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Review

Pediocin-like antimicrobial peptides (class IIa bacteriocins) and their immunity proteins: biosynthesis, structure, and mode of action¶

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Abstract: Pediocin-like antimicrobial peptides (AMPs) form a group of lactic acid bacteria produced, cationic membrane-permeabilizing peptides with 37 to 48 residues. Upon exposure to membrane-mimicking entities, their hydrophilic, cationic, and highly conserved *N*-terminal region forms a three-stranded antiparallel β-sheet supported by a conserved disulfide bridge. This *N*-terminal β-sheet region is followed by a central amphiphilic α-helix and this in most (if not all) of these peptides is followed by a rather extended *C*-terminal tail that folds back onto the central α-helix, thereby creating a hairpin-like structure in the *C*-terminal half. There is a flexible hinge between the β-sheet *N*-terminal region and the hairpin *C*-terminal region and one thus obtains two domains that may move relative to each other. The cationic *N*-terminal β-sheet domain mediates binding of the pediocin-like AMPs to the target-cell surface through electrostatic interactions, while the more hydrophobic and amphiphilic *C*-terminal hairpin domain penetrates into the hydrophobic part of the target-cell membrane, thereby mediating leakage through the membrane. The hinge provides the structural flexibility that enables the *C*-terminal hairpin domain to dip into the hydrophobic part of the membrane. Despite extensive sequence similarities, these AMPs differ markedly in their target-cell specificity, and results obtained with hybrid AMPs indicate that the membrane-penetrating hairpin-like *C*-terminal domain is the major specificity determinant.

Bacteria that produce pediocin-like AMPs also produce a 11-kDa cognate immunity protein that protects the producer. The immunity proteins are well-structured, 4-helix bundle cytosolic proteins. They show a high degree of specificity in that they largely recognize and confer immunity only to their cognate AMP and in some cases to a few AMPs that are closely related to their cognate AMP. The *C*-terminal half of the immunity proteins contains a domain that is involved in specific recognition of the *C*-terminal membrane-penetrating specificity-determining hairpin domain of the cognate AMP.

Keywords: bacteriocins; antimicrobial peptides; immunity proteins; pediocin-like bacteriocins; class IIa bacteriocins

INTRODUCTION

Ribosomally synthesized, membrane-permeabilizing, antimicrobial peptides (AMPs) are widely distributed in nature, being produced by bacteria, plants, and a wide variety of animals – both vertebrates and invertebrates [1–3]. In animals and plants, these peptides are an important defense against microorganisms. Although AMPs produced by animal and plants and those produced by bacteria certainly function in entirely different settings, the production of bacterial AMPs may also be thought of as a type of defense, since the peptides kill invading bacteria that compete with the AMP-producer for nutrients. The AMPs produced by bacteria seem overall to be more potent than the ones produced by eukaryotes, the former peptides being active at pico- to nanomolar concentrations and the latter at micromolar concentrations. Thus, structure–function analysis of AMPs produced by bacteria may especially be useful for elucidating how potent membrane-permeabilizing AMPs function at a molecular level.

One important and well-studied group of bacterial AMPs are the pediocin-like AMPs (often termed the pediocin-like, or class IIa, bacteriocins) produced by a variety of lactic acid bacteria [2,4–6]. The first of these AMPs to be identified and thoroughly characterized were leucocin A, [7], pediocin PA-1 (from which the term pediocin-like bacteriocins/AMPs has been derived) [8–11], sakacin P [12–14], curvacin A [12,15–17], and mesentericin Y105 [18–20]. Since the initial characterization of these peptides in the early nineties, the group has greatly expanded and includes now more than 20 different AMPs (Figure 1). These peptides display antilisteria activity and kill target cells by permeabilizing the cell membrane, thereby disrupting the proton motive force [27,40–44]. They cause a rapid depletion of the adenosine triphosphate (ATP) pool in

