

BACTERIOCINS FROM *LACTOBACILLUS PLANTARUM* – PRODUCTION, GENETIC ORGANIZATION AND MODE OF ACTION

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ABSTRACT

Bacteriocins are biologically active proteins or protein complexes that display a bactericidal mode of action towards usually closely related species. Numerous strains of bacteriocin producing *Lactobacillus plantarum* have been isolated in the last two decades from different ecological niches including meat, fish, fruits, vegetables, and milk and cereal products. Several of these plantaricins have been characterized and the amino acid sequence determined. Different aspects of the mode of action, fermentation optimization and genetic organization of the bacteriocin operon have been studied. However, numerous of bacteriocins produced by different *Lactobacillus plantarum* strains have not been fully characterized. In this article, a brief overview of the classification, genetics, characterization, including mode of action and production optimization for bacteriocins from Lactic Acid Bacteria in general, and where appropriate, with focus on bacteriocins produced by *Lactobacillus plantarum*, is presented.

Key words: *Lactobacillus plantarum*, plantaricin, mode of action, genetic organization

INTRODUCTION

Numerous strains of bacteriocin producing *Lactobacillus plantarum* have been isolated in the last two decades and have also been reviewed by Olasupo (66). Most of these bacteriocins have not been fully characterized. Bacteriocins are biologically active proteins or protein complexes that display a bactericidal mode of action towards usually closely related species (16). Bacteriocins produced by most of the genera of lactic acid bacteria have been reported. In this article, a brief overview of the classification, genetics, characterization, including mode of action and production optimization for bacteriocins from Lactic Acid Bacteria (LAB) in general, and where appropriate, with focus on bacteriocins produced by *Lactobacillus plantarum*, is presented.

CLASSIFICATION OF BACTERIOCINS

Klaenhammer (47) originally defined four distinct classes of lactic acid bacteria bacteriocins. This classification has been

revised and currently bacteriocins are classified as follows (57,62).

Class I: Lantibiotics

Lantibiotics, are divided into type A lantibiotics and type B lantibiotics. Type A lantibiotics are elongated, cationic, pore forming peptides. Type B lantibiotics are compact, with globular structures, are enzyme inhibitors and are immunologically active (16). Lantibiotics (*lanthionine*-containing *antibiotic* peptides) are small (less than 5 kDa, with 19 to 38 amino acids) membrane-active peptides that contain unusual, posttranslationally modified amino acids such as lanthionine (Lan), β -methyl lanthionine (MeLan) and dehydrated residues (47,81). Posttranslational peptide modification usually involves only the amino acids serine, threonine and cysteine, although lysine, aspartate and isoleucine residues may also be found in modified form (81). All lantibiotics currently documented are produced by Gram-positive bacteria (61). The gene cluster encoding lantibiotic peptides usually also contain a gene or genes that encode specific enzymes able to facilitate the dehydration of certain residues in

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the propeptide region, followed by the addition of cysteine residues to form characteristic Lan and MeLan sulfur ring structures (61). Considerable differences in the leader peptide sequence of type A lantibiotics have been observed.

Class II: Small heat-stable bacteriocins

Class II contains small heat-stable non-lanthionine peptides, and is divided into four groups: Class IIa consists of *Listeria*-active peptides with an N-terminal consensus sequence. Class IIb are two-peptide bacteriocins. Class IIc contains *sec*-dependent bacteriocins, and Class IId contains the small heat-stable non-lanthionine bacteriocins that do not belong to any of the three groups within Class II.

These bacteriocins can be defined as small (less than 10 kDa), do not contain any unusual amino acids, are membrane-active and heat resistant up to temperatures of 100°C, or autoclavable. Most of these bacteriocins are characterized by the so-called double glycine (G-G) processing site in the bacteriocin precursor (47). The bacteriocins of Class II share various features, such as the occurrence of a high content of small amino acids such as glycine, being strongly cationic with pI's between 8 and 11, and the possession of hydrophobic and amphiphilic domains (1).

Class IIa: *Listeria*-active bacteriocins

Members of this group, also referred to as pediocin-like bacteriocins, are produced by a wide variety of lactic acid bacteria, and several have been biochemically characterized. Although the antimicrobial spectrum of these bacteriocins is different, they are all active against *Listeria* spp. and share a conserved amino acid sequence, YGNGV, in their structure. The function of the YGNGV consensus motif is not clear, since the mechanism for initial binding of the bacteriocins to the target membranes involves electrostatic interactions between positive amino acid residue groups and negatively charged membrane phospholipid groups, without involvement of the YGNGV motif (11). Pediocin PA-1 is the most characterized bacteriocin within this group (53).

Class IIb: Two-peptide complexes

The activity of these bacteriocins depends on the complementary activity of two peptides. Examples of plantaricins with this type of activity include plantaricin S a and b (42,85), plantaricin J and K (3,19) and plantaricin E and F (3,19). Some two-peptide bacteriocins need both peptides for activity, while one or both peptides of plantaricin S are active. The combined effect of the two peptides of these bacteriocins is much greater than the total activity calculated from the individual effect of these peptides (14).

Class IIc: The *sec*-dependent bacteriocins

Some bacteriocins do not possess a double-glycine leader peptide, but are synthesized with a *sec*-type N-terminal leader

sequence, leading to secretion and processing via the *sec* pathway (62). No plantaricins that belong to this class have been described to our knowledge.

Class IId: Unclassified small heat-stable non-lanthionine bacteriocins

Bacteriocins that do not meet the criteria of the previous sections within the first three Class II bacteriocin classes are included in Class IId.

