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A two-component signal-transduction cascade in *Carnobacterium piscicola* LV17B: two signaling peptides and one sensor-transmitter

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Abstract

In the lactic acid bacterium *Carnobacterium piscicola* LV17B a peptide-pheromone dependent quorum-sensing mode is involved in the regulation of bacteriocin production. Bacteriocin CB2 was identified as an environmental signal that induces bacteriocin production. Here, we demonstrate that a second 24 amino acid peptide (CS) also induces bacteriocin production. Transcription activation of several carnobacteriocin operons is triggered by CB2 or CS via a two-component signal transduction system composed of CbnK and CbnR.

Keywords: Bacteriocin; Signal transduction; Peptide pheromone; Regulation; Quorum sensing.

1. Introduction

Various processes in Gram-positive bacteria are known to be regulated in a cell-density dependent manner. In this group of organisms small secreted peptides, referred to as peptide pheromones, are emerging as the key signal molecules that modulate physiological processes in a cell-density dependent manner (for review see 8, 12, 14). An example of such a system is the production of type II antimicrobial peptides by *Carnobacterium piscicola* LV17, a lactic acid bacterium associated with chill-stored vacuum-packaged meats [1,17]. The LV17 derivative *C. piscicola* LV17B produces at least two bacteriocins, carnobacteriocin B2 (CB2) and carnobacteriocin BM1 (CBM1) [18]. Production of both CB2 and CBM1 is dependent on the presence of a 61-kb plasmid (pCP40) [17]. The genetic determinants of precarnobacteriocin B2 (*cbnB2*) and its immunity protein (*cbiB2*) are located on pCP40, whereas those of precarnobacteriocin BM1 (*cbnBM1*) and its proposed immunity protein (*cbiBM1*) are located on the chromosome [19]. The analyzed region of pCP40 contains four genes (*cbnKRTD*) required for bacteriocin production [17,19] and three additional small ORFs (*cbnS, X* and *Y*) (Fig. 1A). CbnT and CbnD are likely to function as a bacterial *sec*-independent secretion system [10,17], while CbnK and CbnR probably function as a bacterial two-component signal-transduction system [17,27]. The predicted products of *cbnS, X* and *Y* are class II bacteriocin-like precursor peptides containing potential double-glycine type leaders [17]. A peptide-pheromone dependent quorum-sensing mode has been shown to be involved in the regulation of carnobacteriocin production by *C. piscicola* LV17B [17,20,25]. Four different transcripts appeared to be co-regulated in a cell-density dependent manner, encompassing *cbnBM1* and *cbiBM1, cbnB2* and *cbiB2, cbnX* and *cbnY*, and *cbnS*, respectively [17]. This concerted regulation of expression is supported by the presence of homologous direct repeat motifs within their promoter regions (Fig. 1B) [17]. Recently, the characterization of the locus involved in carnobacteriocin A production by *C. piscicola* LV17A (another derivative of *C. piscicola*

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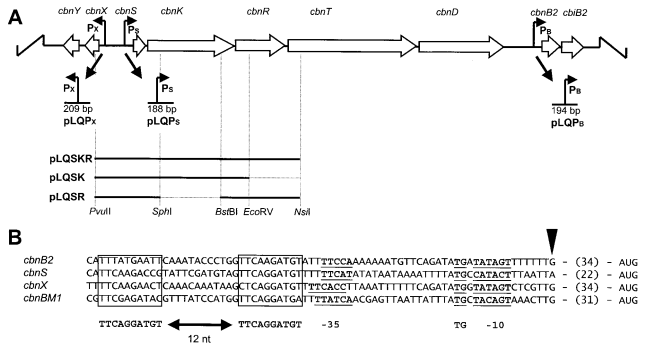

Fig. 1. (A) Schematic representation of the carnobacteriocin B gene cluster. The position of the *cbn* genes and *cbn* promoters as well as the size of the promoter fragments cloned into pNZ273 are indicated. The fragments encoding regulatory elements (*cbnK* and *cbnR*) that were cloned into pIL253 are indicated, together with the relevant restriction sites.
(B) Sequence alignment of the *cbn* promoters. Indicated are the direct repeat elements (boxed) upstream the canonical promoter sequences indicated as –35 and –10 region. The sequence consensus of these direct repeats is indicated in the bottom line. The transcription start site is shown by the arrowhead. Finally, the spacing between the transcription start site and the start-codon (AUG) is indicated between brackets.

