



Lactic Acid Bacteria *Lactobacillus* species MTCC 10093 Strain Produced Bacteriocins.

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Abstract

A Gram (+) bacterium *Lactobacillus* species MTCC 10093 produce during their growth, substances of protein structure (either proteins or polypeptides) owning antimicrobial activities, called bacteriocins. Although bacteriocins could be considered as antibiotics, they are not. The major difference between bacteriocins and antibiotics is that bacteriocins curb their activity to strains of species related to the producing species and particularly to strains of the same species, antibiotics on the other hand have a wider activity spectrum and even if their activity is restricted this does not show any preferential effect on closely related strains. In addition, bacteriocins are ribosomally synthesized and produced during the primary phase of growth, though antibiotics are usually secondary metabolites. Among the Gram (+) bacteria, lactic acid bacteria (LAB) especially, *Lactobacilli* have gained particular attention nowadays, due to the production of bacteriocins.

These substances can be applied in the food industry as natural preservatives. The use of LAB and of their metabolic products is generally considered as safe (GRAS, Grade One). The application of the produced antimicrobial compounds as a natural barrier against pathogens and food spoilage caused by bacterial agents has been proven to be efficient. Nisin is the only bacteriocin that has been officially employed in the food industry and its use has been approved worldwide. Bacteriocins can be applied on a purified or on a crude form or through the use of a product previously fermented with a bacteriocin producing strain as an ingredient in food processing or incorporated through a bacteriocin producing strain (starter culture).

Keywords: Lactic acid bacteria, *Lactobacilli*, Bacteriocins, Nisin, Plantaricins, Lantibiotics

1. Introduction

A great number of Gram (+) bacteria and Gram (-) bacteria produce during their growth, substances of protein structure (either proteins or polypeptides) possessing antimicrobial activities, called bacteriocins. Although bacteriocins could be categorized as antibiotics, they are not. The major difference between bacteriocins and antibiotics is that bacteriocins restrict their activity to strains of species related to the producing species and particularly to strains of the same species. Antibiotics on the other hand, have a wider activity spectrum and even if their activity is restricted this does not show any preferential effect on closely related strains. In addition, bacteriocins are ribosomally synthesized and produced during the primary phase of growth, though antibiotics are usually secondary metabolites [1]. Bacteriocins usually have low molecular weight (rarely over 10 kDa) they undergo posttranslational modification and can be easily degraded by proteolytic enzymes especially by the proteases of the mammalian gastrointestinal tract, which makes them safe for human consumption. Bacteriocins are in general cationic, amphipathic molecules as they contain an excess of lysyl and arginyl residues [3,4]. They are usually unstructured when they are incorporated in aqueous solutions but when exposed to structure promoting solvents such as trifluoroethanol or mixed with anionic phospholipids membranes they form a helical structure[5]. Among the Gram positive (+) bacteria, lactic acid bacteria (LAB) have gained particular attention nowadays, due to the production of bacteriocins [10],[20,21,22]. These substances can be applied in the food industry as natural preservatives. The use of LAB and of their metabolic products is generally considered as safe (GRAS, Grade One). The application of the produced antimicrobial compounds as a natural barrier against pathogens and food spoilage caused by bacterial agents has been proven to be efficient [2].

1.1. Classification of LAB Bacteriocins

Most of LAB bacteriocins are small (<10 kDa) cationic, heat-stable, amphiphilic and membrane permeabilizing peptides. They can be divided into three major classes, their classification was constantly revised throughout the last decade due to the extensive research realized [3, 4]. Many of these bacteriocins appear to exhibit relatively little adsorption specificity. The cell wall of Gram positive (+) bacteria allows passage of relatively large molecules. The anionic cell surface polymers like teichoic and lipoteichoic acids, which are part of the cellular wall, are important in the initial interaction of anionic bacteriocins produced by Gram positive (+) bacteria. The LAB bacteriocins have greater antibacterial activity at lower pH values (below 5) by means that their adsorption to the cell surface of Gram positive (+) bacteria including the producing cells is pH dependent. Within any class of bacteriocin there may be amino acid sequence homologies not only within the mature peptide, but also in the N-terminal leader region and the associated proteins in bacteriocin secretion and processing [2].