Class III: Large heat-labile bacteriocins

Class III consists of large heat labile bacteriocins (57). These bacteriocins are more than 30 kDa in size. The bacteriocin helveticin J is representative of this group. The operon of the bacteriocin has been cloned, and expressed in *Lactobacillus acidophilus* (33).

Most of the bacteriocins belong to Class I or Class II. Research has focused on these two classes, since they are the most abundant and have the best potential for industrial application (62). Although numerous bacteriocins produced by *Lactobacillus plantarum* have been recorded, most cannot be classified for a lack of sufficient information. The majority of these bacteriocins are small heat-stable cationic peptides and therefore the characterization of bacteriocins described in the following chapters will focus on Class I and Class II bacteriocins.

GENETICS OF BACTERIOCINS

Gene location

Bacteriocins may be either chromosomally or plasmid-encoded. Plantaricin 423 (108) is plasmid encoded, while plantaricin ST31 (88) is chromosomally determined. Plasmids associated with bacteriocin production vary considerably in size. Some plasmids are known to carry the genetic determinants for several bacteriocins (40). Where more than one bacteriocin is produced, the bacteriocins can be plasmid (carnobacteriocin B2) and chromosomally (carnobacteriocin BM1) encoded (74).

Genetic organization of Class II bacteriocins

The general genetic structure leading to synthesis of cationic bacteriocins usually encompasses four genes that encode the functions required for production of extracellular antibacterial activity (62). The organization of the gene clusters of Class II bacteriocins, such as plantaricin A (18,19,20), have been studied. Genes that encode Class II bacteriocin production are usually found organized within operon clusters (62,69,81) and usually consist of a structural gene (two genes for the two-peptide bacteriocins) encoding the prepeptide (75), a dedicated immunity gene (19,24), an ABC-transporter gene for transport across the membrane, and a gene encoding an accessory protein needed for export of the bacteriocin (62). In some cases the presence of regulatory genes has been reported (62).

Most class II bacteriocins are synthesized as a biological inactive prepeptide carrying an N-terminal leader peptide and a distinctive double-glycine proteolytic processing site. However, Class IIc bacteriocins differ, because they have a *sec*-type N-terminal signal sequence and are processed and secreted by the general secretory pathway (49,116).

Pediocin PA-/AcH is one of the most extensively studied class II bacteriocin (69). Other plantaricins that have been characterized at a genetic level include plantaricin 423 and those produced by *Lactobacillus plantarum* C11. The plantaricin 423 encoding region on plasmid pPLA4 has a very similar operon structure to pediocin PA-1 (110), with four ORF's (*plaABCD*) encoding the structural gene, immunity gene, accessory protein and ABC transporter. The *plaC* and *plaD* genes of plantaricin 423 are 99% homologous to *pedC* and *pedD* of pediocin PA-1.

L. plantarum C11 produces six bacteriocin-like structures, PlnA, PlnE, PlnF, PlnJ, PlnK, and PlnN. The genes encoding these bacteriocin-like structures, are arranged in five operons, *plnABCD*, *plnEFI*, *plnJKLR*, *plnMNOP* and *plnGHSTUV*. *plnEF* and *plnJK* encode two two-peptide bacteriocins. *plnI*, *plnL*, *plnM* and *plnP* probably encode immunity proteins. *plnB*, *plnC*, and *plnD* encode proteins involved in signal transduction, and *plnG* and *plnH* encode secretion and processing proteins. No function could be determined for the protein encoded by *plnN* (3).

Class II bacteriocins, such as plantaricins of *L. plantarum* C11, also produce an induction factor (a bacteriocin-like peptide exerting no antimicrobial activity) that activate transcription of the regulated genes. The induction factor forms part of the signal transduction system responsible for biosynthesis of class II bacteriocins. This signal transduction system consists of three components, i.e. an induction factor (IF), a histidine protein kinase and a cytoplasmic response regulator (62). To regulate biosynthesis, a prebacteriocin and a bacteriocin-like prepeptide of an induction factor are produced. The induction factor is synthesized as a prepeptide with a double-glycine leader sequence that ultimately undergoes cleavage by a dedicated ABC-transporter. Cleavage of the leader peptide of IF by the ABC-transporter coincides with externalization of the mature peptide from the cell. Following release via the ABC-transporter, the bacteriocin and IF are sensed by the membrane-bound histidine kinase. This leads to autophosphorylation and subsequent transfer of the histidine residue in the extracellular domain to a conserved aspartic acid of the response regulator. This interaction triggers the response regulator to activate transcription of the genes responsible for bacteriocin production (26,62).

The structural prebacteriocin gene

The structural gene encodes a prebacteriocin, called a precursor or prepeptide. These prepeptides contain an N-terminal leader sequence and a C-terminal prepeptide which is

cleaved from the N-terminal leader sequence to form a mature, antimicrobial peptide (40,48). All Class II bacteriocins are produced as precursors with an N-terminal extension (105). Most of the leader peptides differ from typical signal secretion peptides that direct polypeptides into *sec*-dependent secretion pathways (40).

