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MINIREVIEW

Regulation of Class II Bacteriocin Production by Cell-Cell Signaling

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Production of ribosomally synthesized antimicrobial peptides usually referred to as bacteriocins is an inducible trait in several gram positive bacteria, particularly in those belonging to the group of lactic acid bacteria. In many of these organisms, production of bacteriocins is inducible and induction requires secretion and extracellular accumulation of peptides that act as chemical messengers and trigger bacteriocin production. These inducer peptides are often referred to as autoinducers and are believed to permit a quorum sensing-based regulation of bacteriocin production. Notably, the peptides acting as autoinducers are dedicated peptides with or without antimicrobial activity or the bacteriocins themselves. The autoinducer-dependent induction of bacteriocin production requires histidine protein kinases and response regulator proteins of two-component signal transduction systems. The current working model for the regulation of class II bacteriocin production in lactic acid bacteria and the most relevant direct and indirect pieces of evidence supporting the model are discussed in this minireview.

Key words: quorum sensing, antimicrobial peptide, signal transduction, gene regulation

Production of bacteriocins in gram positive bacteria

Many gram positive bacteria, in particular many lactic acid bacteria, produce small (<10 kDa) ribosomally synthesised antimicrobial peptides called bacteriocins. The potential application of many of these bacteriocins as biopreservatives in food systems has propelled research in the field. These alternative biopreservatives can be used in several ways, from the direct application of the bacteriocin-producing strains to foods as starter or protective cultures to the use of purified bacteriocins as food additives. Examples of both kinds of commercial applications, primarily in dairy products, are the worldwide use of preparations of the bacteriocin called nisin A and nisin-producing strains of *Lactococcus lactis* as biopreservative (Ray and Daeschel, 1992; Ross *et al.*, 1999; Ryan *et al.*, 2002)

The work of many research groups around the world has produced a wealth of knowledge in the field of bacteriocins. Today it is widely recognized that bacteriocins are diverse in terms of structure, mode of action, antimicrobial potency, spectrum of antimicrobial activity and potential for commercial application. Most bacteriocins can be included in one of two classes, namely class I and class II (Klaenhammer, 1993). Class I bacteriocins (or lantibiotics) are extensively post-translationally modified by specific enzymes. The modifications include dehydration of amino acid residues, leading to formation of uncommon amino acid residues such as dehydroalanine and dehydrobutyrine, and formation of intramolecular lanthionine and β-methyl-lanthionine bridges (for recent reviews see van Kraaij *et al.*, 1999; Guder *et al.*, 2000; Jack and Jung, 2000; Sablon *et al.*, 2000; McAuliffe *et al.*, 2001). These modifications are essential for the antimicrobial activity of the peptides. An example of this class of bacteriocins is the lactococcal lantibiotic nisin A mentioned above. In contrast to class I bacteriocins, the bacteriocins of class II are unmodified peptides that do not require post-translational modification for antimicrobial activity (for recent reviews see Ennahar *et al.*, 2000; Nes and Holo, 2000; Sablon *et al.*, 2000; van Belkum and Stiles, 2000). Examples of this class of bacteriocins are the carnobacteriocins produced by strains of *Carnobacterium piscicola* LV17 (see below). Both class I and class II bacteriocins are synthesized as precursor peptides containing N-terminal extensions that are not present in the mature and fully active bacteriocins. These N-terminal extensions are usually referred to as leader peptides. The leader peptides of class I bacteriocin precursors are cleaved by membrane-bound dedicated peptidases after the post-translationally modified precursors are translo-

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cated across the membrane by dedicated *sec*-independent transport systems (van der Meer *et al.*, 1993). The leader peptides of class II bacteriocin precursors are cleaved off concomitantly with export by dedicated *sec*-independent transport/processing systems (Håvarstein *et al.*, 1995).

