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REVIEW ARTICLE

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Classification and mode of action of membrane-active bacteriocins produced by gram-positive bacteria

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Abstract Bacteriocins are ribosomally synthesized antimicrobial peptides produced by microorganisms belonging to different eubacterial taxonomic branches. Most of them are small cationic membrane-active compounds that form pores in the target cells, disrupting membrane potentials and causing cell death. The production of small cationic peptides with antibacterial activity is a defense strategy found not only in bacteria, but also in plants and animals. Bacteriocins are classified according to different criteria by different authors; in this review, we will summarize the principal bacteriocin classifications, highlight their main physical and chemical characteristics, and describe the mechanism of some selected bacteriocins that act at the membrane level.

Keywords Bacteriocin · Peptidic antibiotic · Cationic peptides · Membrane-active compound · Electrostatic interaction

Introduction

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by bacteria. The antagonistic interaction between competing bacteria was described early in 1877, when Pasteur and Joubert noticed that some *Escherichia coli* strains interfered with the growth of *Bacillus anthracis* present in infected animals. Bacteriocins were first detected in 1925 by André Gratia, who observed that the growth of some *E. coli* strains was inhibited by the presence of an antibacterial compound, which he called colicin V, released into the medium by *E. coli* V (virulent strain) [26]. Colicin V was later characterized as a heat-stable and dialyzable peptidic compound and, in 1954, Pierre Frédéricq found its genetic determinants in a conjugation-transmissible element similar to the F factor [23]. The production of small antibiotic peptides is a common defense strategy against bacteria that is displayed not only by microorganisms, but also by animals and plants. Magainins, cecropins and defensins are animal [42, 64], and thionins are plant [11, 12] antimicrobial peptides. These antimicrobials vary significantly in their amino acid sequence, but most of them share features such as low molecular weight, heat stability and a cationic and hydrophobic nature. Furthermore, all of them are coded for by structural genes that are ribosomally translated into peptides.

The antimicrobial peptides produced by bacteria have been grouped into different classes on the basis of the producer organism, molecular size, chemical structure and mode of action [21, 31, 37, 38, 55]. These different classifications have produced different names (microcin, colicin, bacteriocin, lantibiotic, thiolbiotic, cystibiotic) that can be misleading, as the same compound can be found under different names in different classifications. To clarify this situation, we will review the different bacteriocin classification systems. Furthermore, because of their potential applications as food preservatives, we will focus on peptidic antibiotics produced by gram-positive bacteria that are active at the membrane level. The most relevant physical and chemical characteristics and the current model explaining the mode of action on the bacterial membrane of this group of antibiotics will be presented.

Bacteriocin classification

Several classification criteria have been used to group the different antimicrobial compounds produced by gram-positive bacteria. Jack et al. [31] considered the presence of disulfide and monosulfide (lanthionine) bonds as the basis for their classification and as a landmark for their spectrum of activity. Accordingly, bacteriocins were classified into four groups: (1) antibiotics containing unusual posttraslationally modified

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amino acids such as dehydroalanine, dehydrobutirine, lanthionine or β-methyl-lanthionine (lantibiotics); (2) antibiotics containing at least one disulfide bridge essential for their activity (cystibiotics); (3) compounds with a single -SH residue that should be in a reduced form for the antibiotic to be active (thiolbiotics); and (4) antibiotics without cysteine residues (Table 1). Jack et al. noted that, although the role of cysteine residues on the activity is not completely understood, the higher the number of disulfide bonds a bacteriocin has, the wider its activity spectrum tends to be [31].

The most thoroughly studied bacteriocins are those produced by lactic-acid bacteria because of their potential use as food preservatives and the increasing incidence of food-borne infectious diseases. According to Klaenhammer [37], bacteriocins can be classified into four groups on the basis of to their molecular mass, thermostability, enzymatic sensitivity, presence of posttranslationally modified amino acids, and mode of action.

Class I bacteriocins

This group comprises lantibiotics and can be further divided into two subgroups on the basis of structure and charge of the compound: (1) group Ia, which consists of screw-shaped, amphipathic, small cationic peptides that produce voltage-dependent pores by unspecific interaction with the membrane of the target cell; and (2) group Ib, which consists of anionic or neutral peptides having a globular shape. The number of thioether bridges (modified amino acids) present in group Ia lantibiotics can vary, and members with either three (pep5) [50], four (epidermin) [3] or five (nisins A and Z, subtilin) [30, 5] monosulfide bonds have been described. The molecular size of these antibiotics ranges from 1,959 (duramycin) to 4,635 (carnocin UI49, which is the largest lantibiotic described up to now) [44]. Group Ib includes antibiotics such as mersacidin [14], actagardin [34], cinnamycin [36], and mutacin A. The latter is produced by *Streptococcus mutans* during the formation of dental plaque [61] and is the most recently described class Ib bacteriocin.