1.1.1. Class I: The Lantibiotics

Class I, the lantibiotics, are a class of peptide substances that contain the characteristic polycyclic thioether amino acids lanthionine or methyllanthionine, as well as the unsaturated amino acids dehydroalanine and 2-aminoisobutyric acid. They are further divided into two types based on structural similarities. Type A comprises of relatively elongated, screw shaped, positively charged, amphipathic, flexible molecules. Their molecular mass varies between 2 to 4 kDa and they generally act through pore formation, through membrane depolarization, of the cytoplasmic membrane of the sensitive target species, nisin and lactacin 3147 are the major representatives of this group. Type B lantibiotics, are globular in structure and interfere with cellular enzymatic reactions. Their molecular mass, lies between 2 to 3 kDa and either they have no net charge or a net negative charge [6],[7]

Class I LAB bacteriocins are small (<5kDa) heat stable peptides, which are extensively modified after translation resulting in the formation of characteristic thioether amino acids lanthionine (Lan) and β -methyllanthionine (MeLan). These arise after a two-step process. Firstly gene encoded serine and threonine can be subject to enzymatic dehydration to give rise to dehydroalanine (Dha) and dehydrobutyrate (Dhb). Subsequently, thiol groups from neighbouring cysteines attack the double bond of Dha and Dhb yielding both Lan and MeLan respectively. This condensation between two neighbouring residues results in the formation of covalently closed rings within the formerly linear peptide conferring both structure and functionality. Also members of this group contain D-alanine. This latter amino acid residue is derived from dehydroalanine resulting from the dehydration of a serine residue [3, 4] [2].

1.1.2. Class II: the Non-Lantibiotics

Class II bacteriocins are also small (<10 kDa) relatively heat stable, non-lanthionine containing membrane active peptides. They are divided into two subclasses. Subclass II a, pediocin-like or listeria active bacteriocins subclass possesses an N-terminal consensus sequence Tyr-Gly-Asn-Gly-Val-Xaa-Cys. They show a high degree of homology (40%-60%) when the corresponding amino acid sequences are aligned and they are synthesized with a leader peptide which is removed by proteolytic processing, usually after a double glycine residue for example like pediocin PA-1, sakacin A [8]. Subclass II b refers to two-component (two separate peptides) bacteriocins by means that require two peptides to work synergistically in order to have an antimicrobial activity. Lactacin F and lactococcin G are members of this group [9].

2. Biosynthesis and Immunity of LAB Bacteriocins

As previously referred bacteriocins are ribosomally synthesized and posttranslational modified peptides [8], [2]. The genes encoding bacteriocin production and immunity are generally organized in operon clusters and can be located on mobilisable elements such as chromosome in association with transposons or on plasmids [7]. The bacteriocins are primarily synthesized as biologically inactive prepeptides containing an N-terminal leader. The prepeptides are then modified by other proteins or amino acids encoded by the bacteriocin gene cluster before export. For example, there are thioether cross-links termed lanthionines (Lans) or methyl lanthionines (MeLans) additionally to amino acids 2,3-didehydroalanine (Dha) and (2)-,2-3-didehydrobutyrine (Dhb). These amino acids are introduced by the dehydration of serine and threonine residues followed by stereo selective intermolecular addition of cysteines onto the unsaturated amino acids [6]. Bacteriocin producing strains have to protect themselves from the action, the toxic effect of their own bacteriocin [7]. This occurs through the production of specific immunity proteins. Genes coding for immunity proteins are in close genetic proximity to other bacteriocin structural and processing genes. Often the structural bacteriocin gene and the immunity gene can be located on the same operon [6]. For the LAB bacteriocins two types of immunity systems have been described. One system is reliant on the specific immunity Lan I, though the second system depends on a separately dedicated multicomponent ABC transporter (Lan EFG) [7]. The Lan I protein is most probably attached to the outside of the cytoplasmic membrane. It provides immunity to the producer cells by preventing pore formation by the bacteriocin molecules that have inserted into the membrane, back to the surrounding medium and thus keeping the concentration of bacteriocin in the membrane under a critical level [20, 21, 22, 23].

