Microbiology (2011), **157**, 3256–3267 DOI 10.1099/mic.0.052571-0

Review

Target recognition, resistance, immunity and genome mining of class II bacteriocins from Gram-positive bacteria

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Due to their very potent antimicrobial activity against diverse food-spoiling bacteria and pathogens and their favourable biochemical properties, peptide bacteriocins from Gram-positive bacteria have long been considered promising for applications in food preservation or medical treatment. To take advantage of bacteriocins in different applications, it is crucial to have detailed knowledge on the molecular mechanisms by which these peptides recognize and kill target cells, how producer cells protect themselves from their own bacteriocin (self-immunity) and how target cells may develop resistance. In this review we discuss some important recent progress in these areas for the non-lantibiotic (class II) bacteriocins. We also discuss some examples of how the current wealth of genome sequences provides an invaluable source in the search for novel class II bacteriocins.

Introduction

Production of antimicrobial compounds is a common trait in many forms of life, ranging from the innate immune systems in multicellular organisms to most micro-organisms. One group of antimicrobial peptides in bacteria is referred to as bacteriocins. These are ribosomally synthesized peptides as opposed to antibiotic peptides such as gramicidin S, polymyxin B and bacitracin, which are made by multienzyme complexes (Hancock & Chapple, 1999). Bacteriocin production can be regarded as an accessory trait as it is not crucial for the normal growth of bacteria; however, it is widely distributed in the bacterial world, probably facilitated by the fact that bacteriocin genetic determinants are often located on mobile genetic elements, such as conjugative plasmids or transposons (Jack *et al.*, 1995). Although the exact biological role of bacteriocins in nature is not well understood, one possible role is to serve as a means for the producers to compete with other bacteria for common resources, hence allowing the producers to dominate or establish growth in certain ecological niches such as the oral cavity or gut environments. Unlike traditional antibiotics such as penicillin and tetracycline, which often have broad inhibitory spectra, most bacteriocins have a relatively narrow spectrum, inhibiting genera or species closely related to the bacteriocin producers. The narrow inhibitory spectrum is sometimes a highly appreciated feature as it provides an excellent means to direct activity toward certain pathogens without disturbing the commensal bacterial flora, which can contribute health-giving benefits to the mammalian host (Rea *et al.*, 2011). Importantly, the emergence of antibiotic-resistant pathogens worldwide also brings a pressing need for novel antimicrobials, and in this context, Gram-positive bacteriocins have been considered to possess great potential as the next era antibiotics (e.g. Gillor & Ghazaryan, 2007; Piper *et al.*, 2009 and references therein). Most bacteriocins are very potent, exhibiting antimicrobial activity at nanomolar concentrations, as opposed to their eukaryotic counterparts, which normally have 10^2–10^3-fold lower activity. Furthermore, bacteriocins have been much studied due to their food-preservative properties; numerous publications have documented that they can be used either as additives or as bioprotective cultures, to prevent growth of food-borne pathogens or food-spoiling bacteria including *Listeria*, *Bacillus* and *Staphylococcus* (De Vuyst & Leroy, 2007; Gálvez *et al.*, 2007 and references therein).

Bacteriocins of Gram-positive bacteria are pore-forming peptides which are very diverse in terms of physicochemical properties, primary sequence, structure and inhibitory spectra. They are generally classified into two

Two supplementary tables are available with the online version of this paper.

main classes: the lantibiotics (class I), which are bacteriocins containing post-translationally modified residues (e.g. the cyclic thioether amino acids lanthionine and methyllanthionine and dehydrated amino acids such as dehydroalanine); and the non-lantibiotics (class II), which are composed of non-modified residues except for the presence of disulfide bridges (Cotter *et al.*, 2005; Nes *et al.*, 2007). Class II bacteriocins have been divided further into several subgroups (Cotter *et al.*, 2005). Class IIa bacteriocins (e.g. pediocin PA-1, mesentericin Y105, enterocin P) contain an N-terminal consensus sequence (YGNGVxCx xxxCxVxWxxA, where x is any amino acid); these peptides are often referred to as pediocin-like bacteriocins after pediocin PA-1, the first characterized member from this group. Class IIb consists of two-peptide bacteriocins whose full activity is dependent on the complementary action of two different peptides (e.g. lactococcin G, enterocin L50, plantaricin S), and models for the interaction between the individual peptides have recently been characterized for some bacteriocins (Oppegård *et al.*, 2008; Soliman *et al.*, 2011). Class IIc bacteriocins are referred to as cyclic bacteriocins whose ring structure is formed in a head-to-end fashion (e.g. enterocin AS-48, lactocyclicin Q, garvicin ML). Lastly, class IId consists of linear non-pediocin-like one-peptide bacteriocins (e.g. lactococcins A and B, enterocin B). Due to their great biochemical diversity, classification of bacteriocins is an unsettled issue, as demonstrated by the different classification schemes suggested over the years (e.g. Cotter *et al.*, 2005; Kemperman *et al.*, 2003; Nes *et al.*, 2007). Additional subgroups have for example been suggested for leaderless peptides and peptides derived from large proteins (Nes *et al.*, 2007), and the classification of a recently discovered new group of bacteriocins, the glycosylated bacteriocins (Oman *et al.*, 2011; Stepper *et al.*, 2011), has not been settled. The debate on this issue will probably continue in the coming years as new bacteriocins with novel features are likely to be identified. It will therefore be important to maintain a uniform classification scheme in the future, preferably based on the bacteriocins' amino acid sequence and structural features.

Despite bacteriocins being studied for decades, only a few (nisin and pediocin PA-1) have been commercialized (De Vuyst & Leroy, 2007). This disappointing lack of progress may partly be due to the general lack of knowledge of how a bacteriocin chooses its target, how the pores in target membranes are formed, how producer cells protect themselves from suicide (self-immunity) and how resistance develops. Detailed knowledge on these issues is of great importance in order to draw up guidelines for applications of bacteriocins in a safe and efficient manner in food or medical treatments. Furthermore, in order to obtain more potent and stable antimicrobial peptides, identification of novel bacteriocins has been and will continue to be a central topic within bacteriocin research. This review will deal with recent progress in cell targeting, immunity and resistance for bacteriocins of class II, with special focus on the class IIa bacteriocins. We will also discuss some genome mining approaches for identification of novel bacteriocins. For a comprehensive overview of other aspects of class II bacteriocins, including bacteriocin production, genetic loci and the biochemical properties of the peptides, we refer the reader to other recent reviews (Drider *et al.*, 2006; Nes *et al.*, 2007; Nissen-Meyer *et al.*, 2009).