Class II bacteriocins

This group comprises heat-stable peptides with molecular masses smaller than 10 kDa and with no modified amino acids. Members of this class can be further subclassified into four groups: (1) Group IIa consists of

Table 1 Antibiotic peptides classified according to Jack [31]

Antimicrobial peptide	Molecular mass (kDa)	Amino acids	Producer microorganism
Lantibiotics			
Actagardine	1.9	19	*Actinoplanes* spp.
Ancovenin	2.0	19	*Streptomyces* spp.
Cinnamycin	2.0	19	*Streptomyces cinnamoneus*
Duramycin	2.0	19	*Streptomyces cinnamoneus*
Epidermin	2.2	22	*Staphylococcus epidermidis*
Gallidermin	2.2	22	*Staphylococcus gallinarum*
Lanthiopeptin	2.0	19	*Streptoverticullum cinnamoneum*
Mersacidin	1.8	19	*Bacillus* sp.
Nisin	3.4	34	*Lactococcus lactis*
Pep5	3.5	34	*Staphylococcus epidermidis*
Subtilin	3.3	32	*Bacillus subtilis*
Cystibiotics			
Pediocin AcH/PA1	4.6	44	*Pediococcus acidilactici* H/PAC 1.0
Leucocin A/UAL 187	3.9	37	*Leuconostoc gelidum* UAL 187
Mesentericin Y 105	3.8	37	*Leuconostoc mesenteroides* Y 105
Sakacin A	4.3	41	*Lactobacillus sake* LB 706
Sakacin P	4.4	43	*Lactobacillus sake* LTH 674
Lactacin F	5.6	57	*Lactobacillus acidophilus* 11088
Carnobacteriocin A	5.1	53	*Carnobacterium piscicola* LV 17 A
Carnobacteriocin BM1	4.5	43	*Carnobacterium piscicola* LV 17 B
Carnobacteriocin B2	4.9	48	*Carnobacterium piscicola* LV 17 B
Cerein 7/8	4.9	56	*Bacillus cereus* Bc7
Thiolbiotics			
Lactococcin B	5.3	47	*Lactococcus lactis* subsp. *cremoris* 9 B4
No cysteine			
Lactococcin A	5.8	54	*Lactococcus lactis* subsp. *cremoris* 9 B4 *L. lactis* subsp. *cremoris* LMG 2130 *L. lactis* subsp. *lactis* bv. *diacetylactis* WM4
Lactococcin M[a]	4.3	48	*L. lactis* subsp. *cremoris* 9B4
Lactococcin N[a]	4.4	47	*L. lactis* subsp. *cremoris* 9B4
Lactococcin Gα[a]	4.3	39	*L. lactis* subsp. *lactis* LMG 2081
Lactococcin Gβ[a]	4.1	35	*L. lactis* subsp. *lactis* LMG 2081

[a]These two pairs of peptides act synergistically

anti-listerial peptides showing the consensus sequence *YGNGV* at their N-terminal sequence. The genetics, structure, synthesis, secretion and mode of action of this group of antibiotics, which includes the bacteriocins pediocin AcH/PA1, mesentericin Y 105, sakacin A, sakacin P, and carnobacteriocin B2, listed in Table 1, have been recently reviewed [21]. These compounds are bactericides that disrupt the integrity of the cytoplasmic membrane, producing ionic imbalance and leakage of organic phosphate to exert their killing action. (2) Group IIb consists of pore-forming complexes requiring two peptides for their activity. These two peptides can be either individually active but synergistic when acting together (enterocins L50A and L50B, [17]), or they may both be necessary for antimicrobial activity (lactococcins Gα/Gβ [46], lactococcins M/N [57], and plantaricins EF and JK [19]). (3) Group IIc includes all class II bacteriocins that do not fall into groups IIa or IIb (Table 2). Two types of bacteriocins can be found within this group: (a) antibiotics with one or two cysteine residues (thiolbiotics and cystibiotics, respectively), and (b) antibiotics without cysteine (lactococcin A and acidocin B). Note that some antibiotics belonging to class IIc are exported via a *sec*-dependent pathway, whereas others are exported by a *sec*-independent mechanism. The export pathway seems to be independent of the chemical structure of the bacteriocin. For instance, both lactococcin A and B are exported by a double-glycine *sec*-independent mechanism, although one of them corresponds to the thiolbiotic group and the other does not contain cysteine residues. Similarly, the primary structure of the mature divergicin A suggests a secondary structure for this antibiotic similar to that of others – such as cerein 7/8, enterocin B or carnobacteriocin A – which are exported by a *sec*-independent mechanism, although divergicin A exports occur via the classical *sec*-dependent pathway (Table 2).