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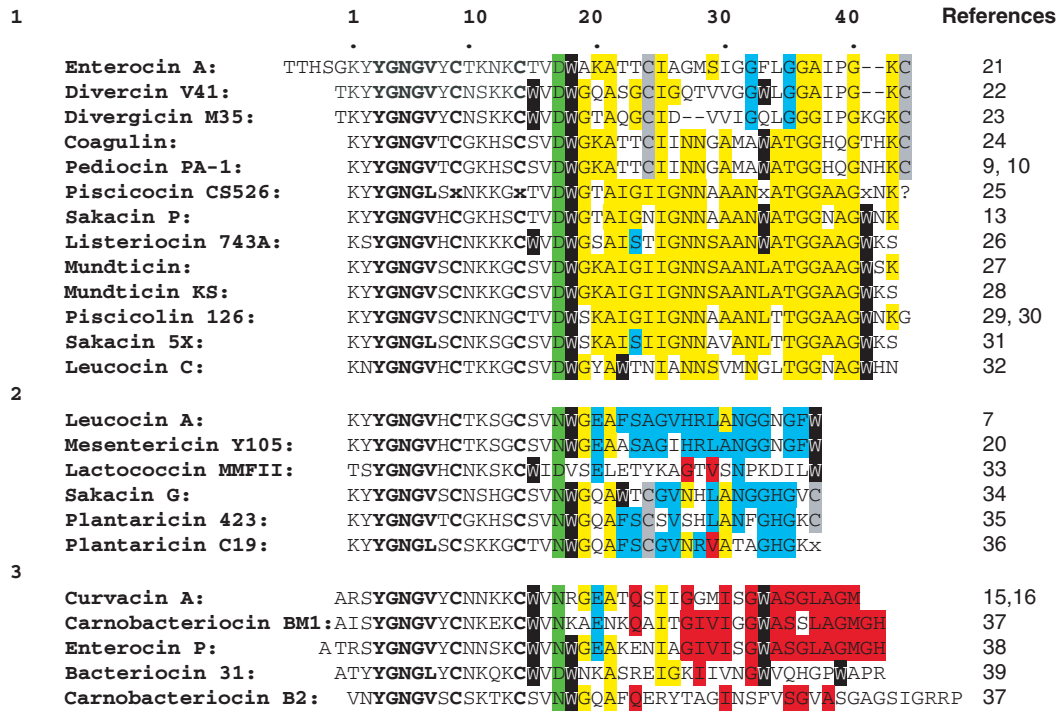

Figure 1 Multiple sequence alignment of pediocin-like AMPs highlighting the YGNGV/L 'pediocin box' motif (bold face) and conserved cysteine residues (bold face) in the *N*-terminal half, tryptophan residues (black boxes), and conserved residues in the *C*-terminal half of the peptides (yellow, blue, gray, green, and red boxes). The *C*-terminal half is more diverse than the *N*-terminal half, and the classification of the peptides into three subclasses is thus based on sequence similarities and differences in the *C*-terminal half of the peptides. There is a flexible hinge at the conserved Asp17 (green boxes) in peptides of subgroup 1, and presumably also at Asn17/Asp17 (green boxes) in peptides of subgroup 2 and 3. This hinge separates the β-sheet *N*-terminal domain and the hairpin-like *C*-terminal domain. Note that in numbering the residues (as indicated above the sequences), residue number 2 before the well-conserved YGNGV motif is in all cases referred to as residue 1, since this residue is the first residue in most – but not all – of the peptides. The PILEUP program of the Genetics Computer Group sequence analysis program package (Wisconsin Package Version 8.1, Genetic Computer Group) was used to set up the sequence alignments.

