp peak at 7 h after inoculation. Transcription of the bacteriocin structural gene, sakTb, reached its highest level 2–3 h later, in accordance with the idea that expression of sakTb involves the gene products of stxK and stxP. This linkage was further corroborated by showing that addition of IP-TX early during growth led to an earlier induction of bacteriocin production. The increase in expression of the bacteriocin structural gene was not transient as for stxP and stxK, but remained elevated from 9 h and onwards. The appearance of the bacteriocin transcript correlated well with the appearance of bacteriocin activity in the culture medium.

Primer extension transcriptional analysis suggests that at least three promoters are involved in sakacin T and sakacin X production. Alignment of the DNA sequences surrounding the transcriptional start sites revealed conserved sequence patterns indicative of three co-regulated promoters. These patterns were not found at other positions in the sakacin TX locus. The two mRNA species that specify the inducing peptide IP-TX (stxP) or the transport and regulatory proteins, (stxTKR), both appear to have two alternative transcription start sites, being T or A, (as confirmed by primer extensions using various primers), while the transcript encompassing sakTc commences at a guanine.

All three promoter regions share common features including a conserved direct repeat sequence, in an organisation that is reminiscent of that observed for the promoter regions of bacteriocin-related genes in other LAB [9,10]. It has been shown that response regulators (RRs), which are homologous to stxR, encoded by plnC and plnD in Lactobacillus plantarum C11 and sppR in L. sakei LTH673, bind as homodimers to conserved direct repeats in these promoters [11–13]. Interestingly, the first nucleotide of the repeated sequence is separated from each other by 21 bp (approx. two turns of the DNA helix), which is the same as in other regulated bacteriocin promoters [9,10] and which enables two molecules of the RR (StxR) to bind on the same side of the DNA helix (Fig. 4). Protein binding may distort the double helical structure of DNA. Several bound regulator molecules may induce a bend in the axis, in a mechanism similar to that found for the transcription regulator Spo0A [21]. The positioning of the direct repeat consensus sequences may indicate that precise spatial DNA-protein binding is essential for the activation and regulation of transcription although this requires further experimental proof.

The stxP and stxT promoters contain what seems to be a third copy of the direct repeat sequence. These less conserved third repeats are located 32 bp downstream of the first nucleotide of the right repeat and show some overlap with the putative –10 promoter elements. Since each turn of the DNA is on average 10.5 nucleotides [22], this would imply that if protein binding was to take place it would be again on the same side of the DNA helix but this time separated by three turns of the helix. A similar observation was made for the promoter region of the ABC-transporter operon plnG in L. plantarum C11, which contains an extra repeat downstream of the right repeat, situated within the site required for RNA polymerase recognition [13]. In this case the first nucleotide of all three repeats is separated from each other by 21 bp. Diep et al. [9] have hypothesised that this third repeat, which is less conserved than the other repeats and which consequently may have a lower affinity for the phosphorylated RR could be occupied by a RR molecule during late exponential growth phase, when the level of phosphorylated RR would be high. A bound RR molecule could interfere with RNA polymerase binding and thus limit transcription. In the present case, it could be speculated that such a scenario might explain the initial transcriptional increase of stxP and stxTKR operons at 7 h is followed by a rapid decline.

Using other Class II bacteriocin regulation systems as a template and the results of this study, the cascade of gene activation and expression can be hypothesised. It appears that inducing peptide and the transport machinery for the peptide (encoded by stxT) are produced constitutively at a low level. At a certain point, the system is activated and a burst in the expression of stxP and the cognate regulatory genes is observed. This leads to the presence of phosphorylated StxR which binds the DNA regulatory elements (left and right direct repeats) of the stx promoters strongly to upregulate gene ex-
pression and weakly (to the third repeat) for downregulation. Some of these activation reactions must take place from 6 h after inoculation onwards, in mid-logarithmic growth phase, since this is when the transcription levels of the structural gene began to noticeably increase until the maximum transcription level is reached 9 h after inoculation. However, it takes another hour for this phenomenon to translate into maximum measurable bacteriocin production. While the transcription of sakT_β continued at the maximum level, the detectable amount of bacteriocin declined. This could be due to degradation of the peptides by environmental factors or the decrease in expression of the genes needed for regulation and processing.

To our knowledge this study is the first to apply LC technology to transcriptional analysis of genes involved in bacteriocin production. So far, studies on the regulation of expression of genes involved in the production of class II bacteriocins have been performed using reporter genes [12,23]. The LC-PCR method is more accurate when it comes to measuring transcription per se, because the signal that is measured is directly proportional to the original number of specific mRNA molecules present in a cell. The present results revealed important aspects of the regulatory cascade, in particular the strong temporal regulation of the stxP and stxT promoters, which were not visible in reporter gene-based studies on comparable systems. Temporal regulation has recently been observed using DNA microarrays, for another quorum-sensing-based regulation of bacteriocin synthesis by quorum-sensing mechanisms [24]. The application of this and other technologies is expected to lead to a more complete understanding of cell density-controlled transcriptional regulation.

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