

Class IIa bacteriocins: biosynthesis, structure and activity

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Received 5 July 1999; received in revised form 19 October 1999; accepted 21 October 1999

Abstract

In the last decade, a variety of ribosomally synthesized antimicrobial peptides or bacteriocins produced by lactic acid bacteria have been identified and characterized. As a result of these studies, insight has been gained into fundamental aspects of biology and biochemistry such as producer self protection, membrane–protein interactions, and protein modification and secretion. Moreover, it has become evident that these peptides may be developed into useful antimicrobial additives. Class IIa bacteriocins can be considered as the major subgroup of bacteriocins from lactic acid bacteria, not only because of their large number, but also because of their activities and potential applications. They have first attracted particular attention as listericidal compounds and are now believed to be the next in line if more bacteriocins are to be approved in the future. The present review attempts to provide an insight into general knowledge available for class IIa bacteriocins and discusses common features and recent findings concerning these substances. © 2000 Published by Elsevier Science B.V. All rights reserved.

Keywords: Bacteriocin; Class IIa bacteriocin; Biosynthesis; Structure; Mode of action; *Listeria*

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Abbreviations: ABC, ATP-binding cassette; *agr*, accessory gene regulator; GSP, general secretion pathway; HPK, histidine protein kinase; IF, induction factor; LAB, lactic acid bacteria; ORF, open reading frames; *pI*, isoelectric point; PMF, proton motive force; RR, response regulator; *sec*, signal sequence; X_{aa} , variable amino acid residue; $\Delta\psi$, transmembrane potential; ΔpH , pH gradient

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1. Introduction

In recent years, numerous food poisoning outbreaks, involving various pathogens and food products, and the increasing concern over the preservation of minimally processed foods have spurred growing awareness of the importance of food safety [1]. This has prompted new approaches to inhibit foodborne pathogens. In particular, there has been a renewed interest in the antimicrobial activity of lactic acid bacteria (LAB), which has been important for centuries in the preservation of food; today, it is the bacteriocin production. As a result of intense investigations on LAB bacteriocins, considerable progress has been made by both basic and applied research disciplines towards a better understanding of these substances and a large number of chemically diverse bacteriocins have been identified. Four classes of bacteriocins have then been defined based on observed common characteristics, mainly structural [2]. New bacteriocins are still being discovered and regularly reviewed and documented in books and reviews, with class I (lantibiotics) and class II (small heat-stable non-lanthionine-containing peptides) bacteriocins being the most abundant and thoroughly studied [3–5]. However, the state of bacteriocin classification requires constant review as the knowledge concerning various aspects of bacteriocin research rapidly accumulates and it appears that the term bacteriocin has been used to cover a wide range of chemically diverse substances which do not necessarily have much in common. These range from low to high molecular mass compounds and from simple unmodified peptides to highly posttranslationally modified peptides, with a wide range of biological activities. However, the data available may not be sufficient to formulate a definite and permanent natural classification scheme, and this requires constant review as more information on existing and novel bacteriocins is acquired.

As a consequence of recurring and serious listeriosis outbreaks [6,7], *Listeria monocytogenes* has come under the focus of bacteriocin investigators during the past decade. The search for bacteriocin-producing LAB has then been directed towards substances whose targets are *Listeria* spp., and consequently this has led to the description of a large number of antilisterial bacteriocins. These belong to either class I or II, but recent reports clearly showed that they are predominantly class IIa bacteriocins, a subgroup of bacteriocins classified on the basis of their strong amino acid sequence similarity, particularly their distinctive N-terminal part, and their strong antilisterial activity

[2,8]. In fact, all class IIa bacteriocins identified so far are highly active against *Listeria* strains, while only part of bacteriocins of other categories are antilisterial. Moreover, several class IIa bacteriocins appear to have potential applications against other spoilage and foodborne pathogenic microorganisms, since they display spectra of activity broad enough to encompass undesirable bacteria, such as spoilage LAB, *Brochotrix* spp., *Clostridium* spp., *Bacillus* spp. and *Staphylococcus* spp. [9–11]. They are now the most promising bacteriocin candidates for various industrial applications, not only due to their biological activity, which is generally higher than that of other bacteriocins [9], but also due to their physicochemical properties.

Fourteen members of the class IIa have been well characterized and their structures described primarily due to the development of efficient and standardized protocols for purification of these hydrophobic and cationic peptides. Though they seem to form a relatively homogeneous group, with properties that can be reasonably matched in a review, these bacteriocins have not yet been thoroughly reviewed.

The present review summarizes the accumulated knowledge on class IIa bacteriocins with a particular emphasis on their structure, synthesis and biological activities. The more explicit discussion deals with some of the most extensively characterized class IIa bacteriocins. For better clarity, we discuss class IIa bacteriocins as a separate subgroup according to the generally admitted classification, though many for the characteristics of this subgroup may as well apply to other bacteriocins, particularly within the class II.

2. General nature of class IIa bacteriocins

2.1. Composition and primary structure

Investigations of a wide range of class II bacteriocins have led to the delineation of two subgroups within this class [2,4]: IIa, *Listeria*-active peptides with a consensus sequence in the N-terminus of YGNGVXaaC and IIb, poration complexes formed by oligomers of two different proteinaceous peptides. The existence of a third subgroup (IIc), which has been successively suggested to contain thiol-activated [2] and *sec*-dependent secreted bacteriocins [4], is uncertain. In fact, it has now been disclosed that the so-called thiol-activated bacteriocins can act with their cysteine residues being oxidized [12] and a *sec*-dependent

Bacteriocin	Amino acid sequence	Reference
Leucocin A	K Y Y G N G V H C T K S G C S V N W G E A F S A G V H R L A N G G N G F W	[15]
Mesentericin Y105	K Y Y G N G V H C T K S G C S V N W G E A S A G I H R L A N G G N G F W	[25]
Mundticin	K Y Y G N G V S C N K K G C S V D W G K A I G I I G N N S A A N L A T G G A A G W S K	[21]
Piscicolin 126	K Y Y G N G V S C N K N G C T V D W S K A I G I I G N N A A A N L T T G G A A G W N K G	[11]
Bavaricin A	K Y Y G N G V H C G K H S C T V D W G T A I G N I G N N A A A N X A T G X N A G G	[99]
Sakacin P	K Y Y G N G V H C G K H S C T V D W G T A I G N I G N N A A A N W A T G G N A G W N K	[23]
Pediocin PA-1	K Y Y G N G V T C G K H S C S V D W G K A T T C I T N N G A M A W A T G G H Q G N H K C	[16]
Bavaricin MN	T K Y Y G N G V Y C N S K K C W V D W G Q A A G G I G Q T V V X G W L G G A I P G K	[17]
Divercin V41	T K Y Y G N G V Y C N S K K C W V D W G Q A S G C I G Q T V V G G W L G G A I P G K C	[20]
Enterocin A	T T H S G K Y Y G N G V Y C T K N K C T V D W A K A T T C I A G M S I G G F L G G A I P G K C	[13]
Enterocin P	A T R S Y Y G N G V Y C N N S K C W V N W G E A K E N I A G I V I S G W A S G L A G M G H	[9]
Carnobacteriocin BM1	A I S Y G N G V Y C N K E K C W V N K A E N K Q A I T G I V I G W A S S L A G M G H	[8]
Sakacin A	A R S Y G N G V Y C N N K K C W V N R G E A T Q S I I G G M I S G W A S G L A G M	[22]
Carnobacteriocin B2	V N Y G N G V S C S K T K C S V N W G Q A F Q E R Y T A G I N S F V S G V A S G A G S I G R R P	[8]
Bacteriocin 31	A T Y Y G N G L Y C N K Q K C W V D W N K A S R E I G K I I V N G W V Q H G P W A P R	[26]
Acidocin A	K T Y Y G T N G V H C T K R S L W G K V R L K N V I P G T L C R K Q S L P I K Q D L K I L L G W A T G A F G K T F H	[27]

Fig. 1. Sequence alignment of class IIa bacteriocins, on the basis of the N-terminal YGNGV consensus motif, and of bacteriocin 31 and acidocin A. White boxes enclose residues conserved in at least 10 of the sequences shown. Shaded boxes enclose C-terminal residues conserved in at least two sequences. Amino acid residues shown in: boldface are positively charged; lowercase are uncertain residues; and Xs are unknown residues. Sequences of the following bacteriocins are respectively identical; piscicolin 126 and piscicocin V1a [13]; carnobacteriocin BM1 and piscicocin B1b [13]; sakacin A and curvacin A [131]; pediocin PA-1, pediocin AcH [68] and pediocin SJ-1 [132].

secreted bacteriocin has recently been discovered among class IIa bacteriocins [9]. The class IIa is the largest and most extensively studied subgroup of class II bacteriocins that are especially strong inhibitors of the foodborne pathogen *L. monocytogenes* [8,9,11,14–24]. Because of this antilisterial effectiveness, class IIa bacteriocins have significant potential as biopreservatives in a large number of foods.

Class IIa bacteriocins that are identified so far contain between 37 (leucocin A and mesentericin Y105 (also called Y 10537, although the term Y105 is used in this review)) and 48 residues (carnobacteriocin B2) and they share considerable sequence similarity (Fig. 1). They were first described based on the presence in their N-terminal halves of the YGNGVXaaC motif, which has been suggested to be part of a recognition sequence for a speculated membrane-bound protein 'receptor' [25]. Fig. 1 shows that as new class IIa bacteriocins emerge the number of residues in the common N-terminal motif increases and now appears as: YGNGVXaaCXaa(K/N)XaaXaaCXaaV(N/D)(W/K/R)-Xaa(G/A/S)(A/N) (i.e. amino acid residues with low variability are inside brackets, residues in capitals can be replaced with residues in lowercase, and those with higher variability are represented by Xaa) [14]. It has been commonly reported that there are low sequence similarities in the C-terminal portion of class IIa bacteriocins, however as new members emerge it appears that subgroups may be defined based on C-terminal sequence similarities. For example: (1) bavaricin MN, divercin V41 and enterocin A; (2) enterocin P, carnobacteriocin BM1 and sakacin A; and (3) bavaricin A, mundticin, piscicolin 126 and sakacin P,

and to a lesser extent pediocin PA-1/AcH. As for carnobacteriocin B2, containing an IXaaXaaXaaXaaSGXaaA motif, and leucocin A (and the very similar mesentericin Y105), containing an AXaaGGXaaXaaXaaW motif, they appear unrelated to the second and the third subgroups, respectively and seem to have a distinctive C-terminal region. Intriguingly, bacteriocins included in each of these subgroups (based upon C-terminal similarity) also share similarities in the sequence that occur in the extreme region of the N-terminal domain prior to the consensus motif. Class IIa bacteriocins share between 34 and 80.5% sequence identity, with the exception of leucocin A and mesentericin Y105, which vary only in the amino acids at positions 22 and 26. Additionally, several independently investigated class IIa bacteriocins have been shown to be identical to others (see Fig. 1, legend).