target cells, presumably due to ATP consumption connected to the cell's attempt to restore the proton motif force. As is the case for most membrane-permeabilizing AMPs, the pediocin-like AMPs are cationic and partly amphiphilic and/or hydrophobic (Figure 1). Their positive charge presumably facilitates interactions with the negatively charged bacterial phospholipid-containing membranes and/or acidic bacterial cell walls, whereas their amphiphilic/hydrophobic character enables membrane-permeabilization. All the pediocin-like AMPs contain between 37 and 48 residues, and in the *N*-terminal region they contain the conserved Y-G-*N*-G-V/L 'pediocin box' motif and two cysteine residues joined by a disulfide bridge (Figure 1). They have very similar primary structures, especially in the *N*-terminal half. They are somewhat more diverse in the *C*-terminal half and they may be grouped into three subgroups according to sequence similarities and differences in this half of the peptides (Figure 1). The peptides in subgroup 2 are clearly somewhat shorter – because of a shorter *C*-terminal half – than the peptides of subgroup 1 and 3, whereas the peptides in subgroup 3 lack the hairpin-stabilizing cysteine and/or tryptophan residues that are present at or near the *C*-terminal end in the peptides of subgroup 1 and 2 (Figure 1). Interestingly, despite similarities in primary structures, the pediocin-like AMPs differ markedly in their target-cell specificity [45–49]. They have been shown to be active – but often to various degrees – against various *Lactobacillus, Pediococcus, Enterococcus, Carnobacterium, Leuconostoc, Lactococcus, Clostridium* and *Listeria strains* (46).

It is often observed that the specificity of similar AMPs and the susceptibility of similar target cells for a given AMP vary much more than one might expect simply on the basis of an interaction between a cationic amphiphilic peptide and the lipids of a cell membrane. Subtle structural differences in peptides may lead to marked differences in specificity, and subtle differences in target cells may lead to marked differences in their susceptibility to a peptide. Elucidation of what governs the specificity of peptides and the susceptibility of target cells is of great importance for future use of peptides as antimicrobial agents. The difference in the target-cell specificity of the pediocin-like AMPs combined with their extensive sequence similarities

make these peptides especially well suited for analyzing the relationship between structure and target-cell specificity. In the following, we will review some recent results on structure–function analysis of the pediocin-like AMPs that reveal interesting aspects about their mode of action.

Biosynthesis and Secretion of Pediocin-Like AMPs

At least four genes found in close proximity (usually organized in one or two operons) are required for production of the pediocin-like AMPs [5,9,14,17,50,51]: (i) the structural gene encoding the AMP precursor, (ii) an immunity gene encoding the immunity protein that protects the AMP-producer from being killed by its own AMP, (iii) a gene encoding a membrane-associated ABC transporter that transfers the AMP across the membrane concomitantly with removal of the leader sequence, and (iv) a gene encoding an assessory protein whose function has not been entirely clarified, although it appears to be necessary for secretion of the AMP. For a more detailed description of the organization of gene clusters involved in production of various pediocin-like AMPs, Ref. 4 is recommended.

The precursor of the pediocin-like AMPs contains a *N*-terminal leader sequence that presumably facilitates interaction with the transporter and/or keeps the AMP inactive until it has been secreted from the cell. The leader sequence contains 15 to 30 residues and a consensus sequence, and is most often of the double-glycine type that is cleaved off at the *C*-terminal side of two glycine residues [5,52,53]. Three pediocin-like AMPs (listeriocin 743 A, bacteriocin 31 and enterocin P), however, have a *sec*-type instead of a double-glycine type leader sequence, and they are presumably secreted by the *sec*-dependent translocation system [26,38,39].

The production of some pediocin-like AMPs (such as curvacin A, sakacin P, carnobacteriocin B2, and enterocin A) is transcriptionally regulated through a three component signal transduction system consisting of an induction factor, a membrane-associated histidine protein kinase, and a response regulator [54,55]. The induction factor is a peptide pheromone, which, upon secretion from bacteria, interacts with the membrane-associated histidine kinase, thereby triggering the kinase to phosphorylate the intracellular response regulator, thus enabling the response regulators to activate the genes needed for AMP-production [54,55].

