Class II bacteriocins: target recognition, resistance and immunity

Klasse II bakteriosiner: målcellespesifisitet, resistens og immunitet

Philosophiae Doctor (PhD) Thesis

Morten Kjos

Dept. of Chemistry, Biotechnology and Food Science
Norwegian University of Life Sciences

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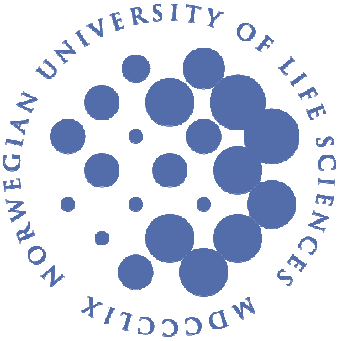

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Morten Kjos

Summary

Bacteriocins are bacterial antimicrobial peptides which are active against strains often closely related to the producer. Bacteriocins produced by food grade lactic acid bacteria are considered promising for applications in food preservation and infection treatment since many of them kill food-spoiling bacteria and pathogens with high potencies. During the last decades, hundreds of different bacteriocins from lactic acid bacteria have been identified, however, the molecular mechanisms underlying target cell recognition, resistance development and producer cell self-immunity are often poorly characterized. The papers I-IV presented in this thesis all shed light on such mechanisms for class II bacteriocins.

In paper I and II the receptor recognition by class IIa bacteriocins and the class IIc bacteriocin lactococcin A was investigated. These bacteriocins bind to the membrane-located protein complex IIC-IID of the mannose phosphotransferase system (man-PTS) to form pores in the membrane of sensitive cells. By phylogenetic analyses and heterologous expression of man-PTSs from different genera, it was demonstrated that class IIa bacteriocins specifically recognize a phylogenetically defined subgroup of man-PTSs. The man-PTSs in this group originated from Gram-positive bacterial genera such as *Listeria*, *Enterococcus* and *Lactobacillus*, which are known to be class IIa bacteriocin sensitive. Moreover, the class IIc bacteriocin lactococcin A was shown to exclusively target the lactococcal man-PTS as receptor, which is in line with the narrow inhibition spectrum observed for this bacteriocin. These results suggest that variation in bacteriocin sensitivity between bacterial species/genera can mainly be ascribed to differences in the sequence/structure of the man-PTS receptor. In order to characterize the species-specific receptor-bacteriocin interaction, the man-PTS genes from the class IIa bacteriocin sensitive *Listeria monocytogenes* (*mpt*) and the non-sensitive *Lactococcus lactis* (*ptn*) were used to construct a series of chimeric man-PTSs and site-directed mutations. It was demonstrated that class IIa bacteriocins specifically recognize an extracellular loop region in the membrane-located man-PTS IIC protein. In contrast, lactococcin A (class IIc) seemed to possess a much more complex receptor interaction by specifically recognizing regions both in the IIC and IID protein.

In paper III the mechanisms by which sensitive bacteria become resistant to the man-PTS targeting bacteriocins (class IIa bacteriocins and lactococcin A) were studied. It has previously been reported that induced resistance to class IIa bacteriocins in *Li. monocytogenes* is linked with downregulation of man-PTS gene expression. By examining natural isolates of *Li. monocytogenes* with varying levels of sensitivity to class IIa bacteriocins as well as lactococcin A resistant *L. lactis* mutants, it was demonstrated that such downregulation of man-PTS gene expression is a general resistance mechanism against different man-PTS targeting bacteriocins. The same resistance mechanism was found both among the natural isolates with low susceptibility and the laboratory

induced resistant mutants. Moreover, it was shown that another, yet unknown resistance mechanism is also involved, since a significant number of resistant cells with normal man-PTS expression were identified.

While the molecular mechanisms underlying target cell recognition and self-immunity for class IIa bacteriocins now have been studied in detail, very little is known about such mechanisms for other class II bacteriocins. Recently it was found that genes belonging to the family Abi, which encode putative transmembrane proteases, are associated with some bacteriocin loci in *Streptococci* and *Lactobacillus*. The Abi genes are thought to encode immunity proteins in these systems, and in paper IV the Abi family was investigated in order to gain more insight into the role of these proteins in self-immunity to class II bacteriocins. Using a bioinformatics approach it was shown that the Abi family is larger than currently annotated in the databases, and by mining sequenced genomes for Abi genes, seven putative new bacteriocin loci were identified. By heterologous expression, two putative bacteriocin genes in one of these new loci (*skkA* and *skkB* of *Lactobacillus sakei* 23K), were indeed shown to be bacteriocinogenic. The associated Abi gene *skkI* conferred immunity to the bacteriocins when expressed in a sensitive strain. Correspondingly, the Abi genes *plnI* and *plnLR* from the plantaricin locus of *Lactobacillus plantarum* were also shown to confer immunity to their cognate class IIb bacteriocins (plantaricin EF and JK, respectively). Most known immunity genes act specifically against their cognate bacteriocins. However, cross immunity between *skkI*, *plnI* and *plnLR* was observed, thus suggesting that different Abi immunity proteins might recognize the same target molecule(s). Proteins of the Abi family are characterized by three highly conserved sequence motifs which are thought to constitute the active site of a proteolytic function, and indeed, site-directed mutations in these motifs in SkkI abolished its immunity function. This finding might suggest that the Abi proteins confer bacteriocin immunity via a proteolytic mechanism.

Sammendrag

Bakteriosiner er antimikrobielle peptider produsert av bakterier som viser aktivitet mot stammer som oftest er nært beslekta med produsenten. Bakteriosiner produsert av melkesyrebakterier har vært spesielt i fokus de siste tiårene. Disse viser antimikrobiell aktivitet mot uønskede bakterier i mat og mot patogene bakterier, og de kan derfor potensielt komme til nytte både innenfor næringsmiddelindustrien og i behandling av infeksjoner. Flere hundre ulike bakteriosiner fra melkesyrebakterier har blitt identifisert, men de molekylære mekanismene som ligger til grunn for antimikrobiell aktivitet, resistensutvikling og produsentimmunitet er for det meste ukjente. De fire arbeidene i denne avhandlinga belyser alle ulike aspekter ved slike mekanismer.

I den første delen avhandlinga (artikkel I og II) ble det undersøkt hvordan bakteriosiner som tilhører klasse IIa og IIc spesifikt gjenkjenner en reseptor på målcellene. Det er kjent fra tidligere at disse bakteriosinene binder til det membranlokaliserte proteinkomplekset IIC-IID i mannose fosfotransferasesystemet (man-PTS) og danner porer i membranen til sensitive celler. I den første artikkelen ble det vist ved fylogenetiske analyser og heterologt uttrykk av man-PTS proteiner fra ulike bakterier at klasse IIa bakteriosiner kun gjenkjenner man-PTSer fra en fylogenetisk definert undergruppe. Man-PTSene i denne gruppa stammer fra Gram-positive bakterier som tidligere har vist seg å være sensitive for klasse IIa bakteriosiner. Bakteriosinet lactococcin A fra klasse IIc viste derimot en helt annen reseptorspesifisitet enn bakteriosinene fra klasse IIa og kunne utelukkende benytte man-PTS fra *Lactococcus* som reseptor. Dette resultatet stemmer overens med at lactococcin A kun er aktiv mot ulike *Lactococcus*-stammer. Til sammen viser disse funnene at forskjeller i sensitivitet for klasse IIa bakteriosiner og lactococcin A mellom ulike bakteriearter/-slekter i stor grad kommer av ulik sekvens/struktur hos man-PTS reseptorene. For videre å karakterisere den spesifikke interaksjonen mellom man-PTS og klasse IIa bakteriosiner, ble en rekke hybride man-PTS systemer konstruert basert på genene fra den klasse IIa-sensitive *Listeria monocytogenes* (*mpt*) og den insensitive *Lactococcus lactis* (*ptn*). Denne framgangsmåten gjorde det mulig å identifisere en ekstracellulær loop i man-PTS IIC proteinet som var ansvarlig for den spesifikke interaksjonen med klasse IIa bakteriosiner. På tilsvarende måte ble det vist at lactococcin A har en mer kompleks reseptorinteraksjon som trolig involverer binding til både IIC og IID proteinene.

I artikkel III ble det studert hvordan sensitive bakterier utvikler resistens mot klasse IIa bakteriosiner og lactococcin A. Tidligere studier har påvist en sammenheng mellom indusert resistens mot klasse IIa bakteriosiner i *Li. monocyotogenes* og redusert genuttrykk av man-PTS. I artikkel III ble naturlige isolater av *Li. monocytogenes* med ulik sensitivitet for klasse IIa bakteriosiner samt lactococcin A resistente *L. lactis* mutanter undersøkt. Det ble vist at nedregulering av man-PTS genene utgjør en generell resistensmekanisme mot ulike typer bakteriosiner som finnes både i naturlige isolater med lav bakteriosinsensitivitet og i resistente mutanter generert ved bakteriosineksponering. I

tillegg ble det også identifisert resistente celler som hadde normalt uttrykk man-PTS. Dette viser at en annen, men hittil ukjent mekanisme også er involvert i resistens mot disse bakteriosinene.

De molekylære mekanismene som ligger til grunn for målcellespesifisitet og immunitet hos klasse IIa bakteriosiner har nå blitt grundig studert. For andre bakteriosiner fra klasse II derimot, er slike mekanismer fortsatt lite kjent. Det ble nylig påvist at noen bakteriosinsystemer i streptokokker og laktobasiller inneholder gener som koder for såkalte Abi proteiner. Abi proteiner er transmembrane proteaser som antas å være involvert i bakteriosinimmunitet i disse bakteriene. I den siste delen av avhandlinga (artikkel IV) ble Abi proteinfamilien studert med spesielt fokus på hvordan disse proteinene er involvert i immunitet mot bakteriosiner fra klasse II. Bioinformatiske analyser viste at Abi familien har flere medlemmer enn det som i dag er kjent ut fra annotering i databasene. Ved å søke etter Abi gener i sekvenserte genomer ble sju nye genklustere, som potensielt koder for bakteriosiner, identifisert. Ved heterologt uttrykk av bakteriosingenene fra ett av genklusterene (*skkA* og *skkB* fra *Lactobacillus sakei* 23K) ble det vist at disse har bakteriosinaktivitet. Det korresponderende Abi genet *skkI* ga immunitet mot bakteriosinene når det ble uttrykt i en sensitiv stamme. På samme måte ble det vist at to andre Abi gener, *plnI* og *plnLR* fra plantaricinlokuset i *Lactobacillus plantarum* ga immunitet mot sine respektive klasse IIb bakteriosiner, plantaricin EF og JK. Kryssimmunitet mellom *skkI*, *plnI* and *plnLR* ble også observert, noe som kan tyde på at de ulike Abi proteinene benytter samme mekanisme for å gi celler immunitet mot disse bakteriosinene. Abi proteiner kjennetegnes ved tre konserverte motiver som antas å utgjøre et aktivt sete med proteolytisk funksjon. Ved å innføre mutasjoner i aminosyrer som er en del av disse motivene, ble det vist at SkkI mister immunitetsfunksjonen. Dette kan tyde på at Abi proteiner gir bakteriosinimmunitet via en proteolytisk mekanisme.

List of papers

List of papers included in this thesis (referred to as paper I-IV in the text):

Paper I

Kjos M, Nes IF & Diep DB (2009) Class II one-peptide bacteriocins target a phylogenetically defined subgroup of mannose phosphotransferase systems on sensitive cells. Microbiology. 155 (9): 2949-61

Paper II

Kjos M, Salehian Z, Nes IF & Diep DB (2010) An extracellular loop of the mannose phosphotransferase system component IIC is responsible for specific targeting by class IIa bacteriocins. J. Bacteriol. 192 (22): 5906-13

Paper III

Kjos M, Nes IF & Diep DB. Resistance mechanisms against bacteriocins targeting the mannose phosphotransferase system. Manuscript.

Paper IV

Kjos M, Snipen L, Salehian Z, Nes IF & Diep DB (2010) The Abi proteins and their involvement in bacteriocin self-immunity. J. Bacteriol. 192 (8): 2068-76

Other relevant papers by the author:

Straume D, Kjos M, Nes IF & Diep DB (2007) Quorum-sensing based bacteriocin production is down-regulated by N-terminally truncated species of gene activators. Mol. Genet. Genomics. 278 (3) 283-93

Kjos M, Straume D, Nes IF & Diep DB (2009) Transposition of IS10R in *Lactococcus lactis*. J. Appl. Microbiol. 106 (1) 288-95

Diep DB, Straume D, Kjos M, Torres C & Nes IF (2009) An overview of the mosaic bacteriocin *pln* loci from *Lactobacillus plantarum*. Peptides. 30 (8): 1562-74

1. Introduction

1.1 Bacteriocins

Production of antimicrobial peptides is a widespread defence mechanism in many forms of life, from the innate immune systems in multicellular organisms to the production of bacteriocins by bacterial cells. Bacteriocins has been defined as "bacterially produced, small, heat-stable peptides that are active against other bacteria and to which the producer has a specific immunity mechanism" (30). Bacteriocin production is ubiquitous in the bacterial world, and the dissemination of this trait has probably been facilitated by the fact that genetic determinants responsible for bacteriocin production often are located on mobile genetic elements, such as conjugative plasmids or transposons (103). Bacteriocins play important roles in the ecology of both Gram-negative and Gram-positive bacteria, where they offer an advantage for the producer over non-producing cells in the competition for common resources. The spectrum of inhibition for bacteriocins is generally rather narrow, as they mainly kill bacteria closely related to their producers, although some peptides show antimicrobial activity across several bacterial genera.

The bacteriocins investigated in this thesis are all produced by lactic acid bacteria (LAB). LAB is a group of low-GC Gram-positive bacteria belonging to the phylum *Firmicutes*. These bacteria are of great economic importance due to their use in food fermentations (e. g., dairy products, meat, vegetables and beverages), where compounds produced by LAB are essential for flavour, texture and preservation. The preservative properties of LAB come both from general growth inhibitory mechanisms, such as pH reduction due to lactic acid production, and more specific antibacterial effects, for example by production of bacteriocins (171).

Due to the widespread bacteriocin production among LAB, mankind has probably unintentionally benefited from the preservative effect of bacteriocins for thousands of years as these microorganisms are commonly found in diverse meat and plant materials. Since LAB are generally regarded as safe for use in food, LAB bacteriocins, still today, certainly hold a great potential for applications in food production. Some bacteriocins can inhibit specific food spoilage bacteria and foodborne pathogens such as clostridia, *Listeria monocytogenes* and *Staphylococcus aureus* but they can also function as agents to manipulate microbial populations in food systems (i. e., promote the growth of strains with desirable properties by inhibiting competing strains). Bacteriocins can be introduced to food systems in at least three different ways; (i) by introduction of a bacteriocin-producing strain in fermented food or (ii) by using purified peptides or (iii) fermentates from bacteriocin producing cultures as food additives (30). The first reports to describe a LAB bacteriocin came in 1928 (157, 158), and deliberate use of bacteriocins in food dates back to the 1950s when nisin was developed as a food additive in England. Nisin has for decades been a common food additive in many countries, however, the success story of the application of nisin has yet to be repeated for other

bacteriocins. The only other bacteriocin-based additive which has been commercialized for use in food is a fermentate from *Pediococcus acidilactici* containing pediocin PA-1 (37, 156). Nevertheless, by proper design of treatment strategies, different bacteriocins may have biopreservative potentials in a variety of foods, including dairy and meat products, vegetables and alcoholic beverages (68, 140).

In the medical field there is great interest in novel antimicrobial compounds, especially in the light of the ever-increasing antibiotic resistance among pathogenic bacteria. Different LAB bacteriocins are known to target many Gram-positive pathogens *in vitro*, including emerging antibiotic resistant bacteria such as *Clostridium difficile*, methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant enterococci (VRE) (146, 152), and recent research has shown that bacteriocin-based therapeutics might be a solution in the fight against these challenging bacteria. For example, studies in distal colon models have demonstrated that the narrow spectrum bacteriocin thuricin CD specifically eliminates *C. difficile* without disrupting the beneficial microbial community in the gastrointestinal tract (151). Such a bacteriocin-based strategy might therefore prove superior to current treatment of *C. difficile*-associated intestinal diseases using broad spectrum antibiotics, which often encounter problems with recurring infections and antibiotic resistance development (5). Furthermore, several *in vivo* studies on animal models have demonstrated that bacteriocins indeed hold a great potential as therapeutic agents; it has been shown that bacteriocins can eradicate infections caused by *Streptococcus pneumonia* and MRSA in mice (71, 118) as well as having preventive effects against tooth diseases in dog (97) and bovine mastitis in dairy cows (180). Also, recent studies have led to increased interest in the use of bacteriocin-producing strains as probiotic bacteria (70). Most compelling, in a study by Corr et al. (28) in 2007 it was demonstrated that *Li. monocytogenes* infections in mice could be treated with a bacteriocin produced by the probiotic strain of *Lactobacillus salivarius* UCC118 supplied in feed. This study highlights the potential of such probiotics to specifically combat infectious agents in the gastrointestinal tract.

1.1.1 Classification of LAB bacteriocins

The research on bacteriocins has exploded during the last 20 years, and bacteriocins today constitute a heterogeneous group of peptides with great variations in size, structure and mode of action. Classification is therefore crucial to get an overview of the characteristics and properties of the various peptides. Peptide bacteriocins produced by Gram-negative species, also known as microcins, are divided into a number of subgroups (47), while bacteriocins from Gram-positive bacteria most often are sorted according to the classification schemes for LAB bacteriocins which was first proposed by Klaenhammer (114) in 1993. This scheme has later been revised several times to accommodate the discovery of new types of bacteriocins (e. g., 30, 112, 132, 133). As LAB bacteriocins are the focus of this thesis, only Gram-positive bacteriocins will be dealt with here.

Bacteriocins from Gram-positive bacteria are divided into two major groups: the lantibiotics (class I) and the non-lantibiotics. The lantibiotics are small peptides of 19-38 amino acids containing post-translational modifications. They are characterized by thioether-based internal ring structures (known as lanthionine or β-methyllanthionine) which are formed by the dehydration of selected serine and/or threonine residues and the subsequent formation of a thiol-bridge between some of the dehydrated residues and neighbouring cysteine residues (16). The lantibiotics may also contain other unusual amino acids formed by post-translational processes, such as D-alanine (169). Because of large structural variations, subclassification of lantibiotics is not straightforward, and up to 11 subclasses have been suggested (14, 29). Nisin is the best known member of the lantibiotics.

The other main cluster of LAB bacteriocins contains the non-lantibiotic bacteriocins, i. e., their residues are not subjected to post-translational modifications (except formation of disulphide bridges and circularisation of cyclic peptides). Some variations in the classification of these peptides are found in the literature; while Cotter et al. (30) suggest that all non-lantibiotics should be assembled in one class (class II) with four subgroups (pediocin-like bacteriocins in IIa, two-peptide bacteriocins in IIb, cyclic bacteriocins in IIc and non-pediocin-like, linear bacteriocins in IId), Nes et al. (133) assigned the cyclic bacteriocins to a separated class (class IV), and separated the remaining class II bacteriocins into five subgroups (pediocin-like bacteriocins in IIa, two-peptide bacteriocins in IIb, unsorted bacteriocins in IIc, leaderless bacteriocins in IId and larger protein-derived bacteriocins in IIe). In this thesis, the bacteriocins are classified according to Nes et al. (133) (Fig. 1), and only peptides belonging to subgroup IIa, IIb and IIc will be dealt with in detail, since these are the peptides which have been investigated.

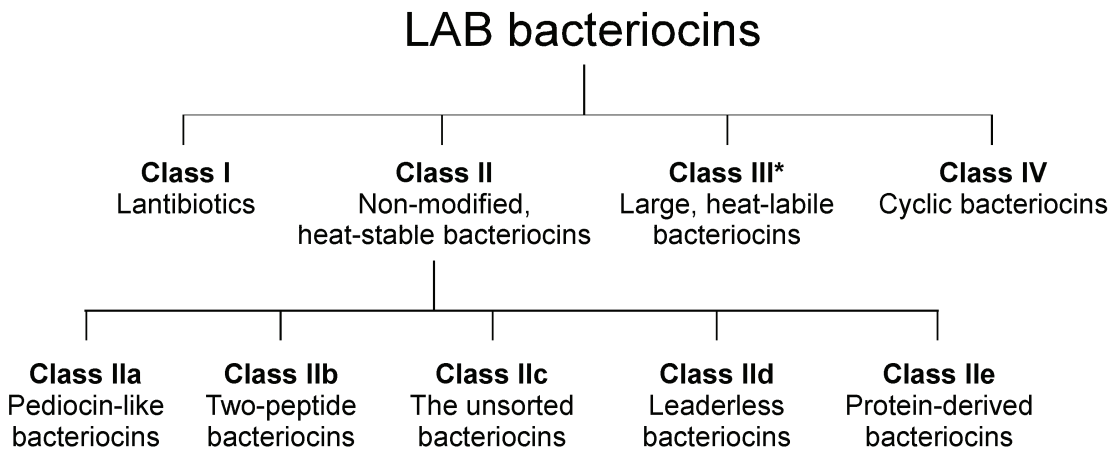

Fig. 1. Classification of LAB bacteriocins according to Nes et al. (133). Class III (*) consists of proteins rather than peptide bacteriocins, however, this class is still included in the classification scheme.

1.1.2 Class IIa bacteriocins

Class IIa bacteriocins, also known as pediocin-like bacteriocins, constitute a large group of peptides with over 35 members (Table 1). Class IIa bacteriocins are particularly active against *Listeria*, but their inhibitory spectra also includes a number of other genera such as *Enterococcus*, *Carnobacterium*, *Lactobacillus*, *Leuconostoc, Pediococcus* and *Clostridium* (49).

Table 1 Subclassification of class IIa bacteriocins based on Nissen-Meyer *et al.* (136).