3. Most Important Bacteriocins

3.1. Plantaricins

L. plantarum has been considered to produce at least 6 distinct bacteriocins. All these peptides were primarily produced as precursors containing a double glycine moiety. *L. plantarum* synthesizes these bacteriocins through the PlnE and PlnF genes. These peptides are then exported and processed by the PlnG and PlnH proteins. The peptide pheromone for this system is encoded by a separate gene (PlnA) and is exported by PlnG and PlnH and detected by the histidine protein kinase PlnB which finally phosphorylates two response regulators PlnC and PlnD. Plantaricins inhibit a broad range of LAB including their natural competitor *L. plantarum* and other bacteria like *Pediococcus*, *Carnobacteria*, *Clostridia* and *Propionobacteria* [7].

3.2. Plantaricins JK, EF

These bacteriocins act as synergetic peptides. They are 30 or 40 residues in length and show little sequence similarity to any other plantaricin approximately. These bacteriocins act with strict specificity and any other combination, except JK or EF results in the complete loss of synergy [5], [10].

3.3. Plantaricin S and Plantaricin W

Plantaricin S is a two-peptide system isolated from *L. plantarum* spp. used to ferment green olives. The structural genes (□, □) indicate that each peptide is initially produced with a leader containing the double glycine motif. These peptides are of 26 and 27 amino residues in length. Plantaricin S is considered to control the fermentation process and preserve the olives [10], [20, 21, 22, 23]. Another two-peptide bacteriocin is plantaricin W comprised by Plwa and Plwb protein molecules. These lantibiotic components consist of 29 and 32 amino acid residues respectively and are considered.

3.4. Nisin

Nisin is the most widely exploited and applied bacteriocin. It is active against Gram (+) positive bacteria including highly pathogenic and food spoilage microorganisms including *S. aureus* and *L. monocytogenes*. In the United States, its use has been approved since 1988 by FDA for use in cheese, heat treated- chill stored soups and pasteurized cheese spreads which are stored in chill temperature. Nisin belongs to the Class I lantibiotics, is composed by thirty-four amino acids and has a pentacyclic structure with one lanthionine residue (Ring A) and four β -methylanthionine residues (rings B, C, D, E) nisin Z, the natural variant of nisin is different only in that the histidine molecule on place 27 is replaced by asparagines [13]. Nisin can be effective at nanomolar concentrations depending on the target strain. Nisin is ribosomally synthesized as a precursor peptide that is later enzymatically modified. This prepeptide is biologically inactive and contains a c-terminal prepeptide domain, following a variety of posttranslational modification reactions, is cleaved from the N-terminal leader sequence to yield the mature antimicrobial compound. It is an auto regulatory two component system which can be activated fully by nisin in very low sub toxic amounts (ng/ml) [15]. Nisin is heat stable at 121°C but for prolonged heating, becomes less heat stable, especially between pH 5 to 7. Nisin is sensitive to β -chymotrypsin but resistant to trypsin, elastase, carboxyl peptidase, pepsin, and erepsin. Nisin is utilised as a food additive, is commercially produced and is assigned under the number E234 (ECCU 1983 EEC Commission Directive 8314631EEC) [15, 16]. NICE system is part of the deeply studied pheromonedependent quorum sensing systems which are widely distributed in the Gram positive (+) bacteria [20, 21, 22, 23].

3.4.1. Mode of Action of Nisin Membrane Insertion & Models of Pore Formation

Gram positive (+) bacteria are characterized by a high content of anionic lipids in the membrane nisin binds rapidly to anionic liposome's and this interaction is strong since nisin has been able to diffuse slowly to other liposome's. Fragments of nisin have been used to identify the regions that are involved in membrane interaction [9]. Nisin has the ability to interact with anionic lipids correlates with its antimicrobial activity. It forms pores in the lipid membrane by interacting with the peptidoglycan precursor lipid II. The presence of lipid II enhances the ability of nisin to depolarize the transmembrane electrical potential and disrupting the lipid bilayer organization when it binds to the membrane [3, 4]. Nisin forms pores through a series of distinct steps. In equilibrium, nisin seems to orient parallel to the surface of the membrane. Nisin induces the transmembrane movement of fluorescent phospholipids suggesting that the membrane insertion of the Cterminus of nisin causes intermonolayer contact of phospholipids, forming pores according to the wedge like model [1]. A wedge-like model can be described as following. Induced pore formation involves a proton motive force driven by co insertion of lipids and nisin domains. The hinge in the nisin molecule might allow bending of the C-terminal part and thus its insertion into the membrane. Multiple inserted nisin molecules may give rise to a large local disturbance of the lipid protein pores. Such structures are intrinsically unstable due to the hydrophobic forces, which are driving the rearrangements of the lipids into their original bilayer organization [27].