The Structure of Pediocin-Like AMPs and their Orientation in Membranes

Based on their primary structures (Figure 1), the peptide chains of pediocin-like AMPs may roughly be divided into two regions: (i) a hydrophilic, cationic and highly conserved *N*-terminal region that contains the 'pediocin box' motif and (ii) a less conserved hydrophobic/amphiphilic *C*-terminal region [45]. Circular dichroism (CD) spectroscopy reveals that the peptides are unstructured in water, but become structured upon contact with membrane-mimicking entities [20,56–58]. Nuclear magnetic resonance (NMR) structural studies of leucocin A [56], carnobacteriocin B2 [59], sakacin P [58] and a mutant of sakacin P [58] in the presence of membrane-mimicking entities have shown that the *N*-terminal region forms a three-stranded antiparallel β-sheet-like structure supported by a conserved disulfide bridge (the β-sheet was not evident in carnobacteriocin B2 [59]). This *N*-terminal β-sheet region is followed by a central amphiphilic α-helix and this in turn by a rather extended *C*-terminal tail that folds back onto the central α-helix, thereby creating a hairpin-like structure in the *C*-terminal half (Figure 2) [56,58,59]. There is a flexible hinge (at the conserved Asp17 in subgroup 1 AMPs, Figure 1) between the β-sheet *N*-terminal region and the hairpin-like *C*-terminal region and one thus obtains two domains that may move relative to each other [58]. Site-directed

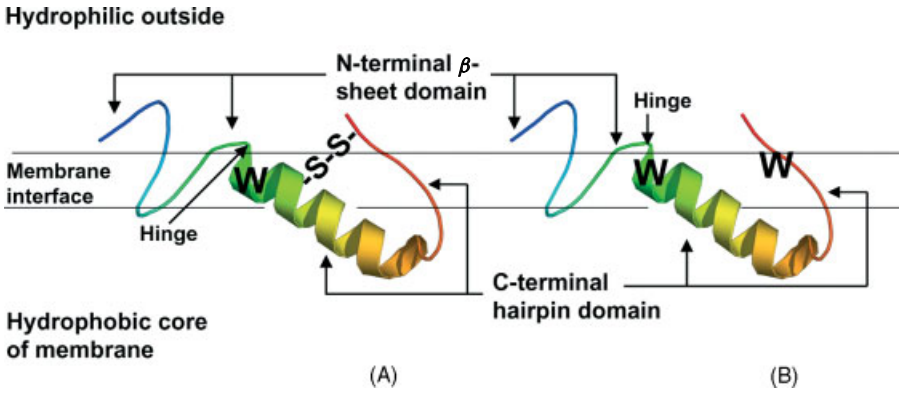

Figure 2 A cartoon depiction of the structure and orientation in membranes of pediocin-like AMPs. A pediocin-like AMP, in which the *C*-terminal hairpin structure is stabilized by (A), a disulfide bridge and (B), an interface-localized tryptophan residue near the *C*-terminal end of the AMP. Tryptophan residues that become localized in the membrane-water interface are indicated by W and the disulfide bridge by –S–S–.

in vitro mutagenesis and peptide-binding studies indicate that the cationic *N*-terminal β-sheet domain mediates binding of the pediocin-like AMPs to the target-cell surface through electrostatic interactions [60,61], whereas the more hydrophobic and amphiphilic *C*-terminal hairpin domain penetrates into the hydrophobic part of the target-cell membrane, thereby mediating leakage through the membrane [45,49,62,63]. The hinge apparently provides the structural flexibility that enables the *C*-terminal hairpin domain to dip into the hydrophobic part of the membrane [58]. The well-conserved central tryptophan residue (at position 18 in most of the peptides; Figure 1) positions itself in the water–membrane interface (as is common for tryptophan residues in membrane-penetrating polypeptides [64]) and thereby helps position the *C*-terminal hairpin domain correctly in the membrane (Figure 2).

The hairpin structure is, in some pediocin-like AMPs (such as enterocin A, divercin V41, coagulin and pediocin PA-1, see Figure 1), stabilized by a disulfide bridge between a *C*-terminal cysteine residue and a cysteine residue in the middle of the α-helix (Figure 2A). Most of the pediocin-like AMPs, however, lack these two cysteine residues, but instead contain a tryptophan residue near the *C*-terminal end of the peptide (exceptions being the subgroup 3 AMPs; Figure 1). Site-directed, *in vitro* mutagenesis have shown that this tryptophan residue also positions itself in the membrane–water interface, thereby stabilizing the hairpin structure of the peptides that lack the structure-stabilizing disulfide bridge in the *C*-terminal domain (Figure 2B) [49]. Consistent with this hairpin structure model is the observation that insertion of a hairpin-stabilizing disulfide bridge into a pediocin-like AMP (sakacin P) that lacks this bridge does not have a detrimental effect on the potency of the peptide, but rather renders it more thermostable (i.e. it functions at higher temperatures) [47].