The function of leader peptides appears to be the prevention of biological activity of the bacteriocin while still in the producer cell, and to provide a recognition signal for the ABC transporter (48,62). Leader peptides may prevent activity of prebacteriocins by increasing the solubility of prebacteriocins in water, causing the peptides to partition into the aqueous phase rather than into the membrane. Leader peptides may also interact with mature peptides and thus reduce their affinity for membranes. Ray *et al.* (76) found the precursor of pediocin AcH to be 80% as active as the mature peptide, suggesting that the leader peptide has little effect on the function of mature domains. This indicates that producer cells with active prebacteriocins need other mechanisms to protect themselves from the prebacteriocin, such as the limitation of prebacteriocins to bind successfully to the putative receptor, limited membrane insertion activity due to the reverse orientation of the membrane electrochemical potential inside the cell, and neutralization of the prebacteriocin by immunity proteins. Since some bacteriocins require disulfide bonds for activity, the cysteinyl thiol groups may be maintained in a reduced state, resulting in inactivity of the prebacteriocin. In this case, the question arises why it is necessary for the leader peptide to be cleaved during secretion. A possible explanation is that the prebacteriocin is more susceptible to proteases produced by target cells (76).

The N-terminal leader peptides of Class IIa bacteriocins are referred to as double-glycine leader peptides. These peptides have two glycine residues at the C-terminus before the cleavage site. Other consensus elements include conserved hydrophobic and hydrophilic regions. The minimum length of the leader peptide of non-lanthionine bacteriocins appears to be 14 amino acids, while the length of the mature bacteriocins identified to date varied from 30 to more than 100 residues (62).

The immunity gene

The immunity gene encodes a protein that protects the producer organism from its own mature bacteriocin (62). Potential immunity proteins have been identified next to or downstream from, all bacteriocin structural genes studied. Immunity genes not directly associated with the bacteriocin cluster have also been identified (22). Variation in the presence and expression of these genes may account for the large variation in sensitivity displayed by lactic acid bacteria towards bacteriocins. Immunity proteins range in sizes from 51 to 150 amino acids. While significant homology exists among the structural genes of the *Listeria* active bacteriocins, this trend does not occur with immunity genes, although some

resemblances do occur (6,57). A close genetic proximity exists between immunity genes and bacteriocin structural and processing genes (83). In the case of Class II bacteriocins, one gene generally encodes for the immunity protein. The mechanisms involved in immunity is poorly understood, but it has been suggested that interaction between the immunity protein and another protein, perhaps a receptor, located at the cytoplasmic side of the cell membrane of the producer, protects it against the action of the bacteriocin (25,62,84).

Several immunity proteins have been identified such as PedB encoded by *pedB* of the pediocin PA-1 operon in *P. acidilactici* (111), LcaB, the immunity protein of leucocin A (105) and PlaB, the immunity protein of plantaricin 423 (109). Although plantaricin 423 shares high sequence similarity with the pediocin PA-1 operon, no cross-reactivity was recorded between the immunity proteins of plantaricin 423 and pediocin PA-1 (109).

The transporter gene

Bacteriocins, similar to other molecules synthesized in the cytoplasm of bacteria and secreted, need to cross one or more membranes to reach their destination. This transport is facilitated via the general *sec* signal secretion pathway, or by using a dedicated export system (27,115).

Bacteriocins containing the double-glycine type leader sequences (G-G) are translocated by a dedicated export system identified as ABC (ATP-binding cassette) transporters (27,62). The gene encoding the bacteriocin ABC transporter is usually part of the bacteriocin operon, or can be found on an operon near the vicinity of the bacteriocin operon (62). ABC transporters facilitate the secretion of a wide range of products in both prokaryotic and eukaryotic organisms. These products include periplasmic permeases (bacterial importers), which transport oligopeptides, amino acids, sugars, phosphate, metal ions and vitamins, eukaryotic exporters, which transport lipophilic drugs, peptides and pigments, and bacterial exporters, which transport molecules such as large protein toxins, small peptide antibiotics, polysaccharides, antibiotics, and possibly heme molecules (27).

The bacteriocin ABC transporters have a dual function, facilitating both the removal of the leader peptide from its substrate and the transport of the substrate across the cytoplasmic membrane (37). Bacteriocin ABC-transporters contain three domains on the same polypeptide, consisting of a cytoplasmic N-terminal proteolytic domain, a hydrophobic integral membrane domain, and a cytoplasmic C-terminal ATP-binding domain (37,62). Two polypeptides appear to be required for the bacteriocin ABC transporter to be functional (37).

A unique feature of bacteriocin ABC transporters is that they carry an N-terminal extension of approximately 150 amino acids, the proteolytic domain, which appears to be involved in the processing of the bacteriocins (62). Two conserved motifs,

the cysteine motif (QX4D/ECX2AX3MX4Y/FGx4I/L) and the histidine motif (HY/FY/VVX10I/LXDP) have been identified in the proteolytic domain and appear to be necessary for translocation (37). Håvarstein *et al.* (37) hypothesized that the proteolytic domain of the double-glycine leader bacteriocins binds the bacteriocin precursor. The processing site is part of the transporter, which indicates that the processes of cleavage and translocation are integrated, and that the leader peptide serves as a recognition signal for the transmembrane transport process of the bacteriocin (62,107).

The membrane spanning domains (MSD's) consist of six membrane spanning segments. ABC transporters (or traffic ATPases) use ATP hydrolysis as a source of energy required for translocation and have a highly conserved ATP-binding cassette (27). The conserved ATP-binding motif can be found in all the ABC transporters of the bacterial export subfamily.

The removal of the leader peptide from its substrate and the subsequent translocation of the bacteriocin across the cytoplasmic membrane effectively prevent the mature and active bacteriocins from remaining in the cytoplasm (37).

The Class IIc bacteriocins are synthesized with a *sec*-type N-terminal leader sequence, and processed and secreted via the *sec* pathway (62).