Regulated bacteriocin production in gram positive bacteria

Production of class I and class II bacteriocins (BAC^+ phenotype) is an inducible trait in several gram positive bacteria, particularly in those belonging to the group of lactic acid bacteria. In these bacteria, the BAC^+ phenotype is expressed only in the presence of peptides in the culture supernatant that induce bacteriocin production (for recent reviews see Kleerebezem *et al.*, 1997; Kleerebezem and Quadri, 2001; Quadri, 2002; Sturme *et al.*, 2002). These inducer peptides are often referred to as autoinducers (AIs). The AI-regulated bacteriocin production systems contrast the more commonly found situation, where bacteriocins are, at least in appearance, produced in a constitutive fashion and without the need for extracellular AIs. The first AI-regulated systems were discovered by serendipity in strains of the lactic acid bacterium *L. lactis* and *C. piscicola* that produced the class I bacteriocin nisin A and the class II bacteriocins called carnobacteriocins, respectively, mentioned above. The initial cue suggesting that an AI-mediated regulatory strategy was involved in the control of nisin A production was the observation that deletion of a 4-bp segment from the gene encoding the bacteriocin precursor (*nisA*) not only rendered a strain deficient for nisin A production, but also suppressed the transcription of the mutated allele (*ΔnisA*) (Kuipers *et al.*, 1993). It was subsequently demonstrated that addition of non-lethal amounts of nisin A to the culture supernatant of the *L. lactis* mutant induced transcription of *ΔnisA* allele (Kuipers *et al.*, 1995; Dodd *et al.*, 1996; van Kraaij *et al.*, 1997). Based on these observations, AI activity was postulated for nisin A (Kuipers *et al.*, 1995; Dodd *et al.*, 1996; van Kraaij *et al.*, 1997). In the case of the carnobacteriocin system of *C. piscicola* LV17, the BAC^+ phenotype was observed to be occasionally lost when the producer strains were grown in liquid medium; however and unexpectedly, colonies of both BAC^+ and BAC^- cultures were all BAC^+. The phenotypic change from BAC^+ to BAC^- observed in liquid culture was ultimately correlated with small-size inocula utilized to start the cultures and the lack of peptide AIs in the culture supernatants of the BAC^- cultures (Saucier *et al.*, 1995). It is clear today that two secreted peptides with no detectable antimicrobial activity and at least one carnobacteriocin have confirmed AI activity in the carnobacteriocin system of *C. piscicola* LV17 (Saucier *et al.*, 1995; Quadri *et al.*, 1997a; Saucier *et al.*, 1997; Franz *et al.*, 2000a; Kleerebezem *et al.*, 2001).

Since the early observations of AI-mediated regulation in the nisin A and carnobacteriocin systems mentioned above, other bacteriocin production systems in gram positive bacteria with similar regulatory characteristics have been identified and studied in different depths (for recent reviews see Kleerebezem *et al.*, 1997; Kleerebezem and Quadri, 2001; Quadri, 2002; Sturme *et al.*, 2002). From these studies, it is becoming apparent that many gram positive bacteria have evolved ways to control production of bacteriocins via a mechanism that involves AI-mediated cell-to-cell communication and a quorum sensing phenomenon. The quorum sensing phenomenon could be considered as a cell-to-cell communication strategy that enables unicellular organisms to behave in a multicellular manner by allowing sensing of cell-density and population-wide synchronised behavioural responses as a function of it. Cell-to-cell communication strategies, including quorum sensing, utilize AIs of different chemical natures (typically homoserine lactones in gram negative bacteria and peptides in gram positive bacteria) as chemical messengers (Dunny and Winans, 1999). In addition to the production of bacteriocins, examples of behavioural responses modulated or influenced by cell-to-cell communication are sporulation and cell differentiation, production of toxins, virulence response, development of genetic competence, bioluminescence, biofilm formation, and conjugative plasmid transfer (for recent reviews see Kleerebezem *et al.*, 1997; Dunny and Winans, 1999; Fuqua *et al.*, 2001; Miller and Bassler, 2001; Whitehead *et al.*, 2001).