Bacteriocin	Producer	Mature sequence	Ref.
Subgroup 1			
Enterocin A	*E. faecium*	TTHSGKYYGNGVYCTKNKCTVDWAKATTCIAGMSIGGFLGGAIPG--KC	(7)
Divercin V41	*C. divergens*	TKYYGNGVYCNSKKCWVDWGCASGCIGQTVVGGWLGGAIPG--KC	(126)
Divergicin M35	*C. divergens*	TKYYGNGVYCNSKKCWVDWGIAQGCID--VVICQLGGGIPGKGKC	(175)
Bavaricin MN	*Lb. sakei*	TKYYGNGVYCNSKKCWVDWGCAAGGICQTVVXGWLGGAIPGK	(108)
Coagulin	*B. cogulans*	KYYGNGVTCGKHSCSVDWGKATTCIINNGAMAWATGGHQGTHKC	(121)
Pediocin PA-1	*P. acidilactici*	KYYGNGVTCGKHSCSVDWGKATTCIINNGAMAWATGGHQGNHKC	(92, 134)
Mundticin	*E. mundtii*	KYYGNGVSCNKKGCSVDWGKAIGIICNNSAANLATGGAAGWSK	(11)
Piscicocin CS526	*C. piscicola*	KYYGNGLSXNKKGCXVDWGKAIGIICNNAAANXATGGAAGxNK	(194)
Piscicocin 126/V1a	*C. piscicola*	KYYGNGVSCNKNGCTVDWGKAIGIICNNAAANLTTGGAAGWNKG	(12, 104)
Sakacin P	*Lb. sakei*	KYYGNGVHCGKHSCTVDWGTAIGNICNNAAANWATGGNAGWNK	(178)
Leucocin C	*Le. mesenteroides*	KNYGNGVHCTKKGCSVDWGYAWTNIANNSVMNGLTGGNAGWHN	(62)
Listeriocin 743A	*Li. innocua*	KSYGNGVQCNKKKCWVDWGSAISTICNNSAANWATGGAAGWKS	(109)
Sakacin 5X	*Lb. sakei*	KYYGNGLSCNKSGCSVDWGKAISIICNNAVANLTTGGAAGWKS	(188)
Enterocin CRL35/Mundticin KS	*E. mundtii*	KYYGNGVSCNKKGCSVDWGKAIGIICNNSAANLATGGAAGWKS	(55, 110)
Avicin A	*E. avium*	AYYGNGVSCNKKGCSVDWGKAISIICNNSAANLATGGAAGWKS	(15)
Mundticin L	*E. mundtii*	KYYGNGLSCNKKGCSVDWGKAIGIICNNSAANLATGGAAGWKS	(57)
Enterocin HF	*E. faecium*	KYYGNGVSCNKKGCSVDWGKAIGIICNNAAANLTTGGKAAWAC	(3)
Bavaricin A	*Lb. bavaricus*	KYYGNGVHXGKHSXSVDWGTAIGNICNNAAANXATGXNAGG	(120)
Ubericin A	*S. uberis*	KTVNYGNGLYCNQKKCWVNWSETATTIVNNSIMNGLTGGNAGWHSGGRA	(93)
Subgroup 2			
Leucocin A	*Leuconostoc spp.*	KYYGNGVHCTKSGCSVNWGEAFSAGVHRLANGGNGFW	(81)
Mesentericin Y105	*Le. mesenteroides*	KYYGNGVHCTKSGCSVNWGEAASAGIHRLANGGNGFW	(87)
Leucocin B	*Le carnosum*	KYYGNGVHCTKSGCSVNWGEAFSAGVHRLANGGNGFW	(56)
Sakacin G	*Lb. sakei*	KYYGNGVSCNSHGCSVNWGCAWTCGVNHLANGGHGVC	(168)
Plantaricin 423	*Lb. plantarum*	KYYGNGVTCGKHSCSVNWGCAFSCSVSHLANEGHGKC	(187)
Plantaricin C19	*Lb. plantarum*	KYYGNGLSCSKKGCTVNWGCAFSCGVNRVATAGHGKX	(6)
Subgroup 3			
Curvacin A/ Sakacin A	*Lb.curvatus/Lb.sakei*	ARSYGNGVYCNNKKCWVNRGEATQSIIGGMISGWASGLAGM	(95, 177)
Carnobacteriocin BM1	*C. piscicola*	AISYGNGVYCNKEKCWVNKAENKQAITGIVIGGWASSLAGMGH	(148)
Enterocin P	*E. faecium*	ATRSYGNGVYCNNSKCWVNWGEAKENIAGIVISGWASGLAGMGH	(25)
Piscicocin V1b	*C. piscicola*	AISYGNGVYCNKEKCWVNKAENKQAITGIVIGGWASSLAGMGH	(12)
Subgroup 4			
Penocin A	*P. pentosaceus*	KYYGNGVHCGKKTCYVDWGCATASIGKIIVNGWTQHGPWAHR	(41)
Bacteriocin 31	*E. faecalis*	AHYYGNGLYCNKQKCWVDWNKASREIGKIIVNGWVQHGPWAPR	(179)
Bacteriocin RC714	*E. faecium*	AHYYGNGLYCNKEKCWVDWNQAKGEIGKIIVNGWVNHGPWAP	(38)
Hiracin JM79/Bacteriocin T8	*E. hirae/E. faecium*	AHYYGNGLYCNKEKCWVDWNQAKGEIGKIIVNGWVNHGPWAPRR	(36, 164)
Enterocin SE-K4	*E. faecalis*	AHYYGNGVYCNKQKCWVDWGRARSEIIDRGVKAYVNGFIKVLGGIGGR	(48)
Carnobacteriocin B2	*C. piscicola*	NYGNGVSCSKTKCSVNWGCAFQERYTAGINAFVSGVASGASIGRRP	(148)
Bacteriocin MC4-1	*E. faecalis*	AHYYGNGVYCNKQKCWVNWGCAWSEGVKRWGDNLFGSFSGGRI	(64)
Lactococcin MMFII	*L. lactis*	TSYGNGVHCNKSKCWIDVSELETYKAGTVSNPKDILW	(58)
SRCAM 1580	*B. circulans*	NVNYGNGVSCSKTKCSVNWGIITHQAFRVTSGVASG	(173)
CONSENSUS		YGNGV C C V W A	

Bacteriocins of this group are characterized by an N-terminal pediocin consensus motif YGNGVxCxxxxCxVxWxxA (where x is any amino acids), in which the first four amino acids (YGNG) and the two cysteins (C) are invariant in all members of class IIa. The lengths of these peptides range from 36 (plantaricin C19) to 49 (ubericin A) amino acids. Based on their sequence, each bacteriocin appears to contain two distinct domains (106, 136); (i) a highly conserved, cationic, hydrophilic N-terminal region containing the pediocin motif and (ii) a less conserved, more hydrophobic C-terminal region. Based on sequence variations at the C-terminus, the class IIa bacteriocins have been further divided into at least four subgroups (Table 1).

Class IIa bacteriocins are unstructured in aqueous solutions but become structured upon contact with membranes or lipid-like environments (66, 85). The three-dimensional structure has been resolved for four class IIa bacteriocins: sakacin P (Fig. 2A) (181), leucocin A (Fig. 2B) (66), curvacin A (Fig. 2C) (85) and carnobacteriocin B2 (190). These studies showed that the two independent domains are separated by a flexible hinge (181): In the conserved, N-terminal part, all peptides contain an anti-parallel β-sheet which is stabilized by a disulphide bridge formed between the two conserved cysteine residues. In the C-terminal region, peptides of the subgroups 1, 2 and 4 contain a hairpin structure consisting of an α-helix followed by an extended tail which folds back to create the hairpin (Fig. 2D). In order to stabilize the hairpin structure, some bacteriocins (enterocin A, divercin V41, divergicin M35, coagulin, pediocin PA-1, sakacin G and plantaricin 423), contain a second disulphide bridge formed between a C-terminal cysteine and a cysteine within the α-helix, while in other class IIa bacteriocins the hairpin is stabilized by two tryptophane residues; one located in the hinge region and the other near the C-terminus of the peptide. The subgroup 3 peptides differ from the others in the C-terminal region. Instead of a hairpin, these peptides contain a functionally equivalent helix-hinge-helix structure (85, 86) (Fig. 2C).

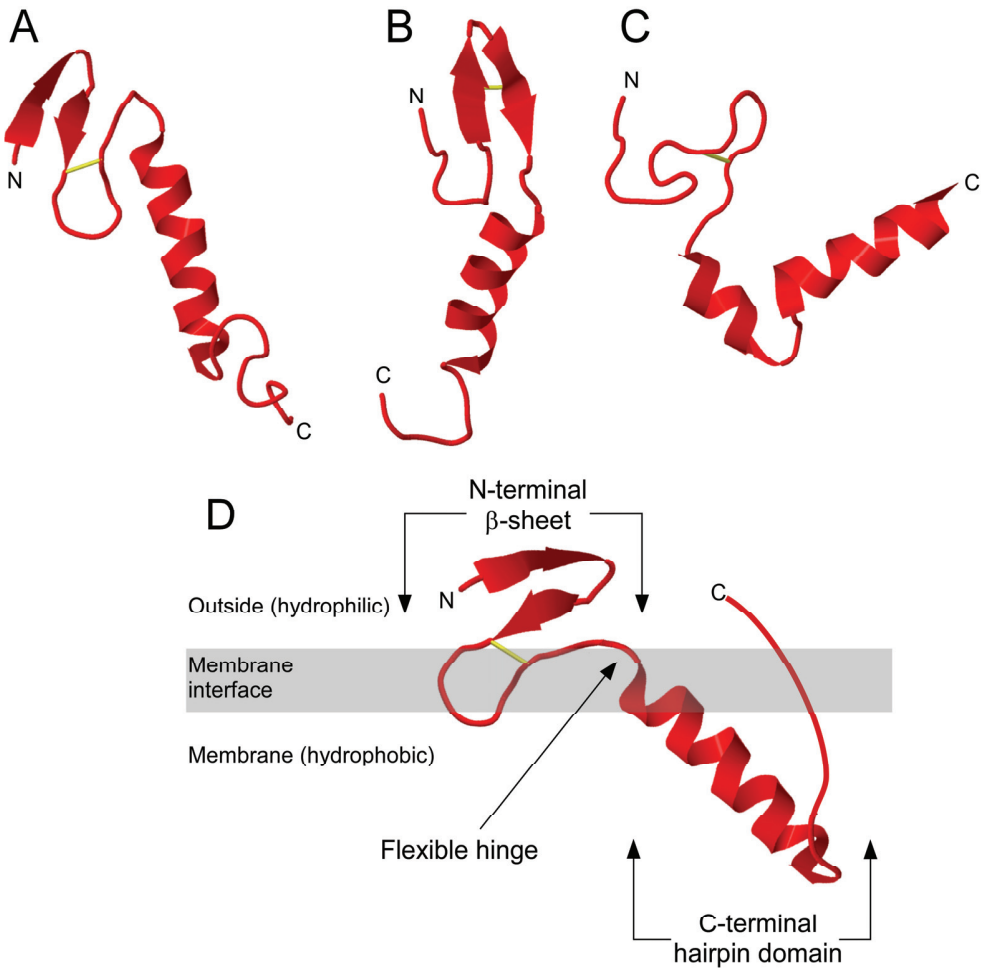

Fig. 2 Three-dimensional structures of class IIa bacteriocins from subgroup 1 (sakacin P; A), subgroup 2 (leucocin A; B) and subgroup 3 (curvacin A; C). The structures were visualized using Protein Explorer (www.proteinexplorer.org). (D) A model depicting the proposed orientation of subgroup 1,2 and 4 class IIa bacteriocins in membranes (based on Nissen-Meyer et al. (136)). Cysteine bridges are shown in yellow. The N- and C-termini are indicated.

1.1.3 Class IIb bacteriocins

The bacteriocins assigned to class IIb (two-peptide bacteriocins) differ conceptually from other class II bacteriocins in that they require the combined action of two different peptides for optimal antimicrobial activity (142). Although they function as one antimicrobial entity, the individual peptides share a number of characteristics with one-peptide bacteriocins. They are mostly cationic and contain both amphiphilic and hydrophobic regions. In some cases, individual peptides may display some antimicrobial activity, however, the activity is significantly higher when both complementary peptides are present (2, 105). The two genes encoding the individual peptides are located next to each

other in the same operon. To date, at least 17 different class IIb bacteriocins have been characterized (141).

The lactococcal bacteriocin lactococcin G from *Lactococcus lactis* (135) along with plantaricins EF and JK (42) produced by *Lactobacillus plantarum*, are the best studied class IIb bacteriocins. The three dimensional structures of the individual peptides (lengths ranging from 25 to 38 aa) constituting these bacteriocins have been resolved (Fig. 3) (63, 159, 160). Similar to class IIa bacteriocins, two-peptide bacteriocins are unstructured in aqueous solutions, but become structured in membrane-mimicking environments. The structures are characterized by a well-defined, central α-helix region with flexible regions in both ends. Although no structure has been determined for the peptide heterodimers, it has been shown that there is direct physical interaction between the complementary peptides when they exert antimicrobial activity (82, 83). Mutational analysis of lactococcin G suggests that the peptides interact by helix interactions to form a parallel transmembrane helix-helix structure (141, 143). The inhibitory spectra of these bacteriocins are narrow; lactococcin G is active only against strains of *Lactococcus* (195), while plantaricins EF and JK, which are co-produced by the same host, display activity only against a few strains of *Lactobacillus* and *Pediococcus* (4).

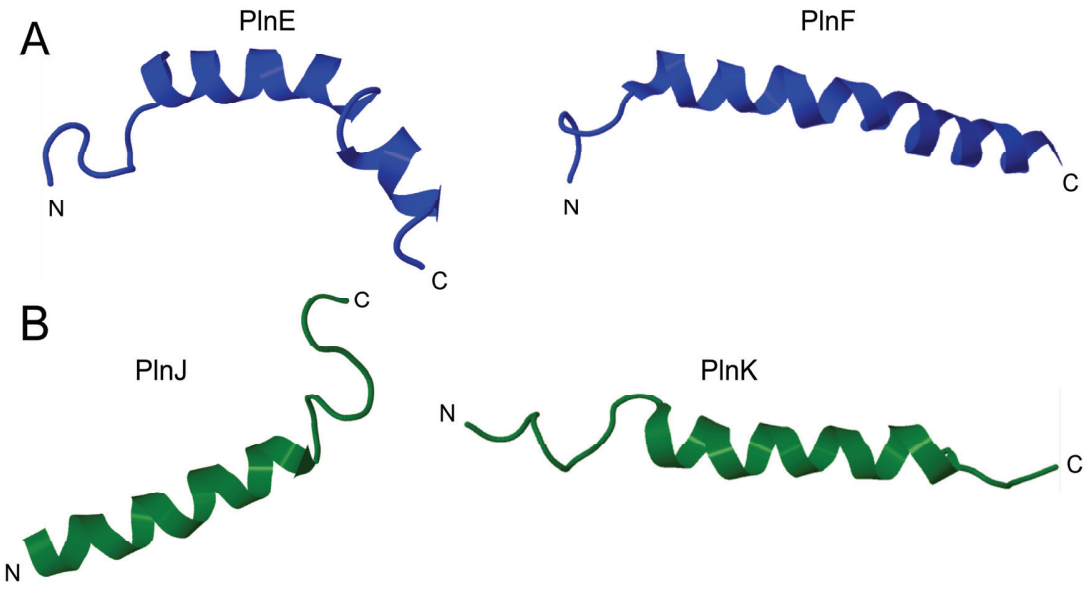

Fig. 3 Three-dimensional structures of the individual peptides constituting the two-peptide bacteriocins plantaricin EF (A) and plantaricin JK (B). The structures were visualized using Protein Explorer (www.proteinexplorer.org). The N- and C-termini are indicated.

1.1.4 Lactococcin A – a class IIc bacteriocin

Lactococcin A is a 54 aa long lactococcal one-peptide bacteriocin without modified amino acids (96). In contrast to class IIa bacteriocins, lactococcin A does not contain the characteristic N-terminal pediocin-motif, and thus belongs to class IIc according to Nes et al. (133). Lactococcin A has a very narrow spectrum of inhibition, as it only kills strains of *Lactococcus* (96). The three-dimensional structure of lactococcin A has not been resolved.

1.1.5 Genes involved in the production of class II bacteriocins

Genes encoding bacteriocins are associated with other genes required for bacteriocin production, such as genes involved in processing, transport, regulation and immunity. The genetic loci for four different class II bacteriocins are displayed in Fig. 4.

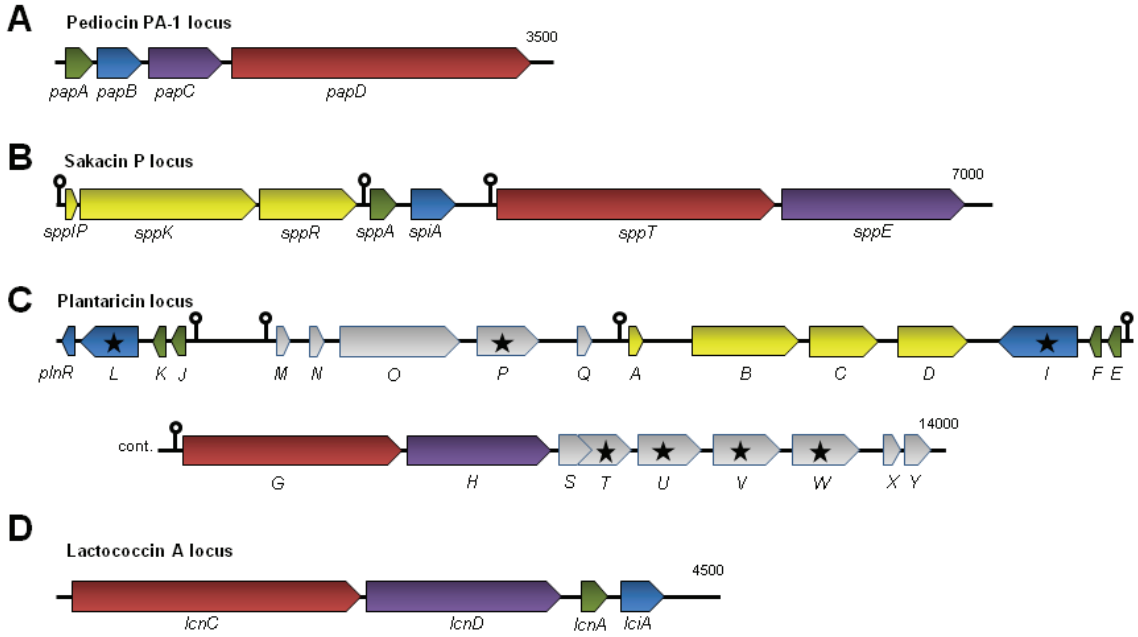

Fig. 4 Genetic organization of the pediocin PA-1 locus of *Pediococcus pentosaceus* (130) (A), sakacin P locus of *Lactobacillus sakei* (101) (B), plantaricin-locus of *Lb. plantarum* (42) (C) and lactococcin A locus of *L. lactis* (172) (D) Gene names are given below the arrows. The loci in A and B encode class IIa bacteriocins, the locus in C encodes two separate class IIb bacteriocins, while the locus in D encodes a class IIc bacteriocin. Bacteriocin structural genes are shown in green, putative immunity genes in blue, ABC-transporter genes in red, accessory transport genes in purple, regulatory genes in yellow and genes of unknown function in grey. Genes in the plantaricin locus encoding proteins belonging to the Abi family are marked with a black asterisk (see section 1.3.2). Regulated promoters are indicated with open circles.

The bacteriocin structural gene usually encodes the bacteriocin itself with an N-terminal extension involved in secretion. This N-terminal extension, normally referred to as the leader sequence, renders the intracellular bacteriocin inactive, and is cleaved off upon externalization to produce the mature and active peptide. The leader sequence of most class II bacteriocins is of the so-called double glycine leader type containing the consensus motif LSxxELxxIxGG (where x is any amino acid), in which the glycine at the second-to-last position (underlined) is fully conserved (132). Double glycine leaders, whose total lengths range between 15 and 30 amino acids, are cleaved off at the C-terminal side of the second glycine. The processing and export of these peptides are facilitated by a dedicated ATP-binding cassette (ABC) transporter and an accessory protein (102, 132). A few bacteriocins from class IIa, such as enterocin P (25) and hiracin JM79 (164), employ the general secretory systems (*sec*) for export, and therefore contain *sec*-dependent leaders. Furthermore, bacteriocins belonging to class IId, such as enterocin L50 (27), enterocin Q (26) and lacticin Q (67), does not contain any leader sequences at all, and the mechanism involved in export of these peptides is largely unknown, although genes encoding ABC-transporter-like proteins are normally found in the vicinity of the bacteriocin structural genes in the genome (31).

Production of bacteriocins is energy consuming, and this process is therefore often under tight regulation. In many LAB, regulation of bacteriocin production is mediated by a quorum sensing based mechanism commonly referred to as a three-component regulatory system (132). The three genes involved encode a secreted inducer peptide, a histidine protein kinase and a response regulator. When the extracellular concentration of inducer peptide exceeds a certain threshold level, it binds to the membrane-located histidine protein kinase and triggers a cascade of phosphorylation reactions that eventually lead to phosphorylation of the response regulator, which in turn binds specifically to selected promoters to activate transcription (132). Such a mechanism allows the bacterial population to communicate and coordinate the production of bacteriocins, as the threshold level of inducer peptide is only achieved at high cell densities.

Bacteriocin producing bacteria also contain genes that confer self-protection or immunity to their own bacteriocins. These genes are often co-regulated with the bacteriocin structural genes, and the mechanisms of action of such proteins will be described in section 1.3.1.

1.2 Bacteriocin mode of action and target recognition

1.2.1 Mode of action

It is generally recognized that LAB bacteriocins kill target cells by creating pores in the membrane, although alternative mechanisms, such as inhibition of cell wall synthesis, have also been described (22, 50). Pore formation causes leakage of low molecular weight compounds (e. g., ions K^+, PO_4^{2-}, H^+) leading to dissipation of the proton motive force (the transmembrane electric potential $\Delta\psi$ and the pH

gradient ΔpH) that is deleterious to cells. The nature of the pores, in terms of size, stability and conductivity of different compounds, can differ considerably between bacteriocins (50). For example, the lantibiotic nisin causes efflux of a wide range of molecules and dissipates both Δψ and ΔpH (161), while the class IIb lactococcin G selectively conducts K^+ ions, causing dissipation of Δψ but not ΔpH (128). Lactococcin A mediated pores cause dissipation of Δψ and partial dissipation of ΔpH by allowing free diffusion of ions and amino acids (185). Similar properties were observed for pediocin PA-1, which induces efflux of K^+, PO_4^{2-} and amino acids (24), while another class IIa bacteriocin, enterocin P, was shown to selectively conduct K^+ to dissipate Δψ but not ΔpH (94). Moreover, the two-peptide plantaricins PlnEF and PlnJK both dissipate Δψ and ΔpH. However, their pores appear to be different since PlnEF preferentially conducts monovalent cations (e. g., K^+, H^+), while PlnJK conducts selected anions (e. g., glutamate) to a larger extent than cations, suggesting that these co-produced bacteriocins (Fig. 4) have evolved complementary modes of action (129).