The accessory protein

Several studies have indicated the presence of an additional gene within bacteriocin operons, called the accessory protein (also accessory factor), that is required for the ABC-transporter dependent translocation process. These additional factors have been identified in several Gram-negative systems to be needed when the secreted product is destined for immediate release into the extracellular medium (27). It is hypothesized that the accessory factor is anchored in the inner membrane and spans the periplasm, probably connecting the inner and outer membranes to facilitate the export of products through both membranes of Gram-negative bacteria. In Gram-positive bacteria, the function of the accessory factor is unclear, since the secreted product only needs to cross one membrane (27,62).

BACTERIOCIN CHARACTERIZATION

Bacteriocins differ widely in molecular weight, pI, and presence and number of particular groups of amino acids, although differences in antimicrobial activity can not be attributed to particular amino acids or sequence of amino acids (40). Most of the low molecular weight bacteriocins are cationic at pH 7, and many of these bacteriocins have greater antimicrobial activity at low pH. Adsorption of bacteriocins to Gram-positive cell surfaces is also pH dependent, with maximum adsorption at or above pH 6 (40). Characterization of bacteriocins include studies such as the spectrum of activity, mode of action, effect of heat, pH, proteolytic enzymes, salt

and detergents on bacteriocin activity, determination of molecular mass, amino-acid composition and sequence, determination of the genetic organization of the bacteriocin production and secretion.

MODE OF ACTION

Class II bacteriocins

The Class II bacteriocins demonstrate a bactericidal mode of action against other closely related organisms. These bacteriocins dissipate the proton motive force by disrupting the transmembrane potential and/or the pH gradient of sensitive cells (59). Some bacteriocins permit the efflux of relatively large molecules (13,51,106). Two-peptide bacteriocins appear to form relatively specific pores, dissipating the transmembrane potential (56). Plantaricin E/F dissipates the pH gradient, causing a pH increase (57).

It is proposed that bacteriocin mediated transmembrane ion flow results in cytotoxic effects, causing a drop in the intracellular pH and inhibiting enzymatic processes. An influx of cytotoxic sodium ions and a depletion of ATP due to futile cycles are caused by ion gradient dissipation. Dissipation of the proton motive force and the transmembrane potential arrest processes dependent on these gradients (9,57).

Bacteriocins form pores in the membranes of target cells (1,2). It is hypothesized that the mode of action involves various steps such as binding, insertion and pore formation (60). Binding of the bacteriocin to the target membrane is necessary for subsequent insertion and pore formation. Although the interaction of a receptor-like factor has been implicated for some bacteriocins (13,32), a protein receptor does not appear to be essential for binding. Chen *et al.* (12) suggested that the binding step primarily involved electrostatic interactions between positive areas of amino acid groups in the bacteriocin and negatively charged phospholipid groups in the target membrane. Jack *et al.* (40) also implied that anionic cell surface molecules in the cell wall of Gram-positive bacteria might play a role in the initial interaction with cationic bacteriocins. Analysis of chimeras that consist of pediocin-like peptides, indicated that the C-terminal part of the molecule is responsible for target specificity (28). A C-terminal fragment of pediocin PA-1 inhibited the activity of pediocin PA-1 peptide, indicating that this fragment competed with the intact peptide for binding sites on the target membrane (29).

Bacteriocins are unstructured in an aqueous solution, but have the ability to form α -helical structures when exposed to structure promoting solvents, or when mixed with anionic phospholipid membranes (57). It is hypothesized that the highly conserved N-terminal of the Class IIa bacteriocins contributes to membrane binding. This allows the low homologous C-terminals to transform from random conformations to defined secondary structures, which are essential for pore formation (60).

Specific amino acids play a role in the antimicrobial activity of Class IIa bacteriocins. The presence of cysteines in the structure of these bacteriocins with subsequent modification of pairs of cysteine residues to form disulfide bridges affects the activity of bacteriocins (55).

Aromatic amino acids are also involved with antimicrobial activity (30,55,73). Loss of activity when small fragments of the N-terminal or the C-terminal are removed suggests that the whole sequence of the bacteriocin is necessary for activity (30,55).

The biological targets of bacteriocins produced by LAB are the anionic lipids of the cytoplasmic membrane, which acts as the primary receptors for initiation of pore formation (1,57,58). Previous findings suggested a protein-‘receptor’-mediated activity (8,13), but recent studies focusing on the effect of class IIa bacteriocins on lipid vesicle systems indicate that protein ‘receptors’ are not the main requirement for pore formation (25). It has been suggested that these receptors act to determine specificity of class II bacteriocins (111).

Pore formation ultimately results in the leakage of inorganic phosphates and an ionic imbalance (17), β -galactosidase and DNA and RNA material (89,97,99,101). The initial disturbance further causes dissipation of the proton motive force (PMF), which encompasses a complete or partial dissipation of either or both the pH gradient and the transmembrane potential (60). For class IIa bacteriocins complete dissipation of the pH gradient occurs readily, while only a partial dissipation of the transmembrane potential usually occurs (25,57). Dissipation of the proton motive force (PMF) by class IIa bacteriocins can be considered their main action to exert lethal activity (1,25,39,111). ATP is depleted as much as 98.9% and active transport involved in the uptake of amino acids is blocked (13,51). Leakage of pre-accumulated amino acids, among various other UV-absorbing materials, has been reported for plantaricin 423 and bacteriocins ST23LD and ST194BZ produced by different strains of *L. plantarum* and this leakage may be due to the diffusion of amino acids through the pores formed by bacteriocins as visualized by Atomic Force Microscopy (97,99).