Bacteriocin target cell recognition and pore formation

It is well established that class II bacteriocins kill target cells by pore formation or by interfering with the integrity of the membrane, but the molecular mechanisms underlying this process are mostly unknown. For class IIa bacteriocins and some members of class IId, however, such a mechanism has been elucidated.

Class IIa bacteriocins target the mannose phosphotransferase system on sensitive cells

Class IIa bacteriocins constitute a large cluster of peptides with lengths between 36 and 49 aa. They are particularly active against *Listeria* species, but their inhibitory spectrum also includes a number of other genera such as *Enterococcus*, *Carnobacterium*, *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Clostridium* (Eijsink *et al.*, 1998). Several studies have revealed that these peptides have some common features in their structure. They normally contain two distinct domains separated by a flexible hinge (Fregeau Gallagher *et al.*, 1997; Haugen *et al.*, 2005; Uteng *et al.*, 2003). The first domain consists of a highly conserved, cationic N-terminal region in which the pediocin consensus motif YGNGVx CxxxxCxVxWxxA (x is any amino acid) is located; this domain is structured as an anti-parallel β-sheet stabilized by a disulfide bridge. The second domain is less conserved and is located in the C-terminal half. This region contains one or two α-helices that form a hairpin or a functionally equivalent helix–hinge–helix structure.

The target receptor for class IIa bacteriocins on sensitive cells has been identified as proteins of the sugar transporter mannose phosphotransferase system (Man-PTS). These sugar permeases belong to a group of transport systems referred to as phosphoenolpyruvate : carbohydrate phosphotransferase systems (PTSs), which are characterized by their ability to couple the import and phosphorylation of incoming sugars (Postma *et al.*, 1993). In addition to the general PTS proteins enzyme I (EI) and HPr, which serve as phospho-donors for different PTS permeases, Man-PTS contains a carbohydrate-specific protein complex (enzyme II, EII) consisting of four subunits: IIA, IIB, IIC and IID. The IIA and IIB subunits, which are often found together on one protein, are located in the cytoplasm along with the general proteins EI and HPr, while the IIC and IID subunits are individual transmembrane proteins which together form a carbohydrate-specific translocation channel

(Postma *et al.*, 1993). Man-PTS has a relatively broad carbohydrate substrate specificity, and members of this family are reported to import and phosphorylate a variety of different hexoses, including glucose, mannose, glucosamine, fructose, galactosamine and *N*-acetylglucosamine (Saier *et al.*, 2005).

Numerous initial studies on bacteriocin-resistant mutants of *Listeria monocytogenes* and *Enterococcus faecalis* provided circumstantial evidence that Man-PTS might act as receptor for class IIa bacteriocins. Two-dimensional gel electrophoresis performed to compare the protein content in bacteriocin-sensitive and -resistant *Li. monocytogenes* isolates revealed the absence of the Man-PTS IIAB protein in the latter cell type (Ramnath *et al.*, 2000). Transposon mutagenesis studies linked class IIa bacteriocin resistance to the regulatory gene *rpoN* in *Li. monocytogenes* (Robichon *et al.*, 1997) and *E. faecalis* (Dalet *et al.*, 2000). This gene encodes the alternative sigma factor σ^{54}, which is involved in the regulation of *mptACD*, an operon encoding the Man-PTS in *Li. monocytogenes* and *E. faecalis* (Héchard *et al.*, 2001). The subsequent inactivation of *mpt* genes led to class IIa bacteriocin resistance in both species studied (Dalet *et al.*, 2001; Héchard *et al.*, 2001). The involvement of Man-PTS in bacteriocin targeting was also confirmed by heterologous expression of the listerial Man-PTS gene *mptC*, which rendered the resistant *Lactococcus lactis* sensitive to class IIa bacteriocins (Ramnath *et al.*, 2004). Conclusive evidence that class IIa bacteriocins physically bind to Man-PTS proteins on target cells was later obtained by co-purification experiments showing that the bacteriocins form a complex with the Man-PTS proteins and the bacteriocin immunity protein in immune cells (Diep *et al.*, 2007).

The Man-PTS family is relatively large, with members present in the genomes of many bacterial genera belonging to the Gram-negative γ-Proteobacteria and the Gram-positive Firmicutes. Interestingly, Man-PTSs are not found in eukaryotes and they could therefore potentially be an ideal drug receptor to target and kill bacterial pathogens without disturbing the mammalian host. Cluster analyses combined with heterologous gene expression of Man-PTSs showed that class IIa bacteriocins target only a phylogenetically defined subgroup of Man-PTSs, and this subgroup (group I) exclusively contains members of the bacteriocin-sensitive Firmicutes (e.g. *Listeria*, *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Carnobacterium*, *Clostridium*, *Pediococcus* and *Streptococcus*) (Kjos *et al.*, 2009). The most potent class IIa bacteriocin receptors are those found in *Listeria* and *Enterococcus* species, and accordingly, these bacteria are often reported as being highly sensitive to class IIa bacteriocins (Diep *et al.*, 2006; Eijsink *et al.*, 1998). An overview of the phylogenetic distribution of Man-PTSs and their receptor potencies is depicted in Fig. 1.

By gene deletion and complementation studies it has been shown that the membrane-located IIC and IID proteins together form the bacteriocin receptor, while the IIAB subunits are dispensable for the receptor function (Diep *et al.*, 2007). Further studies showed that only the IIC protein is involved in species-specific targeting and that an N-terminal extracellular loop-containing region of 40 aa in this IIC protein is the major determinant responsible for the species-specificity, hence potentially serving as an interaction site for the class IIa bacteriocins (Kjos *et al.*, 2010a). Although IID is part of the bacteriocin receptor complex (Dalet *et al.*, 2001; Diep *et al.*, 2007), its role is still unclear. It may be involved in non-specific interactions with the bacteriocin during the pore formation process or possibly may also have a more structural function in this context, i.e. helping its partner (IIC) to fold correctly in the membrane.