In order to form pores, the bacteriocins need to interact with the target cell envelope. The initial attraction of bacteriocins to the cell membrane is probably partly governed by electrostatic interactions between the positively charged peptides and the anionic lipids in the bacterial membranes (50). Other factors, such as membrane potential and pH can also affect binding of bacteriocins to target cells (50). It has long been debated whether bacteriocin-mediated pore formation can occur without a specific receptor on the target cell surface. At high concentrations (μM and higher) it has been shown that bacteriocins can form pores by similar mechanisms as positively charged eukaryotic antimicrobial peptides: bacteriocins bind to anionic lipids and insert unspecifically into the phospholipid bilayer, wherein aggregation of peptides leads to formation of short-lived pore-like structures (18, 186). However, this unspecific pore formation at high peptide concentrations is not the characteristic mechanism of action for most LAB bacteriocins, which typically involves prompt release of intracellular molecules and killing of sensitive cells at very low concentrations (in nM range). Early observations that some bacteriocins were active against whole cells or protein-containing vesicles but not against protein-free vesicles, led to hypotheses that a cellular receptor must be involved in the target recognition (24, 185). However, such cellular targets remained enigmatic until a decade ago when it was demonstrated that nisin and some other lantibiotics (class I) specifically bind to the cell wall precursor molecule lipid II, to form lethal pores and/or inhibit cell wall synthesis in sensitive cells (19, 21). Some years later it was established that the membrane proteins belonging to a sugar transporter, the mannose phosphotransferase system (man-PTS), is the target receptor for pediocin-like class IIa bacteriocins and the class IIc bacteriocins lactococcins A and B (33, 43, 88, 149).

1.2.2 Lipid II as target for lantibiotics

Lipid II is a vital precursor in the bacterial cell wall biosynthesis and consists of a peptidoglycan subunit (N-acetylglucosamine–N-acetylmuramic acid–pentapeptide) coupled to the membrane anchored lipid (bactoprenyl phosphate). Lipid II is synthesized intracellularly and translocated across the phospholipid bilayer to supply peptidoglycan subunits to the growing cell wall (18). Binding to lipid II by antimicrobial compounds appears to be an effective means to kill bacteria, since this molecule is targeted by at least four different classes of antibiotics; glycopeptides antibiotics (vancomycin), mannopeptimycins and ramoplanin as well as some lantibiotics (class I bacteriocins) (18). Nisin and epidermin were the first lantibiotics shown to use lipid II as a docking molecule (21), and the mechanism of action for nisin has been characterized in great detail. Nisin binds lipid II via the characteristic lantibiotic ring structures in the N-terminal part of the peptide, and pores containing both nisin and lipid II are formed (98). In addition, nisin also inhibits target cells by blocking cell wall formation (80). This mechanism is independent of the pore-forming activity and involves relocation of lipid II into patches outside their functional location.

A number of different lantibiotics with N-terminal ring structures similar to nisin kill target cells by lipid II mediated pore formation (18). These include subtilin and members of the epidermin family as well as some two-component lantibiotics, such as lacticin 3147 (18, 191). A lipid II binding mechanism to inhibit cell wall synthesis has also been proposed for the lantibiotic mersacidin family and the non-lantibiotic lactococcin 972, however this activity does not include pore-formation (20, 125).

1.2.3 The mannose-phosphotransferase system as a bacteriocin receptor.

Man-PTSs belong to a group of transport systems known as phosphoenolpuruvate:carbohydrate phosphotransferase systems (PTSs), which are characterized by their ability to couple the import and phosphorylation of incoming sugars (119, 147). The main constituents of a PTS are the general PTS proteins enzyme I (EI) and HPr as well as a carbohydrate specific protein complex known as enzyme II (EII). Most EII complexes consist of three or four subunits; IIA, IIB, IIC and in some cases also IID. The IIA and IIB subunits are located in the cytoplasm along with the general proteins EI and HPr, while the IIC and IID subunits are transmembrane proteins which form a carbohydrate-specific translocation channel (Fig. 5). Based on differences in the EIIs, PTSs are classified into four superfamilies; the glucose-fructose-lactose superfamily, the ascorbate-galactitol superfamily, the mannose family and the dihydroxyacetone family (162). Each class of EII has specificity for a defined set of carbohydrates (162).

The PTS proteins take part in a phosphorylation cascade which involves transfer of a phosphoryl group from phophoenolpyruvate via histidine residues in the proteins EI, HPr, IIA, IIB and

finally to the incoming sugar (Fig. 5) (147). Besides import and phosphorylation of carbohydrates, PTS proteins are also involved in a number of regulatory functions in the cell, such as control of carbon source uptake via carbon catabolite repression and regulation of nitrogen and phosphorous utilization (39). The phosphorylation state of the PTS proteins involved in the phosphorylation cascade (EI, HPr, IIA and IIB) reflects the carbohydrate uptake activity in the cell, and based on this, the PTS proteins can interact and regulate the activity of non-PTS components involved in different metabolic pathways (39). Also, some transcriptional regulators contain the phosphorylatable IIA or IIB subunits (known as PTS-regulated (PRD) domains), and such proteins control transcriptional activity based on their PTS-dependent phosphorylation state (39). PTSs are found exclusively in bacteria, however, their distribution in the bacterial world is very biased (8, 163, 196). In some species, such as members of lactic acid bacteria and enterobacteria, PTSs are highly abundant, while they are totally absent in other species such as cyanobacteria (8). The important role of PTSs in carbohydrate uptake and regulation, as observed for example in *Escherichia coli*, bacilli, streptococci and lactobacilli, can therefore not be generalized to all bacteria. On the other hand, this distinct distribution may allow PTSs to be a useful target for antimicrobials to specifically fight PTS-containing pathogens (163).

The man-PTS family has relatively broad carbohydrate substrate specificity. Members of this family are reported to import and phosphorylate a variety of different hexoses, including mannose, glucose, fructose, galactosamine, glucosamine and N-acetylglucosamine (162). The man-PTS EII complex consists of four subunits which are encoded by three or four genes; the cytoplasmic IIA and IIB subunits are often encoded by a single gene, while IIC and IID, which together form the transmembrane permease, are mostly encoded by separate genes (196). The subunit stoichiometry for the man-PTS EII complex was previously assumed to be $IIAB_2$-IIC_1-IID_2 (153). Recent genetic evidences, however, have shown that both IIC and IID can be encoded by a single gene, which suggest a $IIAB_1$-IIC_1-IID_1 stoichiometry instead (Fig. 5) (Professor Bernhard Erni, University of Bern, personal communication). This notion remains to be verified experimentally. Structural information about man-PTS is limited to the cytoplasmic IIA and IIB subunits from *E. coli* and *Bacillus subtilis* (76, 99, 138, 165-167, 192), and very little is known about the membrane located IIC and IID proteins except for a membrane topology study from 1996 (100). In fact, man-PTS subunits differ from all other PTS proteins (162), and they are thought to have arisen independently and relatively late in the evolutionary history of bacteria (196). Man-PTSs are primarily found in species belonging to *Firmicutes* and *γ-Proteobacteria*, indicating that they have been important for the bacterial adaptation to life on mucosal surfaces in animals, either as symbionts, commensals or pathogens (196).

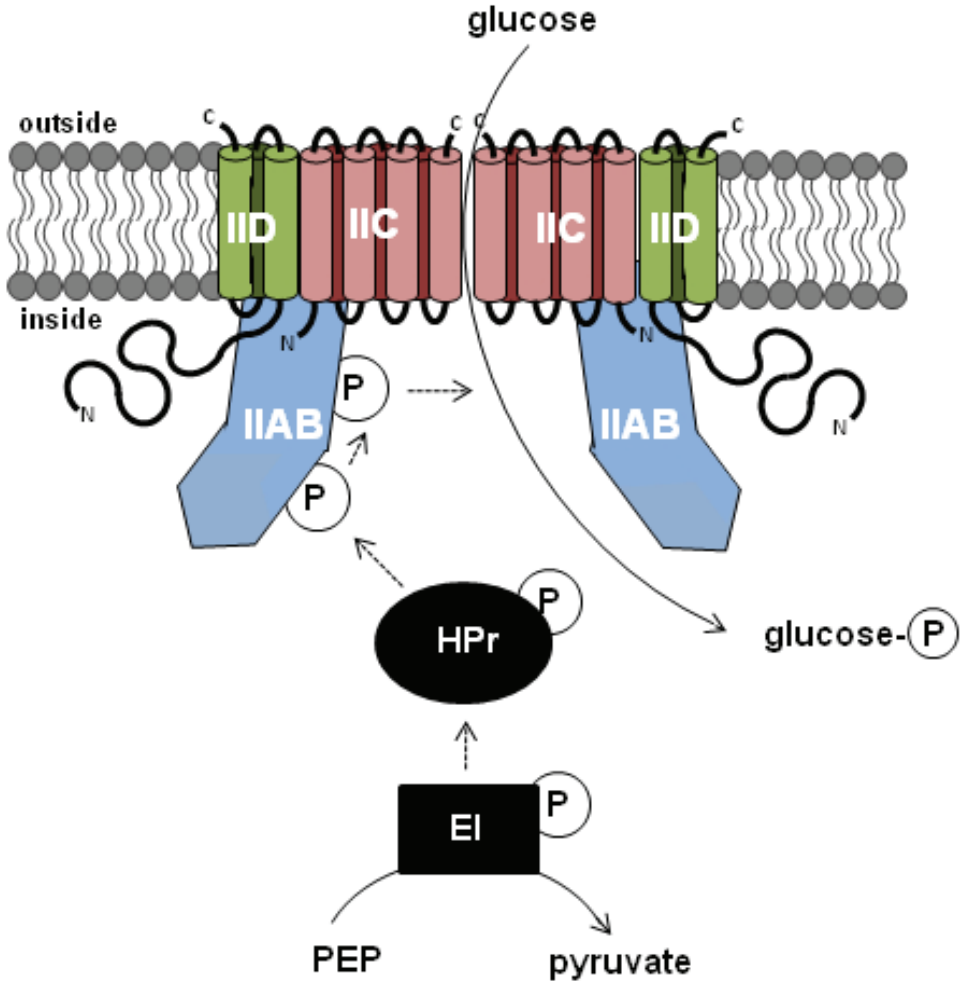

Fig. 5. Schematic model of the mannose-phosphotransferase system. The topological models of IIC and IID are based on prediction of transmembrane helices in the listerial protein MptC using TMHMM v.2.0 (117) and the model proposed by Erni (53). The N- and C-termini are indicated in IIC and IID. The phosphorylation cascade from phosphoenolpyruvate (PEP) via EI, HPr, EIIA, EIIB to the incoming sugar is indicated with dotted arrows.

Numerous early studies on bacteriocin-resistant mutants of *Li. monocytogenes* and *Enterococcus faecalis* provided circumstantial evidence that man-PTS might act as receptor for class IIa bacteriocins. Two-dimensional gel electrophoresis performed to compare the protein content in bacteriocin sensitive and bacteriocin resistant *Li. monocytogenes*, revealed the absence of the man-PTS IIAB protein in the latter cell type (150). Transposon mutagenesis studies linked class IIa bacteriocin resistance to the regulatory gene *rpoN* in *Li. monocytogenes* (154) and the *E. faecalis* (32). This gene encodes the alternative sigma factor σ^{54} (9) which is involved in the regulation of *mptACD* – the genes encoding man-PTS in *Li. monocytogenes* and *E. faecalis* (88). The subsequent inactivation of *mpt*-genes led to class IIa bacteriocin resistance in both species studied (33, 88), supporting the notion that man-PTS proteins could constitute the receptor for these bacteriocins. The involvement of

man-PTS in bacteriocin targeting was also confirmed by heterologous expression of the listerial man-PTS gene *mptC* in resistant *L. lactis* that rendered these cells sensitive to class IIa bacteriocins (149). Conclusive evidence that class IIa bacteriocins physically bind to man-PTS proteins on target cells was obtained when it was shown that man-PTS proteins can be co-purified with the bacteriocin and its cognate bacteriocin immunity protein (43). The same co-purification of bacteriocin, man-PTS proteins and immunity protein was performed for lactococcin A in *L. lactis*, thus showing that the lactococcal man-PTS, named PtnABCD, is the target for lactococcin A (43). By gene-knockouts and cloning it was demonstrated for both class IIa bacteriocins and lactococcin A, that the membrane located proteins IIC and IID together form the bacteriocin receptor, while the IIAB subunits were dispensable for the receptor function (43).

Man-PTS has not only been reported to function as a receptor for class IIa bacteriocins and lactococcin A. It has also been shown that the Gram-negative bacteriocin microcin E492 (MccE492), produced by *Klebsiella pneumonia*, targets sensitive *E. coli* via the man-PTS IIC and IID components (ManY and ManZ, respectively) (13). MccE492 was shown to be toxic both from the cytoplasmic and the extracellular side of the cell envelope, and intriguingly, ManYZ were required for both activities. ManYZ from *E. coli* is also involved in target recognition by bacteriophage lambda (52, 54). Furthermore, recent results have indicated that the large heat-labile streptococcal bacteriocin dysgalacticin (class III) might use man-PTS as target receptor on *Streptococcus pyogenes* (174).

1.2.4 Other cellular targets for bacteriocins

Lipid II and man-PTS are the only well established cellular targets for LAB bacteriocins, and target receptors for most bacteriocins are still unknown. For example, no receptor has been identified for any bacteriocins from class IIb (two-peptide bacteriocins) or class IV (circular bacteriocins). Nevertheless, there are a few reports that give indications of some other receptors for bacteriocin targeting. For example, by investigating the salt dependent activity of the lantibiotic sublancin 168, it was found that this bacteriocin probably targets the membrane located mechanosensitive channel MscL and kills sensitive cells by rendering this channel open (115). For other lantibiotics, specific binding to different membrane lipids has been proposed (124). Moreover, the Gram-negative bacteriocin microcin MccH47 has been shown to target the F_0 component of the ATP synthase complex in the inner membrane of sensitive cells (155), while a putative inner membrane serine transporter protein called SdaC seems to serve as specific receptor for another microcin (MccV) (69). Such results from Gram-negative bacteria might prove interesting also in the context of LAB bacteriocins, since it is known that these bacteriocin groups can employ similar targets (i. e., bacteriocins from both Gram-positive and Gram-negative bacteria target man-PTS). As LAB bacteriocins are active at very low concentrations and display defined inhibition spectra, it is reasonable to believe that most of these

bacteriocins target specific molecules on sensitive cells. Future research should aim at identifying such receptors for different classes of bacteriocins.

1.3 Bacteriocin immunity and resistance

1.3.1 Mechanisms of bacteriocin immunity

In order to protect themselves from their own bacteriocins, producer strains have genes that confer immunity. The genetic determinants encoding immunity are often co-located and co-regulated with the bacteriocin structural gene(s) (Fig. 4), and due to their conserved localization, putative bacteriocin immunity proteins are known for most bacteriocins. However, with a few notable exceptions, the molecular mechanisms by which they confer immunity often remain elusive. In general, putative immunity proteins for different bacteriocin systems vary greatly with respect to size, sequence and structure, suggesting a range of hitherto uncharacterized immunity mechanisms.

Self-immunity is best described for lipid II targeting class I bacteriocins, such as nisin, in which at least two separate immunity mechanisms work together to confer self-protection for the producer: (i) Specialized ABC-transporter systems pump bacteriocins out from the producer membrane (170), while (ii) dedicated lantibiotic immunity proteins (often termed LanI) specifically interact with the cognate bacteriocins on the extracellular side and probably shield the membrane target (lipid II) from the lantibiotic (46). An immunity mechanism involving ABC-transporters has also been proposed for some cyclic bacteriocins (class IV) (40).

For man-PTS targeting bacteriocins of class II (class IIa and lactococcin A), the immunity mechanism has been partly characterized: The immunity protein binds tightly to the man-PTS receptor on the intracellular side of the membrane and this binding prevents pore formation (43). Such binding to man-PTS occurs only in the presence of bacteriocin, suggesting that the bacteriocin induces structural changes in the receptor which allow binding of the immunity protein. These immunity proteins are very specific and by hybrid bacteriocins, it has been shown that the immunity protein specifically recognizes the C-terminal part of class IIa bacteriocins (106). However, no direct physical contact between the immunity protein and the bacteriocin has been demonstrated.

1.3.2 The Abi protein family

Some bacteriocin loci in *Lactobacillus* and *Streptococcus* are reported to encode transmembrane proteins belonging to the protein family Abi (145). These loci include the multibacteriocin *pnc* locus from *S. pneuomoniae* (123), the streptolysin S locus in *S. pyogenes* (137) and the plantaricin locus of *Lb. plantarum* (Fig. 4C) (44). Based on genetic organization as well as gene knockout studies, at least

some of the Abi proteins in these loci are thought to confer self-immunity to bacteriocins by a hitherto unknown mechanism (34, 123).

Proteins belonging to the Abi family (Pfam PF02517) are found both in prokaryotes and eukaryotes, and they are characterized by three conserved motifs. Motif 1 consists of two glutamate residues and an arginine separated by three variable amino acids (EExxxR, where x denotes any amino acid), motif 2 consists of a phenylalanine and a histidine separated by three variable amino acids (FxxxH) while motif 3 consists of a single histidine residue (H). Proteins from this family were first characterized in connection with a lactococcal locus encoding an abortive infection (abi) strategy for protection against bacteriophages (139). However, functional studies of these proteins have been performed only in eukaryotes, where members of this family are known as metal-dependent type II CAAX prenyl endopeptidases or CAAX proteases. CAAX proteases are involved in membrane targeting of proteins with the C-terminal sequence –CAAX (17, 113): The cysteine in the fourth-to-last position in the target protein is first prenylated by a geranylgeranyltransferase or a farnesyltransferase before the CAAX protease acts proteolytically to cleave off the C-terminal tripeptide –AAX. The proteins can then be targeted to membranes via their prenyl group which functions as a lipid anchor. The three conserved motifs are thought to constitute the active site of the metal dependent protease. The second glutamate in motif 1 is predicted to be the catalytic residue, while the first glutamate in motif 1 together with the histidines in motif 2 and 3 possibly co-ordinate a catalytically essential zinc ion in the active site (145).

1.3.3 Resistance to bacteriocins

For some bacteriocin systems, sensitive strains commonly develop bacteriocin resistant mutants upon exposure to bacteriocins. The frequency of resistance development varies greatly between types of bacteriocins and between sensitive strains. For class IIa bacteriocins, resistance frequencies up to 10^{-4} have been reported (72). Most probably, the mechanisms by which mutants become resistant are highly variable for different bacteriocins. In general, changes in the surface properties of bacterial cells seem to be important. For instance, several cell surface associated genes have been linked to nisin resistance (e. g., 1, 73, 116). Furthermore, some class IIa bacteriocin resistant mutants display alterations in cell membrane fluidity and cell surface charge (182, 183). Such cell envelope changes may confer resistance by somehow hindering interactions between the bacteriocin and its target cell.

More bacteriocin-specific resistance mechanisms have also been reported in some systems. For example, *Li. monocytogenes* mutants which are resistant to class IIa bacteriocins have been shown to display reduced expression of man-PTS receptor genes (74, 150, 176). The same observation has also been made for class IIa bacteriocin resistant *E. faecalis* (144).

1.4. Aims of the study

A large number of bacteriocins with a great variety in sequences, structures and inhibitory spectra have been described, in particular during the last two decades. However, the molecular mechanisms underlying killing of target cells, self-protection in producer cells and resistance development in sensitive cells have only been studied in detail for a few groups of bacteriocins. Understanding the molecular basis of these processes is essential both to appreciate the potential applications of these peptides in the food industry and infection treatment, and to gain further knowledge about the ecological role of bacteriocins in nature.

The current study was therefore initiated to increase the understanding of molecular mechanisms involved in class II bacteriocin cell killing, resistance and immunity.

- The target receptor for class IIa bacteriocins and lactococcin A has previously been identified as the man-PTS and the immunity mechanism for these bacteriocins has been (partially) characterized. The aim of this study was to elucidate the basis for the interaction between the bacteriocin and the receptor and to understand why different species and strains harbouring man-PTS display different sensitivity to class IIa bacteriocins and lactococcin A. In addition, we wanted to further investigate the mechanisms by which sensitive strains develop resistance to these man-PTS targeting bacteriocins.
- For two-peptide class IIb bacteriocins, no target receptor or immunity mechanism has been characterized, although a number of putative immunity proteins have been described. It was recently established that a number of these putative immunity proteins belong to the protein family called Abi. We therefore aimed to elucidate the function of Abi proteins in order to gain insight into the mechanisms that protect producer cells.

2. Main results

Paper I

Class II one-peptide bacteriocins target a phylogenetically defined subgroup of mannose phosphotransferase systems on sensitive cells.

Kjos M, Nes IF, Diep DB. (2009) *Microbiology* 155 (9): 2949-61.

Class IIa bacteriocins employ the membrane located man-PTS IIC-IID complex as target receptor on sensitive cells (33, 43, 88). Using homology searches, a large number of man-PTS homologs were found in the genomes of both bacteriocin sensitive and non-sensitive species, and these systems were investigated with respect to receptor functionality. By phylogenetic analysis, it was shown that the man-PTS proteins clustered into three main groups: Group I, II and III, the latter being the largest and most diverse. In order to assess the receptor functionality, selected man-PTSs from all three groups were cloned and heterologously expressed in a *L. lactis* man-PTS deletion mutant and the resulting clones were tested for their sensitivity to four different class IIa bacteriocins (pediocin PA-1, enterocin P, sakacin P, penocin A). Only man-PTSs from the phylogenetic Group I could function as receptor for the class IIa bacteriocins tested, demonstrating that these bacteriocins target a phylogenetically defined subgroup of man-PTS. In addition, within phylogenetic Group I there were variations between clusters of receptors that conferred high sensitivity (man-PTSs from *Listeria* and *Enterococcus*), medium sensitivity (from species of *Lactobacillus*) and low sensitivity (from *Streptococcus*). By sequence comparisons, three regions (region-α and region-β in IIC and region-γ in IID) were identified in which the man-PTSs with receptor function differed markedly from the non-receptor man-PTSs. These regions could thus be important for the specific recognition of class IIa bacteriocins. In contrast to the class IIa bacteriocins, it was found that the class IIc bacteriocin lactococcin A could only use the lactococcal man-PTS, but none of the others, as target receptor on sensitive cells.