It is noteworthy that bacteriocin 31, which possesses an altered N-terminal sequence (YGNGVXaaC) and thereby shares an incomplete consensus motif with the rest of class IIa bacteriocins (Fig. 1), is antilisterial [26], thus rising questions on whether this bacteriocin should be considered a class IIa member and suggesting that the classification scheme will be evolving with the accumulation of knowledge and the appearance of new bacteriocins. For instance, the motif YGNG is already being considered by some authors as the 'real' consensus motif [10]. A similar case is represented by acidocin A in which the YGNGV-XaaC motif is split by a threonine residue (YGTNGV-XaaC) [27] (Fig. 1).

Another unifying feature of class IIa bacteriocins is their net positive charge, with pIs varying from 8.3 to 10.0,

Table 1
Properties of class IIa bacteriocins, bacteriocin 31 and acidocin A

Bacteriocin	Molecular mass (Da)	Number of amino acids	pIa	Net charge	% Of amino acid group				Amino acids absent	Amino acid composition				
					Nonpolar	Polar	Basic	Acidic		K	H	R	D	E
Leucocin A	3390	37	8.8	+4	30	54	13	3	IMQDP	2	2	1	–	1
Mesentericin Y105	3868	37	8.8	+4	30	54	13	3	MPQD	2	2	1	–	1
Mundticin	4287	43	9.7	+4	33	53	12	2	MFPQEHR	5	–	–	1	–
Piscicocin	4416	44	9.3	+3	32	57	9	2	MFPQEHR	4	–	–	1	–
Bavaricin A	3500–4000	41	9.3	+3	27	51	10	2	LMFPQCER	2	2	–	1	–
Sakacin P	4434	43	8.8	+4	30	56	12	2	LMFPQER	3	2	–	1	–
Pediocin AcH	4623	44	8.6	+6	25	57	16	2	LFPER	4	3	–	1	–
Bavaricin MN	4769	42	9.3	+3	33	52	10	2	MFEHR	4	–	–	1	–
Divercin V41	4509	43	8.6	+3	30	58	9	3	MFEHR	4	–	–	1	–
Enterocin A	4829	43	8.9	+4	30	56	12	2	PQER	4	1	–	1	–
Enterocin P	4493	44	8.3	+2	36	50	9	5	FPQD	2	–	1	–	1
Piscicocin V1b	4526	43	9.0	+3	37	46	12	5	FPDR	4	1	–	–	2
Sakacin A	4306	41	9.6	+3	34	54	10	2	FPDH	2	–	2	–	1
Carnobacteriocin B2	4967	48	10.0	+5	31	56	11	2	LMDH	4	–	3	–	1
Bacteriocin 31	5005	43	9.8	+5	37	42	16	5	MF	4	1	2	1	1
Acidocin A	6500	58	10.7	+12	36	40	22	2	ME	9	2	2	1	–

^aValues calculated according to the SDC-GENETYX genetic information processing software.

thereby showing close net charges at various pH values (Table 1). However, this is a rather common feature of all LAB bacteriocins [3]. Class IIa bacteriocins present a number of other close characteristics which relate to the presence of particular groups of residues: a high content of nonpolar amino acid residues and small amino acids such as glycine, which is thought to confer to these bacteriocins a high degree of conformational freedom [17] (Table 1). The highly conserved N-terminal hydrophilic domain clearly contrasts with the moderately conserved hydrophobic and/or amphiphilic C-terminal domain, and this variation affects bacteriocin activity on target membranes [28].

2.2. Predicted secondary and tertiary structures

While it is well established that nisin has a flexible structure in aqueous solutions and several constrained regions in more hydrophobic environments [5], until recently, proposals concerning secondary and the tertiary structures of class IIa bacteriocins have remained quite speculative. Yet, recent studies using nuclear magnetic resonance data, circular dichroism and computer simulation, investigated structural models for class IIa bacteriocins in membrane-mimicking environments [14,17,29–31] (see Fig. 5a). Experimental evidence indicates that class IIa bacteriocins exist primarily in unstructured conformations, generally random coils in watery solutions, whereas in nonaqueous solutions they adopt a partly helical structure with varying amounts of hydrophobicity and other defined secondary structures [2,3,25,29,32].

One important characteristic of class IIa bacteriocins is their cysteine content. In fact, while other non-lanthionine containing bacteriocins may include only one cysteine residue (e.g. the thiol-activated or thiolbiotic lactococcin B

[33,34] or no cysteine residue at all (lactococcins A [35,36], M [34] and G [37], and plantaricin A [38]), class IIa bacteriocins are cystibiotics, i.e. they have at least two cysteines with disulfide bridges. In the alignment of class IIa bacteriocins (Fig. 1), it appears that the two cysteine residues in the N-terminal domain are present in conserved positions, and consequently the disulfide bridge which forms a six-membered ring over these two residues is well conserved in all class IIa bacteriocins. Moreover, pediocin PA-1/AcH, enterocin A and divercin V41 are unique in the sense that they possess an extra disulfide bond involving a second pair of cysteine residues, while other class IIa bacteriocins have only two cysteine residues with a single disulfide bond. The presence of disulfide bonds seems to be crucial for the activity of class IIa bacteriocins, especially those with two disulfide bridges [10] (see below).

The N-terminus of class IIa bacteriocins is believed to contain β -sheets maintained in a β -hairpin conformation that is stabilized by the N-terminal disulfide bridge. This conformation would give class IIa bacteriocins an amphiphilic characteristic in the N-terminal region [31,39]. An N-terminal β -sheet domain, with two strands connected by cysteines to form a β -hairpin at the position YGNGV, has been predicted for pediocin PA-1 (residues 1–18) and leucocin A (residues 2–16) [14,30,31]. Also, secondary structure analysis of piscicocins V1a and V1b suggested that the N-terminal disulfide bond stabilizes a loop which is predicted as a β -hairpin at the YGNGV sequence [14].

The C-terminal half of class IIa bacteriocins has been predicted to adopt an amphiphilic α -helix, spanning similar regions in different molecules and generally leaving a nonhelical portion of only one or two C-terminal residues [2,9,14,25,30,39,40]. This helical portion is believed to be

the transmembrane segment during pore formation in a sensitive cell membrane [2,3,21,28].

A recent study by Bennik et al. [21], who have investigated secondary structure prediction for nine class IIa bacteriocins, indicates that all bacteriocins studied, interestingly contain domains, spanning residues 15,16–27,28, that are predicted to adopt α -helical conformations. These structures meet the criteria of oblique-oriented α -helical peptides, which have been identified in biological processes in which membrane perturbation underlies the action of the peptide [41]. Therefore, as also reported by Bughaloo-vial et al. [14] for piscicocins, class IIa bacteriocins may well contain two different α -helical regions.

2.3. Prevalence among LAB bacteriocins

All class IIa bacteriocins are produced by LAB, such as *Lactobacillus* spp., *Enterococcus* spp., *Pediococcus* spp., *Leuconostoc* spp., and *Carnobacterium* spp. Interestingly, unlike other LAB bacteriocins, all class IIa bacteriocins are produced by food-associated strains, which have been isolated from a variety of food products of industrial and natural origins: mainly from meat and dairy products, but also from vegetables (Table 2).

Some class-IIa-bacteriocin producers produce more than one bacteriocin, a phenomenon which may be quite common among bacteriocin-producing LAB strains. The bacteriocins produced may belong to the pediocin family (Table 2), with only slight differences or no difference at all between their inhibitory spectra [8,14]. One of the bacteriocins produced may belong to another bacteriocin group, like enterocin B, a non class IIa bacteriocin, pro-

duced along with the class IIa enterocin A by *Enterococcus faecium* CTC492 [10,42].

Conversely, LAB strains of different origins, species and genera have been shown to produce the same class IIa bacteriocin (Table 2). This observation suggest that production of class IIa bacteriocins, while confined to LAB, seems to be a quite mobile trait (transfer would be quite common among LAB strains) and no class IIa primary structure seems to be distinctive of a particular LAB genus or species.

3. Genetics of bacteriocin synthesis and secretion

3.1. General genetic organization

Information on the genetic requirements and organization for the expression of most class IIa bacteriocins is still scant, and genetic investigations have yet to be carried out for newly discovered peptides. Fig. 2 shows comparative overviews of the genetic organization of studied DNA fragments encoding class IIa bacteriocins. While bacteriocin production is often correlated with the presence of a plasmid, genes encoding several class IIa bacteriocins have been shown to be located on chromosome fragments [13,20,43,44]. Yet, whether plasmid-borne or chromosomal, the structural gene for class IIa bacteriocins and its surrounding regions reveal one to three operon-like structures, which may be divergently transcribed and are involved in the bacteriocin production and extracellular translocation, the immunity of the producers, and in several cases, the regulation of bacteriocin synthesis [4]. The presence of such regulatory genes has been convincingly

Table 2
Lactic acid bacteria producing class IIa bacteriocins and their origins

Bacteriocin	Producer	Strain	Source	Reference
Leucocin A/B-Ta11a	<i>L. gelidum</i>	UAL187	Vacuum packaged processed meat in Canada	[133]
	<i>Leuconostoc carnosum</i>	Ta11a	Vacuum packaged processed meat in South Africa	[134]
Mesentericin Y105	<i>Leuconostoc mesenteroides</i>	Y105	Goat's milk in France	[24]
Divercin V41	<i>C. divergens</i>	V41	Fish viscera	[20]
Carnobacteriocin B2	<i>Carnobacterium piscicola</i>	LV17B	Fresh pork packaged in modified atmosphere	[135]
Carnobacteriocin BM1		LV17B	Fresh pork packaged in modified atmosphere	[135]
Piscicocin		V1	Fish	[136]
Piscicocin V1a		V1	Fish	[136]
Piscicolin 126		JG126	Spoiled ham	[11]
Mundticin	<i>Enterococcus mundtii</i>	ATO6	Fresh chicory endive	[21]
Enterocin A	<i>E. faecium</i>	CTC492/T136	Spanish dry fermented sausage	[13]
Enterocin P		P13	Spanish dry fermented sausage	[9]
Pediocin PA-1/AcH/SJ-1	<i>Pediococcus parvulus</i>	ATO34/ATO77	Fresh chicory endive	[137]
	<i>Pediococcus acidilactici</i>	H	Fermented sausage	[138]
		PAC 1.0		[139]
		SJ-1	Naturally-fermented sausage in Israel	[140]
Curvacin A	<i>Lactobacillus plantarum</i>	WHE92	Soft cheese in France	[141]
	<i>Lactobacillus curvatus</i>	LTH1174	Fermented sausage	[131]
Sakacin A	<i>Lactobacillus sake</i>	Lb706	Raw meat	[142]
Sakacin P		Lb674	Meat	[143]
Bavaricin A		MI401	Sour doughs	[18]
Bavaricin MN		MN	Meat	[144]