Emerging model for AI-mediated regulation of class II bacteriocin production in gram positive bacteria

From the excellent work of several research groups on the analysis of systems in which bacteriocin production is induced by AIs, a general working model for AI-mediated regulation of class II bacteriocin production in gram positive bacteria is emerging. Except for some differences, a similar model has been proposed for the regulation of class I bacteriocin production in gram positive bacteria. The sections below address only issues pertaining to the regulation of class II bacteriocin production. The reader is referred to more comprehensive reviews in the field for an examination of the regulatory aspects of class I bacteriocin production (Quadri, 2002; Sturme *et al.*, 2002).

The current model for regulation of class II bacteriocin production has been formulated within the frame of the principles governing bacterial quorum sensing. In this model, the peptides functioning as AIs trigger the induction of their own structural genes and other genes needed for production of and immunity to the bacteriocins; *e.g.* bacteriocin precursor genes, immunity genes, secretion/processing genes and regulatory genes (Fig. 1). In most class II bacteriocin production systems, the AIs are dedicated peptides with or without antimicrobial activity. A notable exception is the carnobacteriocin system of *C. piscicola* LV17 mentioned above, where not only dedicated

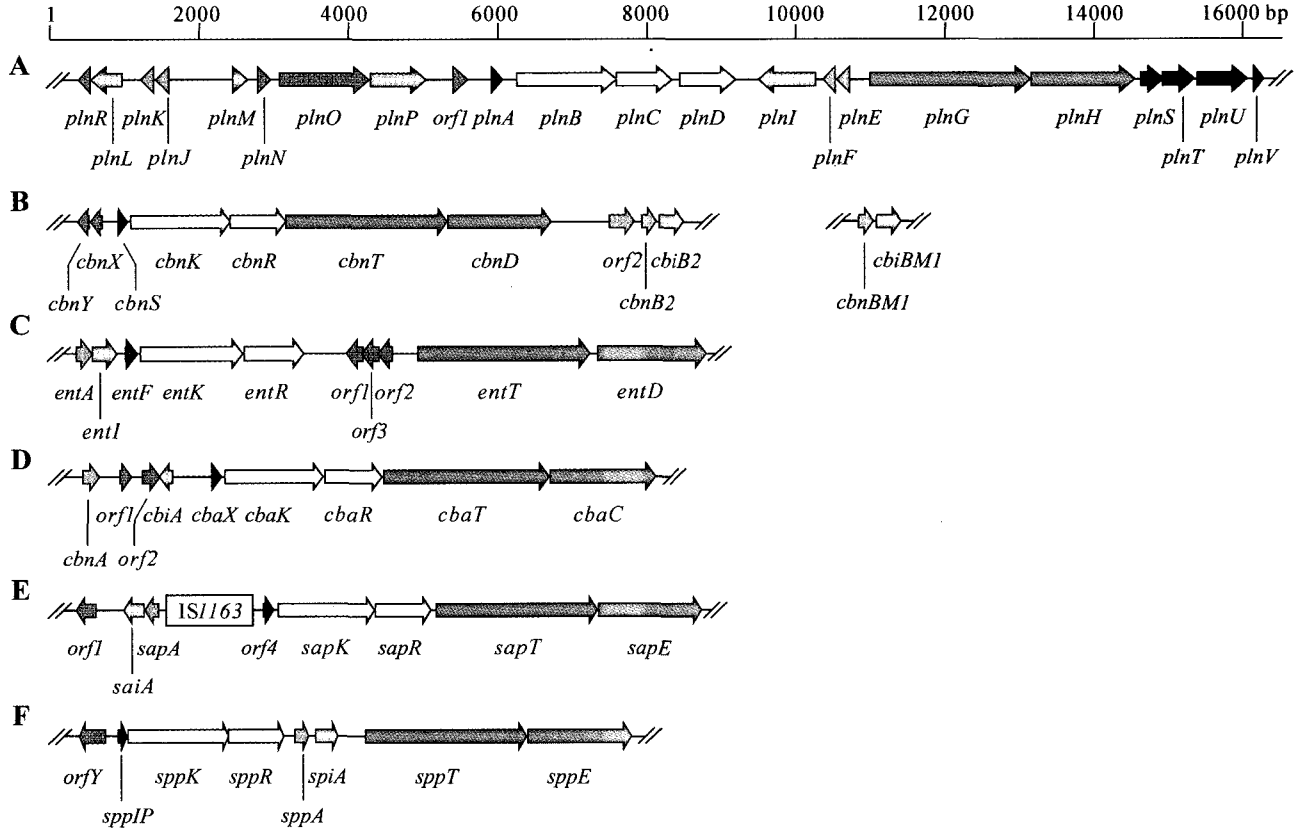

Fig. 1. Gene loci involved in AI-regulated production of class II bacteriocins in gram positive bacteria. The loci represented are those involved in production of: (A) various plantaricins by *Lactobacillus plantarum* C11; (B) carnobacteriocin B2 and BM1 by *Carnobacterium piscicola* LV17B; (C) enterocin A by *Enterococcus faecium* DPC1146; (D) carnobacteriocin A by *Carnobacterium piscicola* LV17A; (E) sakacin A by *Lactobacillus sakei* Lb706; and (F) sakacin P by *Lactobacillus sakei* Lb674/LTH673. The AI precursor genes are coloured in red. The two-component regulatory system genes are coloured in yellow. The bacteriocin precursor genes are coloured in blue. The immunity protein genes are coloured in purple. The secretion/processing genes are coloured in green. Genes without clear functional assignment are depicted in grey.