In addition, we investigated three strains of *Lactobacillus sakei* which displayed variation in class IIa bacteriocin sensitivity despite having identical man-PTSs. It was found that this variation could be ascribed to differences in the level of man-PTS gene expression.

Paper II

An extracellular loop of the mannose phosphotransferase system component IIC is responsible for specific targeting by class IIa bacteriocins.

Kjos M, Salehian Z, Nes IF, Diep DB. (2010) *J. Bacteriol.* 192 (22): 5906-13

The man-PTS from *Li. monocytogenes* (*mpt*) confers high sensitivity to class IIa bacteriocins, while the closely related man-PTS from *L. lactis* (*ptn*) is non-functional as class IIa bacteriocin receptor. Both membrane located proteins IIC (MptC) and IID (MptD) are required for the receptor function. In order to define the region(s) in man-PTS which is involved in the specific bacteriocin recognition, we systematically designed *mpt-ptn* chimeras and introduced site-directed mutations in MptC. The resulting clones were challenged with ten different class IIa bacteriocins. It was shown that a region of 40 amino acids in the N-terminal part of MptC was responsible for the specific recognition by the class IIa bacteriocins. Transmembrane prediction of MptC indicated that this region covers an extracellular loop potentially serving as an interaction site for the bacteriocin. Opposite to class IIa bacteriocins, lactococcin A targets the Ptn-system, but not the Mpt-system, and the *mpt-ptn* chimeric systems were therefore used in an attempt to identify defined region(s) important for specific recognition of lactococcin A. However, no single region responsible for the specificity could be identified, thus indicating that lactococcin A has a more complex receptor interaction pattern involving both the IIC (PtnC) and the IID (PtnD) proteins.

Paper III

Resistance mechanisms against bacteriocins targeting the mannose phosphotransferase system.

Kjos M, Nes IF, Diep DB. Manuscript.

Upon exposure to class IIa bacteriocins, sensitive *Li. monocytogenes* strains develop resistance at high frequencies (up to 10^{-4}). Such resistance has previously been attributed to downregulation of man-PTS gene expression (72, 74, 176). Natural isolates of *Li. monocytogenes* also display differences in class IIa bacteriocin sensitivity, however, the mechanisms underlying such variation has hitherto not been studied in detail. In this work, a collection of *Li. monocytogenes* food industry isolates with high -, intermediate - and low susceptibility to class IIa bacteriocins were compared. It was found that low bacteriocin susceptibility was linked to low expression of the man-PTS genes and reduced growth on glucose. Mutations found in the man-PTS regulatory gene *manR* may be responsible for the lowered man-PTS expression level. On the other hand, the highly - and the intermediately sensitive isolates displayed similar man-PTS expression levels, suggesting that other, unknown factors are also important in determining the degree of bacteriocin sensitivity.

Furthermore, in order to compare the mechanisms of resistance of different types of man-PTS targeting bacteriocins, we also studied lactococcin A resistance in *L. lactis*. Lactococcin A resistant mutants were generated by exposing *L. lactis* to different concentrations of the bacteriocin, resulting in development of resistant mutants at frequencies between 10^{-4} and 10^{-7}. Also here, many of the mutants displayed reduced man-PTS expression, further demonstrating that this is a general mechanism of resistance against man-PTS targeting bacteriocins. Like in *Li. monocytogenes*, downregulation of man-PTS expression in the *L. lactis* mutants was accompanied with reduced growth on glucose and, interestingly, increased growth on galactose compared to wild type cells. The latter may be due to relief of carbon catabolite repression. Moreover, bacteriocin sensitivity in these resistant cells was restored by expression of the man-PTS genes from a plasmid. A significant portion of the lactococcin A resistant *L. lactis* mutants displayed normal man-PTS expression and wild-type-like growth on glucose and galactose, further indicating that also other, yet unknown, mechanisms are involved in the resistance against man-PTS targeting bacteriocins.

Paper IV

The Abi proteins and their involvement in bacteriocin self-immunity.

Kjos M, Snipen L, Salehian Z, Nes IF, Diep DB (2010). *J. Bacteriol.* 192 (8): 2068-76

At least three individual class II bacteriocin loci (in *S. pneumonia*, *S. pyogenes* and *Lb. plantarum*) are reported to encode putative immunity proteins which belong to the Abi family of proteins (34, 44, 123). The Abi family was first investigated *in silico* in order to analyze the dissemination of these genes in bacteriocin loci. By using an improved selection model for identification of Abi proteins, the Abi family was found to contain more members than annotated in the Pfam database. Mining genomes for Abi genes resulted in identification of seven Abi-containing putative bacteriocin loci in genome sequences of different bacterial strains. One of the loci, a putative two-component bacteriocin from *Lb. sakei* 23K was further investigated. Heterologous expression of the bacteriocin genes *skkA* and *skkB* showed that the peptides (SkkA and SkkB) displayed bacteriocin activity individually. The putative Abi immunity determinant, *skkI*, conferred immunity to the bacteriocin when it was expressed in a sensitive strain. Two other Abi genes from the plantaricin locus in *Lb. plantarum* (*plnI* and *plnLR*), were also shown to confer immunity to their corresponding bacteriocins (PlnEF and PlnJK, respectively). Cross-immunity between *skkI*, *plnI* and *plnLR* was observed, suggesting that these immunity proteins might recognize the same target molecule. Proteins belonging to the Abi family are often referred to as CAAX proteases and they are characterized by three conserved sequence motifs which are thought to constitute the proteolytic active site. By site-directed mutagenesis of SkkI, these motifs were shown to be essential for the immunity function.

3. Discussion

The results presented in this thesis shed light on various aspects of target cell recognition, resistance and self-immunity of class II bacteriocins. In the first three papers, class IIa bacteriocins along with the class IIc bacteriocin lactococcin A were studied; the molecular basis for variation in sensitivity between different species and strains was investigated (paper I), it was demonstrated how these bacteriocins interact with the man-PTS receptor (paper II), and the mechanisms of resistance to these bacteriocins were studied (paper III). In paper IV, findings which improve our understanding of the mechanism of self-immunity to a number of different bacteriocins belonging to class IIb and IIc were presented.

3.1 Man-PTS as receptor for class II bacteriocins

From studies of *Li. monocytogenes* (33), *E. faecalis* (88) and *Lb. sakei* (43), it has previously been demonstrated that class IIa bacteriocins employ the membrane components IIC and IID of man-PTS as target receptor on sensitive cells. Man-PTS constitutes a large family of sugar transport systems, and in paper I it was shown that the class IIa bacteriocins target only a phylogenetic subgroup (Group I) of these man-PTSs. Most man-PTS sequences retrieved from databases were either from the Gram-negative *γ-Proteobacteria* or the Gram-positive *Firmicutes*, but only members of the *Firmicutes* were present in Group I. In fact, all species commonly reported to be sensitive to class IIa bacteriocins, e. g., *Listeria*, *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Carnobacterium*, *Clostridium*, *Pediococcus* and *Streptococcus* (41, 49), contain a man-PTS belonging to this phylogenetic subgroup (paper I), strongly suggesting that the presence of a Group I man-PTS is a requirement for displaying sensitivity to class IIa bacteriocins.

Systematic variations between man-PTSs in terms of receptor potency could also be observed within Group I, i. e., man-PTSs which clustered closely together in the phylogenetic tree conferred similar degree of bacteriocin sensitivity (paper I). Based on experimental results from seven man-PTSs, we could divide the members of Group I into subclusters which conferred high-, medium- and low sensitivity to class IIa bacteriocins. This subgrouping corresponded very well with results from sensitivity assays reported previously (41, 49). For example, man-PTSs of *Listeria*, *Enterococcus* and *Carnobacterium* clustered together in the high sensitivity group, and in several comparative studies of class IIa bacteriocins, these bacteria are reported to be the most sensitive. Furthermore, the man-PTSs from *Lactobacillus*, *Clostridium* and *Pediococcus*, genera which are often but not always sensitive to class IIa bacteriocins, clustered together in the medium sensitivity group. Lastly, the man-PTSs from *Leuconostoc* and *Streptococcus*, which only sporadically are reported to be sensitive to class IIa

bacteriocins, were found together in the low sensitivity group. The lactococcal man-PTS system (Ptn), which also belong to phylogenetic Group I, did not confer sensitivity to any of the ten class IIa bacteriocins tested in our assays (paper I and paper II), although a few lactococcal strains have previously been reported to be sensitive (41).

Following the observation that different man-PTSs vary with respect to receptor potency, we looked for differences in their amino acid sequences that could potentially account for this variation. We first identified two sequence regions in IIC (called region-α and region-β) and one in IID (region-γ) that clearly differed between non-sensitive and sensitive receptors (paper I). Next, in order to define the region(s) involved in specific targeting, a collection of hybrid man-PTSs were constructed based on the highly active receptor MptCD from *Li. monocytogenes* and the closely related man-PTS PtnCD from *L. lactis* which is inactive as class IIa bacteriocin receptor (paper II). We found that the N-terminal part of the IIC protein was responsible for specific target recognition by all ten class IIa bacteriocins tested (paper II). Out of the predicted specificity regions (paper I), only region-α is located within this part of IIC. By site-directed mutations and construction of a man-PTS chimera which was identical to PtnCD except for a 40 aa region from MptC, it was confirmed that this region in fact is responsible for the specific target recognition by class IIa bacteriocins, although some of the peptides (e. g., enterocin P and sakacin P) seemed to also require other parts of MptC and/or MptD to obtain full sensitivity (paper II). Accordingly, all man-PTSs that can function as bacteriocin receptors (phylogenetic Group I) hold a MptC-like sequence in the 40 aa region, and this sequence is clearly different from non-receptor man-PTSs (paper I and II). The three dimensional structure of the man-PTS membrane complex is unknown, but sequence-based prediction tools suggest that MptC has seven transmembrane helices and four extracellular loops, and that region-α is located in an extracellular loop between the 3rd and 4th transmembrane segment (paper II). Such a structure implies that the bacteriocin can readily make specific contacts with the loop sequence on the extracellular side. Although only a short region in IIC seems to be important for specific target recognition, it should be highlighted that the IIC and IID proteins together constitute the receptor for class IIa bacteriocins, and deletion or disruption of IID completely abolish the receptor function of man-PTS (33, 43). Most likely, the interaction between intact IIC and IID is essential for the structuring and/or stability of the membrane-located man-PTS complex.

It is not yet known what part(s) of the class IIa bacteriocin which is involved in specific recognition of its receptor. Nonetheless, based on the finding that all of the ten class IIa bacteriocins tested appeared to specifically recognize the same region in the receptor (paper II), it can be hypothesized that the conserved N-terminal pediocin consensus sequence (YGNGVxCxxxxCxVxWxxA), which is shared among all the bacteriocins, is important for this interaction, rather than the variable C-terminal part. It has previously been suggested that the N-terminus, which is cationic due to the presence of several positively charged residues (Lys, His and

Arg), is mainly important to facilitate the binding of pediocin-like bacteriocins to cell surfaces via electrostatic interactions and to position the bacteriocin correctly in the membrane (61, 111). In light of the results presented in paper II, however, it seems likely that this N-terminal part of the peptide is involved in specific interactions with residues in the extracellular loop as well. From another point of view, it has been shown by hybrid peptides that the C-terminal part of class IIa bacteriocins is important for the target cell specificity and specific recognition of the immunity protein (106). Moreover, a 15-mer peptide fragment derived from the helical region in the C-terminal half of pediocin PA-1 has been shown to inhibit the activity of this bacteriocin in a sequence dependent manner, possibly by obstructing the bacteriocin-receptor interaction (60, 84). Both these latter notions strongly suggest that the C-terminal part of the bacteriocin also specifically interacts with the receptor. Although highly speculative at this point, it is conceivable that the conserved N-terminal β-sheet containing part of class IIa bacteriocins initially interacts with the extracellular loop (region-α) of the man-PTS IIC protein, thus enabling the C-terminal α-helix containing part of the bacteriocin to insert into the membrane to engage in helix-helix interactions with transmembrane segments of IIC and/or IID. Such interactions might impose structural changes in the man-PTS which render the permease as an open pore (Fig. 6). Given this mechanism, all class IIa bacteriocins can interact in a similar fashion with the extracellular loop in IIC (paper II), while the helix-helix interactions diverge between bacteriocins due to differences in their C-terminal sequences (106). This may explain why the C-terminal part is responsible for the target cell specificity and for the specific recognition of the immunity protein (Fig. 6) (106). Such a model is also in line with the proposed orientation of class IIa bacteriocins in target cell membranes with the N-terminal β-sheet domain in the membrane interface and the C-terminal hydrophobic domain into the hydrophobic core of the cell membrane (59, 61, 86, 127).

Like class IIa bacteriocins, the class IIc bacteriocin lactococcin A employs membrane-located man-PTS proteins as target molecules on sensitive cells (43). However, in contrast to class IIa bacteriocins, which can target several different man-PTSs, lactococcin A displays an extremely narrow inhibitory spectrum, targeting only the lactococcal man-PTS (paper I). In paper II it was found that this difference in specificity can be explained by the peptides' mode of receptor targeting; while class IIa bacteriocins appear to rely on a single, short region for specific receptor recognition, the specific interaction between lactococcin A and the receptor is more complex, probably involving both the IIC (PtnC) and the IID (PtnD) protein (paper II). In general, man-PTS complexes thus appear to have a number of different sites that are vulnerable for extracellular attack, which may explain why these proteins are targeted by a wide range of different antimicrobials, including bacteriocins of various classes (13, 43, 89, 174) and bacteriophages (54).

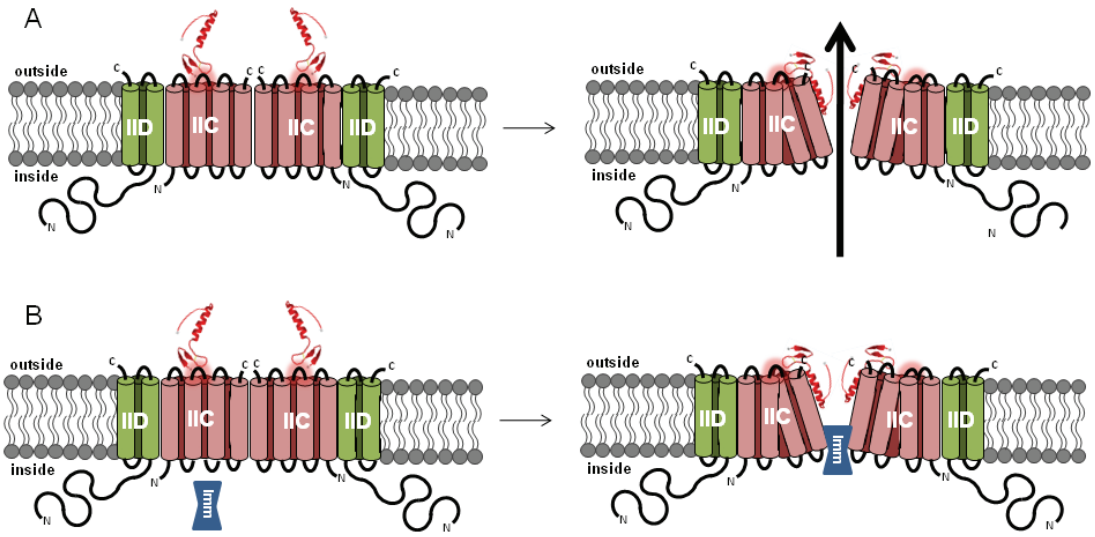

Fig. 6 Proposed model of the mode of action (A) and immunity (B) in class IIa bacteriocin systems. The class IIa bacteriocin is depicted in red. (A) The N-terminal β-sheet containing part of the bacteriocin initially interacts with an extracellular loop (highlighted) of the man-PTS IIC protein. The helix-containing C-terminal part then engages in specific interactions with transmembrane helices of the IIC and/or IID proteins to cause conformational changes which, in turn, lead to pore formation and eventually cell death. (B) In immune cells, the bacteriocin mediates the same conformational changes, but the pore is blocked by a specific immunity protein (blue) which binds tightly to man-PTS.

The findings discussed above together suggest that variation in the man-PTS sequence/structure is the main determinant for genus/species-level variation in bacteriocin sensitivity. For class IIa bacteriocins, the sequence of an extracellular loop region in the man-PTS IIC protein is particularly important in this context (paper II). However, such sequence variations cannot explain the different bacteriocin sensitivities observed between strains of the same bacterial species. For example, in paper I and III we described *Lb. sakei* and *Li. monocytogenes* strains which somehow displayed different susceptibilities to pediocin PA-1 despite harbouring identical man-PTSs. In these cases, the degree of sensitivity seemed to be directly related to the man-PTS expression level. For the *Lb. sakei* strains, a highly sensitive strain displayed an elevated man-PTS expression level compared to the two less sensitive strains (paper I). Correspondingly, a *Li. monocytogenes* isolate with low class IIa bacteriocin susceptibility had clearly reduced man-PTS expression compared to other sensitive isolates (paper III). Furthermore, resistant mutants arise at high frequencies (up to 10^{-4}) when sensitive *Li. monocytogenes* and *E. faecalis* are exposed to class IIa bacteriocins, and previous studies have shown that man-PTS expression is downregulated in many of these mutants (74, 144, 176). In paper III it was shown that the same mechanism applied to lactococcin A resistant *L. lactis*. Hence, downregulation of man-PTS gene expression appear to be a common mechanism of resistance against man-PTS targeting

bacteriocins of different classes, and the same mechanism is found both in laboratory generated resistant mutants and among natural isolates with low bacteriocin susceptibility (paper I and III).

The underlying reasons for reduced man-PTS gene expression in bacteriocin resistant cells are still not fully understood, however, current knowledge indicate that stable mutations in man-PTS regulatory genes might play an important role. This notion is based on results showing that (i) the resistant phenotype is stably preserved for at least hundred generations in non-selective medium (72 and paper III) and (ii) that mutations/polymorphisms in the σ^{54}-associated activator ManR/MptR has been reported both in resistant *E. faecalis* (144) and *Li. monocytogenes* (paper III). ManR/MptR, which is involved in activation of man-PTS transcription in these species (193), might thus represent a genetically variable hot spot which is important for development of bacteriocin resistance. It should be noted that regulation of man-PTS expression in *Listeria* is dependent on several proteins (189). Therefore, there are a large number of sites in the genome where resistance mutations potentially may arise, and such a large sized mutational target might be one of the reasons for the high resistance rate observed for man-PTS targeting bacteriocins. Alternatively, it was also speculated in paper III that stochastic switching (random variation) in the man-PTS gene expression may be an underlying reason for the high frequency of resistant cells. Stochastic gene expression generates phenotypic heterogeneity within a population, and in that way aids bacterial survival in stressful and fluctuating environments (107, 122). Given such a mechanism, exposure to bacteriocins would kill cells with high man-PTS expression, while the part of the population with low man-PTS expression would survive. Heterogeneity with respect to man-PTS expression may potentially be a favourable trait within a culture, since it would render a bacterial population more capable of coping with changes in carbon source availability as well as antimicrobial attacks. In this context, it is interesting to note that the lactococcin A resistant mutants of *L. lactis* displayed reduced growth on glucose but elevated growth on galactose compared to the wild type cells (paper III). Similar changes in metabolic pattern have also been observed for class IIa bacteriocin resistant *Li. monocytogenes* (184) and *E. faecalis* (144). Whether these findings can be explained by relief of carbon catabolite repression due to mutations or if intrinsic heterogeneity actually exists in these cell populations remains to be tested experimentally.

Although downregulation of man-PTS gene expression seems to be a prevalent mechanism of high-level resistance to man-PTS targeting bacteriocins, a significant number of bacteriocin resistant cells show normal or even elevated man-PTS expression (176, 182 and paper III). In these cases, the exact resistance mechanism is unknown, but changes in the cell envelope (e. g., membrane fluidity or cell surface charge) might be involved (182, 183). Some strains may even be resistant because they harbour class IIa bacteriocin immunity genes in their genomes (131). In conclusion, whilst genus/species-level variation in class IIa bacteriocin sensitivity can mainly be attributed to variation in the man-PTS receptor sequence (paper I and II), strain-level variations rather seem to be caused by differences in man-PTS expression level or cell surface properties (paper I and III). Hopefully,

continued research on these issues will result in even better understanding of the genetic basis for strain level variation in bacteriocin sensitivity and bacteriocin resistance development.

Finally, it should be mentioned that during our study of man-PTSs in different bacteria, it was observed that production of class IIa bacteriocins coincided with the presence of man-PTS genes (paper I). Altogether 21 different bacterial species are characterized as class IIa bacteriocin producers (Table 1), including species belonging to the genera *Enterococcus*, *Carnobacterium*, *Listeria*, *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Streptococcus*, *Lactococcus* and *Bacillus*. Genome sequences are available for many of these, and interestingly, from the information available to date, all class IIa bacteriocin producing species appear to contain a Group I man-PTS. Most striking, among the 19 different *Bacillus* species with published genome sequences, *B. coagulans* (along with some strains of *B. thuringiensis*) is the only species that contains a Group I man-PTS, and notably, *B. coagulans* is also the only sequenced *Bacillus* species known to produce a class IIa bacteriocin (coagulin). Thus, the ability to produce class IIa bacteriocins seem to have co-evolved with man-PTS. However, it has not been investigated whether man-PTSs have any important function in class IIa bacteriocin production. In fact, such a functional link seems unlikely since expression of bacteriocins has been performed in a number of heterologous systems, such as Gram-negative bacteria (77, 78) and yeasts (79, 187), without the presence of a Group I man-PTS.