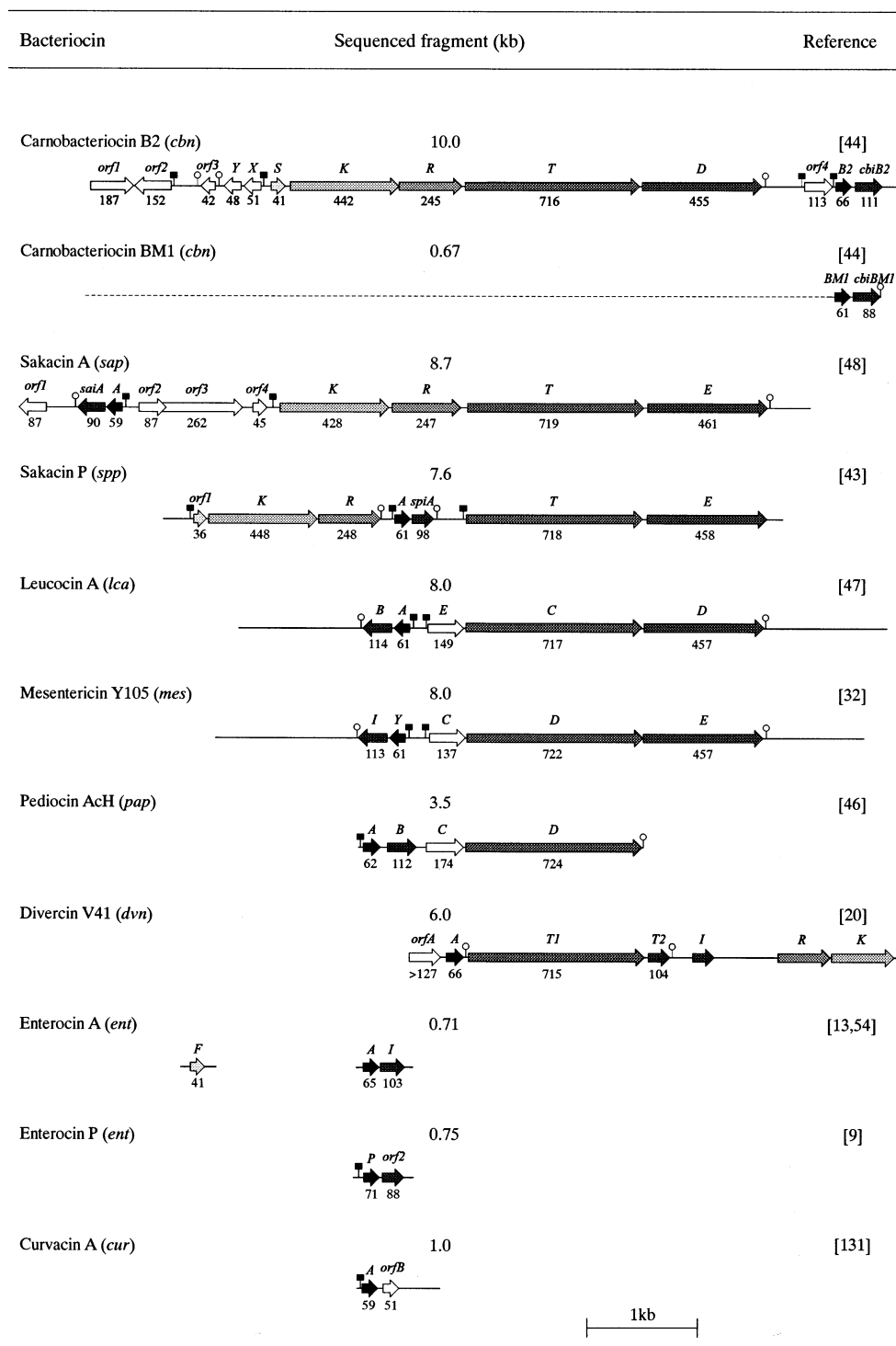


Fig. 2. Organization of the gene clusters involved in the production and immunity of class IIa bacteriocins. Open reading frames (ORFs) encoding the following proteins are marked by arrows with the corresponding shading patterns: bacteriocin's prepeptide (black); immunity protein (darkest gray); ABC-transporter (dark gray); accessory protein (medium dark gray); induction factor's prepeptide (medium light gray); histidine protein kinase (light gray); response regulator (lightest gray); product with unidentified function (white). The number of amino acid residues within each encoded protein is shown below the corresponding ORF. The size of the sequenced fragment is also indicated; in some, only two ORFs have been identified. Promoters (dark boxes), terminators (lollipop-like symbols), and overlapping sequences (overlapping arrows) are shown where information is available. With the exception of the structural and the immunity gene, the same cluster exists for carnobacteriocins B2 and BM1.

demonstrated for six class IIa bacteriocins, and this could probably be the case for all class IIa bacteriocins. In fact, more extensive investigations have to be carried out concerning bacteriocins which revealed no regulatory systems in their the genetic organization [9,32,45–47], mainly because the DNA fragments that have been studied were apparently too short to span the whole bacteriocin locus.

With the exception of the divercin V41 locus, whose unusual genetic organization is ascribed to important DNA rearrangements [20], class IIa bacteriocins show a remarkable conservation of gene arrangement (Fig. 2) [20,32,36,43–49]. In particular, the bacteriocin structural gene encodes a pre-form of the bacteriocin called pre-peptide containing a leader sequence with two glycine residues at its C-terminus, which may serve as a recognition signal for a *sec*-(signal sequence) independent ABC- (ATP-binding cassette) transporter [2,4,50] (see below). In fact, operons of most class IIa bacteriocins possess at least two genes encoding proteins homologous to ABC-transporters and their accessory proteins, which are apparently required for the externalization of the bacteriocins (Fig. 2). Also, the bacteriocin structural gene always precedes and is cotranscribed with an immunity gene as a small operon. For transcriptionally regulated bacteriocins, additional open reading frames (ORFs) are present in the vicinity of the structural gene, always in the same order, and form a putative three-component signal-transduction autoregulatory cassette (see below) encoding a possible induction factor (IF), a histidine protein kinase (HPK) and a response regulator (RR) (Fig. 2).

3.2. Bacteriocin biosynthesis

Characteristically, class IIa bacteriocins, like other low-molecular-mass bacteriocins, are first formed as ribosomally synthesized precursors or pre-peptides, which appear not to be biologically active and contain an N-terminal extension or leader sequence. Subsequent cleavage of the prepeptide at a specific processing site removes the leader sequence from the antimicrobial molecule concom-

itantly with its export to the outside of the cell [51]. The leader peptide's removal during transmembrane translocation, is accomplished by the same protein that is associated with the bacteriocin transport [4,51]. The amino acid sequence of a number of class-IIa-bacteriocin leader peptides, which vary in length from 18 up to 27 residues, has been determined (Fig. 3). One important feature of the majority of these leaders is the presence of two glycine residues in the C-terminus, at positions –2 and –1 relative to the processing site, though this is not distinctive of the class IIa. These leaders are believed to serve as signal peptides for the processing and the secretion of class IIa bacteriocins, independently of the GSP, by a dedicated transport system involving two distinct proteins: an ABC-type translocator and an accessory protein. The two conserved glycine residues may serve as a recognition signal for this *sec*-independent transporter system [2,4,50].

In addition to the two conserved glycine residues, consensus elements found in the double-glycine leaders of class IIa bacteriocins include other residues. Sequence similarity is particularly high for leaders with the same size (Fig. 3). Furthermore, if we consider both the charges and the hydrophobicity of the individual amino acid residues within the double-glycine leaders of class IIa bacteriocins, it appears that these characteristics have been consistently conserved in the corresponding positions. Alignment of class-IIa-bacteriocin leaders reveals a remarkable degree of similarity in their hydropathic profiles; hydrophobic residues are found at positions –4, –7, –12 and –15, while hydrophilic residues are found at positions –5, –6 and –11 (Fig. 3). Also, the net charges of these leaders at pH 7.0 range from –3 for leucocin A to +1 for enterocin A, pediocin AcH and mesentericin Y105. These similarities between double-glycine leaders suggest that the corresponding ABC-transporters and associated proteins are also similar, which may allow the heterologous expression of a class IIa bacteriocin using a secretion machinery, probably more efficient, of another one (see below).

While all other class IIa bacteriocins investigated appear

Bacteriocin	Leader sequence	Reference
Sakacin A/curvacin A	M N N V K E L S M T E L Q T I T G G	[48,131]
Carnobacteriocin BM1	M K S V K E L N K K E M Q Q I I G G	[44]
Carnobacteriocin B2	M N S V K E L N V K E M Q L H G G	[44]
Sakacin P	M E K F I E L S L K E V T A I T G G	[43]
Enterocin A	M K H L K I L S I K E T Q L I Y G G	[13]
Pediocin AcH/PA-1	M K K I E K L T E K E M A N I I G G	[45,46]
Acidocin A	M I S M I S S H Q K T L T D K E L A L I S G G	[27]
Divercin V41	M K N L K E G S Y T A V N T D E I K S I N G G	[20]
Leucocin A	M M N M K P T E S Y E Q L D N S A L E Q V V G G	[15]
Mesentericin Y105	M T N M K S V E A Y Q Q L D N Q N L K K V V G G	[32]
Enterocin P	M R K K L F S L A L I G I F G L V V T N F G T K V D A	[9]
Bacteriocin 31	M K K K L V I C G I I G I G F T A L G T N V E A A T	[26]

Fig. 3. Sequence alignment of leader peptides for class IIa bacteriocins on the basis of the C-terminal double-glycine motif.

to be secreted via a dedicated transport system, the newly characterized enterocin P has been shown to be synthesized without a double-glycine leader, but with a typical *sec*-type signal peptide, thereby being processed and exported by the GSP (Fig. 3) [9]. Evidence that Class IIa bacteriocins can be processed and secreted by two different pathways has then been provided.

3.3. Synthesis regulatory system

Regulatory systems for class IIa bacteriocins typically include an HPK, an RR and an IF, which is required as a signal to induce the transcription of target genes (Fig. 4) [52,53]. Like the corresponding class IIa bacteriocins, the IFs are small, heat-stable, cationic and hydrophobic peptides that are first synthesized as prepeptides with leader sequences of the double-glycine type [4,54]. A three-component system (IF, HPK and RR) has been shown to be responsible for production of most regulated class IIa bacteriocins [4,43,48,54–56]. Only, the recently discovered divercin V41 of *Carnobacterium divergens* V41 has been suggested to depend upon a two-component signal-transduction system [20]. In addition, the production of carnobacteriocins have been interestingly found to involve both systems [44,57].

It is suggested that the three-component system could

be first triggered as a consequence of an excess in IF concentration occurring through slow accumulation, as a consequence of cell growth [4]. Within such a mechanism, the IF may function as a cell density signal, which seems to be largely reported [4,44,54,55]. Some authors however sustain the idea that signal-transducing system for class IIa bacteriocins, may mediate the response to an environmental signal [43,48]. In fact, LAB are known to be more or less capable of producing bacteriocins depending on environmental conditions [58–61], which could possibly be connected to the bacteriocin regulatory system. Nilsen et al. [54] have suggested that environmental factors may affect the binding of the IF to the HPK or the balance of phosphorylation required for the activation of the RR. Nevertheless, still little or nothing is known about the mechanisms and the extent of implication of environmental factors in this system.