In contrast to lantibiotics, class IIa bacteriocins causes no leakage of ATP. This may be due to smaller pore sizes that are formed by the action of the latter. However, ATP depletion does occur and this may result from an increased consumption of ATP in order to restore or maintain the PMF. The depletion may also be due to the efflux of inorganic phosphate that is needed to produce ATP (10,25).

Three pore formation models have been described by which bacteriocins act on the cell membranes of sensitive cells, a wedge-like model, a barrelstave-like model or a carpet mechanism (57). Class I bacteriocins may function by using a wedge-like model to induce pores, whereas Class II bacteriocins may form pores by either following the barrelstave-like model

or a carpet mechanism. The carpet mechanism is accomplished by peptides orientating them parallel to the membrane, thereby interfering with the membrane structure (57). Pore formation by class IIa bacteriocins using the barrelstave-like model may be due to the peptides' putative transmembrane helices, membrane-binding ability and water solubility (1,13,25,111). Thus far, two mechanisms for the initial interaction between class II bacteriocins and the membrane surface have been hypothesized, namely: (A) electrostatic binding of the bacteriocin to the membrane surface mediated by a putative receptor-type molecule bound to the membrane (25), and (B) binding between positively charged amino acids and anionic phospholipid heads in the membrane (25,57). Class II bacteriocins may rely on basically the same type of functional binding due to high structural similarities in their hydrophilic N-terminals (25). A crucial subsequent step in the process of pore formation is the hydrophobic interaction between the amphiphilic region of the C-terminal part of the bacteriocin and the lipid acyl chains (11,12,29,30,43). In contrast to the N-terminal domain that plays a role in the electrostatic interaction between the bacteriocin and the membrane surface, the C-terminal is believed to be the cell-specificity determining region (28,29).

Structural features, such as the YGNGV motif, α -helices, disulfide bonds, and positively charged amino acids, play an important role in cell recognition and activity of class IIa bacteriocins (57). These structural features are found within different domains spanning the bacteriocin peptide, indicating its complex nature (32). The β -sheet domain exerts antimicrobial activity, whereas the α -helix is thought to be responsible for target specificity (57). The YGNGV-motif allows for correct positioning of the bacteriocin on the membrane surface as it is recognized by a putative membrane receptor due to exposure caused by a β -turn structure (7,32,60). The hydrophilic/amphiphilic N-termini of the β -sheet are another component involved in recognition, possibly due to its electrostatic membrane-bacteriocin interaction. However, both the YGNGV motif and N-termini of the β -sheet of class IIa bacteriocins do not determine their specificity of activity (25). The central domain forms a hydrophilic or slightly amphiphilic α -helix and is believed to play a role in destabilization of the phospholipid bilayers. This mediates the insertion of the bacteriocin in the cytoplasmic membrane of the sensitive organism from an initial surface-bound state (57,25). The C-terminal hydrophobic/amphiphilic α -helix contributes to insertion of the bacteriocin into the cytoplasmic membrane of target cells resulting in the formation of water filled pores (57,25). Furthermore, the C-terminal domain plays a role in the target-cell specificity due to its putative transmembrane helices. Another feature that has to be taken in consideration is the presence of disulphide bonds. All class IIa bacteriocins contain at least one disulphide bridge and have been shown to

play a role in activity of the bacteriocin (22,39,60). Studies investigating the spectra of activity of class IIa bacteriocins have shown that bacteriocins with two disulfide bonds displayed a greater and broader spectrum of activity in comparison with those containing only one bond (79).

Class IIb bacteriocins are dependent on two distinct peptides for activity. They are responsible for dissipation of the transmembrane potential, while only a few affect the pH gradient. These two-peptide bacteriocins (class IIb) can be divided into two subgroups based on their ion-selectivity: (A) monovalent cation conducting bacteriocins, e.g. plantaricin EF (57); and (B) anion conducting bacteriocins, e.g. plantaricin JK (57). Class IIc bacteriocins vary in their modes of action, which ultimately leads to membrane permeability, pheromone activity and specific inhibition of septum formation (38).

Plantaricin A (pIA) is a 26-residue bacteria-produced peptide pheromone with membrane-permeabilizing antimicrobial activity (63). In study of Zhao *et al.* (117) the interaction of pIA with membranes is shown to be highly dependent on the membrane lipid composition. PIA bound readily to zwitterionic 1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine (SOPC) monolayers and liposomes, yet without significantly penetrating into these membranes. The presence of cholesterol attenuated the intercalation of pIA into SOPC monolayers. The association of pIA to phosphatidylcholine was, however, sufficient to induce membrane permeabilization, with nanomolar concentrations of the peptide triggering dye leakage from SOPC liposomes. The addition of the negatively charged phospholipid, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-rac-glycerol POPG (SOPC/POPG; molar ratio 8:2) enhanced the membrane penetration of the peptide, as revealed by (i) peptide-induced increment in the surface pressure of lipid monolayers, (ii) increase in diphenylhexatriene (DPH) emission anisotropy measured for bilayers, and (iii) fluorescence characteristics of the two Trps of pIA in the presence of liposomes, measured as such as well as in the presence of different quenchers. Despite deeper intercalation of pIA into the SOPC/POPG lipid bilayer, much less peptide-induced dye leakage was observed for these liposomes than for the SOPC liposomes. Further changes in the mode of interaction of pIA with lipids were evident when also the zwitterionic phospholipid, 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphoethanolamine (POPE) was present (SOPC/POPG/POPE, molar ratio 3:2:5), thus suggesting increase in membrane spontaneous negative curvature to affect the mode of association of this peptide with lipid bilayer. PIA induced more efficient aggregation of the SOPC/POPG and SOPC/POPG/POPE liposomes than of the SOPC liposomes, which could explain the attenuated peptide-induced dye leakage from the former liposomes. At micromolar concentrations, pIA killed human leukemic T-cells by both necrosis and apoptosis. Interestingly, pIA formed supramolecular protein-lipid amyloid-like fibers upon binding to negatively charged phospholipid-

containing membranes, suggesting a possible mechanistic connection between fibril formation and the cytotoxicity of pIA (117)