Class III bacteriocins

This group consists of peptidic antibiotics that are heat-labile proteins with a molecular mass larger than 30 kDa. Most of them are produced by bacteria of the genus *Lactobacillus*. Members of this group are helveticin J [33], produced by *L. helveticus* 481, and lacticin B [6], produced by *L. acidophilus*.

Class IV bacteriocins

This group consists of either glycoproteins (lactocin 27) [56] or lipoproteins (lacstrepcins) [39] that require non-protein moieties for their activity.

Bacteriocins produced by Gram-negative bacteria

Kolter and Moreno [38] were principally interested in bacteriocins produced by gram-negative microorganisms. They introduced a classification scheme that distinguished lantibiotics, non-lantibiotic heat-stable bacteriocins, colicins, and microcins. Colicins and microcins were operationally defined on the basis of their molecular size (smaller than 10 kDa for microcins and larger for colicins), and they include compounds produced by bacteria belonging to the family of *Enterobacteriaceae* that are active specifically against gram-negative microorganisms. Colicins [4], the first bacteriocins discovered, are characterized by their narrow antibiotic spectrum and by displaying a bactericidal activity mediated by interaction with specific membrane receptors. Some of them are peculiar because their synthesis is controlled by a SOS-dependent mechanism that includes suicide of the producer cells [49]. Microcins, on the other hand, have many structural similarities with class II bacteriocins: their molecular size is smaller than 10 kDa, they are synthesized during stationary phase, and they are not under SOS control. Two peculiar members of this class are colicin V, the first bacteriocin described by Gratia in 1925, which can be considered as a microcin because of its molecular mass (6 kDa), and microcin C7, a modified heptapeptide that is considered to be the smallest antibiotic peptide described thus far [25].

Some relevant physical and chemical characteristics of bacteriocins

To perform their lethal activity, bacteriocins belonging to classes I and II must fulfill two principal requirements:

Table 2 Klaenhammer's class IIc bacteriocins

Bacteriocin	Producer strain	Molecular mass (Da)	Amino acids	p*I*	Number of cys-teine residues	Reference
Cerein 7/8	*Bacillus cereus* Bc7	4893	56	8.38	2	[47, 48]
Enterocin B	*Enterococcus faecium* T136	5465	53	9.70	2	[13]
	Enterococcus faecium CECT492					[45]
Carnobacteriocin A	*Carnobacterium piscicola* LV17A	5053	53	9.02	2	[62]
Lactococcin A	*Lactococcus lactis* LMG2130	5778	54	9.21	0	[29]
Lactococcin B	*Lactococcus cremoris* 9B4	5328	47	9.25	1	[58]
	Lactococcus lactis WM4					[54]
Divergicin A[a]	*Carnobacterium divergens* LV13	4224	46	9.96	2	[63]
Acidocin B[a]	*Lactobacillus acidophilus* M46	5754	59	7.38	0	[41]

[a]Bacteriocins exported using a *sec*-dependent mechanism

to be cationic and highly hydrophobic. Most small-size bacteriocins are active over a wide pH range (3.0–9.0), and while resistance to extreme pH values of 1.0 (acidocin B, [41]) and 11.0 (bavaricin A, [40]) has been observed, most of these bacteriocins are cationic at pH 7.0, lactocin S, with a net charge of –1 at neutral pH, being the exception. Their high isoelectric point [31] allows them to interact at physiological pH values with the anionic surface of bacterial membranes. This interaction can suffice, in the case of broad-spectrum bacteriocins, or facilitate, in the case of receptor-requiring compounds, insertion of the hydrophobic moiety into the bacterial membrane. Later, the cooperation between a number of bacteriocin molecules will build up the transmembrane pore responsible for gradient dissipation and cellular death. These features have favored the development of general purification protocols for bacteriocins that include hydrophobic interaction, cationic exchange and reverse-phase chromatographic steps [53].