It should be noted that the amino acid sequences of the *C*-terminal part of the pediocin-like AMPs in subgroup 3 are somewhat different from the corresponding sequences of the AMPs of subgroup 1 and 2, in that most of the subgroup 3 peptides (bacteriocin 31 being an exception) lack both the hairpin-stabilizing disulfide bridge and the well-conserved tryptophan residue near the *C*-terminal end (Figure 1). Consequently, it is not entirely clear whether the subgroup 3 AMPs, in fact, form a hairpin-like structure in their *C*-terminal domain, even though they do have a well-conserved tryptophan residue in the middle of the *C*-terminal half that might stabilize such a structure if it positions itself in the membrane interface along with the conserved tryptophan residue found at positions 16, 17, or 18 (depending on the peptide; Figure 1).

The *C*-Terminal Hairpin Domain That Interacts with the Hydrophobic Part of the Cell Membrane is Important in Determining the Target-Cell Specificity

The structure of the pediocin-like AMPs described earlier implies that the two domains may to some extent function independently of each other. This is consistent with results showing that potent hybrid AMPs may be constructed by joining *N*- and *C*-terminal domains from different pediocin-like AMPs, using the hinge region as the recombination point [65]. The active hybrid peptides have target-cell specificities similar to the peptide from which the *C*-terminal domain is derived, indicating that the membrane-penetrating hairpin-like *C*-terminal domain is the major specificity determinant in the pediocin-like AMPs [65]. This conclusion is consistent with results showing that pediocin-like AMPs that have been altered in the *C*-terminal region by use of site-directed *in vitro* mutagenesis often differ from the wild-type peptide in their target-cell specificity [47,49]. An important specificity-determining step thus apparently involves interactions with lipids and/or proteins in the interphase and/or hydrophobic phase of the cell membrane [65]. One protein that might be involved in such an interaction is the membrane-bound mannose phosphotransferase system permease [66–71]. This protein must apparently be expressed in order for cells to be sensitive to pediocin-like AMPs [66,68–71]. Comparative two-dimensional gel analysis revealed that leucocin A-resistant mutants derived from leucocin A-sensitive listerial strains all lacked the MptA subunit of this protein [69], whereas heterologous expression of the MptC subunit of the mannose phosphotransferase system permease in an insensitive strain of *Lactococcus lactis* rendered the strain sensitive to several pediocin-like AMPs [71]. It has consequently been suggested that one of the permease subunits (MptC) functions as a receptor or docking site for pediocin-like AMPs [71]. Interestingly, 15mer fragments starting from the central hinge region and going towards the *C*-terminal end inhibit pediocin-like AMPs in a specific manner [72,73], suggesting that this region of the *C*-terminal hairpin domain might interact with a receptor.

One might have expected the *N*-terminal domain that interacts with the cell surface to be a major specificity determinant, since the affinity of an AMP to the cell surface will influence the cell's sensitivity to the AMP. Target cells do not, however, seem to discriminate between the *N*-terminal domains of the different pediocin-like AMPs, presumably because the *N*-terminal domains have very similar primary structures (Figure 1).

Immunity Proteins Render Cells Immune to Pediocin-Like AMPs

Bacteria that produce pediocin-like AMPs also produce cognate immunity proteins that protect bacteria from