AIs but also the carnobacteriocins themselves induce bacteriocin production (Fig. 2). The fact that the carnobacteriocins participate in the regulatory circuit by acting as AIs creates a mechanistic overlap between the regulation of carnobacteriocin production and the regulation of class I bacteriocin production, where there are no dedicated AIs and the bacteriocins themselves are the AIs (for recent reviews see Quadri, 2002; Sturme *et al.*, 2002).

The dedicated AIs are unmodified small cationic peptides (19 to 26 amino acids, isoelectric points >9) with or without antimicrobial activity and synthesized as class II bacteriocin-like precursors with N-terminal leader peptides of 16 to 22 amino acids (Fig. 3). These leader peptides, which contain a double-glycine sequence motif preceding the cleavage site, are believed to be cleaved upon AI export from the cell by the same *sec*-independent machinery involved in possessing and secretion of class II bacteriocins. The genes encoding the precursor peptides of the dedicated AIs have been invariably found forming an operon with regulatory genes encoding histidine protein kinases (HPKs) and response regulators (RRs) of two-component signal transduction systems (Fig. 1). The AI-HPK-RR triad is frequently referred to as a three component regulatory system (Nes *et al.*, 1996). The HPK and RR pairs in class II bacteriocin systems have similarity to the AgrC-AgrD signal transduction systems involved in quorum sensing-dependent regulation of gene expression in *Staphylococcus aureus* (Novick, 1999). It is generally assumed that the HPKs encoded in the class II bacteriocin production loci are the receptors of the AIs, them being dedicated peptides or both dedicated peptides and bacteriocins in the case of the carnobacteriocin system. It is believed that the HPKs modulate the activity of their cognate RRs via phosphorylation in an AI-dependent manner and that the activated (phosphorylated) RRs control gene transcription through binding to conserved sequences in the regulated promoters of target genes in the bacteriocin production loci (Fig. 2).