3.4. Processing and secretion

With the exception of enterocin P, transmembrane translocation of class IIa bacteriocins is mediated by an ABC-transporter and an accessory protein, which are two membrane-bound proteins that form their own dedicated transport system [4,8,13,20,32,43–48,62]. Deletion mutations in the genes encoding one of these proteins generally

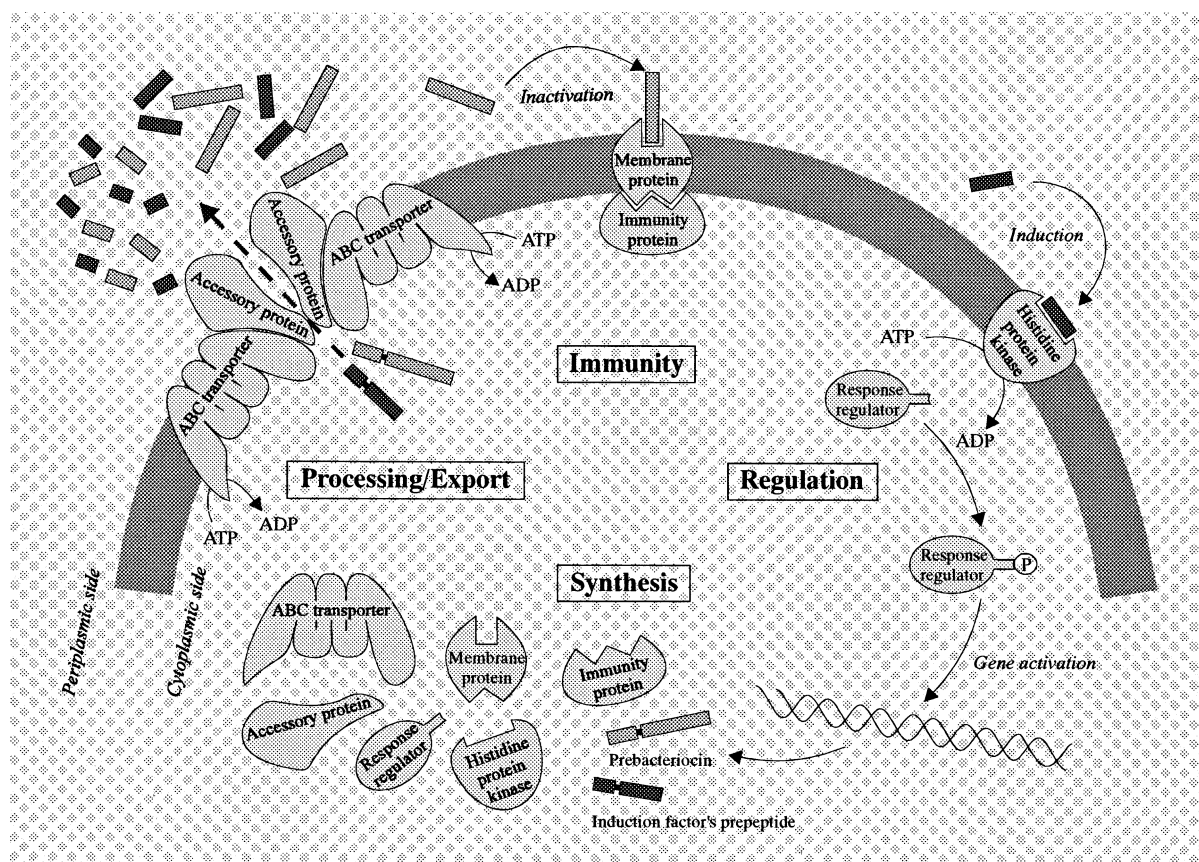


Fig. 4. Schematic overview of the suggested machinery for production of class IIa bacteriocins: three-component regulatory system, synthesis, processing, excretion and immunity.

results in complete loss of bacteriocin production [20,32,43–48].

Class-IIa-bacteriocin ABC-transporters/associated proteins are involved in the translocation of proteins lacking typical N-terminal signal peptides. The highest similarities have been shown with proteins of the ABC-transport systems HlyB/HlyD and ComA/ComB, responsible for the secretion of hemolysin A in *Escherichia coli* and a competence factor in *Staphylococcus pneumoniae*, respectively [20,32,43–48,63–66]. High homology of class-IIa-bacteriocin dedicated transport system has also been reported with the LcnC/LcnD system, where LcnC is an ABC-transporter and LcnD an accessory protein, both necessary for the externalization of the class II bacteriocin lactococcin A [32,36,43,44].

Putative class-IIa-bacteriocin ABC-transporters are proteins ranging from 715 for divercin V41 to 724 amino acids for pediocin PA-1/AcH (Fig. 2), which display particularly high degrees of homology in their N-terminal and C-terminal parts [20,45,46]. Yet, there are no distinctive structural characteristics of class-IIa-bacteriocin ABC-transporters, since most of their features are shared by a wide range of ABC-transporters. The N-terminal and C-terminal regions are structurally different and have been reported to be crucial for the activity of ABC-transporters. The C-terminal part of ABC-transporters contains a highly conserved ATP-binding domain, within the 200 terminal amino acids, unique to ABC-transporters [32,47,67]. The N-terminal region is a hydrophobic integral membrane domain, which displays a unique characteristic by carrying an extension of approximately 150 amino acids, also present in HlyB, and ComA [20,51]. This 150-amino acid peptide has been shown to be able of cleaving off the leader peptide, specifically at the C-terminus of the double-glycine motif [51]. A functional study by Venema et al. [62] on the N-terminal part of the ABC-transporter PedD (for pediocin PA-1) has further confirmed the role of this domain in proteolytic removal of the leader peptide and suggested that this region is not involved in the secretion process. According to Håvarstein et al. [51], the leader peptide serves as a recognition signal for both the cleavage of the bacteriocin prepeptide and the transmembrane translocation of the mature molecule, which are naturally closely integrated processes (Fig. 4). The proteolytic domain of the ABC-transporter may bind to a prepeptide's leader sequence, which triggers the ATP hydrolysis and subsequent conformational changes in the transporter, resulting in leader's removal and translocation of the mature bacteriocin across the cytoplasmic membrane [51].

Accessory proteins for export of class IIa bacteriocins consist of about 460 amino acids, with sequences sharing significant homology [32,43,44,47,48]. An exception is PedC/PapC (see Fig. 2), which has been speculated to have a function comparable to that of an accessory protein, while consisting of only 174 amino acids [45,46]. Accessory proteins consist of a large hydrophilic C-terminal

region and a hydrophobic N-terminal section that might span the membrane [45–47,68]. They are postulated to facilitate the membrane translocation and/or help in the processing of the leader peptide (Fig. 4), and have been shown to be required for successful externalization of class IIa bacteriocins [47,62]. However, their specific role of the in the translocation process is still not fully understood.

Expression of class IIa bacteriocins using heterologous secretory pathways, with and without substituting the signal peptides, has been investigated in recent studies. Indeed, similarities between the double-glycine leaders and between the corresponding transport proteins of class IIa and other class II bacteriocins, allow the production of a bacteriocin using the dedicated transport system of a different one and the production of multiple bacteriocins with the dedicated transport system of a single bacteriocin [32,69,70–74]. On the other hand, by substituting their double-glycine leader with an appropriate signal peptide, class II bacteriocins could be produced by the GSP rather than a dedicated transport system, which offers the advantage of decreasing the amount of genetic information required for independent bacteriocin expression [69]. This reveals a new trend of bacteriocin research which may prove of paramount interest in enhancing antimicrobial efficiency of bacteriocin producers in food. Particularly, this would allow the development of LAB producing multiple bacteriocins, each one having its specific range of target bacteria, in order to encompass a broad range of undesirable organisms.

Heterologous expression of class IIa bacteriocins using secretion systems of other class II bacteriocins, and vice versa, could be achieved to a limited extent. For instance, secretion of lactococcin A in *Leuconostoc gelidum* UAL187 could be partially achieved using the ABC-transporter and associated protein of leucocin A, while the transporters of lactococcin A failed to complement the deficiency of a translocatory apparatus for secretion of leucocin A [47]. Similarly, another class IIa bacteriocin (pediocin PA-1) could hardly be secreted in a lactococcal host using the lactococcin A secretion apparatus, since bacteriocin yield did not exceed 1% of that displayed by the parental *Ped-iococcus* strain [75]. Low levels of mesentericin Y105 have also been obtained when this bacteriocin was expressed in a *Lactobacillus* strain using the lactacin F dedicated transport system [32]. Yet, relatively high pediocin PA-1 yield (75% of the production level in the parental strain) could be achieved by the lactococcin A secretion machinery, when the secretion was directed by the lactococcin A signal peptide [71]. This shows that some double-glycine leaders may be poorly recognized by heterologous ABC-transporters and further demonstrates the importance of the leader sequence in recognition of the prepeptide by the secretion apparatus. Most certainly, the accessibility by a bacteriocin of a dedicated transport system of another bacteriocin is closely related to the degree of similarity between their leader peptides.

On the other hand, the flexibility of the secretory system of class IIa bacteriocins could be illustrated by their expression via the GSP. Processing and export of carnobacteriocin B2 in the absence of its specific secretion genes could be achieved in a lactococcal host using the genetic information, including the leader peptide, necessary for the secretion of divergicin A, a bacteriocin that utilizes the GSP [69,76]. Using the same heterologous secretion pathway, mesentericin Y105 could be expressed in *Leuconostoc* hosts, but not in lactococcal hosts [70]. However, the expression of both carnobacteriocin B2 and mesentericin Y105 by the GSP has been shown to be less efficient than by the native dedicated transport system, which has been ascribed mainly to possible higher effectiveness of a dedicated transport system as compared to the GSP. Conversely, the secretion machinery and leader peptide of class IIa bacteriocins could be used to direct secretion of bacteriocins that normally utilize the GSP [74,76].

Yet, there is no conclusive evidence regarding the reason why substitution of the dedicated transport system of a class IIa bacteriocin generally leads to a lower level of bacteriocin production than with the native dedicated transport system and why, despite using the bacteriocin's whole dedicated transport system, heterologous expression could not be achieved in certain host bacteria [70]. Further study of the factors that determine recognition, secretion, and processing of class IIa bacteriocins by the secretion apparatus will facilitate the development of more efficient heterologous secretion systems for these compounds.