Figure 3 Sequence alignment and grouping of immunity proteins of pediocin-like AMPs. Black and grey boxes indicate regions of sequence similarity. The following amino acids were considered similar: D and E; F and Y; I, V and L; N and Q; K and R; S and T. Subgroup A consists of the immunity proteins for the following pediocin-like AMPs (with abbreviated name of the immunity proteins in parenthesis): leucocin A (leuA-im), mesentericin Y105 (mesY-im), divercin V41 (divI-im), enterocin A (entA-im), an orphan immunity protein with no corresponding AMP (orfY-im), pediocin PA-1 and coagulin (ped-im). Subgroup B consists of the immunity proteins for the following pediocin-like AMPs (with abbreviated name of the immunity proteins in parenthesis): two orphan immunity proteins with no corresponding AMPs (orfβ3-im and orf285-im), piscicolin 126 (pisc-im), sakacin 5X and P (sakX-im and sakP-im), mundticin KS (munKS-im), enterocin CRL35 (entCL-im), listeriocin 743A (lisA-im), and an orphan immunity protein with no corresponding AMP (divT2-im). Subgroup C consists of the immunity proteins for the following pediocin-like AMPs (with abbreviated name of the immunity proteins in parenthesis): carnobacteriocin B1 (cbm1-im), curvacin A (curA-im), enterocin P (entP-im), bacteriocin31 (bac31-im), and carnobacteriocin B2 (cbnB2-im). The PILEUP program mentioned in legend to Figure 1 was used to set up the sequence alignments.

being killed by their own AMPs [14,17,50,51,65,74–77]. The functionality of the immunity proteins was demonstrated by showing that heterologous expression of their genes in sensitive bacteria strains rendered the strains less sensitive to pediocin-like AMPs. Presently, the primary structures of at least 20 immunity proteins for pediocin-like AMPs have been deduced from DNA sequences (Figure 3). They are well-structured α-helical proteins that consist of between 88 and 115 amino acid residues and display 5 to 85% sequence similarities [77,83–85]. They show a high degree of specificity in that they largely recognize and confer resistance only to their cognate pediocin-like AMP and in some cases to a few AMPs that are closely related to the cognate AMP [65,76,77].

The gene encoding an immunity protein is generally located close to and often on the same operon as the gene encoding the cognate pediocin-like AMP. Expression of the two genes is consequently often coregulated, and bacteria may thus be sensitive to their own AMP when in a nonproducing state [46]. Orphan immunity genes/proteins have, however, also been identified [14,22,37,76]. These orphan immunity proteins are not directly associated with a particular pediocin-like AMP and they may render bacteria that do not produce AMPs resistant to some pediocin-like AMPs.

The mode of action of the immunity proteins has not been elucidated, although it has been suggested that they might act by interfering with the interaction between pediocin-like AMPs and a (putative) membrane-located receptor [74,83]. The immunity proteins for pediocin-like AMPs are located intracellularly, a small proportion (about 1%) possibly being associated with the cell membrane [74,75]. The recently reported NMR solution structure of the immunity protein for carnobacteriocin B2 (cbnB2-im; see Figure 3 for its primary structure) has revealed that the protein consists of an antiparallel four-helix bundle (helix 1–4) with the *C*-terminal region (containing a fifth helix and an extended strand) being packed approximately in a perpendicular manner across helix 3 and 4 [83]. We have recently obtained the crystal structure of the enterocin A immunity protein (entA-im; see Figure 3 for its primary structure), which was also shown to contain an antiparallel four-helix bundle (helix 1–4) with a flexible *C*-terminal tail [85] (Figure 4). Structural modeling based on sequence similarities indicates that other immunity proteins for pediocin-like AMPs (leuA-im, divI-im, and mesY-im) have similar three-dimensional structures [85].

Using hybrid immunity proteins and hybrid pediocin-like AMPs, it has been demonstrated that the *C*-terminal half of the immunity proteins contains a region that specifically recognizes the *C*-terminal hairpin domain of the cognate AMP [65]. It has, however, not been possible to demonstrate direct physical contact between immunity proteins and AMPs [83]. The fact that there is some strain-dependent variation in