3.2 Abi proteins and bacteriocins

As discussed above, the molecular mechanisms underlying target cell killing and self-immunity for class IIa bacteriocins and lactococcin A are now relatively well understood. This is, however, not the case for other bacteriocins of class II. In order to gain more insight into the molecular mechanisms of self-immunity for some of these bacteriocins, we investigated the protein family known as Abi proteins or CAAX proteases (Pfam PF02517). Abi genes have previously been found in bacteriocin loci in *S. pneumonia*, *S. pyogenes* and *Lb. plantarum* (34, 44, 123) and by mining sequenced genomes for Abi genes, seven new putative bacteriocin loci were identified in Gram-positive bacteria (paper IV). Among these, a putative two-peptide bacteriocin (class IIb) was found in the genome of *Lb. sakei* 23K. No bacteriocin production was detected in the endogenous strain, however, heterologous expression of the bacteriocin structural genes (*skkA* and *skkB*) confirmed that they encode peptides with antimicrobial activity and the identified bacteriocin was named sakacin 23K (paper IV). Surprisingly, the two peptides (called Skkα and Skkβ) displayed bacteriocin activity individually and no significant synergy was observed when they were combined, suggesting that this locus encodes two one-peptide bacteriocins of class IIc. This is not the first time new antimicrobial peptides have been identified by means of genome mining for bacteriocin-associated genes (10, 41). Since bacteriocin structural genes are small and display large sequence variations, searching genomes for these genes

directly is often difficult. One approach to circumvent this problem is to utilize conserved, bacteriocin-associated genes as a means to localize novel bacteriocin genes. For example, in order to identify new lantibiotics, Begley *et al.* (10) mined genomes for conserved lanthionine synthetase genes. Likewise, as Abi genes are found in several characterized bacteriocin genetic loci, we used these genes as a guide to identify novel bacteriocin loci (paper IV). Given the large amount of genome data currently available in public databases and knowing that this wealth of sequence information will continue to expand in the coming years, such *in silico* approaches for identification of novel antimicrobial compounds will become increasingly important in the future. A dedicated genome mining tool for identification of bacteriocin genes in sequenced bacterial genomes (BAGEL) is available (35), and the accuracy of this tool will continue to improve as further knowledge about bacteriocin genetic loci is revealed. For example, the observed association of Abi genes with bacteriocin loci (paper IV) may be worth implementing in such a genome mining tool.

Abi genes are reported to be involved in bacteriocin self-immunity in streptococci (34, 123), and we demonstrated that the Abi genes *plnI* and *plnL* from *Lb. plantarum* conferred immunity to their cognate bacteriocins (plantaricin EF and JK, respectively) when expressed in a sensitive host (paper IV). Similarly, the Abi gene of the novel sakacin 23K locus, *skkI*, conferred immunity to both cognate antimicrobial peptides Skkα and Skkβ (paper IV). All three immunity genes, *plnI*, *plnL* and *skkI*, are located immediately downstream of the bacteriocin determinants, a genetic organization which is common among class II bacteriocins. However, while expression of the single genes *plnI* and *skkI* was sufficient to confer immunity, *plnL* required co-expression of the downstream gene *plnR* in order to function as an immunity determinant. Thus, the gene pair *plnLR* seems to be functionally equivalent to *plnI* and *skkI*. Perhaps, *plnL* and *plnR* formerly constituted a single open reading frame which has maintained its function despite being disrupted into two genes. In general, dedicated bacteriocin immunity proteins are very specific and confer immunity only to their cognate bacteriocins (30). We were therefore intrigued to observe extensive cross-immunity between the three Abi immunity proteins (paper IV); SkkI protected sensitive cells not only against sakacin 23K but also partially against plantaricins EF and JK. Similarly, PlnI also partially protected the cells against sakacin 23K and plantaricin JK in addition to conferring full immunity against the cognate bacteriocin plantaricin EF. This cross-immunity suggests that the three Abi proteins confer immunity via similar mechanisms. It could thus be speculated that sakacin 23K and plantaricins EF and JK hold similar mode of action, for example by recognizing a common receptor on target cells.

How do the Abi proteins then act to confer immunity against bacteriocins? The mechanisms of action for Abi proteins have hitherto been studied mainly in eukaryotes, where they act as zinc dependent proteases that cleave off the tripeptide –AAX from proteins with the C-terminal sequence CAAX (145). The Abi proteins contain three conserved motifs (EExxxR in motif 1, FxxxH in motif 2 and H in motif 3) which are thought to constitute the active site of the protease. This is supported by

site-directed mutagenesis of motif 1, 2 and 3 in the yeast Abi protein RCE1, which resulted in inactivation of the protease function (45). Likewise, mutations of motif 1 or 3 in SkkI abrogated the immunity function of this protein (paper IV). Thus, the conserved motifs are indeed essential for the Abi immunity function, and this might also indicate that a proteolytic mechanism is involved. No Abi protein of bacterial origin has hitherto been shown to possess a proteolytic function. However, it has been demonstrated that an Abi-like protein from *B. subtilis*, called PrsW, acts proteolytically against the protein RsiW (51, 90, 91). PrsW differs from the Abi proteins in motif 2, where the conserved histidine is replaced with a glutamate (FxxxE instead of FxxxH), while the two conserved glutamates in motif 1 and the conserved histidine in motif 3 are identical. We therefore suggested in paper IV that PrsW should be included in the Abi protein family. Interestingly, site-directed mutation in motif 1 or 3 of PrsW both caused inactivation of the proteolytic function in a similar manner as observed for the eukaryotic Abi protein RCE1 (51), however unlike the eukaryotic protein which acts proteolytically on the C-terminal end of the target -CAAX protein, PrsW cleaves RsiW 40 amino acids from the C-terminal end (90). These findings in *B. subtilis* underline the possibility that an enzymatic activity may be responsible for the Abi mediated bacteriocin immunity mechanism. Due to the presumed transmembrane nature of Abi proteins, they could potentially act by degrading bacteriocins extracellularly, however, no such activity could be observed in our assays (paper IV). Importantly, it is also possible that the Abi proteins confer bacteriocin immunity via a non-proteolytic mechanism, in which the conserved motifs are essential for correct folding/structuring, rather than constituting an active site.

The Abi proteins constitute a family with members from all domains of life, the majority being bacterial proteins (99 %). By January 2011, the number of Abi proteins in the Pfam database of protein families is 4443. Using a bioinformatics approach we demonstrated that this number should be even higher (paper IV), as several proteins with conserved Abi motifs (e. g., PlnL, SkkI and PrsW) had escaped detection and not been annotated as Abi proteins. Mining bacterial genomes for bacteriocin loci showed that only a small fraction (~1 %) of the identified Abi genes were associated with bacteriocin loci. This biased distribution implies that the vast majority of Abi proteins fulfil functions in bacteria other than being dedicated bacteriocin immunity proteins. Recent reports have for instance suggested an important role for these proteins in bacterial extracellular biology. For example, in *S. aureus*, three proteins with Abi domains (SpdA, SpdB, SpdC) are involved in secretion of surface proteins with so-called YSIRK signal sequences (65). Deletion of these Abi genes in *S. aureus* also led to an increase in the thickness of cross walls in dividing cells, suggesting that Abi proteins may represent a link between cell division and protein secretion (65). Furthermore, deletion of the Abi gene LyrA (identical to SpdC) in *S. aureus* increased resistance to the bacteriolytic enzyme lysostaphin, an effect that was attributed to altered cell envelope properties (75). Also the *B. subtilis* protein PrsW, discussed above, is involved in alteration of the cell surface in an indirect manner, by activating a

sigma factor (σ^W) which regulates genes of extracytoplasmic function (23, 51, 90, 91). In addition, Abi genes often seem to be genetically linked with genes involved in transport (e. g., PlnTUVW in the plantaricin locus, paper IV). Thus, current knowledge suggests that Abi proteins are important for the extracellular biology of bacteria, including involvement in mechanisms that confer protection against antimicrobial peptides, and future research will certainly unveil novel roles of these proteins in bacterial cells.

4. Concluding remarks and future perspectives

The results presented in this thesis have improved our understanding of the molecular mechanisms involved in cell killing, resistance and immunity of class II bacteriocins. While the first part (paper I, II and III) focused on expanding the understanding of target cell recognition, receptor interaction and resistance development for the well studied class IIa bacteriocins, the last part (paper IV) reported pioneer work on a protein family that confers immunity to a number of other class II bacteriocins.

The molecular basis for the defined inhibitory spectra of class IIa bacteriocins was revealed by pinpointing a small region of the man-PTS receptor which is essential for specific targeting by this group of bacteriocins. By combining our results with other findings, a model for the molecular nature of the class IIa bacteriocin pore formation was also proposed. Yet, important questions remain unanswered and continued research efforts focusing on class IIa bacteriocins and man-PTS will hopefully lead to an even more detailed insight into the molecular mechanisms governing the bacteriocin mode of action and immunity. A complete understanding of these issues will largely depend increased structural understanding of the man-PTS proteins, such as confirmation of the man-PTS subunit stoichiometry and transmembrane topology. The ultimate goal would be to resolve the structure of the bacteriocin : man-PTS : immunity protein tripartite complex. However, determining such a structure is an extremely complicated task, and to date no structures on any membrane spanning PTS component has been reported.

Sensitive cells frequently become resistant to man-PTS targeting bacteriocins, and it was shown that downregulation of man-PTS gene expression is a major mechanism of resistance in many these cases. However, other types of resistant cells also exist, and future research should aim at deciphering the molecular and genetic basis of such alternative resistance mechanisms. Identification of differences in the cell envelope between sensitive and resistant cells should be a primary focus in this research.

The Abi proteins constitute a large family of proteins, and the results presented here are among the first to shed light on this large but yet underexplored protein family in prokaryotes. It was demonstrated that Abi proteins can function as bacteriocin immunity determinants, and shown that the conserved Abi protein motifs are essential for this function. Future research should aim at unravelling the molecular mechanisms underlying this immunity function, with particular focus on the putative enzymatic function of the Abi proteins. Further studies on the immunity proteins might also elucidate other aspects of class II bacteriocin biology, such as mode of target cell recognition. Continued investigation into the Abi protein family will also reveal the roles of these proteins in other cellular processes.

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PAPER I

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Class II one-peptide bacteriocins target a phylogenetically defined subgroup of mannose phosphotransferase systems on sensitive cells

Morten Kjos, Ingolf F. Nes and Dzung B. Diep

Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, PO Box 5003, 1432 Ås, Norway

Correspondence
Dzung B. Diep
dzung.diep@umb.no

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Membrane-located proteins (IIC and IID) of the mannose-phosphotransferase system (man-PTS) have previously been shown to serve as target receptors for several bacteriocins. Although many bacteria contain at least one such man-PTS in their genome, most bacteriocins display a narrow inhibitory spectrum, targeting predominantly bacteria closely related to the producers. In the present study we have analysed the receptor spectrum of one-peptide bacteriocins of class II. A phylogenetic analysis of 86 man-PTSs from a wide range of bacterial genera grouped the man-PTSs into three main clusters (groups I–III). Fourteen man-PTSs distributed across the phylogenetic tree were selected for experimental analysis in a heterologous host. Only members of group I could serve as receptors for class IIa bacteriocins, and the receptor efficiencies varied in a pattern directly related to their phylogenetic position. A multiple sequence alignment of IIC and IID proteins revealed three sequence regions (two in IIC and one in IID) that distinguish members of the bacteriocin-susceptible group from those of the other groups, suggesting that these amino acid regions confer the specific bacteriocin receptor function. Moreover, we demonstrated that variation in sensitivity might also exist within the same species due to differential expression levels of the receptor, since three strains of *Lactobacillus sakei* harbouring identical man-PTSs were shown to display different expression levels of a man-PTS gene that corresponded to the variation in bacteriocin sensitivity. Together, the results of our study show that the level of bacteriocin susceptibility for a bacterial cell is primarily determined by differences in its man-PTS proteins, although the expression levels of the corresponding genes also play an important role.

INTRODUCTION

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by a wide range of Gram-positive bacteria, of which lactic acid bacteria (LAB) constitute the major part. Most of these antimicrobial substances direct their activity toward bacteria closely related to the producers, but some also have broader inhibitory spectra, including important pathogens such as the food-borne *Listeria monocytogenes* and pathogenic species of *Enterococcus*. The LAB bacteriocins are classified into two major classes (Nes *et al.*, 2007): the class I lantibiotics, containing post-translationally modified amino acids such as lanthionine, dehydrated amino acids and thioether cross-linked amino acids; and the class II non-lantibiotics, containing only non-modified amino acids. The class II bacteriocins are further categorized into several subgroups. The subclass IIa bacteriocins have a conserved sequence (YGNG[V/L]) and a disulfide bridge in their N-terminal region and are known for their strong anti-listerial activity (Nissen-Meyer *et al.*, 2009); these peptides are normally referred to as pediocin-like bacteriocins after pediocin PA-1, the first member characterized from this subclass (Henderson *et al.*, 1992; Nieto Lozano *et al.*, 1992). Subclass IIb contains bacteriocins whose full activity is dependent on the complementary action of two different peptides (e.g. lactococcin G and plantaricin EF and JK), and subclass IId contains bacteriocins without leader sequences (e.g. enterocin L50 and lacticin Q). Finally, the bacteriocins that do not belong to any of the above subgroups (e.g. lactococcin A and B) are placed in class IIc (Nes *et al.*, 1996, 2007).

Most bacteriocins are believed to kill sensitive cells by disrupting the integrity of target membranes, which leads

Abbreviations: aLRT, approximate likelihood-ratio test; BU, bacteriocin unit; 2-DG, 2-deoxy-D-glucose; man-PTS, mannose-phosphotransferase system.

A supplementary figure, showing a multiple sequence alignment of all 86 man-PTSs used in the phylogenetic analysis, and a supplementary table, listing man-PTSs used in the phylogenetic analysis, are available with the online version of this paper.

to dissipation of the proton motive force, depletion of intracellular solutes, and eventually cell death (Drider *et al.*, 2006). The fact that each bacteriocin displays a defined inhibitory spectrum strongly suggests that the individual bacteriocins recognize specific receptor molecules on target cells. In fact, it has been shown that several of the class I lantibiotics as well as the non-lantibiotic lactococcin 972 employ lipid II, a precursor in cell wall synthesis, as a docking molecule, and interaction between a bacteriocin and lipid II leads to inhibition of cell wall synthesis and/or pore formation, depending on the structure of the bacteriocin and its concentration (Brotz *et al.*, 1998; Martinez *et al.*, 2008; Wiedemann *et al.*, 2001). Similarly, some studies have shown that the mannose-phosphotransferase system (man-PTS) might serve as a receptor for some pediocin-like bacteriocins, based on the observations that resistant mutants have an altered expression pattern of man-PTS and that heterologous expression of cloned man-PTS genes renders resistant cells sensitive (Dalet *et al.*, 2001; Gravesen *et al.*, 2002; Héchard *et al.*, 2001; Ramnath *et al.*, 2000, 2004). More recently, it has been conclusively demonstrated for several bacteriocins from subclass IIa and some from subclass IIc that the membrane components (ManM/PtnC and ManN/PtnD) of the man-PTS are directly involved as receptors, and that in bacteriocin-producing cells, a cognate immunity protein tightly binds the receptor in a bacteriocin-dependent manner, to prevent killing by the bacteriocin (Diep *et al.*, 2007).

The man-PTS transporter family is responsible for the concomitant import and phosphorylation of carbohydrates such as mannose and glucose in bacteria (Postma *et al.*, 1993). A PTS transporter normally consists of three major components: enzyme I (EI), HPr and enzyme II (EII). The first two are cytoplasmic proteins involved in the transfer of a phosphoryl group to EII, which in turn relays the phosphoryl group to imported sugar molecules. EI and HPr serve as common phosphoryl group suppliers for different EIIs, while the individual EIIs are specific for each PTS family and are responsible for the sugar specificity. EII in man-PTS consists of four subunits, IIA, IIB, IIC and IID, in which the first two appear as one single (IIAB) or two separate (IIA and IIB) proteins, and the last two (IIC and IID) normally are separate proteins. Subunits IIA and IIB are located in the cytoplasm, while IIC and IID together form a membrane-located complex through which the sugar entities enter the cell (Postma *et al.*, 1993). Expression of the genes encoding these four subunits is coordinated, as they are commonly clustered in one operon (Deutscher *et al.*, 2006).

Hitherto, a few reports have linked sensitivity to class IIa bacteriocins to specific man-PTSs. For instance, the *mpt* operons in *Enterococcus faecalis* V583 and *Li. monocytogenes* EGD-e have been shown to be required for sensitivity to mesentericin Y105 (Dalet *et al.*, 2001; Héchard *et al.*, 2001). Another man-PTS of *Li. monocytogenes* EGD-e, encoded by the *mpo* operon, has also been shown to be involved in sensitivity to class IIa bacteriocins via regulation of the *mpt* operon (Arous *et al.*, 2004). Furthermore, in a recent study of bacteriocin sensitivity and immunity in our laboratory (Diep *et al.*, 2007), it was shown that the *man* operon of *Lactobacillus sakei* 23K confers sensitivity to a number of class IIa bacteriocins, while the *ptn* operon of *Lactococcus lactis* IL1403 confers sensitivity to the class IIc bacteriocins lactococcin A and lactococcin B. However, for several bacteria of both bacteriocin-sensitive and non-sensitive species, a large number of homologous putative man-PTS transporter genes are found in the genomes (Zúñiga *et al.*, 2005) and whether these homologous man-PTS proteins can serve as receptors for bacteriocins has not been evaluated. Starting from a bioinformatics approach, we systematically selected a number of man-PTSs from different genera, and showed that only a defined phylogenetic group of the man-PTSs confers sensitivity to class IIa bacteriocins and, more importantly, that variation in bacteriocin sensitivity can, to a large extent, be predicted on the basis of man-PTS phylogenetic positions.

METHODS

Phylogenetic analysis. To screen for man-PTS proteins in databases, the proteins ManM (YP_395063) and ManN (YP_395064) from *Lb. sakei* 23K were used as query sequences; these two proteins are members of the conserved IIC and IID protein families. Amino acid sequences of man-PTS proteins were retrieved from the Entrez Gene database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene) using the Basic Local Alignment Search Tool (BLASTP; http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) with standard settings. All PTS transporters selected for further study, as listed in Supplementary Table S1, have been classified as man-PTSs (Zúñiga *et al.*, 2005). Phylogenetic analysis was performed using the web service Phylogeny.fr (http://www.phylogeny.fr; Dereeper *et al.*, 2008): Sequences were aligned using MUSCLE v3.7 (Edgar, 2004) in default mode. The phylogenetic tree was constructed with the maximum-likelihood algorithm implemented in PhyML v3.0 (Guindon & Gascuel, 2003) with the following settings: (i) initial tree: BIONJ; (ii) amino acid substitution model: WAG; (iii) proportion of invariant sites: estimated; (iv) number of substitution rate categories: 4; (v) gamma shape parameter: estimated. The reliability of the branches was assessed using bootstrap analysis with 100 replicates and an approximate likelihood-ratio test (aLRT) (Anisimova & Gascuel, 2006) with the Shimodaira–Hasegawa-like (non-parametric) procedure. The phylogenetic trees were visualized using TreeDyn (Chevenet *et al.*, 2006).

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. *Lc. lactis* IL1403 and clones derived therefrom were routinely grown at 30 °C in M17 medium (Oxoid) supplemented with 0.4 % (w/v) glucose or 0.4 % (w/v) galactose, *Lactobacillus plantarum* WCFS1 and *Lactobacillus delbrueckii* ATCC 11842 in MRS medium (Oxoid) at 30 and 37 °C, respectively, *Li. monocytogenes* EGD-e and *Ent. faecalis* V583 in BHI medium (Oxoid) at 37 °C, *Streptococcus thermophilus* LMG 18311 in Todd–Hewitt broth (BD) supplemented with 0.8 % (w/v) glucose and *Escherichia coli* in LB medium (Oxoid) at 37 °C with agitation (250 r.p.m.). When appropriate, erythromycin (5 μg ml^{-1}) and chloramphenicol (5 μg ml^{-1}) were added to the growth medium. Growth analysis was performed using Bioscreen C (Oy Growth Curves); overnight cultures were diluted 100-fold, and OD_{600} was measured at intervals of 20 min.

Table 1. Plasmids and bacterial strains used in this study

Plasmid or strain	Characteristics*†	Reference or source
Plasmids		
pNZ9530	Vector expressing *nisRK* (nisin regulatory genes), EryR	Kleerebezem *et al.* (1997)
pNZ8037	Expression vector in lactococci, nisin-inducible, CamR	de Ruyter *et al.* (1996)
p513	pNZ8037 with man-PTS Lsak (*manL*, *manM*, *manN*) of *Lb. sakei* LMGT2313	This study
p515	pNZ8037 with man-PTS Lsak (*manL*, *manM*, *manN*) of *Lb. sakei* 23K	Diep *et al.* (2007)
p517	pNZ8037 with man-PTS Lsak (*manL*, *manM*, *manN*) of *Lb. sakei* Lb790	This study
p423	pNZ8037 with man-PTS Llac (*ptnAB*, *ptnC*, *ptnD*) of *Lc. lactis* IL1403	Diep *et al.* (2007)
pEcol1	pNZ8037 with man-PTS Ecol1 (*agaB*, *agaC*, *agaD*) of *E. coli* BL21	This study
pEcol2	pNZ8037 with man-PTS Ecol2 (*manX*, *manY*, *manZ*) of *E. coli* BL21	This study
pEfae1	pNZ8037 with man-PTS Efae1 (*EF0020*, *EF0021*, *EF0022*) of *Ent. faecalis* V583	This study
pEfae12	pNZ8037 with man-PTS Efae12 (*EF3136*, *EF3137*, *EF3138*, *EF3139*) of *Ent. faecalis* V583	This study
pLdel	pNZ8037 with man-PTS Ldel (*Ldb1799*, *Ldb1800*, *Ldb1801*) of *Lb. delbrueckii* ATCC 11842	This study
pLmon1	pNZ8037 with man-PTS Lmon1 (*lmo0781*, *lmo0782*, *lmo0783*, *lmo0784*) of *Li. monocytogenes* EGD-e	This study
pLmon2	pNZ8037 with man-PTS Lmon2 (*lmo0021*, *lmo0022*, *lmo0023*, *lmo0024*) of *Li. monocytogenes* EGD-e	This study
pLmon3	pNZ8037 with man-PTS Lmon3 (*lmo1997*, *lmo2000*, *lmo2001*, *lmo2003*) of *Li. monocytogenes* EGD-e	This study
pLmon4	pNZ8037 with man-PTS Lmon4 (*lmo0096*, *lmo0097*, *lmo0098*) of *Li. monocytogenes* EGD-e	This study
pLpla1	pNZ8037 with man-PTS Lpla2 (*pts9A*, *pts9B*, *pts9C*, *pts9D*) of *Lb. plantarum* WCFS1	This study
pLpla2	pNZ8037 with man-PTS Lpla2 (*pts19A*, *pts19D*, *pts19C*, *pts19B*) of *Lb. plantarum* WCFS1	This study
pSthe	pNZ8037 with man-PTS Sthe (*manL*, *manM*, *manN*) of *S. thermophilus* LMG18311	This study
Strains		
Ent. faecalis V583	Source of man-PTSs	Sahm *et al.* (1989)
Enterococcus faecium P13	Enterocin P producer	LMGT strain collection‡
E. coli BL21	Source of man-PTS	Invitrogen
Lb. delbrueckii ATCC 11842	Source of man-PTS	S. Orla-Jensen (unpublished)
Lb. plantarum WCFS1	Source of man-PTS	Kleerebezem *et al.* (2003)
Lb. sakei		
B316	*Lb. sakei* Lb790 clone producing penocin A	Diep *et al.* (2006)
B317	*Lb. sakei* Lb790 clone producing sakacin A	Diep *et al.* (2006)
LTH673	Sakacin P producer	Tichaczek *et al.* (1992)
Lb790	Source of man-PTS	Schillinger & Lucke (1989)
23K	Source of man-PTS	Lauret *et al.* (1996)
LMGT 2313	Source of man-PTS	LMGT strain collection
Lc. lactis		
IL1403	Indicator strain for lactococcin A	Chopin *et al.* (1984)
B488	*ptn* deletion mutant of *Lc. lactis* IL1403 carrying pNZ9530 with *nisRK*, EryR	Diep *et al.* (2007)
B515	B488 with p423, CamR, EryR	Diep *et al.* (2007)
B520	B488 with pNZ8037, CamR, EryR	Diep *et al.* (2007)
B628	B488 with p513, CamR, EryR	This study
B630	B488 with p515, CamR, EryR	Diep *et al.* (2007)
B632	B488 with p517, CamR, EryR	This study
M127	B488 with pLpla1, CamR, EryR	This study
M128	B488 with pLpla2, CamR, EryR	This study
M129	B488 with pEcol1, CamR, EryR	This study
M130	B488 with pEcol2, CamR, EryR	This study
M148	B488 with pLmon1, CamR, EryR	This study
M149	B488 with pLmon2, CamR, EryR	This study

Table 1. cont.