3.5. Immunity of the producers

Evidence for the production of immunity proteins, consisting of 88 to 114 amino acids, by producers of class IIa bacteriocins has been reported in all the cases investigated (Fig. 2). In addition to providing total immunity against the producer's bacteriocin, these immunity proteins appear to also provide 'partial' protection against other class IIa bacteriocins [10]. This suggests a priori a connection between the, so far, two distinct notions of bacteriocin resistance and immunity. The existence of 'cross-immunity' between class IIa bacteriocins is further supported by reports about the presence in class-IIa-bacteriocin-producing LAB of copies of immunity genes not necessarily associated with a gene encoding a cognate bacteriocin [8,20,43,77,78]. In light of these observations, it can be suggested that LAB genera which contain the class-IIa-bacteriocin producers generally possess one or more immunity genes for class IIa bacteriocins. These genes may show various degrees of homology and may be expressed to various extents [10].

The comparative study of Eijsink et al. [10], which included a dozen class-IIa-bacteriocin (putative) immunity proteins, has shown that the homology between these proteins is surprisingly low when considering the similarity between the corresponding bacteriocins. This low similar-

ity has also been observed by Aymerich et al. [13]. Moreover, the highest similarity between immunity proteins does not necessarily mean the highest similarity between the corresponding bacteriocins. Considering the high homology rates of class IIa bacteriocins as compared to the low degree of sequence homology between their immunity proteins and also reports about cross-immunity, it may be suggested that the immunity proteins do not interact directly with their respective bacteriocins but interact indirectly through some kind of common target or 'receptor' (Fig. 4), whose existence has been suggested for the lactococcal A immunity protein [79,80].

Despite the low sequence similarities between immunity proteins protective against class IIa bacteriocins, several common characteristics of these proteins have been observed. They are cationic and principally hydrophilic molecules [10,46,77,81]. A certain similarity between these proteins has also been suggested by secondary structure predictions [82,83], which indicated that all class-IIa-bacteriocin immunity proteins were largely α helical [10]. However, amino acids sequences of class-IIa-bacteriocin immunity proteins, though they may include several hydrophobic segments, are devoid of apparent putative transmembrane segments, which suggests that these molecules are either secreted to the outside or retained inside the cell [10,46,77,81]. A study by Abdel-Dayem et al. [81], using immunofluorescence and electron microscopy, has shown that the major part of the immunity protein MesI of mesentericin Y105 produced is found in the cytoplasm compartment, with only a small proportion detected in the membrane. Similar data have been reported by Quadri et al. [77] for the immunity protein CbiB2 of carnobacteriocin B2. These authors have further shown that, while the expression of CbiB2 within the cells provided immunity against carnobacteriocin B2 applied externally, the addition of CbiB2 to the culture medium failed to protect sensitive cells. This further confirms that CbiB2 is not an extracellular protein. Additional experiments by Quadri et al. [77] have demonstrated that CbiB2 has poor affinity for carnobacteriocin B2 and that no direct interaction occurs in aqueous solution between the two proteins. These observations suggest that class-IIa-bacteriocin immunity proteins would preferably be free intracellular molecules, which would prevent bacteriocin action at the membrane site indirectly via a membrane-bound protein (Fig. 4). However, the presence of such a protein is still speculative and the molecular entity has yet to be identified.

4. Antibacterial activity of class IIa bacteriocins

4.1. Bactericidal effects

Like other LAB bacteriocins, class IIa bacteriocins are bactericidal peptides which act primarily by permeabilizing the membranes of susceptible microorganisms, probably

through the formation of poration complexes, causing an ionic imbalance and leakage of inorganic phosphate [2,3,28,31,84–88]. This has been convincingly demonstrated for pediocin PA-1 [84,86,89], mesentericin Y105 [88], and bavaricin MN [17].

A consequence of such disruptions is the dissipation of proton motive force (PMF), which involves the partial or total dissipation of either or both the transmembrane potential ($\Delta\psi$) and the pH gradient (ΔpH) [39]. Unlike lantibiotics, which totally dissipate both $\Delta\psi$ and ΔpH [39], class IIa bacteriocins readily provoke a total dissipation of ΔpH , but only a partial dissipation of $\Delta\psi$ [17,84,85,88]. Only the newly discovered mundticin has been shown to cause a complete dissipation of $\Delta\psi$ [21].

The lethal activity of class IIa bacteriocins is thus mainly ascribed to the dissipation of the PMF [3,28,49]. Particularly, the intracellular ATP is depleted by rates of up to 98.9% [21,89] and the uptake of amino acids, which is mediated by active transport, is blocked [86,88]. Moreover, a leakage of preaccumulated amino acids, among other UV-absorbing materials, has been reported for pediocin PA-1 [84,86] and mesentericin Y105 [88]. The efflux of amino acids caused by these two bacteriocins may occur by diffusion through the bacteriocin pores, probably combined with reflux via PMF transport systems [39]. However, the very rapid efflux of amino acids due to the action of mesentericin Y105 suggests that this resulted from simple leakage [88].

Unlike lantibiotics [90–92], no leakage of ATP seems to be caused by class IIa bacteriocins [21,89], which is thought to be due to smaller pore sizes formed by the latter than by the former. The observed depletion of intracellular ATP may therefore result from an accelerated consumption of ATP in order to maintain or restore PMF and/or the inability of the cell to produce ATP due to phosphate efflux. By comparing the rates of pediocin-PA-1-induced ATP depletion and inorganic phosphate efflux, Chen and Montville [89] have suggested that the observed depletion of ATP is most likely due to attempts of the cell to regenerate the decreased PMF, rather than a shift in the ATP hydrolysis equilibrium due to the loss of inorganic phosphate. Again, contrasting results have been reported for the lantibiotic nisin Z [91].

4.2. Models for bacteriocin–membrane interactions

The presence in class IIa bacteriocins of amphiphilic segments which are putative transmembrane helices (Fig. 5a), their water solubility and membrane-binding ability suggest that they may form poration complexes following a ‘barrel-stave’ model. The initial step of class-IIa-bacteriocin interaction with the membrane surface is generally believed to be an electrostatic binding mediated by a putative membrane-bound receptor-type molecule [28,49,86]. Yet, in contrast to earlier findings suggesting a protein-‘receptor’-mediated activity [84,86], recent studies investi-

gating the effect of class IIa bacteriocins on lipid vesicle systems indicate that protein ‘receptors’ may not be absolutely required for pore formation [17,31]. On the other hand, it has been suggested that functional binding of the positively charged and polar residues of class IIa bacteriocins occurs primarily in conjunction with anionic phospholipid head groups in the membrane [17,31,88,93,94] (Fig. 5b). Considering the cationic nature of class IIa bacteriocins (Table 1) and the high structural similarity of their hydrophilic N-terminal half (Fig. 1), which is thought to mediate the initial binding, it is most likely that all class IIa bacteriocins rely at least in part on the same type of functional binding.

As a subsequent step, hydrophobic interactions would occur between the hydrophobic/amphiphilic domain within the C-terminal half of the bacteriocin and the lipid acyl chains, and have been shown to be crucial for the pore formation process [17,25,93,95] (Fig. 5b). In fact, the C-terminal half of class IIa bacteriocins, which is more hydrophobic than the N-terminal half, contains a domain which appears to be involved in hydrophobic interactions with the membrane [40,95]. It has been suggested that this domain may be the cell-specificity-determining region for class IIa bacteriocins, in contrast to the N-terminal domain which interacts electrostatically with the membrane surface probably in an unspecific manner [40,95] (see below). In such a case, hydrophobic interactions might occur between residues in the specificity-determining region of the bacteriocin and a membrane component, thereby rendering the membrane more susceptible to permeabilization [95]. Alternatively, membrane destabilization, occurring due to increased bacteriocin binding to the cell surface resulting in a higher concentration of bacteriocin molecules in the vicinity of the membrane, appears less likely. Kaiser and Montville [17] hypothesized that, following hydrophobic interactions, the bacteriocin may be reoriented into a more energetically favorable orientation, which could simply be its insertion into the membrane followed by aggregation.

As far as insertion of bacteriocins in the membrane bilayer is concerned, *in vitro* models exist for the lantibiotic nisin in various lipid membranes [39,92], while concerning class IIa bacteriocins, it was only recently that a number of models have been proposed suggesting their possible orientations in lipid bilayers [14,25,31,96,97]. Class IIa bacteriocins are in fact believed to insert into the target membrane via their hydrophobic and/or amphiphilic C-terminal domain, and aggregate to form water-filled pores [14,25,93] (Fig. 5b,c).

4.3. Factors affecting bacteriocin activity

Class-IIa-bacteriocin-induced cell death, has been shown to occur in concentration- and time-dependent manners and it is further influenced by other factors related either to the target cell or to the medium [17,89]. In a

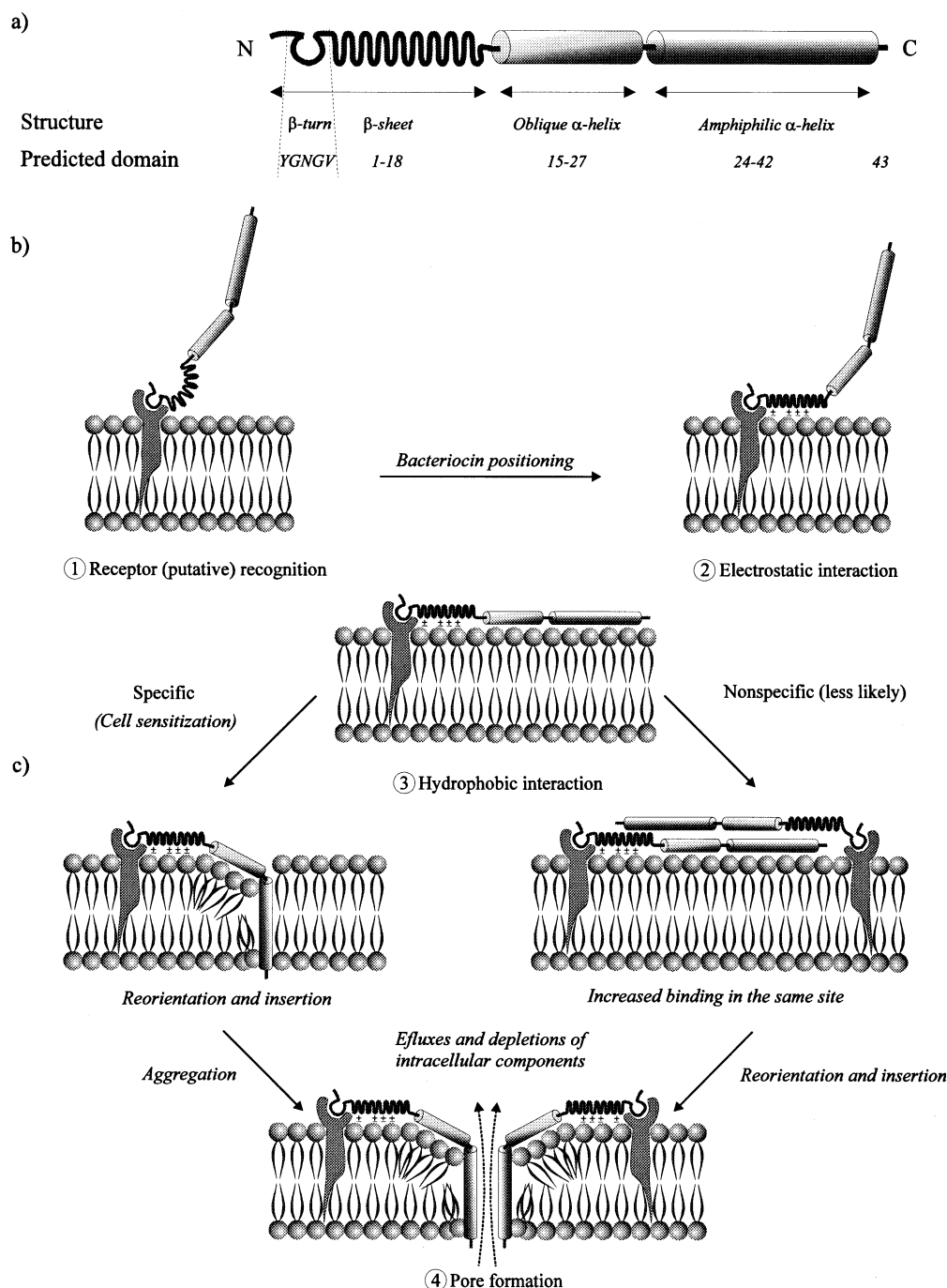


Fig. 5. Schematic representation of the structure of a model class-IIa bacteriocin and the predicted location its domains with respect to target cell membrane: (a) bacteriocin predicted structural domains; (b) possible interactions of each domain with the membrane surface; (c) bacteriocin insertion and formation of hydrophilic pores. The hydrophobic face of the peptide is shaded dark and hydrophilic face is shaded light.