Experimental evidence supporting the model for AI-mediated regulation of class II bacteriocin production in gram positive bacteria

Many aspects of the regulatory mechanism represented in

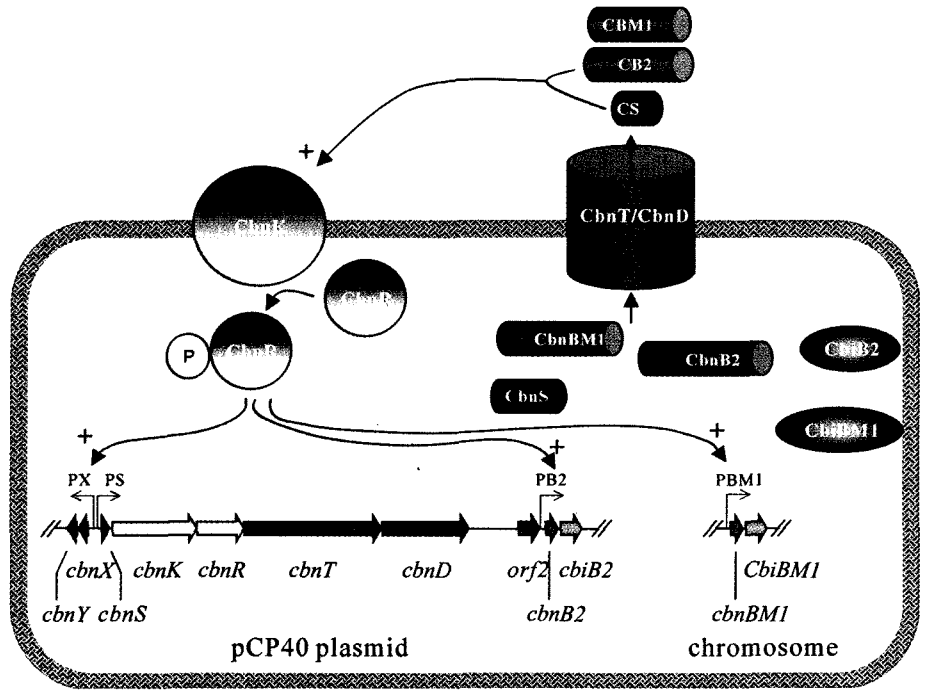

Fig. 2. Model for AI-medicated regulation of class II bacteriocin production in gram positive bacteria. The model is represented using the regulatory strategy involved in the production of bacteriocins in *Carnobacterium piscicola* LV17B. In this organism, not only the dedicated AI (CS) part of the three-component regulatory system triggers bacteriocin production as in all other regulated class II bacteriocin systems, but also at least one of the bacteriocins (carnobacteriocin B2) produced by the bacterium. Both the pCP40 and the chromosomal *cbn* loci are represented. The *cbn* promoters are shown with open arrows.