Plasmid or strain	Characteristics*†	Reference or source
M150	B488 with pLmon3, CamR, EryR	This study
M151	B488 with pLmon4, CamR, EryR	This study
M158	B488 with pSthe, CamR, EryR	This study
M159	B488 with pLdel, CamR, EryR	This study
M160	B488 with pEfae1, CamR, EryR	This study
M161	B488 with pEfae12, CamR, EryR	This study
Li. innocua LMGT 2785	Indicator strain for class IIa bacteriocins	LMGT strain collection
Li. monocytogenes EGD-e	Source of man-PTSs	Glaser *et al.* (2001)
Pediococcus acidilactici PAC 1.0	Pediocin PA-1 producer	Henderson *et al.* (1992)
S. thermophilus LMG 18311	Source of man-PTS	Bolotin *et al.* (2004)

*EryR, erythromycin resistance; CamR, chloramphenicol resistance.
†Locus tags of the man-PTS genes are given in parentheses. Protein accession numbers are given in Supplementary Table S1.
‡Laboratory of Microbial Gene Technology, Ås, Norway.

Genetic cloning. The constructs used in this study are listed in Table 1. Man-PTS genes from different strains were amplified by PCR, using the primers listed in Table 2. Total genomic DNA from the respective strains was used as template in all PCRs. The PCR products were cleaved with SphI and *Xho*I, except Lmon3, which was cleaved with *Sph*I and *Sal*I (all restriction endonucleases from New England Biolabs), and ligated into the nisin-regulated pNZ8037-derivative plasmid p519 (Diep *et al.*, 2007) between the *Sph*I and *Xho*I sites, resulting in the plasmids listed in Table 1. All cloned genes were confirmed by sequencing. Transformation into the man-PTS-free strain *Lc. lactis* B488 (Diep *et al.*, 2007) was performed using the electroporation protocol of Holo & Nes (1989).

RNA isolation, cDNA synthesis and RT-PCR. RT-PCR was performed on three strains of *Lb. sakei* (23K, Lb790 and LMGT 2313). Cells in exponential growth (OD_{600} ~0.5) were harvested by centrifugation and stored at −80 °C. The cell pellet was dissolved in 700 µl buffer RTL (Qiagen) supplemented with 0.1 % (v/v) β-mercaptoethanol (Sigma), and the suspension was transferred to 2 ml screw-capped FastPrep tubes (MP Biomedicals) containing 0.5 g acid-washed glass beads (<106 µm, Sigma), 300 µl phenol and 300 µl chloroform. Cells were lysed using Fp120 Fastprep (Bio 101) for three times 25 s at 4 m s^{-1} at 4 °C. Following a short centrifugation (10 000 ***g*** for 1 min), the water phase was transferred to a fresh Eppendorf tube containing 500 µl ethanol. The lysate was then applied to RNeasy spin columns (Qiagen), and RNA was purified as described by the manufacturer (Qiagen). To remove remnants of DNA, RNA was treated with RNase-free DNase I (Qiagen). cDNA was synthesized using the Superscript III Reverse Transcriptase set (Invitrogen), and RNA was then removed by treatment with RNase H (Takara Bio). RT-PCR was carried out using primers mk86 (5′-CCATGTCTTAT-CTTAGGCGG-3′) and mk87 (5′-ACCATCTGTTAACCATACTGG-3′) for *manM* (encoding the IIC subunit), and mk96 (5′-CGTG-CTTCCATACCTTCAAC-3′) and mk97 (5′-ACCTCAAGTTGC-TTACCGTG-3′) for the housekeeping gene *fusA* (encoding translation elongation factor EF-G). Primers were designed based on the annotated genome sequence of *Lb. sakei* 23K (Chaillou *et al.*, 2005).

Bacteriocin assays. Bacteriocins (pediocin PA-1, enterocin P, sakacin P, penocin A and lactococcin A) were concentrated from supernatants of overnight cultures by ammonium sulphate precipitation as described previously (Diep *et al.*, 2007). The bacteriocin activity in the concentrated supernatants was determined using *Listeria innocua* LMGT 2785 and *Lc. lactis* IL1403 as indicator strains for class IIa bacteriocins and lactococcin A, respectively. Bacteriocin sensitivity of the different *Lc. lactis* clones was determined using a microtitre plate assay. Overnight cultures were diluted 100-fold in a medium containing 0.1 ng nisin ml^{-1} to induce expression of the cloned genes, and growth inhibition was measured spectrophotometrically at 600 nm after 15–18 h. One bacteriocin unit (BU) was defined as the amount of bacteriocin required to produce 50 % growth inhibition in a 200 µl culture of the indicator strain. MIC was defined as the minimum bacteriocin concentration needed to produce at least 50 % growth inhibition of tested clones.

RESULTS AND DISCUSSION

Bioinformatic analysis of man-PTS family proteins

A large number of homologous man-PTS genes are found in the genome sequences of different bacterial species (Zúñiga *et al.*, 2005). To get an overview of different members of the man-PTS family in the context of being a reservoir of potential bacteriocin receptors, we first analysed these proteins with a phylogenetic approach. We chose to focus only on the membrane-located subunits (IIC and IID), because expression of these genes (without IIAB) has previously been shown to be sufficient to confer sensitivity to several class IIa bacteriocins and class IIc lactococcins A and B (Diep *et al.*, 2007). By using the proteins ManM (IIC) and ManN (IID) from *Lb. sakei* 23K as query sequences, a large number of homologous man-PTS IIC and IID protein sequences were retrieved from the Entrez Gene database (sequence identity ranging from 84 to 22 %) using BLAST. Members of the man-PTS family were found predominantly in species belonging to two separate phylogenetic taxa, the Gram-positive Firmicutes and the Gram-negative Proteobacteria. This observation is in line with the evolutionary analysis of man-PTS transporters of Zúñiga *et al.* (2005), who propose that these transporters have been distributed to species of Firmicutes and Proteobacteria by horizontal gene transfer. The *in silico* analysis of published genomes revealed that

Table 2. Primers used in this study

Amplified product	Primer	Sequence (5′→3′); restriction site*
Lpla1	mk54	AGCTGCATGCCACTGTAACGAAAAATAGGAGG; *Sph*I
	mk46	ATCGCTCGAGCCCAATATTATGTTAACAATAGC; *Xho*I
Lpla2	mk53	ACGTGCATGCCGTAGGTTAATTACTATCCGC; *Sph*I
	mk48	ATGCCTCGAGCAACAGATAACTTGTTAATCGG; *Xho*I
Ecol1	mk49	ACGTGCATGCAGTGCCTTAATGGAAAAGGAG; *Sph*I
	mk50	ATCGCTCGAGCACCAGACGCAGTGC; *Xho*I
Ecol2	mk51	ACGTGCATGCGACGATTCAAAAATACATCTGG; *Sph*I
	mk52	ATCGCTCGAGGCCAAAAGGCCCCGGTAG; *Xho*I
Lmon1	mk56	ACGTGCATGCCTTATCCACGCTTAAGGAGG; *Sph*I
	mk57	ATGCCTCGAGAGAACCGGAATTTTAATCCCG; *Xho*I
Lmon2	mk58	ACGTGCATGCAACCCTATGAATATATTGAAAGC; *Sph*I
	mk59	ATCGCTCGAGACCCATTTTGTCTATTCCTCC; *Xho*I
Lmon3†	mk62	ACGTGCATGCAGTTGATTTTAACTAATACTAAGG; *Sph*I
	mk63	GTACAAACAAAAATTGCTTTCATTCTTATTTTAATAAACCTGTGAACG
	mk60	ACGTGTCGACAAAGACAGCCGCAGCTGTC; *Sal*I
	mk61	CGTTCACAGGTTTATTAAAATAAGAATGAAAGCAATTTTTGTTTGTAC
Lmon4	mk64	ACGTGCATGCGCAATAAATATAGCGGGTAGC; *Sph*I
	mk65	ATCGCTCGAGTCGGTGAATATTGCACCAGC; *Xho*I
Sthe	mk72	ACGTGCATGCAAGAAGCAATATATAAAAGGAGG; *Sph*I
	mk73	ATGCCTCGAGTCAGCTTTGCTAAACTCTTGC; *Xho*I
Ldel	mk74	ACGTGCATGCTACCGAAGTTTTTGAGGAGG; *Sph*I
	mk75	ATGCCTCGAGACGGTCTTATTCATTGATTGAG; *Xho*I
Efae1	mk76	AGCTGCATGCAAAAACATCAATTATACAGGAGG; *Sph*I
	mk77	ATGCCTCGAGTTGATTAGAAGTAATAAACTTACC; *Xho*I
Efae12	mk80	AGCTGCATGCCCAAAACCAAGGATAAGAAGG; *Sph*I
	mk81	ATGCCTCGAGTCCCCATTTCATTGCTTTCAC; *Xho*I
Lsak	pr205	CATTATAATTGCATGCGTTTTTATCAGTGTG; *Sph*I
	pr206	AGTTAATCCTCGAGATATAAACACCTGC; *Xho*I

*Restriction sites are underlined in the sequences.
†In Lmon3, the gene encoding the IIA subunit is separated from the other genes by 2112 bp. To remove this sequence, Lmon3 was amplified using a two-step PCR approach (Higuchi, 1990), with mk60 and mk62 as outer primers and mk61 and mk63 as inner primers.

the number of different man-PTSs within a strain is highly variable between different genera, e.g. only one is found in *Lc. lactis*, *Lb. sakei* and *S. thermophilus*, four in *Li. monocytogenes* and a total of 13 in *Ent. faecalis* V583. Some of the man-PTS operons are incomplete, i.e. missing one or two of the four subunits (e.g. the *agaBCD* operon in *E. coli*, encoding IIB, IIC and IID, but no IIA subunit). Based on an initial phylogenetic clustering of all collected sequences, 86 man-PTSs from the Entrez Gene database were selected for further phylogenetic analysis, and the man-PTSs were designated using the following four-letter system: the first letter was derived from the first letter of the genus name, the next three from the first three letters in the species name, and a digit when more than one man-PTS was present in the bacterial species, e.g. Lmon1 for one of the man-PTSs from *Li. monocytogenes* (an overview of the proteins used in the phylogenetic analysis can be found in Supplementary Table S1). The phylogenetic trees of protein families IIC and IID (Fig. 1) show that their members are clustered into three distinct groups in both families, named groups I, II and III, and this grouping is supported by significant branch support values (bootstrap value >0.85, aLRT value >0.9). Group III is the largest and most diverse. In all cases, the phylogenetic position of a IIC protein and its cognate IID protein (from the same man-PTS) correspond well with each other, i.e. both occupy a similar position in their respective trees, suggesting that the pairwise IIC and IID subunits have evolved in parallel. Notably, we observed that the man-PTSs previously suggested to be involved as receptors for class IIa bacteriocins Efae1 (*mpt* operon from *Ent. faecalis*; Héchard *et al.*, 2001), Lmon4 (*mpt* operon from *Li. monocytogenes*; Dalet *et al.*, 2001) and Lsak (*man* operon from *Lb. sakei*; Diep *et al.*, 2007) are clustered in group I.

Class IIa and IIc bacteriocins target only a phylogenetically defined subset of man-PTSs

To evaluate which man-PTSs from different loci or different bacteria act as receptors for bacteriocins of class

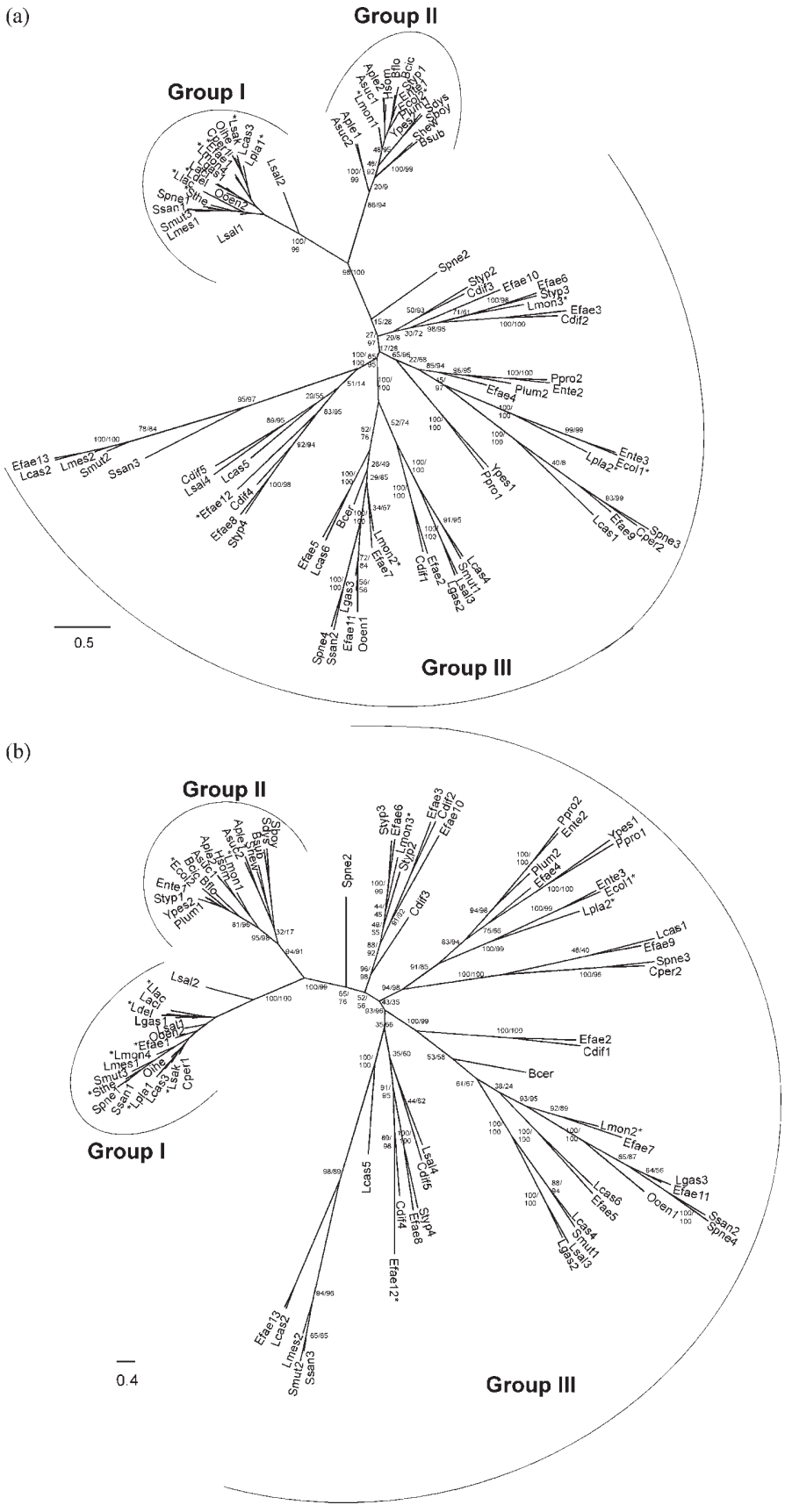
(a)
Group I
Group II
Group III
0.5
(b)
Group I
Group II
Group III
0.4

Fig. 1. Phylogenetic analysis of the man-PTS protein families IIC (a) and IID (b). Altogether, 86 protein pairs were analysed. The trees were constructed using the maximum-likelihood algorithm implemented in PhyML. Bootstrap values from 100 replicates (first value) and the aLRT branch support values (second value) are shown for most branches (some of the values are not shown due to space limitations). Asterisks indicate man-PTSs selected for the experimental analysis.

IIa and the class IIc bacteriocin lactococcin A, 14 different man-PTSs, distributed all over the phylogenetic tree, were selected for experimental analysis. These included all four man-PTSs from *Li. monocytogenes* (Lmon1–4), two (of 13) from *Ent. faecalis* (Efae1 and Efae12), two (of two) from *Lb. plantarum* (Lpla1–2), two (of two) from *E. coli* (Ecol1–2), and the single man-PTSs found in *Lb. sakei* (Lsak), *Lc. lactis* (Llac), *Lb. delbrueckii* (Ldel) and *S. thermophilus* (Sthe). The nisin-controlled expression (NICE) system (Kuipers *et al.*, 1998) was used to express the individual man-PTS operons in an *Lc. lactis* mutant clone (B488) in which the single indigenous man-PTS locus in the genome has been deleted (Diep *et al.*, 2007), thus containing no background man-PTS that might interfere with our bacteriocin receptor assays. The different homologous man-PTSs were first assessed for their ability to transport glucose in *Lc. lactis* by adding the non-metabolizable glucose analogue 2-deoxy-D-glucose (2-DG) to the growth medium (Thompson & Chassy, 1982); growth inhibition with 2-DG is an indication of a functional man-PTS transporter. The results from the growth analysis, as summarized in Table 3, showed that all the man-PTSs of group I (Llac, Lsak, Efae1, Lmon4, Ldel, Sthe and Lpla1), in addition to Ecol2 (group II), can take up 2-DG when expressed in the heterologous host *Lc. lactis*. On the other hand, none of the remaining man-PTSs of group III (Lmon2, Lmon3, Ecol1, Lpla2 and Efae12) or Lmon1 (group II) was functional as a glucose transporter in *Lc. lactis*, which suggests that these homologous transporters fulfil roles in the cells other than glucose transport (e.g. uptake of other sugars) or are not functional at all. Alternatively, these man-PTSs could be dependent on species-specific factors to be functional. It should be noted that a functional man-PTS in terms of sugar uptake is not a prerequisite for being a bacteriocin receptor, as we have previously shown that expression of the man-PTS IIC and IID genes in the absence of the man-PTS IIAB gene can provide a functional receptor (Diep *et al.*, 2007).

To examine whether the expressed man-PTS genes could serve as bacteriocin receptors, all 14 *Lc. lactis* clones were challenged with four different class IIa bacteriocins, pediocin PA-1 (Marugg *et al.*, 1992), enterocin P (Cintas *et al.*, 2000), sakacin P (Tichaczek *et al.*, 1994) and penocin A, (Diep *et al.*, 2006) and one class IIc bacteriocin, lactococcin A (Holo *et al.*, 1991). The MIC values determined by microtitre plate assays are given in Table 3. In group I, six out of the seven selected man-PTSs were able to confer sensitivity to the class IIa bacteriocins but not to lactococcin A, while the last man-PTS from this

Table 3. Experimental analysis of selected man-PTS family members

Group	man-PTS (clone)	MIC value* (BU ml^{-1}) for bacteriocin					2-DG inhibition†	Genetic org.‡
		Ped PA-1	EntP	SakP	PenA	LcnA		
I	Lsak (B630)	25	10	10	35	NI	+	AB-C-D
	Lpla1 (M127)	35	25	140	30	NI	+	AB-C-D
	Lmon4 (M151)	20	5	<0.5	10	NI	+	AB-C-D
	Efae1 (M160)	20	5	5	5	NI	+	AB-C-D
	Ldel (M159)	35	10	35	35	NI	+	AB-C-D
	Sthe (M158)	95	95	25	50	NI	+	AB-C-D
	Llac (B515)	NI	NI	NI	NI	190	+	AB-C-D
II	Lmon1 (B148)	NI	NI	NI	NI	NI	−	A-B-C-D
	Ecol2 (M130)	NI	NI	NI	NI	NI	+	AB-C-D
III	Lmon2 (M149)	NI	NI	NI	NI	NI	−	A-B-C-D
	Lmon3 (M150)	NI	NI	NI	NI	NI	−	A-i-B-C-D
	Ecol1 (M129)	NI	NI	NI	NI	NI	−	B-C-D
	Lpla2 (M128)	NI	NI	NI	NI	NI	−	B-C-D-A
	Efae12 (M161)	NI	NI	95	190	NI	−	C-D-B-A

*The numbers indicate the minimum concentrations (in BU ml^{-1}) required to produce at least 50 % growth inhibition. MIC values were determined at least twice with virtually the same results. NI, No inhibition observed at the highest bacteriocin concentration tested (400 BU ml^{-1}).
†+, Growth of the *Lc. lactis* clone was inhibited by 2-DG; −, no inhibition.
‡Organization of the genes encoding the man-PTS subunits. Separated genes are indicated with hyphens. In Lmon3, an inserted gene (i) is found between the man-PTS IIA and IIB genes.

group, the *ptn* operon from *Lc. lactis* (Llac), conferred sensitivity to lactococcin A but not to any of the class IIa bacteriocins. Of these, the man-PTSs Lsak (*man* operon), Lmon4 (*mpt*) and Efae1 (*mpt*) from *Lb. sakei*, *Li. monocytogenes* and *Ent. faecalis*, respectively, have previously been shown, in a direct or indirect manner, to be associated with sensitivity to bacteriocins (Dalet *et al.*, 2001; Diep *et al.*, 2007; Héchard *et al.*, 2001), and we finally confirmed here that these man-PTSs indeed act as receptors for several class IIa bacteriocins. In addition, the group I man-PTS systems Lpla1 (*pts9*) from *Lb. plantarum*, Ldel from *Lb. delbrueckii* and Sthe (*man*) from *S. thermophilus* were shown for the first time to function as receptors for bacteriocins. Remarkably, all selected members of groups II and III were unable to confer sensitivity to the different bacteriocins, except for Efae12 (from *Ent. faecalis*), which seemed to cause some sensitivity when the resulting clone was exposed to relatively high concentrations of sakacin P and penocin A (20- to 40-fold higher concentrations than for Efae1, the other enterococcal man-PTS tested). At present we cannot explain why Efae12 has this function, but as both Efae1 and Efae12 are derived from the same host (*Ent. faecalis* V583), the biological function of Efae12 as a bacteriocin receptor is probably insignificant, as it will be overshadowed by Efae1.