LV17, containing plasmid pCP49) was reported. Interestingly, besides the previously identified *cbnA* structural gene [30], the cluster also contained genes encoding CbnS, -K, -R, -T, and -D homologues (*cbaXKRTD;* 11). Remarkably, the promoter regions upstream of *cbnA* and *cbaX* contain similar direct repeat sequence motifs as those found in the *cbn* promoter regions described above. The observed similarities between these carnobacteriocin systems suggests that cross talk between the various regulatory systems present in the parent strain *C. piscicola* LV17 could generate an elaborated pattern of growth phase dependent production of different antimicrobial peptides. However, experimental evidence supporting regulatory cross talk has not been presented to date. The regulatory direct repeat sequence motifs appears to be conserved among several antimicrobial peptide biosynthesis systems, as they are also found in the promoter regions of genes involved in production of bacteriocins by various *Lactobacillus* species (*L. plantarum* C11, *L. curvatus* LTH1174, *L. Sakei* Lb706, Lb674) [7,14,15]. Moreover, for some of these repeats it has been shown that they act as the target site for the corresponding response regulator [21,22].

Previous studies have shown that the bacteriocin CB2 acts as a peptide-pheromone, inducing bacteriocin production [20,24]. However, we have recently found that the culture supernatants of *Carnobacterium* strains transformed with plasmids harboring the carnobacteriocin B2 gene cluster, but lacking a functional copy of *cbnB2* gene itself (due to a specific deletion), induced bacteriocin production in cultures of *C. piscicola* LV17B that had lost the ability to produce bacteriocin (Bac^- culture) after dilution below 10^4 colony forming units per ml [17,24]. This indicates that at least one as yet unidentified inducer molecule was present in these supernatants.

2. Identification of a second peptide pheromone encoded by pCP40

In general the peptide pheromones known to induce class II bacteriocin production are small (~20 amino acids) bacteriocin-like peptides that are produced as precursors containing double-glycine type leaders. The encoding genes are generally located directly upstream of the genes encoding the two-component regulatory system that is supposed to be triggered by these peptides (for reviews see 8, 11, 14, 15). Based on these characteristics, the *cbnS* gene represents the most likely candidate to encode a second peptide pheromone involved in regulation of carnobacteriocin production (Fig. 1A). The *cbnS* gene encodes a 41 amino acid product (MKIKTITKKQLIQIKGG-SKNSQIGKSTSSISKCVFS-FFKKC) that is probably processed between Gly17 and Ser18, resulting in a mature peptide (CS) of 24 amino acids. CS was ordered using standard solid phase Fmoc (9-fluorenylmethoxycarbonyl) methodology on a PS3 peptide synthesizer from Rainin Instruments followed by reverse-phase HPLC purification to homogeneity of the reduced form of CS (95% purity; dissolved in 0.1% TFA and stored at -70°C). Addition of CS to a Bac^- culture to a final concentration of 3 nM induced bacteriocin production to 400 arbitrary inhibitory units per ml (data not shown), as was determined by a spot-on-lawn test [1]. This level of induction is comparable to that reached by the addition of 2% of heat-treated (65°C, 15 min) Bac^+ culture supernatant. In contrast, when CS was added to a final concentration of 0.4 nM or lower, no induction of bacteriocin production could be detected (data not shown). Interestingly, CS appears to be far more potent as an inducer compared to CB2, of which a concentration of 60 nM was required to induce bacteriocin production [20]. This result clearly demonstrates that CS can indeed act as the postulated second peptide pheromone involved in regulation of bacteriocin production in *C. piscicola* LV17B. Despite the resemblance of CS to type II bacteriocins, it could not inhibit the growth of any *Carnobacterium* sp. tested in this study (20 μl of a 2 μg/μl solution of CS spotted on lawns of *Carnobacterium* strains).