4. Applications of Bacteriocins

Nowadays, bacteriocins have been widely utilised especially in the field of food preservation. The use of bacteriocins in food industry especially on dairy, egg, vegetable and meat products has been extensively investigated. Among the LAB bacteriocins nisin A and its natural variant nisin Z has been proven to be highly effective against microbial agents causing food poisoning and spoilage. Furthermore, nisin is the only bacteriocin that has been officially employed in the food industry and its use has been approved worldwide [5], [7]. Numerous preservation methods though, have been used in order to prevent food poisoning and spoilage. These techniques include thermal treatment (pasteurization, heating sterilization), pH and water activity reduction (acidification, dehydration) and addition of preservatives (antibiotics, organic compounds such as propionate, sorbate, benzoate, lactate, and acetate). Although these methods have been proven to be highly successful, there is an increasing demand for natural, microbiologically safe products providing the consumers with high health benefits [7]. Bacteriocins can be applied on a purified or on a crude form or through the use of a product previously fermented with a bacteriocin producing strain as an ingredient in food processing or incorporated through a bacteriocin producing strain (starter culture). The incorporation of a

bacteriocin producing strain has the disadvantage of the lack of compatibility between the bacteriocin producing strain and the other cultures required for fermentation [10]. However, it has been proven that a bacteriocin alone in a food is not likely to ensure complete safety; especially in the case of Gram negative (-) bacteria this has been apparent. Then the use of bacteriocins has to be combined with other technologies that are able to disrupt the cellular membrane so bacteriocins can kill the pathogenic bacteria [9]. For example the use of non-thermal treatments such as pulsed electric field (PEF) is advantageous as it does not have any effect on food functionality and nutritional qualities. This technique may not be financially viable when used alone, but in lower levels and combined with other treatments such as bacteriocins may be highly effective. Furthermore, bacteriocins could be combined with other antimicrobial compounds such as sodium acetate and sodium lactate resulting in enhanced inactivation of bacteria. Bacteriocins can also be used to improve food quality and sensory properties, for example increasing the rate of proteolysis or in the prevention of gas blowing defect in cheese. Another application of bacteriocins is bioactive packaging, a process that can protect the food from external contaminants. For instance, the spoilage of refrigerated food commonly begins with microbial growth on the surface that reinforces the attractive use of bacteriocins being used in conjunction with packaging to improve food safety and self-life [10]. Bioactive packaging can be prepared by directly immobilizing the bacteriocin to the food packaging or by addition of a sachet containing the bacteriocin into the packaged food, which will be released during storage of the food product. The gradual release of bacteriocins from a packaging film on the food surface may have an advantage over dipping and spraying foods with bacteriocins, because antimicrobial activity may be lost or reduced due to inactivation of

the bacteriocins by food components or dilution below active concentration due to migration into the foods [10]. There are several methods to prepare packaging films with bacteriocins. One method is to incorporate bacteriocin directly into polymers for example incorporation of nisin into biodegradable protein films. The incorporation of nisin or any other bacteriocin can be achieved through heat press and casting into films made from soy proteins or corn zein. Another method is to coat or adsorb bacteriocins to polymer surfaces; examples include nisin methylcellulose coatings for polyethylene films for the use on poultry meat, adsorption of nisin on polyethylene, ethylene, vinyl acetate, polypropylene, polyamide, polyester acrylics and polyvinyl chloride [7]. **References** such as propionate, sorbate, benzoate, lactate, and acetate). Although these methods have been proven to be highly successful, there is an increasing demand for natural, microbiologically safe products providing the consumers with high health benefits [7]. Bacteriocins can be applied on a purified or on a crude form or through the use of a product previously fermented with a bacteriocin producing strain as an ingredient in food

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