It is not yet known what part of a class IIa bacteriocin is directly involved in specific receptor recognition. A panel of ten class IIa bacteriocins covering the range of variation within this class, i.e. they all have a similar N-terminal consensus sequence (YGNGVxCxxxxCxVxWxxA) but vary greatly in the C-terminal half, were all shown to display similar specificity against clones expressing different hybrid receptors (Kjos *et al.*, 2010a). This suggests that the conserved N-terminal consensus sequence (which is shared among all class IIa bacteriocins) is probably involved in receptor interaction. In contrast, a different study using hybrid class IIa bacteriocins in which the conserved N-terminal half and the variable C-terminal half were combined from different bacteriocins (obtained by gene fusions) provided evidence that the C-terminal half is most likely involved in the target cell specificity (Johnsen *et al.*, 2005). Moreover, peptide fragments derived from the helical region in the C-terminal part of pediocin PA-1 have been shown to inhibit the activity of the full-length peptide in a sequence-specific manner, possibly by obstructing the bacteriocin–receptor interaction (Fimland *et al.*, 1998; Haugen *et al.*, 2011). Similar inhibition of activity was also seen when two different class IIa bacteriocins (leucocin A and carnobacteriocin B2) were mixed (Yan *et al.*, 2000). The latter observations strongly suggest that the C-terminal part of the bacteriocin also specifically interacts with the receptor. These apparently conflicting results may indicate that the target recognition involves different regions of the bacteriocin to bring about different steps during receptor recognition and the subsequent pore formation. It is tempting to speculate that the conserved N-terminal β-sheet-containing part of class IIa bacteriocins initially interacts with the extracellular loop region of the Man-PTS IIC protein, which in turn enables the C-terminal α-helix-containing part of the bacteriocin to insert into the membrane to engage in helix–helix interactions with transmembrane segments of IIC and/or IID. Such interactions might impose structural changes in the Man-PTS, in effect rendering the permeases open as pores, thereby causing leakage of solutes, disruption of membrane integrity and eventually cell death (Fig. 2a). Given this model, all class IIa bacteriocins might interact in a similar fashion with the extracellular loop in IIC, while

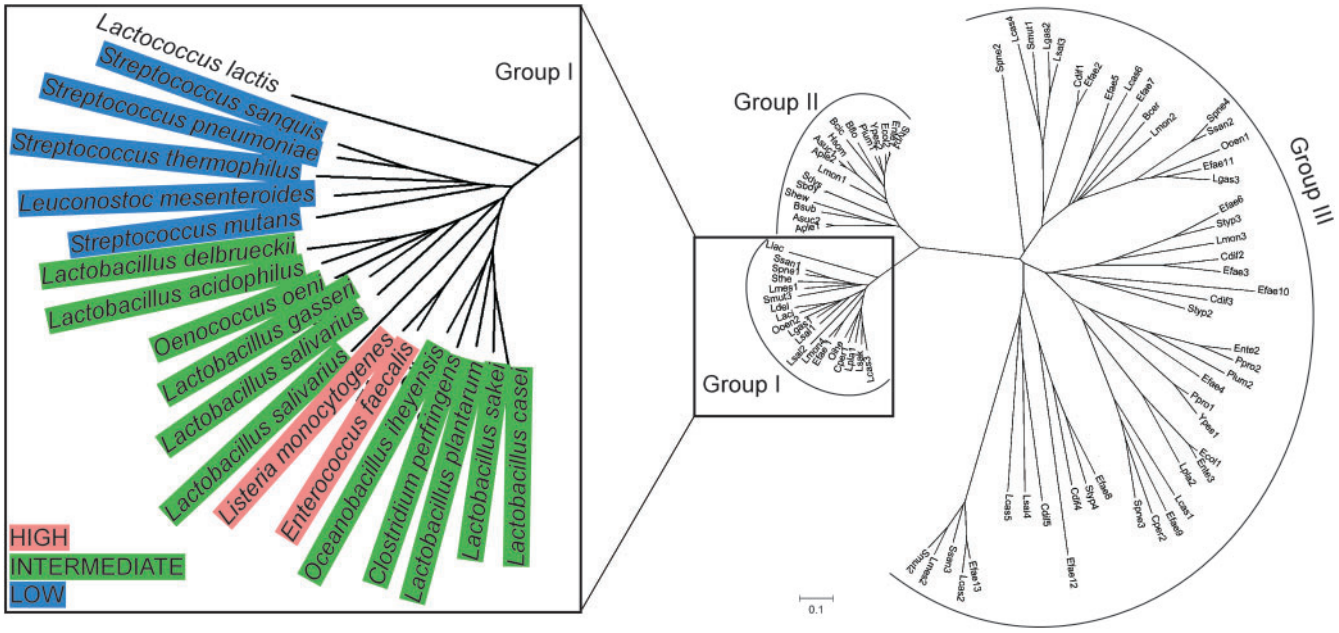

Fig. 1. Phylogenetic distribution of selected Man-PTSs derived from sequenced genomes (right panel). Analysis was performed using the PhyML interface at Phylogeny.fr (Dereeper *et al.*, 2008). Each node represents a Man-PTS IIC protein (see Supplementary Table S1, available with the online version of this paper, for full list of proteins), but similar clustering is seen also for IID proteins (Kjos *et al.*, 2009a). The scale bar represents 1 mutation per 10 amino acids. Man-PTSs are classified into three different phylogenetic groups; only members of group I are functional receptors for class IIa bacteriocins (Kjos *et al.*, 2009). In the box, bacterial species carrying group I Man-PTSs are indicated; based on experimental evidence (Kjos *et al.*, 2009) these can be further subgrouped into receptors conferring high, intermediate and low sensitivity to class IIa bacteriocins. The Man-PTS of *L. lactis* is a member of group I, but does not confer sensitivity to class IIa bacteriocins. A comprehensive list of bacterial species whose genome encodes a group I Man-PTS (and thus may be sensitive to class IIa bacteriocins) is given in Supplementary Table S2.

the helix–helix interactions diverge between bacteriocins due to differences in their C-terminal sequences (Johnsen *et al.*, 2005). This may explain why the C-terminal part is responsible for the target cell specificity and also for the specific recognition of the cognate immunity protein (Johnsen *et al.*, 2005) (see below for discussion regarding the immunity proteins). Such a model is also in line with the proposed orientation of class IIa bacteriocins in target cells with the N-terminal β-sheet domain in a hydrophilic environment and the C-terminal hydrophobic/amphiphilic domain in a more hydrophobic environment (Fimland *et al.*, 2002b, 2006; Haugen *et al.*, 2008; Miller *et al.*, 1998). Future studies should aim at detailed characterization of interactions between the bacteriocin and the receptor as this may allow us to design bacteriocins (e.g. by genetic engineering) with increased potency and specificity towards the receptors of different target bacteria.