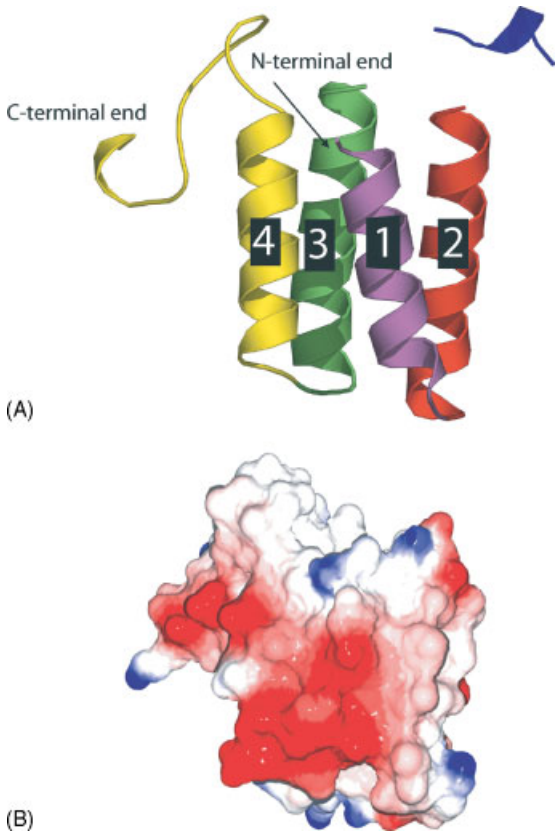

Figure 4 The three-dimensional structure of the immunity protein (entA-im) for enterocin A (A) shown as a ribbon diagram (the helices are numbered from 1 to 4 starting from the *N*-terminal end) and (B) showing the molecular surface and surface charge distribution (red indicates negative charge and blue positive charge). The loop structure shown in blue (upper right-hand corner) is a ribbon diagram of the 7-residue loop that connects helix 2 and 3 (see below). The structure shown in B is in the same orientation as that shown in A. The structure of the immunity protein in two crystals with different space groups was determined [85]. In one of the crystals, there was one molecule per asymmetric unit and the structure of this molecule was determined to 2.2 Å resolution, whereas in the other crystal there were three molecules per asymmetric unit and the structure of each of these molecules was determined to 1.6 Å resolution. The four molecules had identical structures except that the *C*-terminal loop that extends from helix 4 appears to be flexible and its structure was thus only well defined in one of the four molecules (the one shown in this figure). In this molecule, however, the loop connecting helix 2 and 3 (from and including residue 40 to and including residue 46) apparently fluctuated and could not be determined, and it is, consequently, not included in the structure shown in the figure. This 7-residue loop structure was well defined and was identical in the three other molecules. The 7-residue loop contained a one-turn α-helix in the middle and it is illustrated in a ribbon diagram in blue in the upper right-hand corner (see Ref. 85 for more details).

immunity protein functionality [76,77] suggests that the immunity proteins may interact indirectly with the AMPs via cell components that vary somewhat between strains. This cell component could, for instance, be an AMP receptor. The immunity proteins could then act by binding to the cytoplasmic side of the receptor and thereby block the receptor's ability to interact with the AMP. Such a mechanism of action necessitates, however, that the strain-dependent variation in the receptor is large enough to cause sufficient variations in the receptor-binding of (i) the various pediocin-like AMPs (in order to account for the different target-cell specificities of the pediocin-like AMPs), and/or (ii) the various immunity proteins (in order to account for specificity of immunity proteins for their cognate AMP).

Although the pediocin-like AMPs are among the best-characterized AMPs produced by lactic acid bacteria, there are still many aspects concerning these AMPs that need further elucidation. The peptides of subgroup 3 differ conceptually somewhat in their primary structures from the peptides of subgroup 1 and 2. It is thus unclear to what extent the three-dimensional structure discussed in this review also describes the structure of the AMPs of subgroup 3. It is also unclear to what extent the three-dimensional structure that has been determined for some of the immunity proteins may be generalized and thus describes the structure for all the pediocin-like immunity proteins. It remains to be clarified how the immunity proteins (directly or indirectly) specifically interact with the pediocin-like AMPs and thereby render cells immune, and we lack detailed understanding of how the AMPs bind to and permeabilize the target-cell membrane. Further research on the pediocin-like AMPs and their immunity proteins will clarify these questions, and the answers obtained should reveal some general mechanistic aspects relevant also to other cationic membrane-permeabilizing AMPs.

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