Out of the 14 man-PTSs selected, only the lactococcal Llac of group I could function as a receptor for the class IIc bacteriocin lactococcin A. This lactococcal species-specificity, noticed more than 15 years ago (Henderson *et al.*, 1992; Holo *et al.*, 1991; Kok *et al.*, 1993), that lactococcins A and B only target lactococcal species, while pediocin-like bacteriocins do not target these species, can now be ascribed to differences in the amino acid sequences of the IIC and IID subunits of their man-PTSs.

Notably, the selected bacteriocins seemed to differ greatly from each other in their potency in targeting the various man-PTSs. While pediocin PA-1 appeared to target all non-lactococcal receptors (from group I) with a similar range of efficiency (i.e. having only a fivefold difference between the highest and the lowest), a significantly larger variation was seen for the other bacteriocins; the difference between the highest and the lowest was about 19-fold for enterocin P, 280-fold for sakacin P and 10-fold for penocin A. Two of the man-PTSs, Lmon4 and Efae1, were the most active receptors for all four bacteriocins, and not surprisingly, these two systems are most closely related to each other according to the phylogenetic trees (Fig. 1). This correlation became even more evident in a separate phylogenetic analysis of concatenated IIC and IID proteins from 31 group I man-PTSs (including 12 man-PTSs that were left out of the first phylogenetic analysis due to space limitations), in which receptors conferring high, medium and low sensitivity appeared to be organized into distinct domains (Fig. 2). For instance, Lsak and Lpla1, both conferring medium sensitivity, are located next to each other within a domain, and both are well separated from the domain that contains the highly efficient receptors Lmon4 and Efae1. Similarly, Sthe, which confers low sensitivity, is defined within another domain. These results are in line with previous comparative analyses of the inhibitory spectra of class IIa bacteriocins: (i) species of *Enterococcus*, *Listeria* and *Carnobacterium* are highly sensitive to pediocin-like bacteriocins (low MIC values); (ii) species of *Lactobacillus*, *Pediococcus* and *Clostridium* are also frequently inhibited by these bacteriocins, although they are often less sensitive (higher MIC values); and (iii) strains of *Streptococcus* and *Leuconostoc* are occasionally reported to be sensitive to class IIa bacteriocins at a low level (very high MIC values) (Diep *et al.*, 2006; Eijsink *et al.*, 1998).

It is also worth noting that the *in silico* analysis of man-PTSs also revealed an interesting link between man-PTS receptors and bacteriocin producers. From the available sequence information, all bacterial species producing class IIa bacteriocins (Drider *et al.*, 2006; Fimland *et al.*, 2005) seem to have genes encoding a man-PTS belonging to phylogenetic group I. For example, *Bacillus coagulans* is the only *Bacillus* species known to produce a pediocin-like bacteriocin (Le Marrec *et al.*, 2000), and *B. coagulans* is also the only *Bacillus* species harbouring a group I man-PTS. This notion implies that there is an evolutionary link between the production of class IIa bacteriocins and the presence of a group I man-PTS in the same cell, although the biological significance of this observation has yet to be understood.

Group I receptors contain three distinct regions in their sequences

Given that the receptor function of a man-PTS is related to its phylogenetic position (Figs 1 and 2), we looked for important sequence differences between the different man-PTSs that might be responsible for the observed variation in receptor potency. Multiple sequence alignments of the 14 man-PTSs used in the experimental analysis revealed at least three regions in the IIC and IID proteins, termed region-α, region-β and region-γ, that clearly distinguish IIC and IID proteins with good receptor function from those with poor or no receptor function (Fig. 3 and Supplementary Fig. S1). Region-α, which is located in the N-terminal half of IIC of group I, is characterized by several conserved residues and an additional sequence stretch (3–4 aa); this region contains the conserved sequence GGQGXXG in the man-PTSs belonging to the high- and medium-sensitivity group, while a related motif (GG[D/K]FXXXG) is found in the man-PTSs with low sensitivity. Interestingly, Llac, which confers sensitivity only to the class IIc lactococcin A but not to class IIa bacteriocins, lacks such a sequence, suggesting that the motif in region-α is important for interaction with class IIa bacteriocins only. Region-β, which is located at the C-terminal end of the IIC proteins, is characterized for group I man-PTSs by an enrichment in glycine residues and the presence of a conserved sequence DP[I/L/V]GDI[I/L][D/E/

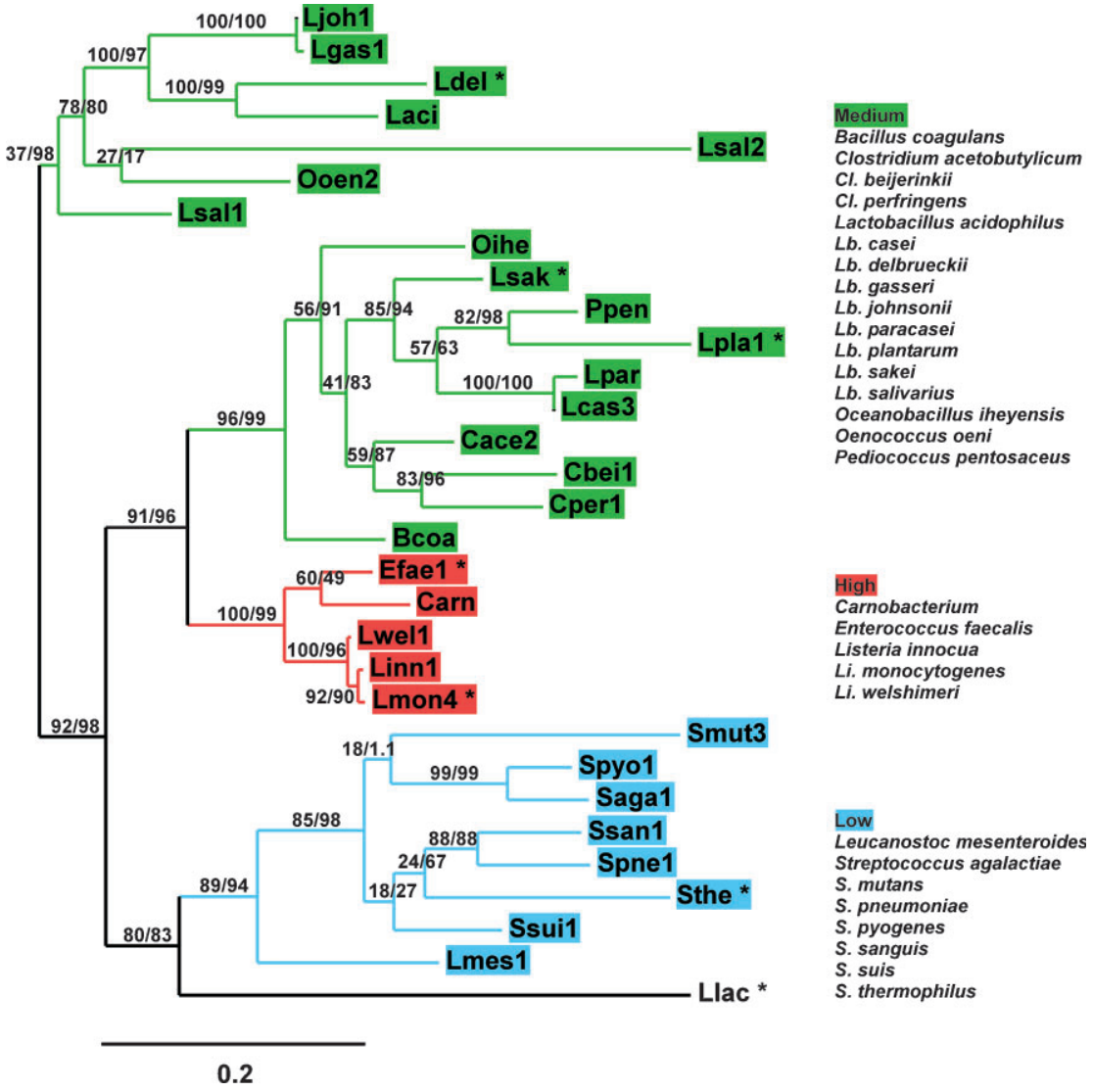

Fig. 2. Phylogenetic clustering of concatenated man-PTS IIC and IID proteins from group I using the maximum-likelihood algorithm in PhyML. Bootstrap support values from 100 replicates (first value) and aLRT branch support values (second value) are shown. The man-PTSs are separated into high- (red), medium- (green) and low-sensitivity (blue) groups based on the receptor potency. Asterisks indicate man-PTSs included in the experimental analysis. The bacterial species carrying these proteins are listed.

N]XY, in which P, I and Y are unique to group I man-PTS. Finally, region-γ designates a location in the IID proteins in which members of phylogenetic group I contain an additional sequence of 35–40 aa which is absent in their counterparts from groups II and III. Interestingly, transmembrane prediction of the IIC and IID proteins using the TMHMM Server 2.0 (Krogh *et al.*, 2001) suggested that all these three regions discussed here are located on the extracellular side of the membrane, and thus might serve as binding sites for bacteriocins on the surface of sensitive cells.

The fact that two of these distinct regions are located in IIC and the last one in IID suggests that the bacteriocin binding site(s) on the receptor are composed of sequences from different proteins. This notion is in line with our recent study (Diep *et al.*, 2007), which showed that expression of individual IIC or IID genes derived from a potent man-PTS (such as Lsak or Llac) could not confer sensitivity. Furthermore, we have also found that, while the pairwise IIC and IID from Lsak and Llac are very potent receptors for pediocin-like bacteriocins and lactococcin A, respectively, the combination of one component from one system

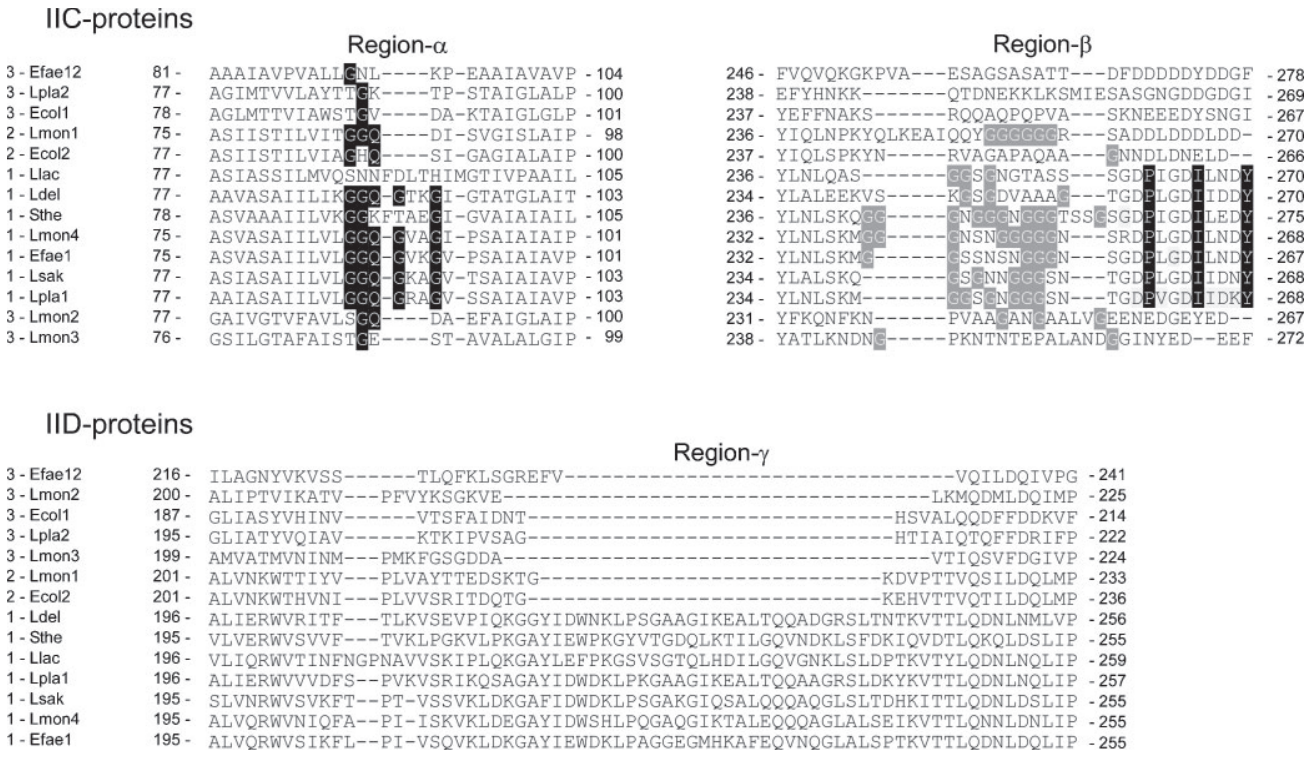

Fig. 3. Multiple sequence alignments of the IIC and IID proteins from the 14 man-PTSs used in the experimental analysis. Shown here are three regions which clearly separate group I from group II and III sequences: region-α in the N-terminal half of IIC (residues in the putative GGQGXXG motif are highlighted with a black background), region-β in the C-terminal end of IIC (the glycine-rich region is indicated by a grey background, and residues that are conserved only in group I man-PTSs are shown with a black background) and region-γ in the C-terminal half of IID. The number in front of the man-PTS names indicates the phylogenetic group (1, 2 or 3), and the numbers next to the sequences indicate amino acid positions in each protein. Alignments of region-α, region-β and region-γ, including all 86 IIC and IID proteins used in the phylogenetic analysis, can be found in Supplementary Fig. S1. The alignment was constructed using MUSCLE.

with a non-cognate partner from another system leads to little or no sensitivity to either type of bacteriocin (data not shown). As the pairwise IIC and IID proteins seem to have evolved in a parallel manner (Fig. 1), creation of such hybrids from two phylogenetically divergent subunits probably forms a complex that has become defective for bacteriocin binding. However, we cannot exclude the possibility that the bacteriocin binding site is located in one subunit but that its functionality needs to be stabilized by the presence of the cognate partner. It should also be mentioned that region-γ in IID has previously been associated with sensitivity to class IIa bacteriocins, because an in-frame deletion of this region in *mptD* renders *Li. monocytogenes* EGD-e cells resistant to mesentericin Y105 (Dalet *et al.*, 2001). However, it is not known whether region-γ is directly involved in the interaction with the bacteriocin, or whether this deletion perturbs the structure of MptD. Intragenic and intergenic chimeric receptors designed to address the nature of the bacteriocin receptor-specificity, and the role of region-α, region-β and region-γ in this context, are currently under investigation.

Differential expression of man-PTS causes variation in bacteriocin sensitivity

It has been frequently observed that different strains of the same bacterial species can vary greatly in sensitivity to a given bacteriocin (Eijsink *et al.*, 1998; Katla *et al.*, 2003). In most cases, the reason for this variation is not known. For instance, *Lb. sakei* strains 23K, Lb790 and LMGT 2313 display a great variation in sensitivity toward pediocin PA-1, with LMGT 2313 being the most sensitive (Fig. 4a). By DNA sequencing, all three strains were found to harbour almost identical man-PTS genes in their genomes (except for a few silent mutations in the DNA sequences that have no effect on the amino acid sequences). Furthermore, subcloning of the man-PTSs of *Lb. sakei* 23K, Lb790 and LMGT 2313 in the man-PTS-free strain *Lc. lactis* B488 showed that all three clones display more or less the same degree of sensitivity to pediocin PA-1 (Fig. 4b), illustrating that these silent mutations do not account for the observed variation in sensitivity.

To examine whether the variation in sensitivity among these strains could be due to differential gene expression,

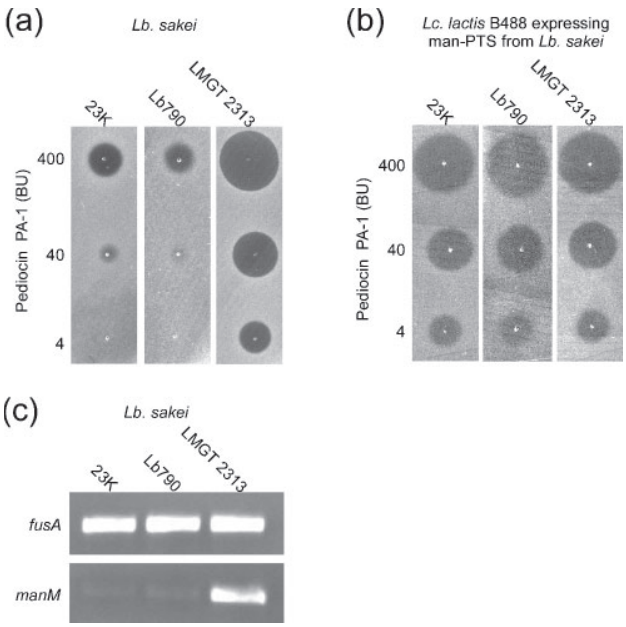

Fig. 4. Variation in bacteriocin sensitivity is due to differential expression levels of the man-PTS. Soft agar overlay assay with the three *Lb. sakei* strains 23K, Lb790 and LMGT 2313 (a), and with the three *Lc. lactis* clones expressing the man-PTSs derived from the *Lb. sakei* strains (b). Cells were challenged with 5 μl of three different concentrations of pediocin PA-1 (400, 40 and 4 BU) and inhibition is seen as clear zones. (c) RT-PCR analysis of *manM* (encoding the man-PTS IIC component) in the *Lb. sakei* strains. The housekeeping gene *fusA* (encoding the translation elongation factor EF-G) was included as a control.

the relative transcription levels of *manM* (encoding the receptor IIC subunit) in the three *Lb. sakei* strains were determined by RT-PCR (Fig. 4c). These results clearly indicate that the *manM* expression levels are higher in the more sensitive strain LMGT 2313 than in 23K and Lb790. Thus, the three *Lb. sakei* strains harbouring the same man-PTSs displayed highly variable sensitivity to pediocin PA-1 because of differential expression levels of their man-PTS genes. This is in line with previous observations, which show that genes involved in the regulation of man-PTS expression, such as *rpoN* (encoding a σ^{54} subunit of RNA polymerase) and *mptR*/*manR* (encoding σ^{54}-associated transcription factors), influence sensitivity to the pediocin-like bacteriocin mesentericin Y105 in *Ent. faecalis* and *Li. monocytogenes* (Dalet *et al.*, 2000, 2001; Héchard *et al.*, 2001). In addition, it should also be kept in mind that other strain-specific factors, including cell-surface differences (Vadyvaloo *et al.*, 2002, 2004) and relics of bacteriocin loci containing immunity genes (Moretro *et al.*, 2005), can be responsible for the observed variability in bacteriocin sensitivity between strains from the same bacterial species.

Concluding remarks

Class IIa bacteriocins are considered promising antimicrobial agents for use in medicine and food preservation. Most studies performed hitherto have been conducted by assaying the susceptibility of randomly selected bacterial strains to various bacteriocins, and these results are often difficult to compare due to variations in growth conditions (of both the bacteriocin producer and the indicator strain), bacteriocin assays and choice of indicator strains. The present work provides a systematic overview of the functionality of receptors for pediocin-like bacteriocins, showing that only one subgroup of man-PTS proteins are efficient receptors. The results highlight the great potential of these bacteriocins to improve therapeutic strategies, in particular against enterococcal and listerial infections. Variation in receptor sequences seems to account for much of the variation in bacteriocin susceptibility between different bacterial species, and we have identified three sequence regions that clearly distinguish man-PTSs that could serve as bacteriocin receptors from those that could not. Whether these three sequence regions are directly involved in the bacteriocin binding sites awaits further investigation. In addition, other factors such as gene expression level also seem to be of high importance in determining the degree of sensitivity of a strain.