Lactococcin A – another Man-PTS-targeting bacteriocin

Lactococcin A is a linear, non-pediocin-like lactococcal bacteriocin belonging to class IId (Holo *et al.*, 1991). Like the class IIa bacteriocins, lactococcin A also employs the membrane-located Man-PTS proteins (IIC and IID) as target on sensitive cells (Diep *et al.*, 2007). However, in contrast to class IIa bacteriocins, which can target Man-PTSs from different genera, lactococcin A has an extremely narrow inhibitory spectrum, targeting only the lactococcal Man-PTS (Ptn system) (Kjos *et al.*, 2009). This difference in target specificity can be attributed to the mechanisms by which the peptides bind their receptors: while class IIa bacteriocins rely on a short defined region of IIC for specific receptor recognition, lactococcin A appears to interact with several regions of both IIC (PtnC) and IID (PtnD) (Kjos *et al.*, 2010a). The related class IId lactococcal bacteriocin lactococcin B also employs Man-PTS as receptor on sensitive cells (Diep *et al.*, 2007); however, details of the mechanism of target recognition have not been investigated for this bacteriocin.

Self-immunity mechanisms of class II bacteriocins

The genetic determinants encoding self-immunity for class II bacteriocins are often located just downstream of and within the same operon as the bacteriocin structural gene(s). Due to this conserved organization, putative immunity genes for most bacteriocins are readily identified

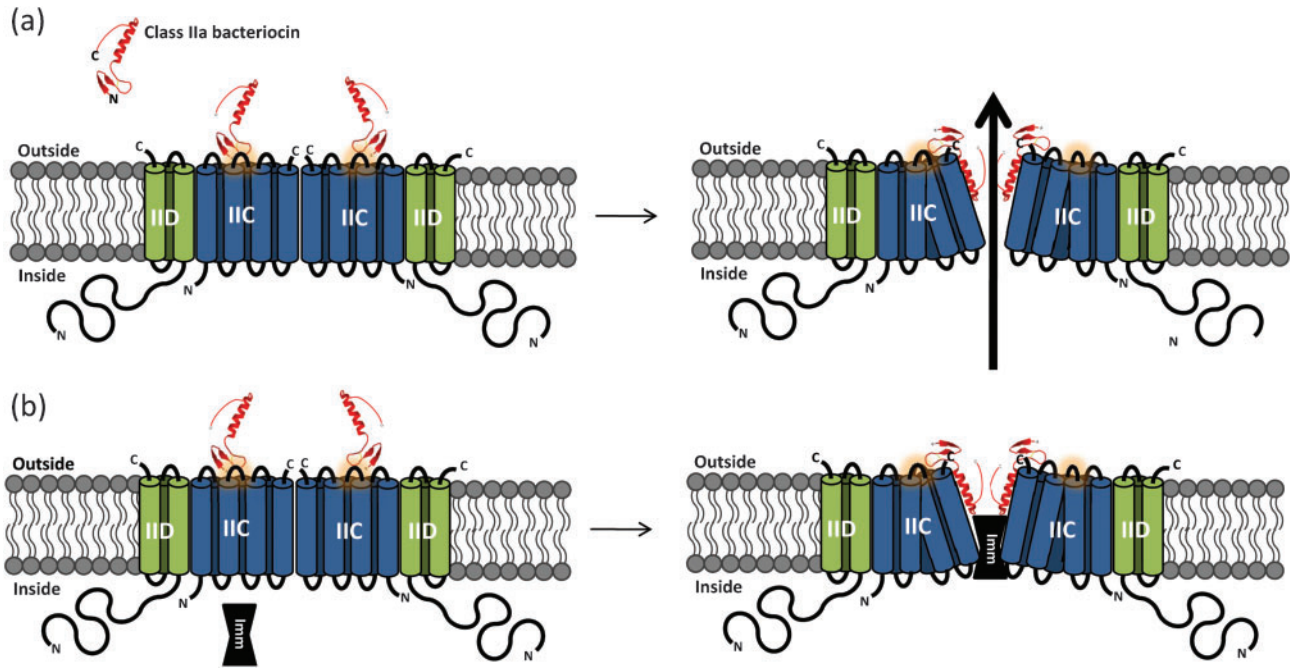

Fig. 2. Proposed model of the mode of action (a) and immunity (b) for class IIa bacteriocin systems. (a) The N-terminal β-sheet containing part of the bacteriocin (red) initially interacts with an extracellular loop (highlighted in yellow) of the Man-PTS IIC protein (left panel), before the helix-containing C-terminal part engages in specific interactions with transmembrane helices of the IIC and/or IID proteins (right panel) to cause conformational changes which, in turn, lead to pore formation and eventually cell death. (b) In immune cells, the bacteriocin mediates the same conformational changes, but the pore is blocked by a specific immunity protein (black) which binds tightly to Man-PTS.

and their function has in many cases been confirmed by heterologous expression in sensitive cells, rendering these cells immune to cognate bacteriocins. Examples of this include the immunity protein EntqC of the class IId bacteriocin enterocin Q (Criado *et al.*, 2006) and LagC of the class IIb bacteriocin lactococcin G (Oppegård *et al.*, 2010). In general, immunity proteins for class II bacteriocins vary greatly with respect to their sizes and amino acid sequences; however, the molecular mechanisms underlying immunity remain elusive for most bacteriocins. Recently, some reports have shed light on immunity mechanisms for some class II bacteriocins (Diep *et al.*, 2007; Gajic *et al.*, 2003; Kjos *et al.*, 2010b).