recent investigation, Chen et al. [94] have shown that the lipid composition of the target membrane is a determinant factor in modulating the pediocin PA-1 action, particularly the affinity of this bacteriocin for lipid vesicles increases with the increase in their anionic lipid content [94].

The binding affinity of class IIa bacteriocins to target membranes has also been found to be affected by pH. For instance, decreasing the pH from 7.5 to 6.0 has been shown to improve both membrane binding and permeabi-

lization abilities of pediocin PA-1 [31]. Moreover, pore formation by bavaricin MN is optimal at pH 6.0 and less efficient at other pH values [17].

The positive net charge of class IIa bacteriocins presumably allow them to adhere to negatively charged phospholipid head groups. Therefore, altering the charge properties of either the bacteriocin, by changing the medium pH, or the membrane, by changing its lipid composition, obviously influences this adhesion by affecting the dissocia-

tion constant of peptide-lipid interactions. Both kinds of alterations further confirm the involvement of electrostatic binding in the interactions of class IIa bacteriocins with target membranes.

Class IIa bacteriocins are generally opposed to nisin in the sense that they interact with the cytoplasmic membranes of sensitive cells regardless of their degree of prior energization, suggesting that the loss of permeability of the cytoplasmic membrane occurs in a voltage-independent manner [3,49,85,86], while nisin acts in a membrane-potential-dependent manner [5,92]. However, recent investigations have shown, on the one hand, that nisin's dependency on $\Delta\psi$ varies with the experimental system used: while a threshold level of $\Delta\psi$ is required for activity in *Listeria* cells and black lipid membranes, nisin can, however, display activity on lipid vesicles and sensitive lactococcal cells in the absence of $\Delta\psi$, though the presence of $\Delta\psi$ increases its membrane permeabilization ability [5,39,85,92,98]. On the other hand, the antimicrobial activity of the class IIa bacteriocins, pediocin PA-1 and bavaricin MN, has also been shown to be enhanced by $\Delta\psi$ (66% and 88% increase, respectively), although it is not fully dependent on it [17,31]. It has been suggested that these bacteriocins would function in an energy-enhanced manner [17]. The presence of $\Delta\psi$ is speculated to increase the size or the number of poration complexes and/or help in reorienting membrane-bound bacteriocin molecules into a more energetically favorable orientation, thereby promoting their insertion [17,31].

4.4. Spectrum of activity

The bactericidal activity of class IIa bacteriocins seems to be targeting primarily *Listeria* strains, since all these peptides show antilisterial activity, but it is also commonly directed against several other Gram-positive bacteria. In fact, in addition to all species of *Listeria*, species belonging to the following genera have been reported to be sensitive to class IIa bacteriocins: *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, *Carnobacterium*, *Enterococcus*, *Micrococcus*, *Staphylococcus*, *Streptococcus*, *Clostridium*, *Bacillus* and *Brochothrix* [2,3,9,10,13,14,21,25,40,99]. Furthermore, some class IIa bacteriocins have been shown to prevent the outgrowth of spores and vegetative cells of *Clostridium* spp. [21]. Yet the extent of sensitivity to class IIa bacteriocins vary from species to species and from strain to strain. In other respects, it is noteworthy that class IIa bacteriocins display overall narrow inhibitory spectra, as compared to bacteriocins of other groups, such as nisin.

Despite large regions of sequence identity and close homology in the N-terminus, class IIa bacteriocins have been reported to display considerable disparity in the spectra of antimicrobial activity [10,13,25,40]. In fact, the only apparent common characteristic of class IIa bacteriocins regarding their inhibitory spectra is their strong bactericidal

action against strains of the genus *Listeria*. Therefore, the determination of factors accounting for the observed disparity would require detailed knowledge of various structural features of class IIa bacteriocins, such as their three-dimensional structures, which would have to be compared with their antibacterial profiles. However, studies which have matched antibacterial spectra generally used a maximum of four class IIa bacteriocins, which does not integrate sufficiently variable structural aspects, and/or a limited number of indicator strains. Moreover, the fact that the indicator species and strains used often differ from one study to another, makes it difficult to suggest reasonable comparisons.

So far, only the general features of the mode of action of class IIa bacteriocins, described above, have been identified, while factors underlying the specificity of action of these peptides are not yet clarified (see below). Nevertheless, based on observations of antibacterial spectra, several interesting proposals have been made that can help structure-function studies to move forward. For instance, pediocin PA-1 and enterocin A have been shown to exhibit spectra of activity broader than those of sakacin P and curvacin A, which has been ascribed to the presence of an extra disulfide bond in the two former bacteriocins [10]. It has also been speculated that bacteriocins with fewer amino acid residues would tend to have relatively broader antibacterial spectra than those with larger numbers of residues [3]. Furthermore, bacteriocins with slight differences in their structures, like mesentericin Y105 and leucocin A naturally display antibacterial spectra with insignificant differences or no difference at all [24,25,32]. Surprisingly, class IIa bacteriocins from the same bacterium: carnobacteriocin BM1 and B2 produced by *Carnobacterium piscicola* LV 17B, and piscicocin V1a and V1b produced by *C. piscicola* V1, also exhibit spectra with only slight differences or no difference at all, although their respective structures are less related as compared to other members of the group [8,14]. Moreover, it has been recently shown that very few discrepancies in activity exist between four class IIa bacteriocins (pediocin AcH/PA-1, enterocin A, curvacin A and sakacin P) when acting against strains within the genus *Listeria*, while within LAB genera, results were far less consistent, as they varied considerably within each genus and species [10]. In this regard, both specificity and potency of the activity of class IIa bacteriocins have been suggested to be influenced by the lipid composition of target membranes which would determine the occurrence and the degree of interactions with bacteriocin molecules [17,31,88]. Therefore, the comparable activities of class IIa bacteriocins against strains of *Listeria* could be due to the homogeneous membrane lipid composition within this genus.

4.5. Structure-function relationships

Understanding the relationships between structure and

function is probably the greatest challenge in ongoing bacteriocin research. Investigations concerning class IIa bacteriocins are in progress in several laboratories and involve comparisons of the activity spectrum, amino acid sequences and predicted secondary structures, with the aim of identifying regions playing a critical role in cell recognition and/or bactericidal action of these peptides. So far however, almost all structural features of class IIa bacteriocins have been suggested to play a part in at least one of the two processes (Fig. 5). These features include the N-terminal consensus motif, forming a β -turn, a hydrophilic N-terminal part following this motif, forming amphiphilic β -sheets, a central domain forming a hydrophilic/slightly amphiphilic α -helix, the C-terminal hydrophobic domain forming amphiphilic α -helix, the disulfide bridges, the two or three amino acids at the N-terminal extreme prior to the consensus motif, the positively charged amino acids, and other basic and aromatic amino acids such as Lys and Trp. This is indicative of the complex nature of bacteriocin-membrane interactions, as it is now well established that the pore formation process occurs through, not one, but a series of recognition steps which would involve different domains and structural features in the bacteriocin. In fact, it appears that the whole sequence of a class IIa bacteriocin is required for antibacterial activity, since bacteriocin fragments, no matter how large, display weak or no activity at all [8,25,96,100,102]. Stringent structural requirements for cell recognition may thus account for the relatively narrow spectrum of activity of class IIa bacteriocins. However, one molecular recognition event might be more critical than the others in determining cell specificity.

4.5.1. Role of the YGNGV motif

Due to a β -turn structure, the YGNGV motif would be easily exposed to and recognized by a putative membrane 'receptor', which would allow correct positioning of the bacteriocin on the membrane surface, necessary for activity [14,30,31,39] (Fig. 5b). A current view assumes that the YGNGV consensus motif could be involved in a recognition step of the mechanism of action of class IIa bacteriocins, but is unlikely to be involved in the specificity of action [30,40]. However, it has been coined the *Listeria* active part of class IIa bacteriocin. Although, no clear evidence for this has yet been reported, modifications and/or deletions in the YGNGV seem to have serious consequences on the anti-*Listeria* activity of class IIa bacteriocins [25,96,102]. On the other hand, it has been shown that substitutions within the YGNGV motif also drastically reduces bacteriocin activity against a variety of LAB strains [100,102]. In addition, the presence of this motif is not a requirement for the anti-*Listeria* activity of two class-IIa-related bacteriocins, which have an altered YGNGV motif: bacteriocin 31 (YGNGL) [26] and acidocin A (YGTNGV) [27] (Fig. 1). Therefore, it is possible that modifications within the YGNGV perturb the β -turn structure and consequently the N-terminal β -sheet confor-

mation (see below), which ultimately affects activity not only against *Listeria* strains, but also against other target strains.

4.5.2. Role of the N-terminal hydrophilic/amphiphilic β -sheet

The N-terminal β -sheet structures are believed to give class IIa bacteriocins an amphiphilic characteristic in this region, which is of great significance for bacteriocin-membrane primary interaction [14,30,31,39]. It has been reported that the predicted N-terminal β -sheet of pediocin PA-1 contains a positively charged patch consisting of residues Lys-1, Lys-11 and His-12, which mediates bacteriocin binding to target membranes [31,93], but also a hydrophobic patch consisting of residues Val-7, Cys-9 and Cys-14, Val-16 and Trp-18, which may be involved in the membrane insertion process [31]. Chen et al. [31] have shown that it is the positive patch at the tip of pediocin PA-1 hairpin loop, rather than the YGNGV, that mediates the initial binding (electrostatic) of this bacteriocin to target membranes. Nonetheless, as only binding has been assessed by Chen et al. [31], the critical role of the YGNGV for the activity of class IIa [14,96], which may occur through another form of cell recognition, is far from being ruled out.