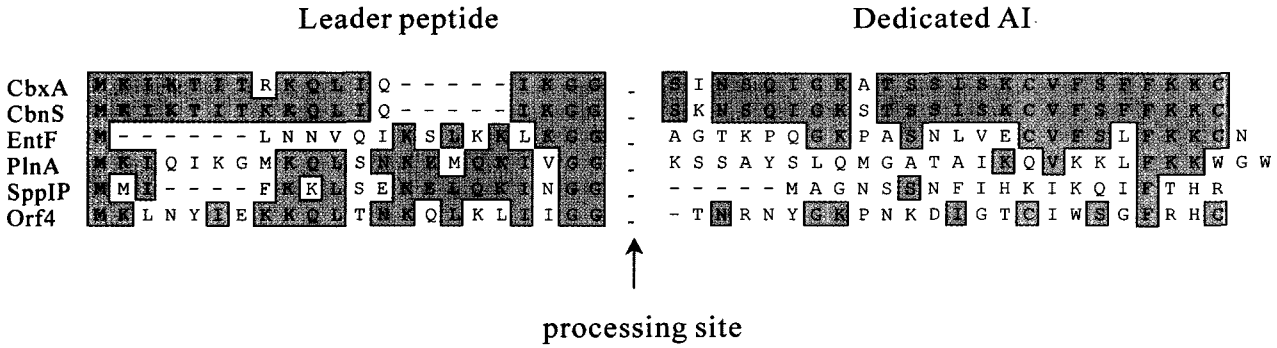

Fig. 3. Multiple sequence alignment of precursors of dedicated AIs involved in regulation of class II bacteriocin production. The alignment includes CbxA from *Carnobacterium piscicola* LV17A, CbnS from *Carnobacterium piscicola* LV17B, EntF from *Enterococcus faecium* CTC492/DPC1146, PlnA from *Lactobacillus plantarum* C11, SppIP from *Lactobacillus sakei* Lb674/LTH673 and Orf4 from *Lactobacillus sakei* Lb706. Identical amino acids are boxed. The processing site, preceded by the Gly-Gly conserved motif, is indicated.

the model described above have been established experimentally. First, the ability of peptide AIs, with or without antimicrobial activity, to induce class II bacteriocin production has been clearly demonstrated in several gram positive bacteria, including strains of *C. piscicola* LV17B (Saucier *et al.*, 1995; Franz *et al.*, 2000a; Kleerebezem *et al.*, 2001), *Lactobacillus plantarum* C11 (Diep *et al.*, 1995), *Lb. sakei* Lb706 (Diep *et al.*, 2000), *Lb. sakei* LTH673 (Eijsink *et al.*, 1996), *Enterococcus faecium* CTC492 (Nilsen *et al.*, 1998) and *E. faecium* DPC1146 (O'Keeffe *et al.*, 1999). In these organisms, cultures that have been made BAC$^-$ (for example by starting a new culture with a highly diluted inoculum or by incubating the culture under suboptimal conditions) can be stimulated to produce bacteriocins by treatment with the corresponding AIs.

Second, it is well documented that the AIs induce transcription of their own genes and of genes required for bacteriocin production and bacteriocin immunity. AI-induced transcription has been shown by transcriptional analysis and the use of transcriptional fusions to reporter genes in many of the bacteria mentioned above, including *C. piscicola* (Quadri *et al.*, 1997b; Saucier *et al.*, 1997; Kleer-

ebezem *et al.*, 2001), *Lb. plantarum* (Diep *et al.*, 1995; Diep *et al.*, 1996) and *Lb. sakei* (Eijsink *et al.*, 1996; Brurberg *et al.*, 1997; Diep *et al.*, 2000; Risoen *et al.*, 2000).

Third, the involvement of genes encoding HPKs and/or RRs of two-component signal transduction systems in the regulatory mechanism has been established by genetic analysis in some of the class II bacteriocin-producing bacteria. The RR CbnR and the HPK SakB/SapK have been shown to be involved in regulation of bacteriocin production in *C. piscicola* LV17B (Kleerebezem *et al.*, 2001) and *Lb. sakei* Lb706 (Axelsson *et al.*, 1993), respectively. Similarly, involvement of the HPK PlnB and the RRs PlnC and PlnD has been established in *Lb. plantarum* C11 (Diep *et al.*, 2001; Diep *et al.*, 2003). Notably, in the later bacterium, the two phosphorylated RRs have opposite activities on the same regulated promoters of the plantaricin gene cluster; while PlnC activates gene transcription, PlnD appears to repress it (Diep *et al.*, 2003). Additional mutational and genetic analysis in *Lb. sakei* Lb706 (Axelsson and Holck, 1995), *Lb. sakei* Lb674 (Huhne *et al.*, 1996) and strains of *C. piscicola* (Franz *et al.*, 2000b; Quadri *et al.*, 1997b) suggests the requirement of the regulatory genes encoded in the bacteriocin production loci of these bacterial for bacteriocin production; however, the possibility that the BAC^- phenotype of the regulatory mutants was due to polar effect was not ruled out in these studies.