3. CS and CB2 control *cbn* promoter activity via CbnK and CbnR

To obtain insight into the molecular mechanism of regulation of bacteriocin production, the previously mapped *cbnS* (PS), *cbnX* (PX) and *cbnB* (PB) promoters (Fig. 1B) [17] were cloned upstream of the promoterless β-glucuronidase encoding gene (*gusA*) of pNZ273 [16]. PX- and PS-containing DNA fragments were generated by PCR using *Pwo* polymerase (Boehringer Mannheim), restriction site introducing primer pairs (*Bam*HI and *Eco*RI sites are underlined: PX-reverse: 5′-CGC*GGATCC*TTGTTTAAT-TAAAGTATGGCATAAAA-3′, PX-forward: 5′-GCG-*GAATTC*AAAATTAACCTCCTTTGGTTTAT-3′, and PS-

reverse: 5′-CCG*GGATCC*AACGAGACTATACCATAT CTGAA-3′, PS-forward: 5′-GCG*GAATTC*TATTCCCC TCTCGCTTGTTTA-3′) and pCP40 as a template. The 209- and 188-bp fragments (Fig. 1A), respectively, were digested with *Bam*HI and *Eco*RI and cloned into similarly digested pNZ273, resulting in pLQPX and pLQPS, respectively. Similarly, a 194-bp PB containing fragment (Fig. 1A) was generated by PCR (primer pair: PB-reverse: 5′-CCGA*AGATCT*ATTGTATATACTATTTTTACTATG-3′, PB-forward: 5′-CTAAATCAAACTCCTTTATTTTATAT 3′), digested with *Bgl*II and cloned in *Hin*cII-*Bam*HI digested pUC19 from which it was re-isolated as a *Sma*I-*Pst*I fragment and cloned in *Pvu*II-*Pst*I digested pNZ273, resulting in pLQPB. pLQPX, pLQPS and pLQPB were introduced separately into *C. piscicola* LV17B (harboring pCP40) and its plasmidless derivative *C. piscicola* LV17C, and transformants were selected on APT-agar plates containing chloramphenicol (5 μg/ml). All *C. piscicola* LV17B transformants developed an intense blue color on plates containing the chromogenic β-glucuronidase substrate X-Gluc (5-bromo-4-chloro-3-indolylglucuronide), whereas all *C. piscicola* LV17C transformants remained white on similar plates (data not shown). This phenotypic difference demonstrates that the cloned fragments (PX, PS and PB) contain functional, regulated promoters that are only active in pCP40-containing *C. piscicola* LV17B. Moreover, β-glucuronidase activity in *C. piscicola* LV17C transformants could not be induced by treating the cells with culture supernatant of a Bac^+ culture of *C. piscicola* LV17B (2% v/v), indicating that the lack of PX, PS, and PB activity in *C. piscicola* LV17C was not due to a lack of inducing factor(s) (peptide pheromone(s)) in the supernatant of this strain. These observations indicate that pCP40 encodes one or more trans-acting factors required for PX, PS and PB transcription activation. Likely candidates for such a role are the *cbnK* and *cbnR* gene products that, from sequence homology with other regulatory proteins, appear to constitute a two-component regulatory system. To evaluate this putative role of *cbnK* and *cbnR*, a *Pvu*II-*Nsi*I (2843 bp) fragment containing *cbnSKR* from pCP40 was cloned into the pNZ273-compatible broad host-range vector pIL253 [26,29] digested with *Sma*I and *Pst*I to create pLQSKR. In this construct the *cbnSKR* genes are constitutively transcribed by read-through of the plasmid-encoded *rep* promoter [13,26]. Derivative pLQSK lacks a functional *cbnR* gene and was constructed by religation of pLQSKR after its digestion with *Eco*RV and *Xba*I (ends were blunted using Klenow). Derivative pLQSR that lacks 1051 bp of the *cbnK* gene was constructed by digestion of pLQSKR with *Sph*I and *Bst*BI (ends were blunted using T4 DNA polymerase) followed by religation. In the latter plasmid, polar effects of the *cbnK* deletion (out of frame) on the expression of *cbnR* can not be excluded, although the *cbnR* coding region is preceded by a typical ribosome-binding site. The plasmids pLQSKR, pLQSK, pLQSR or the vector pIL253 were introduced separately into *C. piscicola* LV17C already harboring one of the *cbn* promoter constructs (pLQPX, pLQPS, or pLQPB), or the vector pNZ273, and double-transformants were selected on APT-agar plates containing chloramphenicol (5 μg/ml) and erythromycin (5 μg/ml). These *C. piscicola* LV17C double transformants were grown at 24°C in APT to an OD_{600} of 0.3 before carnobacteriocin B2 (1 μg/ml), the peptide CS (1 μg/ml) or heat-treated culture supernatant from a Bac^+ culture of *C. piscicola* LV17B (2% v/v) was added. After continuing growth for an additional 2 h, β-glucuronidase activities were determined as previously described [4] (Fig. 2). The presence of pLQSKR was found to be essential for induction of transcription of *gusA* from PX (Fig. 2A), PS (Fig. 2B) and PB (Fig. 2C), and transcription from each of these promoters was specifically induced by all three of the treatments analyzed. Moreover, no GusA-response was observed in these double transformants after treatment with culture supernatants of *C. piscicola* LV17B (Bac-), or *C. divergens* LV13 (producing the class II bacteriocin divergicin A) [31], or with 0.1% TFA (data not shown). Transcription from none of the *cbn* promoters could be induced in cells harboring pLQSK, dem-