Immunity against Man-PTS-targeting bacteriocins

Immunity proteins for class IIa bacteriocins and lactococcin A and B (all bacteriocins targeting Man-PTS as receptor) vary between 88 and 118 amino acids in length and they show relatively low sequence similarities among each other as compared to the higher sequence similarities among their bacteriocin counterparts (Eijsink *et al.*, 1998; Fimland *et al.*, 2002a). In general, the immunity proteins act specifically toward their cognate bacteriocins. Studies on lactococcin A have uncovered important details concerning how these proteins confer immunity. Cloning of the lactococcin A immunity gene *lciA* renders the sensitive strain *Lc. lactis* IL1403 immune (Holo *et al.*, 1991). During purification of the immunity protein from the bacteriocin producer, it was found that a pool of LciA was associated with the membrane fraction in addition to the remaining pool in the cytosol (Venema *et al.*, 1994), suggesting that LciA might possess different conformations in cells. Indeed, when using a recombinant LciA containing an N-terminal tag (fLciA) for immunopurification, the immunity protein was found to behave differently depending on the presence or absence of the cognate bacteriocin: in the absence of lactococcin A the recombinant protein remained mostly in unassociated form, but upon addition of the bacteriocin, the immunity protein tightly associated with the membrane-located Man-PTS receptor and the bacteriocin (Diep *et al.*, 2007). From this study it was suggested that lactococcin A is locked onto Man-PTS by its immunity protein, thereby being prevented from proceeding any further to form lethal pores (Fig. 2b) (Diep *et al.*, 2007). Virtually the same bind-and-lock mechanism is true for proteins conferring immunity to class IIa bacteriocins (Diep *et al.*, 2007), indicating that, despite the divergence in amino acid sequence of the bacteriocins and their cognate immunity proteins, lactococcin A and the class IIa bacteriocins display functional convergence in the mechanisms of receptor targeting (both using Man-PTS) and immunity (both involving a bind-and-lock mechanism).

By using hybrid class IIa bacteriocins it has been shown that the C-terminal, flexible part of immunity proteins specifically recognizes the C-terminal part of class IIa bacteriocins; however, no direct physical contact between immunity proteins and bacteriocin has been demonstrated (Johnsen *et al.*, 2005; Sprules *et al.*, 2004). As the immunity proteins differ greatly in their amino acid sequence and confer immunity specifically (i.e. only to their cognate bacteriocins), it is reasonable to believe that the variations in the C-terminal part of class II bacteriocins create pores with different physico-chemical properties (e.g. size, hydrophobicity and acidic character) and that the variations in the immunity proteins have evolved in parallel in order to match and block the pores created by the cognate bacteriocins (Fig. 2b). However, the nature of the pores created by Man-PTS-targeting class II bacteriocins remains to be investigated. We do not know whether (model 1) the bacteriocin employs Man-PTS as a docking molecule in order to oligomerize and form pores in the same manner as found for several lantibiotics using lipid II as docking molecule, or if (model 2) the bacteriocin interferes with the gate of the Man-PTS, causing it to open irreversibly in a manner depicted in Fig. 2(a). The fact that the bacteriocin is tightly associated with the Man-PTS in immune cells strongly implies that the bacteriocin is entrapped within the Man-PTS, thus favouring model 2. In further support of this notion is the observation that cloned immune cells (expressing an immunity protein) display reduced growth on glucose when they are challenged with bacteriocin, probably as a consequence of the Man-PTS being inhibited by the complex formation with the bacteriocin and the immunity protein (Diep *et al.*, 2007).

Putative proteases of the Abi family confer immunity

For some members of the class IIb bacteriocins (two-peptide bacteriocins), recent findings suggest that a proteolytic mechanism may be involved in self-immunity (Kjos *et al.*, 2010b). Genes encoding putative transmembrane proteases of the family CPBP (Caax proteases and bacteriocin-processing enzymes, also known as the Caax protease family or the Abi family, Pfam PF02517; Pei & Grishin, 2001; Pei *et al.*, 2011), are involved as immunity determinants in several bacteriocin loci encoding class IIb bacteriocins, such as the *pnc* locus of *Streptococcus pneumoniae* (Lux *et al.*, 2007), the *pln* locus of *Lactobacillus plantarum* and the *skk* locus of *Lactobacillus sakei* (Kjos *et al.*, 2010b). The Abi family proteins are characterized by three conserved motifs (Fig. 3). In eukaryotes, these proteins have been shown to have a proteolytic function, in which the three conserved motifs are thought to constitute the active site. Correspondingly, the Abi bacteriocin immunity protein SkkI was shown to lose its immunity function by mutagenesis in the conserved motifs (Kjos *et al.*, 2010b). Also interesting, while class II bacteriocin immunity proteins are generally highly specific, three different Abi immunity proteins (SkkI from the *skk* locus, and PlnI and PlnLR from the *pln* locus) were shown to confer cross-immunity across different bacteriocin systems (Kjos *et al.*, 2010b). These results suggest that Abi proteins probably confer immunity via a common, proteolytic mechanism. At present the substrates for this group of proteases remain enigmatic in the bacterial world; however, results obtained so far indicate that the bacteriocin itself is not the target of the proteolytic activity as it was not degraded in the study of Kjos *et al.* (2010b).

Transport systems confer immunity

For some class II bacteriocin systems, transporter proteins seem to be involved in the immunity function. For example, the production of two bacteriocins, designated LsbA and LsbB, synthesized with and without an N-terminal extension, respectively, was shown to be mediated by LmrB, a multidrug resistance (MDR) transporter protein which was involved in the secretion of and in conferring immunity to both antimicrobial peptides (Gajic *et al.*, 2003). Furthermore, producers of some cyclic bacteriocins (e.g. enterocin AS-48) are known to be protected by specialized ABC transporters which probably pump bacteriocins out from the membrane (Diaz *et al.*, 2003).