Forth, the relevance of conserved sequences motif in the promoter region of the inducible transcripts has been confirmed by deleting/mutating the conserved sequences and measuring the effect of the modifications in gene expression. This has been done in *Lb. plantarum* C11 (Risoen *et al.*, 2001) and *Lb. sakei* Lb706 (Axelsson and Holck, 1995). Furthermore, *in vitro* binding of the RRs to the conserved sequences in the AI-responsive promoters has been shown in *Lb. plantarum* C11 (Risoen *et al.*, 1998; Risoen *et al.*, 2001) and *Lb. sakei* LTH673 (Risoen *et al.*, 2000).

Finally, the role of the *sec*-independent processing/secretion systems of the class II bacteriocins in cleavage of the leader peptide of the AI precursors and export of the dedicated mature AIs is supported by the sequence similarity between the leader peptides of the precursors of the AIs and the precursors of the bacteriocins and experiments in *C. piscicola*, where inactivation of the genes of the processing/secretion system results in mutants that lack AIs in the culture supernatants (Quadri *et al.*, 1997b).

Sensing the AIs

One important aspect of the regulatory mechanism reflected in the current model for AI-mediated regulation of class II bacteriocin production in gram positive bacteria and requiring further experimental exploration is the proposed function of the HPKs encoded in the bacteriocin production loci as AI receptors. The observation that both L and D enantiomers of plantaricin A (the AI of plantaricin production in *Lb. plantarum* C11) have antimicrobial activity but only the L-enantiomer has AI activity suggests that the latter activity is dependent on a chiral interaction, a property consistent with a AI-receptor interaction (Hauge *et al.*, 1998). Since the HPKs of many two-component regulatory systems function as receptors, the HPKs shown to be required for bacteriocin production are good candidates to be the AI receptors. Studies based on genetic reconstitution of the signal transduction pathways in heterologous hosts support the receptor functions of the HPKs CbnK (L.E.N. Quadri, unpublished results) and SppK (Brurberg *et al.*, 1997), which are required for production of carnobacteriocins B2 and BM1 (by *C. piscicola* LV17B) and sakacin P (by *Lb. sakei* LTH673/Lb674), respectively. However, experimental evidence towards establishing whether there is a direct interaction between the HPKs and their cognate AIs is needed to validate the receptor hypothesis.

The genetic dissection of the carnobacteriocin system of *C. piscicola* LV17B has shown that CbnK and CbnR are necessary and sufficient for the activation of the promoters regulated by both carnobacteriocin B2 and the dedicated AI (named CS) in an homologous *Carnobacterium* host and in an heterologous lactococcal host (Kleerebezem *et al.*, 2001; L.E.N. Quadri, unpublished results). The fact that peptides as different as carnobacteriocin B2 and CS function as AIs in *C. piscicola* LV17B raises intriguing questions regarding the nature of the stimulus sensed by the HPK CbnK and the mode and specificity of recognition between CbnK and the AIs. In this regard, both carnobacteriocin B2 and CS are cationic peptides with solution structures that display amphipathic α-helixes in membrane mimicking environments and potential for membrane interactions (Wang *et al.*, 1999; L.E.N. Quadri and D. Eliezer, unpublished results). It is possible that carnobacteriocin B2 and the peptide CS are recognized by CbnK in their membrane associated conformation, in which the peptides may display common three-dimensional features key to CbnK recognition.

Topology predictions suggest that CbnK has six transmembrane segments (TMSs) at the N-terminal half of the protein. This analysis has been used to propose a topological model that consists of six TMSs with short and hydrophilic periplasmic and cytoplasmic connecting loops, in which both N-and C-termini are cytoplasmic. The proposed model also considers that the C-terminal kinase domain should be in the cytoplasm (L.E.N. Quadri, unpublished results). Multi-TMS topology arrangements are common in AgrC-type HPKs (Diep *et al.*, 1994; Lyristis *et al.*, 1994; Magnuson *et al.*, 1994; Axelsson and Holck, 1995; Solomon *et al.*, 1995; Håvarstein *et al.*, 1996; Huhne *et al.*, 1996), where the N-terminal TMSs form the so-