FERMENTATION OPTIMIZATION

Many studies have focused on optimization of media and growth conditions for increased bacteriocin production. Verellen *et al.* (112), Powell *et al.* (72) Todorov and Dicks (93,94,95,96), Todorov *et al.* (87,100,101,102,103) and Todorov (89) reported higher bacteriocin production levels for *L. plantarum* ST194BZ, *L. plantarum* ST13BR, *L. plantarum* ST414BZ, *L. plantarum* ST664BZ, *L. plantarum* ST23LD, *L. plantarum* ST341LD, *L. plantarum* 423, *L. plantarum* AMA-K, *L. plantarum* ST26MS, *L. plantarum* ST28MS, *L. plantarum* ST8KF, *L. plantarum* ST31 in optimized growth media.

BACTERIOCINS PRODUCED BY *L. PLANTARUM*

Numerous small, heat-stable plantaricins have been described in the literature that have only been partially characterized. These include the following, of which the producing organisms have been isolated from various fermented food products:

Meat: Several bacteriocin-producing *L. plantarum* strains have been isolated from fermented sausages obtained from different manufacturers at different times of ripening (35). Schillinger and Lücke (82) isolated various bacteriocin-producing lactobacilli including *Lactobacillus plantarum* from fresh meat and different meat products. Plantaricin UG1 (23) is produced by *L. plantarum* UG1 isolated from dry sausage.

Plantacin 154 (44) is produced by *L. plantarum* LTF 154 isolated from fermented sausage. Bacteriocin-deficient mutants obtained after treatment of cells with acriflavin, coincided with the loss of a plasmid of 9.5 mDa, designated pLP1542. The bacteriocin-deficient mutants are immune to plantacin 154, suggesting that the genes coding for immunity are located on the chromosome.

Plantaricin SA6, produced by *L. plantarum* SA6 was isolated from fermented sausage (78).

Plantaricin 1.25L (21) is a thermostable class two bacteriocin produced by *L. plantarum* TMW1.25 isolated from sausage fermentation. It is co-produced with several other bacteriocin-like peptides. Using oligonucleotides derived from previously determined peptide sequences, a 3.8 kb DNA fragment could be amplified. A neighboring 1.8 kb fragment was amplified using ligation-anchored single-specific-primer PCR. Sequencing of the complete 5.6 kb stretch revealed that the structural gene for plantaricin 1.25L, plnB, was located downstream of another bacteriocin gene, plnC. Seven other open reading frames were detected, including plnK encoding a

bacteriocin-like peptide, but not including any putative immunity genes. Interestingly, the gene cluster contained an IS30-like insertion sequence, designated IS125, as well as an ISS1 homolog (21).

Messi *et al.* (54) isolated lactic acid bacteria (more than hundred) from Italian sausages that were tested for the production of antimicrobial substances (bacteriocins). Six percent of these showed antibacterial activity against one or several closely related microorganisms used as indicators.

Fish: Plantaricin F, produced by *L. plantarum* BF001 isolated from spoiled catfish fillets, can only be detected after growth of the producing organism on solidified medium, or after a 50-fold concentration of liquid medium (34,70).

Fruit and vegetables: Olasupo (67) reported the production of plantaricin NA, produced by a *L. plantarum* sp. isolated from vegetable origin (67), Plantaricin C19 (4,5) is produced by *L. plantarum* C19 isolated from fermented cucumbers (Table 1).

L. plantarum C-11, isolated from fermented cucumbers (15), produces two two-peptide bacteriocins, plantaricin EF and plantaricin JK (3). Plantaricin A, previously incorrectly identified as the bacteriocin responsible for inhibitory activity by *L. plantarum* C11 (63), induces the production of these two two-peptide bacteriocins (Table 1).

Plantaricins S and T are produced by *L. plantarum* LPCO10, isolated from green olive fermentations (41,42). Plantaricin S is produced during the logarithmic phase of growth. A second bacteriocin, plantaricin T, is secreted once the producing organism reaches the stationary phase of growth. Plantaricin T exhibits the same heat resistance as plantaricin S, but is not inactivated by α -amylase or lipase A. Plantaricin T also exhibits a lower level of inhibition against the various organisms tested than plantaricin S. Plantaricin S is 2.5 kDa in size, while plantaricin T is slightly smaller. The genetic determinants for both bacteriocins do not appear to be plasmid encoded. Amino acid sequence of plantaricin S was determined (Table 1).

The genes plsA and plsB encoding for production of plantaricin S (Pls), a two-peptide bacteriocin produced by *L. plantarum* LPCO10, are commonly distributed among wild-type *L. plantarum* strains isolated from olive fermentations. Among 68 independent isolates from different olive processing plants in South Spain, 15 of them were shown to produce bacteriocins that were active against other lactic acid bacteria, as well as spoilage and pathogenic bacteria. On the basis of PCR amplification and hybridization with specific probes, the Pls operon was detected in all the bacteriocin producer strains but not in the non-producer ones. Purification and subsequent amino acid sequencing of the bacteriocin produced by some of the 15 isolates yielded both the a and h peptides from Pls. These results suggest that bacteriocin production contributes an ecological advantage for the wild-type *L. plantarum* strains in the colonization of the spontaneous, traditional olive fermentation process (52).