On the other hand, class IIa bacteriocins with a blocked N-terminus, either with the leader peptide (prebacteriocins) or with the maltose-binding protein (for instance MBP-pediocin ACh), have been reported to display antibacterial activity, which indicate that the N-terminal part does not insert the phospholipid bilayer of target membranes [51,96,100]. However, the observed activity of prebacteriocins is weak as compared to that of the mature peptides, probably because of possible interference of the leader region in the adoption of correct conformations by the bacteriocin and/or the membrane binding [51,100]. These observations support the view that the N-terminal region, generally under a β -sheet conformation, is involved in a membrane-surface recognition step (Fig. 5b), most likely through electrostatic interactions, which is common to all class IIa bacteriocins and is not determinative of their specificity of activity.

4.5.3. Role of the central hydrophilic/slightly amphiphilic α -helix

Nine class IIa bacteriocins have been investigated by Bennik et al. [21] and shown to possess central oblique-oriented α -helical structures (spanning residues 15,16–27,28), able to insert into hydrophobic-hydrophilic interfaces at a 30–60° angle relative to these interfaces. The oblique orientation of α -helical regions is believed to contribute to destabilization of the phospholipid bilayers, thereby facilitating the insertion of bacteriocin molecules in the cytoplasmic membrane of the target organism [21]. Bennik et al. [21] have also observed that among the hybrid class IIa bacteriocins constructed by Fimland et al.

[40], Ped-Sak (combining residues 1–21 of pediocin PA-1 and residue 22 to the end of sakacin P) was the only peptide which have kept an oblique-oriented α -helical central region, and, interestingly, the only hybrid bacteriocin to retain full activity.

Nonetheless, the central hydrophilic or slightly amphiphilic α -helix predicted by Bughaloo-vial et al. [14] for piscicocins V1a and V1b, which almost entirely spans the oblique-oriented helical portion described for these two bacteriocins [21], has been suggested to rather anchor the bacteriocins to the surface of lipid bilayers, without inserting the membranes [14], probably due to its low amphiphilicity and/or central position. The study of Bennik et al. [21] opens a new perspective in the current research on structure-activity relationships of class IIa bacteriocins: the oblique orientation of the α -helical region may also account for the activity of these peptides (Fig. 5b,c). However, since only the potency of class IIa bacteriocins has been investigated by these authors and not their specificity of activity, it is still unknown whether the central oblique-oriented α -helix may have a cell recognition function.

4.5.4. Role of the C-terminal hydrophobic/amphiphilic α -helix

Presumably, the ability of class IIa bacteriocins to form a C-terminal amphiphilic α -helix in membrane-mimicking environments accounts for their mode of action, particularly the likely formation of the so-called 'barrel-stave' poration complex [2,14,31,40,101]. Due to this property, the C-terminal domain would easily insert into the membrane of target cells and form water filled pores (Fig. 5c).

Additionally, as opposed to the N-terminal region, which is mainly common to all class IIa bacteriocins, the C-terminal region, containing putative transmembrane helices, is more heterogeneous and may play a critical role in the target-cell specificity of these bacteriocins. Recent investigations of structure-function relationships have indeed shown that the specificity of action of class IIa bacteriocins may be attributed at least in part to their C-terminal region [30,40,95]. Fimland et al. [40], through construction of hybrid bacteriocins, have observed that the C-terminal part is the main determinant of target cell specificity and would also act as the key membrane-recognition region. This is supported by the fact that a pediocin-PA-1-derived C-terminal fragment (residues 20–34) interfered with pediocin PA-1 activity, probably by occupying a specific interacting entity, most likely hydrophobic, on the target membrane [95]. The pediocin-PA-1-derived fragment also inhibited the activity of enterocin A, a bacteriocin with a relatively long continuous sequence identical to that of pediocin PA-1 in the region spanned by the fragment, but was not so inhibitory to less related class IIa bacteriocins in the same region [95]. Based on these findings, one may expect that class IIa bacteriocins with similar C-terminal sequences, i.e. belonging to the same C-terminus-based subgroup, defined in Fig. 1, would display

similar spectra of antimicrobial activity. However, this is apparently not the case for the two piscicocins, which have been shown to display very similar spectra of activity [14], though their C-terminal parts are largely different.

Studies by Bhugaloo-vial et al. [14] and Fleury et al. [25], respectively on piscicocins and mesentericin Y105, assume that the C-terminal domain only modulates the activity potency and has no critical role in cell targeting, while the N-terminal domain mediates cell recognition and is responsible for the inhibitory spectrum. A critical role of the N-terminal KYY motif, particularly the N-terminal Lys residue in interactions with membrane phospholipids has been suggested for pediocin AcH and mesentericin Y105 [25,96,102]. A possible joint involvement in a key recognition process for membrane activity of both the N-terminal extreme (prior to the consensus motif) and the C-terminal part of a class IIa bacteriocin should be considered. With this regard, it is an interesting observation that class IIa bacteriocins with the most similar C-terminal parts are also those with the same N-terminal amino acid and the most similar N-terminal extremes (Fig. 1). However, one should keep in mind that in addition to the primary sequence, hydrophobic/amphiphilic properties and correct spatial arrangements of key amino acid side chains, would be determinant factors for both specificity and potency of bacteriocin molecules.

4.5.5. Role of disulfide bonds

It is remarkable that all class IIa bacteriocins contain at least one disulfide bridge and that those with two disulfide bonds have broader spectra of activity than those with a single bond [3,10,39]. In a comparative study, Eijsink et al. [10] have in fact recently shown that the two-disulfide-bond bacteriocins pediocin AcH and enterocin A are more efficient antimicrobials than sakacin P and curvacin A, which possess a single disulfide bond, specially against *Listeria* strains, and that they display an overall broader spectrum of activity. Also pediocin AcH or PA-1 has a broader spectrum of antibacterial activity than leucocin A (with a single disulfide bond) [3].

Reducing the disulfide bond with β -mercaptoethanol or dithiothreitol dramatically reduced the activities of pediocin PA-1 [10,86] and enterocin A [10]. A study by Miller et al. [102] has even reported a complete loss pediocin PA-1/AcH activity upon reducing treatment or Cys residues mutation. As for bacteriocins with a single disulfide bond, reducing treatments has been reported to result only in a moderate decrease of bactericidal activity, which indicates that the single disulfide bond present is not crucial for activity. This has been shown for leucocin A [15], sakacin A [22], sakacin P [10,19], curvacin A [10] and carnobacteriocin B2 [8]. In contrast, Fleury et al. [25] have observed a dramatic decrease (up to 20 000-fold) of mesentericin Y105 activity upon substitution of Cys-9 and Cys-14 with Ser residues, but no explanation is available for this apparent inconsistency.

These findings interestingly suggest, on the one hand, a possible correlation between the presence of disulfide bonds and the potency of class IIa bacteriocins, and on the other hand, that an extra disulfide bond in the C-terminal region may allow higher levels of activity [10]. It is likely that disulfide bridges could promote intramolecular rigidity within class IIa bacteriocins, which may affect activity or heat stability under certain conditions [16]. Recently, Chen et al. [31], through a model of the three-dimensional structure for pediocin PA-1, have suggested that each of its two disulfide bridges would bring the positively charged Lys and His residues closer together to form a patch with positively charged side chains. As indicated above, such a patch would allow a tighter junction between the bacteriocin and the negatively charged lipid head groups of target membranes, thereby enhancing activity [31]. Nevertheless, the exact role of disulfide bridges in the antibacterial effectiveness of class IIa bacteriocins has not yet been fully defined.

4.5.6. Role of positively charged amino acids

Site directed mutations for the positively charged amino acids Lys-1, His-42 and Lys-43 in pediocin AcH result in a decrease in activity, which is ascribed to a less efficient binding of the bacteriocin to the membrane surface [102]. In fact, another study has shown that a mutated pediocin PA-1 fragment lacking the charged residues Lys-11 and His-12 does not bind to target membranes [31]. In addition, the protonation of the three His residues of pediocin PA-1 is believed to contribute significantly to its adsorption to target membranes, but unlike nisin, the deprotonation of these residues does not enhance membrane insertion [31,39]. Furthermore, the Lys-11 of pediocin PA-1 may play a more important role in the pore formation, perhaps by interfering with bacteriocin insertion, since its replacement by a Glu residue results in a significant increase in the antimicrobial activity of pediocin PA-1 [102].

4.5.7. Role of other particular amino acids

Single-residue mutation and modification through chemical synthesis have shown that the presence of aromatic residues, particularly Trp, in class IIa bacteriocins is crucial for the antimicrobial activity. Substitution of Arg for Trp-18 in pediocin PA-1 results in the loss of activity by two orders of magnitude [102]. A Trp at this position is a well conserved residue in the majority of class IIa bacteriocins. Similarly, mesentericin Y105 shows a dramatic drop in its anti-*Listeria* activity upon removal of its N-terminal Trp residue [25]. Residues Trp-18 and Trp-33 in pediocin PA-1 have been shown to penetrate the hydrophobic core of lipid bilayers, while the bacteriocin is bound to target membranes [31,102]. Bhugaloo-Vial et al. [14] who have identified Trp residues at both ends of the C-terminal putative helical domain of piscicocin VIa, suggested that these residues would strengthen the helix

and help orienting the bacteriocin into the target membrane. Trp residues may also help establish the interfacial boundaries of the bacteriocin's transmembrane portion [103]. Another aromatic residue, phenylalanine, also seems important for activity, as the replacement of Phe-33 in carnobacteriocin B2 with Ser results in total loss of activity, which may be due to a change in the α -helix amphiphilicity [100].

Furthermore, it has been demonstrated that all substitutions of residues within the hydrophobic C-terminal part of pediocin AcH (residues 25–37), which induce an increase in polarity, result in a complete loss of activity [102]. In contrast, conservative modifications in terms of hydrophobicity, charge and structural variation have been shown to generate bacteriocin variants with activities comparable to that of the native peptide [25,100]. This seems to be the case for mesentericin Y105 and its natural variant leucocin A, which vary only slightly in their potency and specific activity [25].

4.6. Occurrence of bacteriocin tolerance

The occurrence of bacteriocin tolerance and/or resistance among food spoilage and pathogenic bacterial species that are normally bacteriocin-sensitive is a major concern with regard to the practical use of bacteriocins, since it may compromise the antibacterial efficiency of these compounds. Within a particular species, highly tolerant and/or resistant strains may exist naturally or result from exposure to bacteriocins.