called membrane-associated domain (Håvarstein *et al.*, 1996). It has been hypothesized that the 80 amino acids at the N-terminus of the membrane-associated domain of the AgrC-type HPK ComD is involved in the binding of the competence-stimulating peptide (CSP), which is the AI that stimulates competence in Streptococci (Håvarstein *et al.*, 1996). It is possible that AI recognition by the sensor domain of AgrC-like HPKs takes place in the membrane environment, a situation that is analogous to that observed for several G-protein-couple receptors, where the peptide ligands bind to sites that are partially or entirely located within the plane of the lipid bilayer between the transmembrane segments of the receptors (Bockaert, 1991; Håvarstein *et al.*, 1996; Pozzi *et al.*, 1996; Henry *et al.*, 2002; Boucard *et al.*, 2003).

Structure-activity relationship analysis of AIs involved in class II bacteriocin production

Structure-activity relationship analysis has permitted identification of a carnobacteriocin B2 variant (CB2.Phe33-Ser) without detectable antimicrobial activity but with AI activity in *C. piscicola* LV17 (Quadri *et al.*, 1997a). This finding suggests that the AI function of carnobacteriocin B2 does not require the antimicrobial activity of the peptide. Similar conclusion has been recently reached for the activity of the dedicated AI plantaricin A of *Lb. plantarum* C11, which also has antimicrobial activity (Hauge *et al.*, 1998). The recently determined solution structure of carnobacteriocin B2 reveals that the bacteriocin has a helical structure (residues 18-39), where the side chains of the hydrophobic residues Trp18, Phe22, Tyr26, Ile30, Phe33, Val34 and Val37 form a hydrophobic surface on one side of the helix and the side chains of the hydrophilic residues Asn17, Gln20, Glu24, Arg25, Asn31, Ser35 and Ser39 form a hydrophilic surface on the opposite side (Wang *et al.*, 1999). The activity of CB2.Phe33-Ser may suggest that the integrity of the hydrophobic surface is essential for antimicrobial activity but not for AI activity. A C-terminally truncated carnobacteriocin B2 variant preserving only the first 28 residues of peptide has neither AI nor antimicrobial activity, suggesting that the C-terminal half of carnobacteriocin B2 is important for both activities, whereas the carnobacteriocin B2 precursor (CbnB2) has marginal antimicrobial activity and also functions as AI, suggesting that the free N-terminus of the mature peptide is not essential for its AI function (Quadri *et al.*, 1997a). More recent structure-activity relationship analysis of the dedicated AI (CS) of the *C. piscicola* LV17B system demonstrated that CSCys24-Ala and CSCys16-Ala variants lack AI activity as determined using a CbnKR-dependent β-glucuronidase reporter system (L.E.N. Quadri, unpublished results). These results suggest that the conserved Cys residues are key for biological activity of the dedicated AI.

Conclusions

Many bacteriocins are of relevance for the food, agricultural and pharmaceutical industries as biopreservatives and alternative therapeutics. In particular, the association of bacteriocin-producing lactic acid bacteria with food products makes these bacteria and their bacteriocins of interest for the food industry as potential biopreservatives to extend the shelf life and enhance the bacteriological safety of selected food products. Deconvolution of the molecular basis of AI-mediated cell-to-cell communication mechanisms modulating bacteriocin production is important for understanding and manipulating the behavior of bacteriocin-producing bacteria in natural and artificial niches. The practical implication of a better understanding the molecular mechanisms of AI-mediated regulation of bacteriocin production could be foreseen in areas as diverse as production of food and feed, biological control of crop and animal pathogens and probiotics. Although tremendous progress has been made towards the understanding of regulation of bacteriocin production, more research is needed to gain further insights into yet unexplored or poorly understood aspects of the regulatory mechanisms controlling the production of bacteriocins in gram positive bacteria.

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