Table 1. Primary structure of bacteriocins produced by different strains of *Lactobacillus plantarum*.

Bacteriocin	amino-acid sequence	reference
Plantaricin ST31	KRKKH RXQVY NNGMP TGMYS	(88)
Plantaricin S α	XNKLA YNMGW YAGXA TIFGL AAXAL L	(42)
Plantaricin S ν	KKKKQ SWYAA AGDAI VSFGE GFLN	(42)
Plantaricin A α	YSLQM GATAI KQVKK LFKKW G	(63)
Plantaricin A β	AYSLQ MGATA IKQVK KLFKK WG	(63)
Plantaricin C	KKTKK NXSGD I	(36)
Plantaricin 149	YSLQM GATAI KQVKK LFKKK GG	(45)
Plantaricin WHE92	KYYGN GVYCG KHSCS VDWGK ATTCI IINNG AMAWA TGGHQ GNHCK	(26)
Plantaricin C19	KYYGN GLSCS KKGCT VNWGQ AFSCG VNRVA TAGHG K	(4)
Plantaricin 423	KYYGN GVTCT KHSCS VNWGQ AFSCS VSHLA NFGHG KC	(108,110)

Plantaricin-149, produced by *L. plantarum* NRIC 149 and isolated from pineapple has been partially sequenced (Table 1) (45).

Plantaricin D is produced by *L. plantarum* BFE 905 isolated from “Waldorf” salad (31).

Detection and characterization of bacteriocin production by *L. plantarum* strain J23, recovered from a grape must sample in Spain, have been carried out by Rojo-Bezarez *et al.* (80). Bacteriocin production was detected in liquid media only when J23 was cocultivated with some inducing bacteria. The presence of ethanol or acidic pH in the media reduced bacteriocin production in the cocultures of J23 with the inducing bacteria. The presence of plantaricin-related plnEF and plnJ genes was detected by PCR and sequencing. Nevertheless, negative results were obtained for plnA, plnK, plnC8, plnS and plnW genes (80).

Bacteriocin-producing strains of *L. plantarum* ST23LD and ST34ILD were isolated from the brine of spoiled black olives (92) and their production optimized (95). Mode of action and effect of the temperature, pH and different chemicals on adsorption of bacteriocin ST23LD to *Latobacillus sakei* and *Enterococcus* sp. were determined (99).

Two bacteriocins, ST28MS and ST26MS, produced by two different strains of *L. plantarum* were isolated from molasses and partially characterized (94). Both bacteriocins showing unusual activity against Gram-negative bacteria, including *Pseudomonas aeruginosa*, *Escherichia coli* and *Acinetobacter baumannii* (94).

Milk products: Plantaricin C (36), is produced by *L. plantarum* LL441, isolated from Cabrales cheese. The sequence of the first 11 amino acids of plantaricin C is KKTKKNXSGDI. Turner *et al.* (104) recently identified plantaricin C as a lantibiotic.

Plantaricin LC74, produced by *L. plantarum* LC74 isolated from crude goat’s milk (77).

Strain AMA-K, isolated from naturally fermented milk produced in Gwanda, Kafusi area, Zimbabwe. Bacteriocin

production is stimulated by the presence of *Listeria innocua*. *L. plantarum* AMA-K grows in milk, but produces only 800AU bacteriocin per ml after 24 h (100).

L. plantarum ST8KF, isolated from kefir, produces a 3.5 kDa bacteriocin (bacST8KF) active against *Lactobacillus casei*, *Lactobacillus salivarius*, *Lactobacillus curvatus*, *Enterococcus mundtii* and *Listeria innocua* (72). Potential application of *L. plantarum* ST8KF for control of *Enterococcus* spp. in situ in kefir was shown (71).

Cereal: Olukoya *et al.* (68) reported the production of plantaricin K, produced by *L. plantarum* DK9 isolated from “fufu”, a fermented cassava product, and pentocin D, produced by *L. pentosus* DK7, isolated from “ogi”, a fermented maize product. Plantaricin ST31 (88) is produced by *L. plantarum* ST31 isolated from sourdough. Amino acid sequence of plantaricin ST31 was determined (Table 1).

Plantaricin KW30 (46) is produced by *L. plantarum* strain KW30 isolated from fermented corn (Kaanga Wai).

L. plantarum 423 produces a small heat-stable antimicrobial protein designated plantaricin 423. This protein is bactericidal and has been characterized at a genetic and protein level, including amino-acid sequence (Table 1) (108,110).

L. plantarum strains ST194BZ, ST414BZ and ST664BZ, isolated from boza, (98). From a total population of 9×10^6 cfu/ml lactic acid bacteria from Bulgarian boza two isolates (JW3BZ and JW6BZ) identified as *L. plantarum* by biochemical and biomolecular methods produced bacteriocins active against a broad spectrum of Gram-positive bacteria. (113).

The bacteriocin ST13BR, produced by *L. plantarum* ST13BR was isolated from beer and partially characterized (90,91).

Unknown: Several plantaricins produced by *L. plantarum* Lb 75, *L. plantarum* Lb 592 and *L. plantarum* BN were reported (50,64,65).