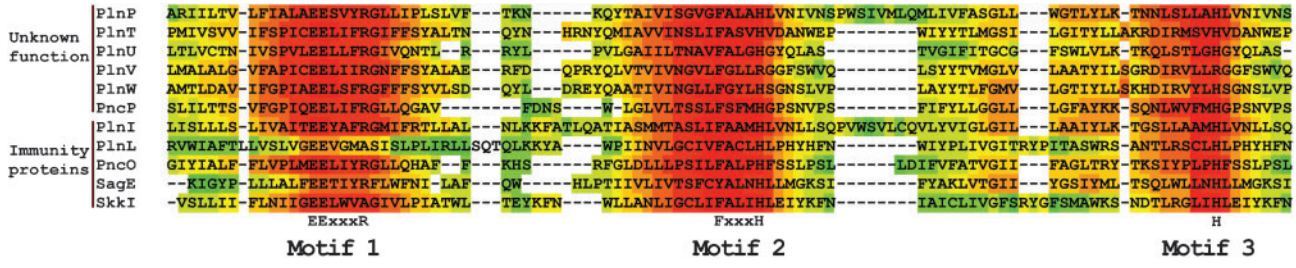

Fig. 3. Alignment of the amino acid sequences containing the three conserved motifs of Abi proteins. Motif 1 consists of two glutamate residues and an arginine separated by three variable amino acids (EExxxR), motif 2 consists of a phenylalanine and a histidine separated by three variable amino acids (FxxxH), while motif 3 consists of a single histidine residue (H). Five of the proteins (PlnI, PlnL, PncO, SagE and SkkI) have been shown to have a bacteriocin immunity function, while the remaining proteins have unknown function(s). The alignment was constructed using T-COFFEE (http://tcoffee.vital-it.ch/).

Resistance to class II bacteriocins

For some class II bacteriocin systems, sensitive strains can easily develop resistance upon exposure to bacteriocins. The frequency of resistance development varies greatly between types of bacteriocins and between sensitive strains. For class IIa bacteriocins, resistance frequencies up to 10^{-4} have been reported in *Li. monocytogenes* (Gravesen *et al.*, 2002a). Most probably, the mechanisms by which mutants become resistant are highly variable for different bacteriocins, but to date, only cells resistant to class IIa bacteriocin and lactococcin A have been investigated in detail.

For class IIa bacteriocins and lactococcin A, a number of studies have shown that Man-PTS expression is downregulated in resistant mutants of *Li. monocytogenes, E. faecalis* and *Lc. lactis* (Gravesen *et al.*, 2002b; Kjos *et al.*, 2011; Opsata *et al.*, 2010; Tessema *et al.*, 2009). Correspondingly, natural isolates of *Li. monocytogenes* with low bacteriocin sensitivity have been shown to display low Man-PTS expression compared to sensitive isolates (Kjos *et al.*, 2011). Hence, downregulation of the receptor genes is a common mechanism of resistance against Man-PTS-targeting bacteriocins. Man-PTS is a major player in carbon catabolite repression in bacteria (Postma *et al.*, 1993), and resistant mutants have thus been shown to display major shifts in the expression of genes involved in sugar metabolism. For example, in resistant mutants of *E. faecalis*, a transcriptional shift from genes involved in the glycolytic pathway to genes involved in transportation and degradation of secondary source sugars was observed and the metabolism was shifted from lactic acid fermentation to a mixed fermentation pattern (Opsata *et al.*, 2010). Similar phenotypes, i.e. reduced glucose consumption, reduced production of lactate and elevated production of formate, acetate and ethanol compared to wild-type cells, have also been observed in resistant mutants of *Li. monocytogenes* (Naghmouchi *et al.*, 2007; Vadyvaloo *et al.*, 2004a). In resistant cells of *Lc. lactis*, reduced growth on glucose was compensated with elevated growth on galactose (Kjos *et al.*, 2011).

The underlying reasons for reduced Man-PTS gene expression and the regulatory consequences thereof in bacteriocin-resistant cells are still not fully understood; however, current knowledge indicates that in some cases, stable mutations in Man-PTS regulatory genes might play an important role. This notion is based on the observations that (i) the resistant phenotype is stably preserved in non-selective medium (Gravesen *et al.*, 2002a) and that (ii) mutations/polymorphisms in the σ^{54}-associated activator ManR/MptR have been reported in resistant strains of both *E. faecalis* (Opsata *et al.*, 2010 and unpublished work) and *Li. monocytogenes* (Kjos *et al.*, 2011). ManR/MptR, which is involved in the activation of Man-PTS transcription in these species (Xue & Miller, 2007), might thus represent a genetically variable hot-spot which is important for the development of bacteriocin resistance. It should be noted that regulation of Man-PTS expression in *Li. monocytogenes* is dependent on several proteins (Vu-Khac & Miller, 2009). Therefore, there are a large number of sites in the genome where resistance mutations potentially may arise, and such multiple mutation sites may explain the high frequencies of bacteriocin resistance often observed in these cells. However, other mechanisms leading to resistance may also exist. For example, stochastic gene expression in monocultures is a relatively common phenomenon, such as during competence development and spore formation (Leisner *et al.*, 2008). Heterogeneity with respect to Man-PTS expression may potentially be a favourable trait, since it could allow a subpopulation of cells to cope with changes in carbon source availability as well as antimicrobial attacks. In this context, it is interesting to note that class IIa bacteriocin-resistant *Li. monocytogenes* (Vadyvaloo *et al.*, 2004b) and *E. faecalis* (Opsata *et al.*, 2010) display altered metabolic profiles. Whether such a stochastic mechanism contributes to bacteriocin resistance development remains to be investigated experimentally.

Moreover, there are also cases where class IIa bacteriocin-resistant cells show normal or even elevated Man-PTS expression (Kjos *et al.*, 2011; Tessema *et al.*, 2009; Vadyvaloo *et al.*, 2004b). The exact resistance mechanism in these cases is unknown, but changes in the cell envelope (e.g. membrane fluidity or cell surface charge) have been found to be associated with these resistant cells (Vadyvaloo *et al.*, 2002, 2004b). Probably, such changes can mask or interfere with the bacteriocin–receptor interaction, thereby rendering cells resistant to bacteriocins. Changes in the surface properties of bacterial cells may be a common bacteriocin resistance strategy, since such changes have also been implicated in resistance to lantibiotics (class I bacteriocins) (Abachin *et al.*, 2002; Gravesen *et al.*, 2004).