Heat-stability is another major feature of low-molecular-weight bacteriocins. The complex pattern of monosulfide and disulfide intramolecular bonds helps in the stabilization of secondary structures by reducing the number of possible unfolded structures (entropic effect). From a structural point of view, the effect of the intramolecular bonds is additive, and the higher their number, the higher the global stability of the peptide [10]. In this context, Cintas et al. [18] observed that most supernatants of bacteriocin-producing strains are resistant to autoclaving conditions and to heat treatment (100 and 121 °C). However, some bacteriocins produced by *Lactobacillus* strains (helveticin J [33]) were inactivated by10- to 15-min treatments of 60–100°C. Bacteriocins can be easily recovered not only from classical cultures (Fig. 1), but also from complex sources such as rice-hull ash, where pediocin PA-1 was recovered by acid treatment [32]. More recently, divercin VH1 has been purified by a cationic-exchange step using the culture supernatant, which had been previously treated with a non-ionic detergent (Triton X-114) [43].

The most extensively studied bacteriocin, nisin, was discovered in 1928 [51]. It is produced by some *Lactococcus lactis* strains, consists of 34 amino acids and has five lanthionine bonds. This antibiotic shows a high correlation between pH and heat stability: at pH 2.0 it shows high solubility, antimicrobial activity, and thermostability (being active after a heat treatment of 100°C for 10 min [30]). Nisin is inactivated, on the other hand, at pH 7.0 and shows a low solubility at this physiological pH. These facts and the sensitivity of nisin to digestive enzymes discouraged the clinical application of this compound but made it a product of choice as food preservative [7].

Mode of action of bacteriocins

Due to the great variety of their chemical structures, bacteriocins affect different essential functions of the living cell (transcription, translation, replication, and cell wall biosynthesis), but most of them act by forming membrane channels or pores that destroy the energy potential of sensitive cells. The different modes of action of various types of bacteriocin produced by gram-positive bacteria have been reviewed by several authors [1, 21, 52]. In this section, we will summarize these studies and propose a mode of action for bacteriocins classified within Klaenhammer's class IIc group on the basis of their chemical structure and properties.

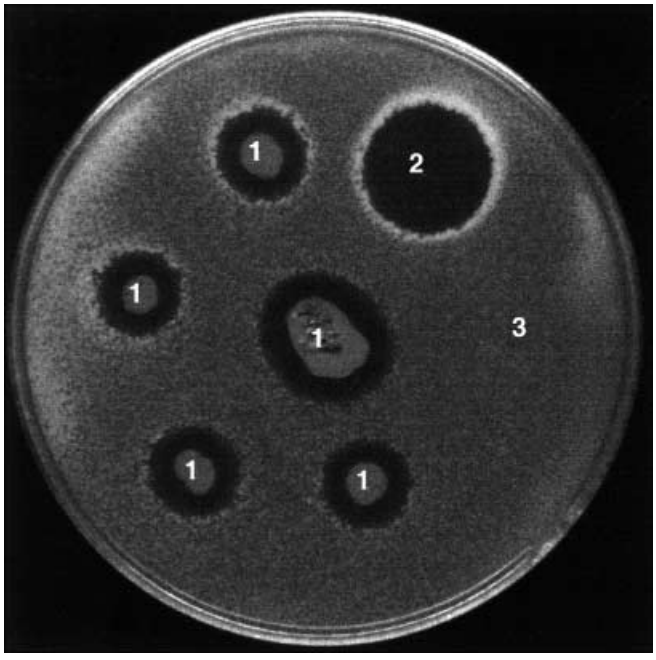

Fig. 1 Cerein 7/8 plate activity assay. *1* Picks of colonies of the producer strain *Bacillus cereus* Bc7, *2* spot of purified cerein 7, *3* spot of protease-treated cerein 7 on a lawn of sensitive *Listeria innocua*

Nisin (a compound belonging to group Ia, according to Klaenhammer's classification) is the bacteriocin whose mode of action has been studied the best. This cationic lantibiotic associates electrostatically with the negatively charged membrane phospholipids [2, 20], which favors subsequent interaction of bacteriocin's hydrophobic residues with the target cytoplasmic membrane. Lysine is the cationic amino acid involved in this electrostatic interaction – similar to the membrane interaction of mammal defensins mediated by arginine [24]. The interaction between the hydrophobic part of nisin and the bacterial target membrane generates unspecific ionic channels whose formation is aided by the presence of high transmembrane potentials, and by the presence of anionic and absence of cationic lipids [27]. Pore formation, on the other hand, decreases in the presence of divalent cations (Mg^{2+} or Ca^{2+}) because they neutralize the negative charges of the phospholipids, reducing the fluidity of the membrane. Nisin-generated membrane pores allow the passive efflux of ions (K^+ and Mg^{2+}), amino acids (glutamic acid, lysin), and ATP, but not of larger cytoplasmic proteins,