4.6.1. Adaptional tolerance

Most studies on the emergence of highly tolerant and/or resistant mutants of sensitive bacteria have dealt with *Listeria* spp. exposed to nisin. It has been particularly shown that *L. monocytogenes* strains spontaneously develop resistance towards this bacteriocin at relatively high frequencies (10^{-6} – 10^{-8}) in both laboratory media and foods [104–107]. Nisin-resistant isolates have recently been reported to emerge, at even higher frequencies (10^{-3} – 10^{-4}), from a single exposure of *L. monocytogenes* Scott A to nisin at 100 IU ml⁻¹ [108]. Mutation frequencies appear to depend on the strain and the conditions used, particularly the bacteria/bacteriocin ratio. As far as class IIa bacteriocins are concerned, the development of tolerance among *Listeria* strains has only recently attracted attention from investigators. The generation of *Listeria* mutants with enhanced tolerance or resistance towards class IIa bacteriocins could be achieved easily in laboratory media containing these compounds [106,109].

For a long time, the great structural diversity of LAB bacteriocins has been claimed to be an opportunity to circumvent the problems of resistance of *Listeria* strains to class IIa bacteriocins [2,3], since no cross-resistance has been observed between class IIa bacteriocins and the class I nisin for instance [106,108–110]. Nonetheless, contradic-

tory evidence has recently been provided by independent studies reporting cross-resistance between class IIa bacteriocins, nisin and the class IV leuconocin S for *L. monocytogenes* and *Clostridium botulinum* strains [111–113]. Consequently, no conclusive evidence has yet been achieved regarding the existence of cross-resistance between class IIa bacteriocins and other bacteriocins, particularly nisin. Further studies have to be carried out in order to determine whether class IIa bacteriocins may in fact be effective in preventing the emergence of spontaneous nisin-resistant subpopulations, particularly within *L. monocytogenes*.

It is generally admitted that each bacteriocin-sensitive bacterial population includes potentially tolerant and/or resistant cells with structural modifications or at least with a high predisposition to such modifications, which would allow them to spontaneously emerge in case of exposure to the bacteriocin [110,114,115]. The mechanisms implicated in bacteriocin resistance appear to be complex and involve various structural and physiological changes in the bacterial cell. In particular, modifications in the cytoplasmic membrane composition are often investigated to explain bacteriocin resistance, considering the primordial role of the membrane in the activity of bacteriocins. In the case of nisin, *L. monocytogenes* resistance has been ascribed to alterations in both the fatty acid composition [105,107] and the phospholipid composition [116,117]. Specific changes include a lower ratio of C-15 to C-17 fatty acids than the wild-type strains [107], more zwitterionic phosphatidylethanolamine and less anionic phosphatidylglycerol and cardiolipin [112,117]. Changes in the fatty acid composition are supposed to render the membrane less fluid, thereby preventing the insertion of nisin molecules. In addition, to compositional alterations in the membrane, modifications in the cell wall [118] and a requirement for divalent cations [112] may also account for nisin resistance in *L. monocytogenes*. Nonetheless, the link between cell alterations and the mechanisms of resistance remain undetermined for nisin and investigations have yet to be carried out for class IIa bacteriocins.

4.6.2. Natural tolerance

It is well established that within a given species, strains differ greatly in their relative sensitivity to a particular bacteriocin [119,120]. However, so far, little information is available concerning the occurrence of naturally-tolerant or resistant strains, as most investigations on bacteriocin sensitivity have been conducted with single or very few strains. In fact, experience shows that it is important to examine a wide variety of strains in order to determine the likely occurrence of tolerant or resistant ones. Furthermore, data obtained in different studies are generally difficult to compare, because the strains used as indicators often vary from one study to another and the absolute values of the minimum inhibitory concentrations (MIC) depend on the assay and the conditions used.

Data available for nisin indicate that some *L. monocytogenes* strains may be sensitive to nisin concentrations below 200 IU ml⁻¹, whereas others are relatively resistant and require much higher amounts of nisin to be inhibited [121,122]. As far as class IIa bacteriocins are concerned, the occurrence of naturally tolerant or resistant *L. monocytogenes* strains has been observed to be by far more frequent than with nisin [123]. Significant differences in sensitivity of *Listeria* strains towards class IIa bacteriocins, have been observed in independent studies [124–126]. However only two attempts have been made to compare a large number of strains within the same study. Larsen and Nørrung [18] have found that three (1.2%) of 245 strains were tolerant to bavaricin A, using a non-quantitative agar spot assay. More recently, Rasch and Knöchel [123] have examined the sensitivity of 381 *L. monocytogenes* strains to different concentrations of pediocin PA-1. Twenty strains have been found to be totally resistant to all bacteriocin concentrations, while 34 other strains have been characterized as having enhanced tolerance, since growth was inhibited at the highest bacteriocin concentration used (1600 AU ml⁻¹). Furthermore, correlation has been reported between the sensitivity of 22 *L. monocytogenes* strains to pediocin PA-1 and to another class IIa bacteriocin, bavaricin A [123], whilst no cross-resistance between these two bacteriocins and nisin could be observed.

It has been hypothesized that, over time, the occurrence of naturally tolerant and/or resistant strains of *L. monocytogenes* may result from a sustained exposure to bacteriocin in natural environments [18]. However, up until now, this remains quite speculative and the mechanisms of natural tolerance and/or resistance of *L. monocytogenes* to class IIa bacteriocins have yet to be determined. Yet, a recent investigation by Robichon et al. [127] has uncovered a gene (*rpoN*), encoding a protein with homologies to the transcriptional σ 54 factor found in Gram-positive bacteria, which is responsible for the sensitivity of *L. monocytogenes* to mesentericin Y105. In fact, transposon insertion in *rpoN* generated resistant mutants, which suggested that resistance-associated genes may be controlled by σ 54 [127].

4.6.3. Significance of bacteriocin resistance

The main limiting factors for bacteriocin effectiveness in food systems reported in the literature are related to the likely occurrence of highly tolerant and/or resistant strains [7]. As far as the adaptional resistance is concerned, studies have clearly demonstrated that this resistance is relatively weak, as it does not generally exceed a 10-fold increase in comparison with the wild-type, and unstable, since it is generally easily lost if the variants are cultured in the absence of the bacteriocin [105,106,111,128,129]. In addition, it has been shown that the resistant *Listeria* mutants had a lower growth rate than the wild-type strain and were unable to outgrow populations of the sensitive

strain [129,130], which decreases the likelihood of spontaneous bacteriocin-resistant mutants proliferating widely in stable form in natural environments. Furthermore, since the frequency of emergence of resistant mutants greatly depends on the medium, strategies can be developed in order to minimize the risk associated with the adaptional resistance, e.g. by lowering the pH or adding NaCl [108,113]. Hence, the transient and relatively low degree of resistance with mutants derived from exposure to bacteriocins, coupled with lack of genetically based resistance and low fitness of the obtained variants, may act together to limit the risks related to acquired resistance. Nevertheless, the effect of long-term exposure to bacteriocins has to be carefully monitored, since it may possibly result in resistant populations as stable and fit as the naturally occurring resistant strains, which would be of significant concern with regard to the use of bacteriocins as food preservatives.

5. Perspectives

Class IIa bacteriocins are apparently abundant antimicrobials among LAB, which are highly effective in controlling the growth of microorganisms and attractive as food preservatives. The use of several class IIa bacteriocins in food preservation is still in experimental stages and may have future applications in enhancing the safety and extending the shelf life of many inherently perishable foods. Pediocin PA-1 is a representative example of potential practical applications of these peptides as a food preservative. This bacteriocin's use in meat, cheese and salad is actually covered by several US and European patents (Gonzalez et al., European Patent 88101624, 1988; Vandenberg et al., European Patent 89101125.6, 1989; Bourdreaux, et al., United States Patent 5137319, 1992; Werner-Aoude et al., French Patent 9411188, 1994). The need for expanded use of class IIa bacteriocins is obvious, especially in light of consumer demands for minimally processed, safe foods of adequate shelf-life and convenience, and the global need for increasing the supply of food. Consequently, class IIa bacteriocins are believed to be the next in line if more bacteriocins are to be approved as food additives in the future. For the use of bacteriocins to increase to the level of commercial development and economic production, there should be the concomitant development of multihurdle preservative systems and technology transfer. We suggest that future research should be concerned with the following challenges:

5.1. Heterologous-, over- and multi-expression

The limited efficiency of bacteriocin-producing cultures in fermented foods may be ascribed to various factors such as low production, regulatory systems, genetic instability, inactivation, and occurrence of resistance among

target bacteria. The recent trend in bacteriocin research, involving heterologous expression of bacteriocins among various LAB strains, offers an excellent tool that may help overcoming such obstacles. In particular, cloning and expression of class-IIa-bacteriocin genes in new hosts have allowed constitutive production and even over-expression of bacteriocins, therefore overcoming bacteriocin regulation systems [32,69–71,75]. Also, various food-grade LAB strains can be selected, based on their characteristics relevant to specific food systems, for use as hosts for defined bacteriocins of interest. Therefore bacteriocin-producing LAB strains can be constructed that are adapted to each type of food, which may further help overcoming colonization and bacteriocin production problems. Moreover, the use of the heterologous expression of bacteriocins to develop LAB producing multiple bacteriocins, each one having its specific range of targets, may prove of great interest in enhancing antimicrobial efficiency of LAB in food. In fact, this would yield bacteriocin-producing strains active against a broad range of undesirable organisms and would possibly reduce risks of bacteriocin-resistance development among target bacteria. Hence, heterologous expression creates interesting possibilities for further development and extension of bacteriocin applications as preservatives in various food industries.

5.2. Engineering of bacteriocins

The engineering of bacteriocins, through genetic or chemical modifications, offers the possibility of developing new biologically important peptides with improved activity and stability. It is generally reported that sequence modifications, including single-residue substitutions, of class IIa bacteriocins result in peptides with diminished inhibitory activity compared to the native bacteriocins [25,31,39,40,100]. However, a recent study by Miller et al. [102] showed a significant increase in pediocin PA-1 activity upon substitution of a Glu residue for Lys-11, which suggests possible interesting future developments in the field of bacteriocin engineering.

5.3. Multihurdle concept

Obviously, food preservation can be assisted not only by a class IIa bacteriocin with an individual antimicrobial effect, but, more interestingly, by a combination of bacteriocins in order to enhance the overall effectiveness against target organisms. Class IIa bacteriocins are particularly suitable for use in multihurdle food preservation systems by interactions with multiple bacteriocins resulting in additive or synergistic effects that can better prevent or delay undesirable microbial activity. In fact, while being all anti-listerial, class IIa bacteriocins largely vary in their spectra of activity, which allow many possibilities of combinations of class IIa bacteriocins for *Listeria* elimination procedures adapted to each food product. Combinations with

bacteriocins from other classes may also be interesting when targeting multiple undesirable bacteria. Finally, as mentioned above, for optimal effectiveness against food-borne pathogenic and spoilage bacteria, bacteriocins, including class IIa, have to be used as a part of a general multihurdle food preservation system, which involves a set of other antimicrobial factors.

Acknowledgements

Financial support for research in the authors' laboratory and the preparation of this manuscript, as well as a fellowship for S.E. were kindly provided by the Japan Society for the Promotion of Science.

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