In some cases bacterial strains may become bacteriocin-resistant due to so-called immune mimicking mechanisms. For example, several non-producing strains have been shown to contain immunity genes with no corresponding bacteriocin gene (Fimland *et al.*, 2002a; Møretrø *et al.*, 2005). These immunity genes, which are probably remnants of bacteriocin gene clusters, may render the strains resistant to some bacteriocins if they are properly expressed. The presence of transporters that pump peptides out from the cell envelope may also be involved in bacteriocin resistance, in a similar manner as has been shown for lantibiotics (Collins *et al.*, 2010; McBride & Sonenshein, 2011). Finally, it is also reasonable to believe that non-specific extracellular proteases that degrade the peptides can confer bacteriocin resistance. This may, for instance, be the case for gelatinase of *E. faecalis*, which is known to degrade class IIa bacteriocins (Sedgley *et al.*, 2009).

Bacteriocin discovery

Traditionally, new bacteriocins have been identified by screening of bacterial isolates for antimicrobial activity

followed by purification and identification of the bacteriocin and its genetic determinants. Such screening strategies are still fundamental for detection and identification of potent bacteriocins of various subclasses, and recent examples of this include (i) a class IIa bacteriocin named avicin A that was identified from *Enterococcus avium* strains isolated from faecal samples of healthy human infants from both Ethiopia and Norway (Birri *et al.*, 2010), (ii) a class IIb bacteriocin, enterocin X, isolated from an *E. faecalis* strain from sugar apples (Hu *et al.*, 2010), (iii) a circular bacteriocin named garvicin ML produced by a *Lactococcus garvieae* strain isolated from mallard duck (Borrero *et al.*, 2011b) and (iv) a glycosylated bacteriocin (glycocin F) from *Lb. plantarum* isolated from fermented corn (Kelly *et al.*, 1996; Stepper *et al.*, 2011).

The large and rapidly growing number of available bacterial genome sequences in public databases offers another source in the search for novel bacteriocins. Recent examples of such *in silico*-based approaches include (i) the discovery of the class IIa bacteriocin penocin A in the genome of *Pediococcus pentosaceus* (Diep *et al.*, 2006) and (ii) identification of a fourth bacteriocin operon (plantaricin J51) in the plantaricin locus of *Lb. plantarum* (Navarro *et al.*, 2008 and D. B. Diep, unpublished data). Some bacteriocin groups contain sequence signatures that can readily be used in screens for new bacteriocins. However, many bacteriocin structural genes are small and display large sequence variations. Searching genomes for these genes directly is therefore often difficult. This problem can be circumvented by using conserved, bacteriocin-associated genes (e.g. immunity genes, transport genes, regulatory genes) as guides to localize new bacteriocin loci. Such an approach has successfully applied to identify a novel bacteriocin termed sakacin 23K in the genome of *Lb. sakei* 23K by means of mining for bacteriocin loci associated with Abi immunity genes (see above) (Kjos *et al.*, 2010b). Furthermore, Dirix *et al.* (2004) identified over 20 bacteriocins or bacteriocin-like peptides by screening 45 fully sequenced Gram-positive bacterial genomes for the presence of peptide genes with double glycine leaders as well as the corresponding peptidase C39 domain in the ABC transporter genes. Also, a new family of glycopeptide bacteriocins (glycocins) has recently been identified by mining bacterial genomes for the co-location of glycosyltransferases and C39-ABC transporter genes (Stepper *et al.*, 2011). It is important to note that in order to conclude that a novel bacteriocin has been identified, the *in silico* discovery of bacteriocin genes needs to be followed by experimental confirmation of antimicrobial activity, and this has indeed been done in several of the works mentioned above (Diep *et al.*, 2006; Kjos *et al.*, 2010b; Stepper *et al.*, 2011).

As another example of how powerful simple surveys of sequenced bacterial genomes can be to identify new bacteriocins *in silico*, we performed BLAST searches using class IIa immunity proteins as queries (the immunity proteins for leucocin A, sakacin P and enterocin P), and the sequence upstream of the immunity gene was examined for the presence of potential class IIa bacteriocin genes. Eight new class IIa bacteriocin sequences were revealed and their characteristics are summarized in Table 1. Importantly, an automated genome mining tool for identification of bacteriocin genes in sequenced bacterial genomes (BAGEL2) is available (de Jong *et al.*, 2010). This tool may be used to screen annotated genomes for potential bacteriocin genes, and is based on identification of conserved bacteriocin domains as well as analysis of the genomic context of putative bacteriocin genes. The BAGEL2 web server (http://bagel2.molgenrug.nl/) also offers reannotation of sequence genomes using Prodigal (Hyatt *et al.*, 2010). As exemplified by EquX in *Streptococcus equi* (Table 1), bacteriocins are often omitted during annotation of sequenced genomes; however, implementing BAGEL2 as part of the annotation pipeline would solve much of this problem.

Identification of novel antimicrobial peptides is not the only means to obtain more potent bacteriocins. The antimicrobial properties of bacteriocins may also be further improved by genetic engineering approaches using available peptide sequences as templates. Such techniques are particularly suitable for class II bacteriocins, given their gene-encoded nature. Mutational analysis and gene shuffling of class II bacteriocins has been performed in several studies (Haugen *et al.*, 2011, 2008; Oppegård *et al.*, 2007, 2008; Quadri *et al.*, 1997; Tominaga & Hatakeyama, 2006, 2007); however, systematic engineering efforts to improve antibacterial potencies have so far mainly been conducted for the lantibiotic bacteriocins (Field *et al.*, 2008). Notably, the specific antimicrobial activity of some bacteriocins has been increased through cloning and expression in other producer hosts. For example, a larger production, antimicrobial activity and specific antimicrobial activity of the class IIa bacteriocin enterocin P was obtained after heterologous expression in *Lc. lactis* (Borrero *et al.*, 2011a).