Plantacin B (114) is produced by *L. plantarum* NCDO 1193. Since the molecule cannot be isolated in liquid media, the mode

of action and size of the protein has not been determined and no DNA or fermentation studies have been reported.

L. plantarum NCIM 2084 produced an antibacterial substance when grown at 40°C for 36 h in a laboratory medium (86).

APPLICATION OF PLANTARICINS

Bacteriocins are antimicrobial peptides or proteins produced by strains of diverse bacterial species. A several bacteriocins produced by different strains of *L. plantarum* isolated from food products have been described (4,15,18,21,23,31,34,36, 41,42,44,52,54,63,66,68,70, 72,77,80,85-98,104,108,113). This bacteriocinogenic strains of *L. plantarum* are naturally presented in the good products and contribute not only to the organoleptic characteristics of the products, but play an essential role in natural biopreservation of this products.

The antimicrobial activity of this group of natural substances against foodborne pathogenic, as well as spoilage bacteria, has raised considerable interest for their application in food preservation. Application of bacteriocins may help reduce the use of chemical preservatives and/or the intensity of heat and other physical treatments, satisfying the demands of consumers for foods that are fresh tasting, ready to eat, and lightly preserved. In recent years, considerable effort has been made to develop food applications for many different bacteriocins and bacteriocinogenic strains. Depending on the raw materials, processing conditions, distribution, and consumption, the different types of foods offer a great variety of scenarios where food poisoning, pathogenic, or spoilage bacteria may proliferate.

Application of plantaricins or *L. plantarum* bacteriocin-producers strains have a important potential for control of *L. monocytogenes*. Plantaricin C-11 (4), plantaricin NA (67), bacteriocin AMA-K (89,100) was showing a strong anti-*Listaria* activity and they may have a future application in food preservation (4,67,89,100). In a model system high cell numbers of *L. plantarum* AMA-K (producer of bacteriocin AMA-K) and *L. innocua* F were recorded on MRS plates when co-cultured (89). However, results recorded on selective LEB medium indicated that the cell numbers of *L. innocua* F decreased from 3.4×10^4 CFU/mL to 7.0×10^2 CFU/mL in 12 h and to undetectable levels after 24 h (89). This indicated that the high cell numbers recorded on MRS plates were *L. plantarum*. Inhibition of *L. innocua* F cannot be ascribed to lactic acid production or a decrease in pH, since a much more prominent decline in cell numbers was recorded after 12 h of fermentation and an increased production of bacteriocin AMA-K. Was shown that bacteriocin AMA-K has a strong adsorption to cells of *L. monocytogenes*, *L. ivanovii* subsp. *ivanovii* and *L. innocua* pointing that this bacteriocin can be successfully applied in bio-control of this food-borne pathogens (100).

Plantaricin ST8KF is a bacteriocin produced by *L. plantarum* ST8KF, a strain isolated from kefir (71,72). A kefir artificially inoculated with *E. mundtii* was used as a model for *in situ* monitoring effect of plantaricin ST8KF. Low cell numbers of *E. mundtii* were recorded in kefir produced with *L. plantarum* ST8KF (71). However, high cell numbers of *E. mundtii* (28 cells per 10 µL sample) were recorded in kefir produced with *L. plantarum* ST8KF⁽⁻⁾ (bacteriocin negative mutant). Concluding from these results, the kefir grains were successfully enriched with *L. plantarum* ST8KF, with the level of bacST8KF production high enough to inhibit the growth of *E. mundtii*. *Lactobacillus plantarum* ST8KF could be used as a starter culture in kefir production and contribute to the bio-preservation of this product (71).

CONCLUSION

Numerous strains of bacteriocin producing *L. plantarum* have been isolated in the last two decades from different ecological niches including meat, fish, fruits, vegetables, and milk and cereal products. Several of these plantaricins have been characterized and the amino acid sequence determined. Different aspects of the mode of action, fermentation optimization and genetic organization of the bacteriocin operon have been studied.

Application of bacteriocins may help reduce the use of chemical preservatives and/or the intensity of heat and other physical treatments, satisfying the demands of consumers for foods that are fresh tasting, ready to eat, and lightly preserved. Several bacteriocin producer strains of *L. plantarum* have been isolated from different food products and they may be successfully applied in the food fermentation processes, contributing not only to organoleptic characteristics, but increasing the shelf-life and safety of the final products.

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RESUMO

Bacteriocinas de *Lactobacillus plantarum* - produção, organização genética e modo de ação

Bacteriocinas são proteínas ou complexos protéicos biologicamente ativos que apresentam atividade bactericida contra espécies relacionadas. Nas últimas duas décadas, várias cepas de *Lactobacillus plantarum* produtoras de bacteriocinas

foram isoladas de diferentes nichos ecológicos como carnes, peixes, frutas, vegetais e produtos lácteos e de cereais. Várias plantaricinas foram caracterizadas e suas seqüências de aminoácidos determinadas. Diferentes aspectos do modo de ação, otimização da fermentação e organização genética já foram estudados. Entretanto, muitas bacteriocinas produzidas por diferentes cepas de *Lactobacillus plantarum* ainda não foram completamente caracterizadas. Nesse artigo, apresenta-se uma breve revisão sobre a classificação, genética, caracterização, modo de ação, e otimização da produção de bacteriocinas de bactérias lácticas em geral, e, quando apropriado, de bacteriocinas de *Lactobacillus plantarum*.

Palavras-chave: *Lactobacillus plantarum*, plantaricina, modo de ação, organização genética.

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