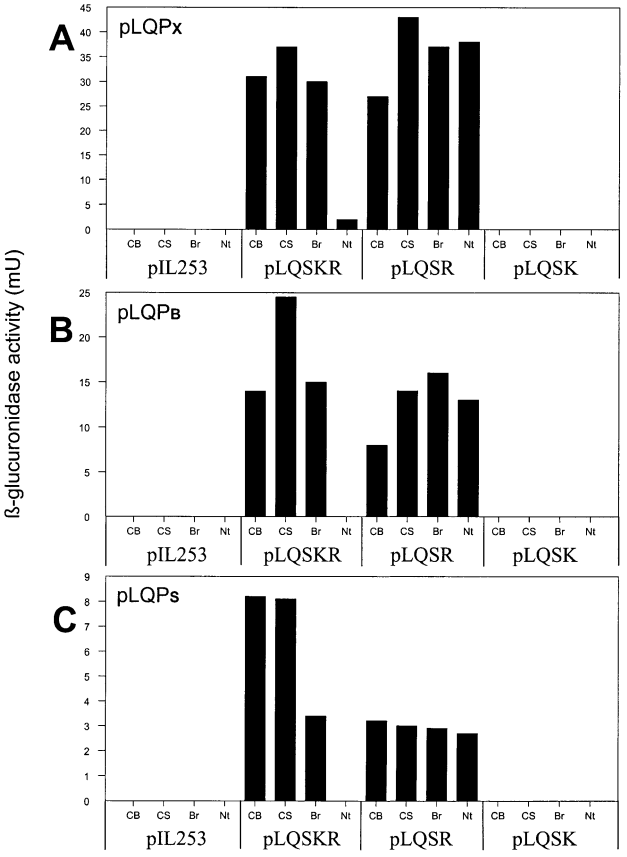

Fig. 2. β-glucuronidase activity determined in cell extracts from double transformants of *C. piscicola* LV17C harboring pLQPX (plot A), pLQPB (plot B), or pLQPS (plot C), in combination with pIL253 or one of the pIL253 derivatives (pLQSKR, pLQSK, or pLQSR). Cultures were treated with 1 μg/ml (~0.2 μM) of CB2 (CB bars), 1 μg/ml (~0.3 μM) CS (CS bars), 2% v/v of culture supernatant of a Bac^+ culture (Br bars), or not treated (Nt bars).

onstrating that CbnR is essential for promoter activation. In contrast, since cells harboring pLQSR in combination with one of the *cbn* promoter-plasmids exhibited constitutive expression of *gusA* the role of CbnK in the regulatory circuit remains less clear. With this last plasmid combination it can not be excluded that the constitutive expression driven by the *cbn* promoters is caused by an unnaturally high level of expression of *cbnR* due to the relatively high copy number of pLQSR. Nevertheless, it is tempting to speculate that CbnK acts as a peptide pheromone sensor involved in regulation of CbnR activity, probably by modulation of its phosphorylation state. The results described here would then suggest that CbnK maintains CbnR in a non-activated form under non inducing conditions, which has been reported for other response regulators [2,23,28].

Taken together the data presented here suggest a model for regulation of carnobacteriocin production where at least two peptide pheromones (CS and CB2) are involved in activation of transcription of several *cbn* promoters. This observation is in contrast to what has been described for the production of bacteriocins in lactobacilli, where only a specific type II bacteriocin-like peptide (CS functional homolog) appears to be involved in regulation of production [5,6,8,14,15]. Activation of transcription of the genes under control of *cbn* promoters requires a two-component signal transduction system composed of the putative peptide pheromone receptor CbnK and its corresponding response regulator CbnR. It is tempting to speculate that CbnR-mediated *cbn* promoter activation involves the similar sequence motifs found within these promoters, which could represent CbnR binding sites.

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