Future perspectives

The emergence of antibiotic-resistant pathogens causing serious problems in medical treatments and the increasing demand by consumers for more natural foods with fewer chemical additives have led to a crucial need for novel sources of antimicrobials and better strategies in food safety. During the last two decades bacteriocins have been the subject of much study worldwide because of their great potential (i) as natural preservatives to prevent the growth of food-spoiling bacteria or food-borne pathogens, (ii) as second-generation antibiotics to deal with pathogens that have acquired multi-resistance to traditional antibiotics, and also (iii) as probiotics with health-giving effects for man and animals. Nevertheless, only a few bacteriocins have made it into practical use. One reason for this is the lack of detailed knowledge of their modes of inhibition. Curiously, up to now Man-PTS is the only known receptor for class II bacteriocins. For the majority of class II bacteriocins, the receptors are virtually unknown.

Table 1. Characteristics of class IIa bacteriocins identified *in silico*

Bacteriocin*	Sequence†	Closest homologue	Subgroup‡	Immunity protein*	Leader type§	Origin‖
ZP_03958670	MENKKKLTKADLAKVTGG-SRYYGNGVTCGKHKCTVNWGQAWTCG VNRLANFGHGNC	Plantaricin C19	2	ZP_03958671	GG	*Lactobacillus ruminis* ATCC 25644
EquX¶	MNTTLMKQFEIIDADKLAHVEGG-KTTYYGNGLYCNTQKCWVNWSEAVNIIL NNSVMNGLTGGNAGWHSGGII	Ubericin A	4	YP_002123864	GG	*Streptococcus equi* MGCS 10565
ZP_07467492	MNTKTFDQFDVMTDAELSTVEGG-KTIYYGNGLYCNANKCWVNWSQTATTIA NNSVMNGLTGGNAGWHSGGRA	Ubericin A	4	ZP_07467491	GG	*S. bovis* ATCC 700338
ZP_07463259	MNLKMMEQFEIMDTEMLASKVGG-KTIYYGNGLYCDNSKGCWVNWPEAINKI LTNSIVNGFSGGNAGWNSGGPL	Ubericin A	4	ZP_07463258	GG	*S. mitis* ATCC 6249
YP_002996928	MSSCNRQNKQIQEIFNVNTK IFEKFDFMDNEKLAYIDGG-AGSGKTVYQGNGLYCNKVKCWVNWAET WTTIANNSVMNVLTGGNAGWHSGGAL	Ubericin A	4	YP_0029 969287	GG	*S. dysgalactiae* GGS_124
ZP_05831736	MKHCVILGILGTCLAGIGTGIDVDA-ATYYGNGLYCNKEKCWVNWGQS WSEGLKRWGDNLFGSFSGGR	Bacteriocin MC4-1	4	ZP_05831735	Sec	*Enterococcus faecium* E1162, C68 and 1,231,408
ZP_06674180	MKKKLVKGSVICSMIGIGFIVIGTNVEA-ATYYGNGVYCNKQKCWVNWGQA WSKGVKRWGDNLFGSFSGGRI	Bacteriocin MC4-1	4	ZP_06674181	Sec	*E. faecium* E1039 and 1,231,408
ZP_05665533	MILGIVLLSVSTLGITVDA-ATYYGNGVYCNTQKCWVDWSRA RSEIVDRGVKAYVNGFTKVLGGVGGR	Hiracin JM79	4	ZP_05665532	Sec	*E. faecium* TX1330, Com12 and 1,231,501

*Protein accession number.
†Amino acid sequence of the full-length bacteriocin. For each bacteriocin, the proposed cleavaage site between the leader peptide and the mature peptide is indicated with a hyphen.
‡Class IIa bacteriocin subgroup according to the classification by Nissen-Meyer *et al.* (2009).
§Type of leader sequence: double glycine type (GG) or general secretion leader (Sec).
‖Sequenced bacterial strain(s) whose genome contains the bacteriocin gene.
¶This bacteriocin gene is not annotated in the genome sequence of *S. equi* MGCS 10565.

However, the fact that each bacteriocin has a defined inhibitory spectrum strongly implies that they recognize specific receptors on target cells. In this context it is interesting to note that Campelo *et al.* (2011) found a link between sensitivity to the bacteriocin Lcn972 and uptake of cellobiose via the membrane-bound cellobiose-PTS protein CelB. We have also identified mutants that are resistant to different class II bacteriocins, which often have impaired metabolism of certain sugars, including lactose, arbutin and gentiobiose (unpublished data). These preliminary data suggest that targeting sugar uptake systems might be a general feature for class II bacteriocins, and it will be of great interest to reveal the nature of the receptors for these bacteriocins and learn how they differ from each other in their modes of receptor recognition. As receptors play a key role in defining inhibitory spectra for bacteriocins and also serve as potential drug targets, identification of these receptors and detailed understanding of the molecular mechanisms underlying the specific recognition by bacteriocins are crucial steps for successful design of strategies in combating food-spoiling bacteria and pathogens efficiently and safely without problematic resistance development.

In addition to the topics highlighted in this review, future studies should also focus on unravelling the biological role

of bacteriocins in nature. Bacteriocins have long been thought of as a useful means for bacteriocin producers to compete with other bacteria for nutrition and establish niches in diverse environments, and this is definitely true in many cases. However, the often extreme narrowness in target spectra of many class II bacteriocins suggests that they may have a role beyond killing. Several studies have shown the involvement of bacteriocins and bacteriocin-like peptides in for example competence development and fratricide in *Streptococcus* (Guiral *et al.*, 2005), quorum sensing (Diep *et al.*, 1995) and remodelling of sugar metabolism in lactic acid bacteria (Opsata *et al.*, 2010), suggesting that these peptides play a role in communication between cells. Another aspect worth mentioning here is that the production of class IIa bacteriocins has up to now only been observed in species containing genetic determinants encoding a group I Man-PTS (Fig. 1), the only group of Man-PTSs that is known to be targeted by class IIa bacteriocins (Kjos *et al.*, 2009). Whether the genetic link between these two apparently unrelated traits (sugar transport and bacteriocin production) is of any biological importance awaits further investigation. In general, more functional and ecological assessment of the roles of bacteriocins in complex environments (such as the mammalian gut) is probably a key approach to reveal their true biological roles in nature.

Acknowledgements

The authors would like to thank the Research Council of Norway for financial support.

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