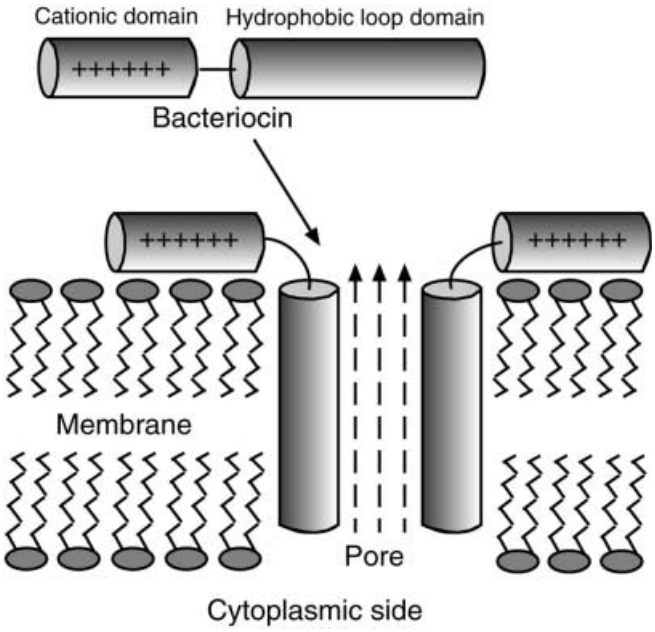

Fig. 2 Killing mechanism proposed for class IIc bacteriocins

yielding membrane potential and proton-motive-force dissipation and subsequent cell death [9].

The mode of action Klaenhammer's class IIa bacteriocins has been recently reviewed by Ennahar et al. [21]. The general scheme is similar to that just discussed for nisin. Class IIa bacteriocins show a pronounced anti-listerial specificity due to the presence of the sequence YGNGV in their N-terminal region [15]. The current mechanistic hypothesis to explain the mode of action of bacteriocins belonging to this class includes electrostatic binding of the antibiotic to the target membrane mediated by a putative membrane-bound receptor molecule [1, 60], although the necessity of this specific receptor is still controversial [16, 35]. The hypothetical receptor would be responsible for the recognition of the YGNGV anti-listerial motif present in these peptides.

As discussed above, bacteriocins belonging to Klaenhammer's class IIc can be subdivided into two different groups on the basis of the presence or absence of intramolecular disulfide bonds. Accordingly, the mode of action of both subtypes could be quite different. Membrane studies carried out on lactococcin A (a bacteriocin lacking cysteine residues) indicate that this antibiotic is a membrane-active protein whose primary mechanism of action is pore formation on sensitive cell membranes [59]. Similarly, the activity of cerein 7/8 (a cystibiotic) decreases as the osmolarity of the culture medium increases [47, 48], as expected for bacteriocins acting at the membrane level [52]. The wide antibiotic spectrum of these compounds suggests that the presence of a specific membrane-bound bacteriocin receptor is not necessary. In all class IIc bacteriocins, the presence of positively charged amino acids and tryptophan in the hydrophilic N-terminal region could facilitate an unspecific interaction with the negatively charged phospholipids of the target membrane further stabilizes the transmembrane pore [8] (Fig. 2). In the case of cerein 8, for instance, three tryptophan residues and one lysine residue are located in the eight amino-acid-long N-terminal region, providing a docking function in a minimal sequence (Oscáriz, unpublished results). This region is followed by a highly hydrophobic C-terminal portion that could participate in the formation of transmembrane pores in a similar way to that proposed for pediocin PA1 (class IIa) [22]. The cystibiotics belonging to class IIc (cerein 7/8, enterocin B, carnobacteriocin A and divergicin A) should have a secondary structure characterized by the presence of a large loop covering nearly the entire hydrophobic moiety. In all cases, the first cysteine residue is found after a positively charged lysine residue at the end of the hydrophilic stretch, and the second residue at the three last sequence positions. The disulfide bond, which is required for the antibacterial activity, should fold the C-terminal part of the protein into a compact hydrophobic structure very rich in glycine residues that should be structurally flexible. This flexibility would permit molecules to switch from β to α structures in response to the environmental hydrophobicity [28]. The length of the hydrophobic loop is enough to expand through the cytoplasmic membrane, and the aggregation of a number of loop structures would create the lethal pore.

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