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Supplementary Table S1. Man-PTSs used in the phylogenetic analyses

Name	Species	Operon name*	IIC	IID	Group
Aple1	*Actinobacillus pleuropneumoniae* L20		YP_001054350	YP_001054349	II
Aple2			YP_001054081	YP_001054082	II
Asuc1	*Actinobacillus succinogenes* 130Z		YP_001344240	YP_001344239	II
Asuc2			YP_001343600	YP_001343601	II
Bcer	*Bacillus cereus* E33L	*levDFGE*	YP_245827	YP_245828	III
Bcic	*Baumannia cicadellinicola* str. Hc		YP_588891	YP_588890	II
Bcoa	*Bacillus coagulans* 36D1		ZP_01696349	ZP_01696350	I
Bflo	*'Candidatus' Blochmannia floridanus*	*manXYZ*	NP_878732	NP_878733	II
Bsub	*Bacillus subtilis* subsp. *subtilis* str. 168	*levDEFG*	NP_390583	NP_390582	II
Cace2	*Clostridium acetobutylicum* ATCC 824	*manYptnD*	NP_149231	NP_149232	I
Carn	*Carnobacterium sp. AT7*		ZP_02184141	ZP_02184140	I
Cbei1	*Clostridium beijerinckii* NCIMB 8052		YP_001307852	YP_001307853	I
Cdif1	*Clostridium difficile* 630		YP_001089584	YP_001089583	III
Cdif2			YP_001087561	YP_001087562	III
Cdif3			YP_001089793	YP_001089792	III
Cdif4			YP_001086757	YP_001086758	III
Cdif5			YP_001086967	YP_001086968	III
Cper1	*Clostridium perfringens* SM101		YP_698130	YP_698131	I
Cper2			YP_697727	YP_697728	III
Ecol1	*Escherichia coli* K-12	*agaBCD*	NP_417608	NP_417609	III
Ecol2		*manXYZ*	NP_288254	NP_288255	II
Efae1	*Enterococcus faecalis* V583	*mptACD*	NP_813832	NP_813833	I
Efae2			NP_814322	NP_814323	III

Name	Species	Operon name*	IIC	IID	Group
Efae3			NP_816643	NP_816642	III
Efae4			NP_816655	NP_816654	III
Efae5			NP_814239	NP_814240	III
Efae6			NP_815632	NP_815631	III
Efae7			NP_814559	NP_814560	III
Efae8			NP_816595	NP_816594	III
Efae9			NP_815928	NP_815927	III
Efae10			NP_816814	NP_816815	III
Efae11			NP_815501	NP_815500	III
Efae12			NP_816747	NP_816746	III
Efae13			NP_815527	NP_815526	III
Ente1	*Enterobacter* sp. 638		YP_001177107	YP_001177108	II
Ente2			YP_001178283	YP_001178284	III
Ente3			YP_001178289	YP_001178290	III
Hsom	*Haemophilus somnus* 129PT	*manXYZ*	YP_718820	YP_718819	II
Laci	*Lactobacillus acidophilus* NCFM	*manLMN*	YP_193373	YP_193374	I
Lcas1	*Lactobacillus casei* ATCC 334		YP_807827	YP_807826	III
Lcas2			YP_805851	YP_805852	III
Lcas3			YP_807983	YP_807982	I
Lcas4			YP_805700	YP_805701	III
Lcas5			YP_805667	YP_805667	III
Lcas6			YP_807934	YP_807933	III
Ldel	*Lactobacillus delbrueckii* ATCC 11842		YP_619526	YP_619525	I
Lgas1	*Lactobacillus gasseri* ATCC 33323		YP_815622	YP_815620	I
Lgas2			YP_814351	YP_814352	III
Lgas3			YP_813968	YP_813969	III
Ljoh1	*Lactobacillus johnsonii* NCC 533		NP_965751	NP_965750	I
Linn1	*Listeria innocua* Clip11262		NP_469489	NP_469490	I

Name	Species	Operon name*	IIC	IID	Group
Llac	*Lactococcus lactis* subsp. *lactis* IL1403	*ptnABCD*	NP_267863	NP_267864	I
Lmes1	*Leuconostoc mesenteroides* ssp. *mesenteroides* ATCC 8293		YP_819227	YP_819226	I
Lmes2			YP_818741	YP_818740	III
Lmon1	*Listeria monocytogenes* EGD-e	*mpoABCD*	NP_464309	NP_464308	II
Lmon2			NP_463556	NP_463557	III
Lmon3			NP_465525	NP_465524	III
Lmon4		*mptACD*	NP_463630	NP_463631	I
Lpar	*Lactobacillus paracasei* 1195		ABK27639	ABK27640	I
Lpla1	*Lactobacillus plantarum* WCFS1	*pts9ABCD*	NP_784349	NP_784350	I
Lpla2		*pts19BCDA*	NP_786053	NP_786052	III
Lsak	*Lactobacillus sakei* subsp. *sakei* 23K	*manLMN*	YP_395063	YP_395064	I
Lsal1	*Lactobacillus salivarius* subsp. *salivarius* UCC118		YP_535548	YP_535549	I
Lsal2			YP_534991	YP_534992	I
Lsal3			YP_536600	YP_536599	III
Lsal4			YP_536837	YP_536836	III
Lwel1	*Listeria welshimeri* serovar 6b str. SLCC5334		YP_848286	YP_848287	I
Oihe	*Oceanobacillus iheyensis* HTE831		NP_694296	NP_694295	I
Ooen1	*Oenococcus oeni* PSU-1		YP_810011	YP_810012	III
Ooen2			YP_810087	YP_810088	I
Plum1	*Photorhabdus luminescens* subsp. *laumondii* TTO1	*manXYZ*	NP_929933	NP_929934	II
Plum2		*agaBCD*	NP_928179	NP_928180	III
Ppen	*Pediococcus pentosaceus* ATCC 25745		YP_804990	YP_804989	I
Ppro1	*Photobacterium profundum* SS9		YP_131817	YP_131818	III
Ppro2			YP_132711	YP_132712	III
Saga1	*Streptococcus agalactiae* 2603V/R	*manLMN*	NP_687394	NP_687393	I
Sboy	*Shigella boydii* Sb227	*sorFBAM*	YP_410307	YP_410306	II
Sdys	*Shigella dysenteriae* Sd197		YP_405701	YP_405702	II

Name	Species	Operon name*	IIC	IID	Group
Shew	*Shewanella* sp. W3-18-1		YP_961836	YP_961835	II
Smut1	*Streptococcus mutans* UA159		NP_720577	NP_720578	III
Smut2			NP_722259	NP_722258	III
Smut3		*ptnACD*	NP_722189	NP_722190	I
Spne1	*Streptococcus pneumoniae* TIGR4		NP_344821	NP_344820	I
Spne2			NP_346576	NP_346575	III
Spne3			NP_344860	NP_344861	III
Spne4			NP_344611	NP_344612	III
Spyo1	*Streptococcus pyogenes* M1 GAS	*manLMN*	NP_269762	NP_269763	I
Ssan1	*Streptococcus sanguinis* SK36		YP_001035844	YP_001035845	I
Ssan2			YP_001034071	YP_001034072	III
Ssan3			YP_001034232	YP_001034233	III
Ssui1	*Streptococcus suis* 05ZYH33		YP_001199145	YP_001199146	I
Sthe	*Streptococcus thermophilus* LMG 18311	*manLMN*	YP_138867	YP_138866	I
Styp1	*SaLmonella typhimurium* LT2	*manXYZ*	NP_460787	NP_460788	II
Styp2			NP_459567	NP_459566	III
Styp3			NP_463396	NP_463397	III
Styp4			NP_462669	NP_462668	III
Ypes1	*Yersinia pestis* KIM		NP_670519	NP_670520	III
Ypes2		*manXYZ*	NP_669856	NP_669857	II

*If assigned in Entrez Gene (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene).

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SUPPLEMENTARY FIGURE LEGEND

Supplementary Fig. S1. Multiple sequence alignment of all 86 man-PTSs used in the phylogenetic analysis. Three segments of the alignments are shown: region-α in the N-terminal half of IIC (a), region-β in the C terminus of IIC (b) and region-γ in the C-terminal half of IID (c). Residues are highlighted as explained in the legend for Fig. 3. The alignment was constructed using MUSCLE. The number in front of the protein names indicates whether the man-PTS belongs to phylogenetic group 1, 2 or 3, and the numbers next to the sequences indicate amino acid positions in each protein.

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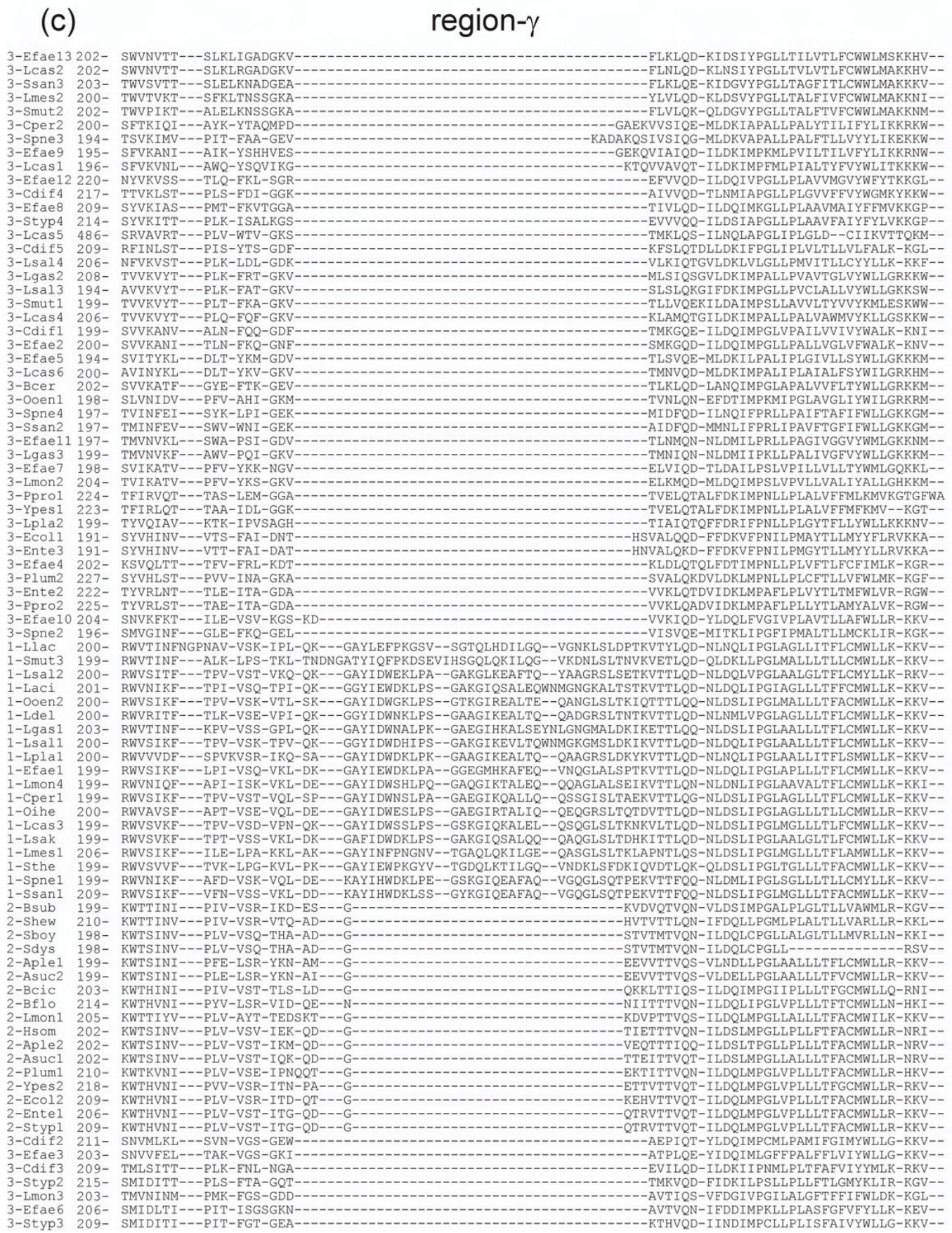
Supplementary Fig. S1


(c)
region-γ

PAPER II

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An Extracellular Loop of the Mannose Phosphotransferase System Component IIC Is Responsible for Specific Targeting by Class IIa Bacteriocins[▽]

Morten Kjos, Zhian Salehian, Ingolf F. Nes, and Dzung B. Diep*

Laboratory of Microbial Gene Technology and Food Microbiology, Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, N-1432 Ås, Norway

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Class IIa bacteriocins target a phylogenetically defined subgroup of mannose-phosphotransferase systems (man-PTS) on sensitive cells. By the use of man-PTS genes of the sensitive *Listeria monocytogenes* (*mpt*) and the nonsensitive *Lactococcus lactis* (*ptn*) species to rationally design a series of man-PTS chimeras and site-directed mutations, we identified an extracellular loop of the membrane-located protein MptC that was responsible for specific target recognition by the class IIa bacteriocins.

Bacteriocins are small, ribosomally synthesized antimicrobial peptides that normally kill bacteria closely related to the bacteriocin producers, but some also target a wider spectrum of bacteria, including a number of pathogens and food spoilage bacterial species (5, 28). Class IIa (pediocin-like) bacteriocins display a broad antimicrobial spectrum, including important pathogens such as *Listeria monocytogenes* and *Enterococcus faecalis*. These peptides consist of 37 to 48 nonmodified amino acids, contain a conserved pediocin-box sequence (Y-G-N-G-V/L) in the N-terminal region, and have defined secondary features in their structure: a cationic β sheet at the conserved N terminus and a helix-containing domain at the less-conserved C terminus (16, 30). Class IIa bacteriocins target sensitive cells by using the mannose phosphotransferase system (man-PTS) as a receptor (6, 10, 17, 19, 33). This sugar uptake system is the major glucose transporter for many bacteria, particularly *Firmicutes* and *Gammaproteobacteria* (39). Each man-PTS complex consists of four structural domains: IIC and IID, represented by two membrane-located proteins, and IIA and IIB, which are normally represented by a single cytoplasmic protein that can form reversible contacts with its membrane-located partners (31).

It has previously been shown that coexpression of the IIC and IID genes is needed to confer sensitivity to class IIa bacteriocins as well as to the lactococcal bacteriocin lactococcin A and that the cytoplasmic IIAB partner is not involved in this process (10). However, while lactococcin A (belonging to class IIc) targets only the lactococcal man-PTS, the class IIa bacteriocins target man-PTSs of species of diverse genera (e.g., *Listeria*, *Enterococcus*, and *Lactobacillus*) but somehow not those of the *Lactococcus* genus (24). This genus specificity has been recognized for almost 2 decades (20, 23, 26); still, the molecular nature underlying the specificity has remained very enigmatic. In the present report we clarify this issue by demonstrating that these two types of bacteriocins exhibit different binding patterns on their receptors: class IIa bacteriocins specifically interact with a defined region of 40 amino acids in the IIC protein whereas lactococcin A has a more complex interaction involving regions from both IIC and IID.

* Corresponding author. Mailing address: Laboratory of Microbial Gene Technology and Food Microbiology, Dept. of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, N-1432 As, Norway. Phone: 47 6496 5910. Fax: 47 64 96 59 01. E-mail: dzung.diep@umb.no.

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MATERIALS AND METHODS

Bacterial strain and growth conditions. *Lactococcus lactis* B488 (10), a man-PTS null mutant derived from *L. lactis* IL1403, was used as an expression host in this study. Lactococcal clones were grown at 30°C in M17 medium (Oxoid) supplemented with 0.4% (wt vol^{-1}) galactose and with 5 μg ml^{-1} erythromycin and 5 μg ml^{-1} chloramphenicol, when appropriate.

Construction of hybrid man-PTS genes. *ptnCD* of *L. lactis* IL1403 and *mptCD* of *Listeria monocytogenes* EGD-e were used as sources for the construction of hybrid man-PTS genes. Different combinations of the man-PTS IIC (*mptC* and *ptnC*) and IID (*mptD* and *ptnD*) genes were fused using a two-step PCR approach (21). In this procedure, two separate fragments were amplified in the first step by using one outer primer and one inner primer for each fragment. Overlapping sequences were introduced by the inner primers, and the two fragments were fused in a second PCR using the outer primers. The primers and template DNA used to construct hybrids H1 to H16, H2X1, H2X2, and H2X3 are outlined in Table 1, and primer sequences are given in Table 2. Plasmid pH1, a pNZ8037 derivative containing *mptACD* with an XmaI site inserted between *mptA* and *mptC*, was constructed first and was used as a cloning cassette to introduce different versions of man-PTS IIC and IID genes downstream of *mptA* between the XmaI and XhoI restriction sites. All constructs were verified by sequencing. An overview of the plasmids used in this study is found in Table 3.

Site-directed mutagenesis. Site-directed mutagenesis was performed using the *mptC* gene with plasmid pH1 and a two-step PCR procedure. Mutations were introduced by the use of mutagenic inner primers in combination with outer primers mk236 and mk65. Mutagenic primers mk270 and mk271 were used for mutation G86S, mk272 and mk273 for G87N, mk180 and mk181 for Q88F, mk266 and mk267 for G89H, mk277 and mk278 for G89A, and mk279 and mk280 for G92A. All primer sequences are given in Table 2.

Transformation and heterologous expression. The nisin-inducible two-plasmid system based on pNZ9530 (25) and pNZ8037 (8) was used to express various hybrid combinations of man-PTS genes. pNZ8037-derived plasmids were propagated in *Escherichia coli* (34) prior to electroporation into *L. lactis* B488 (22), which is an *L. lactis* IL1403 *ptnABCD* deletion mutant carrying plasmid pNZ9530, which contains genes necessary for nisin-induced gene expression. Expression of man-PTS genes was induced by the addition of 0.1 ng ml^{-1} nisin to the growth medium. The expression and functionality of the man-PTS hybrids in terms of sugar transport were assessed by growing cells in M17 medium (Oxoid) with and without 1% (wt vol^{-1}) 2-deoxy-D-glucose, a nonmetabolizable glucose analogue. The M17 complex medium (containing 5 g of tryptone, 5 g of soya peptone, 5 g of meat digest, 2.5 g of yeast extract, 0.5 g of ascorbic acid,

TABLE 1. Outline of the cloning procedure[a]

Construct	Outer primer	Inner primer	Template (reference)
pH1	mk64	mk236	pLmon4 (24)
	mk65	mk237	pLmon4 (24)
pH2	mk239	—[b]	p423 (10)
	mk240	—[b]	p423 (10)
pH3	mk236	mk147	pH1
	mk240	mk148	pH2
pH4	mk239	mk151	pH2
	mk65	mk152	pH1
pH5	mk236	mk153	pH1
	mk240	mk154	pH2
pH6	mk239	mk155	pH2
	mk240	mk156	pH3
pH7	mk236	mk153	pH1
	mk65	mk154	pH4
pH8	mk239	mk155	pH2
	mk65	mk156	pH1
pH9	mk236	mk157	pH1
	mk240	mk158	pH2
pH10	mk236	mk159	pH3
	mk65	mk160	pH1
pH11	mk239	mk157	pH4
	mk240	mk158	pH2
pH12	mk239	mk159	pH2
	mk65	mk160	pH1
pH13	mk236	mk157	pH7
	mk240	mk158	pH2
pH14	mk236	mk153	pH1
	mk65	mk154	pH12
pH15	mk239	mk155	pH2
	mk240	mk156	pH12
pH16	mk239	mk155	pH2
	mk65	mk156	pH10
pH2X1	mk239	mk165	pH2
	mk240	mk166	pH2
pH2X2[c]	mk239	mk282	pH2
	mk240	mk281	pH5
pH2X3	mk239	mk284	pH2X2
	mk240	mk283	pH2

[a] Primer sequences are listed in Table 2.
[b] —, no two-step PCR was necessary for this construct.
[c] pH2X2 is an intermediate for construction of pH2X3.

0.25 g of magnesium sulfate, and 19 g of disodium β-glycerophosphate per liter) supports growth of *L. lactis* even without the addition of sugar. Growth inhibition by 2-deoxy-D-glucose provides evidence for the presence of a functional sugar transporter (36).

Bacteriocins and bacteriocin assay. All bacteriocins were concentrated from spent supernatants by precipitation with 30% ammonium sulfate (see Table 4 for a list of the producer strains), except for curvacin A, leucocin A, and leucocin C, which were obtained as purified fractions (kindly provided by Helen S. Haugen and Jon Nissen-Meyer). Bacteriocin sensitivity was measured using microtiter plate assays. Stationary-phase cultures of the indicator strains (10^7 CFU ml^{-1}) were diluted 50-fold and exposed to 2-fold dilutions of the bacteriocins in a total volume of 200 μl in each well. The plates were incubated for 7 to 8 h at 30°C before the growth inhibition was scored spectrophotometrically at 600 nm. The MIC was defined as the amount of bacteriocin required to produce a 50% growth inhibition.

RESULTS AND DISCUSSION

The IIC protein is the major specificity determinant for class IIa bacteriocins. The individual subunits within the man-PTSs are well conserved across the different bacterial phyla. Nevertheless, our recent study revealed that subtle differences within their primary sequences can group IIC and IID proteins into phylogenetically defined subgroups and that, more importantly, this subgrouping corresponds well with their relative levels of sensitivity to class IIa bacteriocins (24). Thus, the man-PTSs of *Listeria monocytogenes* (*mpt*) and *E. faecalis*, which belong to the same phylogenetic subgroup, are both highly potent receptors for class IIa bacteriocins, while the corresponding man-PTS of *Lactococcus lactis* (*ptn*), which belongs to a more distantly related subgroup, confers no sensitivity to class IIa bacteriocins. To localize the part(s) of IIC and/or IID that is responsible for specific recognition by class IIa bacteriocins, we constructed hybrid man-PTSs with components derived from the highly sensitive *mpt* and the nonsensitive *ptn* genes (Table 3) and then assessed their sensitivities to a panel of 10 different class IIa bacteriocins. Different combinations of the class IIC (*mptC* and *ptnC*) and IID (*mptD* and *ptnD*) genes were fused and coexpressed with *mptA* (encoding the IIAB subunits from *Listeria monocytogenes*) in the *L. lactis* B488 host strain (10), from which the endogenous man-PTS genes *ptnABCD* have been deleted to prevent background interference.

As expected, expression of the wild-type *mptCD* (H1) genes of *Listeria* conferred sensitivity to all class IIa bacteriocins tested whereas expression of the lactococcal *ptnCD* (H2) genes did not (Fig. 1A). Combinations of *mptC* with *ptnD* (H3) and *ptnC* with *mptD* (H4) revealed that only the former could confer strong sensitivity. H1 and H3 displayed similar degrees of sensitivity for most bacteriocins, with the exception of sakacin P, which was over 30-fold less active against H3 (Fig. 1A). This suggests that the listerial class IIC protein (MptC) is the main determinant for specific targeting by these bacteriocins. This result also holds for the man-PTS genes *manM* and *manN* (encoding IIC and IID, respectively) of *Lactobacillus sakei*, which is known to be sensitive to class IIa bacteriocins (10). Here, only the combination of *manM* (encoding IIC) with *ptnD* (IID) rendered cells sensitive to class IIa bacteriocins whereas the reciprocal combination, i.e., *ptnC* (IIC) with *manN* (IID), did not (data not shown), confirming that IIC is the main specificity determinant.

In a previous study, Ramnath et al. (32) showed that heterologous expression of *mptC* alone was sufficient to render resistant *L. lactis* strains sensitive to class IIa bacteriocins; this result appeared contradictory to both previous and later studies showing that both IIC and IID are necessary for the receptor function (6, 10). However, given that MptC can form a potent receptor together with PtnD (H3; Fig. 1A), this discrep-

TABLE 2. Primer sequences

Primer	Sequence (5′→3′); restriction site[a]
mk64	ACGTGCATGCGCAATAAATATAGCGGGTAGC; SphI
mk65	ATCGCTCGAGTCGGTGAATATTGCACCAGC; XhoI
mk147	GAGTTACTTTATTTTCAGACATTTTTCTCCTCCTTTTTTTATTAATAG
mk148	CTATTAATAAAAAAAGGAGGAGAAAAATGTCTGAAAATAAAGTAACTC
mk151	CTGCCATTCTATTCTCCTCCCTCCTTTCTTAGTAGTCGTTC
mk152	GAACGACTACTAAGAAAGGAGGGAGGAGAATAGAATGGCAG
mk153	GTCCAGCAGGGATAGCAATACGAACACCTTGCATACAAATTG
mk154	CAATTTGTATGCAAGGTGTTCGTATTGCTATCCCTGCTGGAC
mk155	CTGCAGCTGGAATCGCGATACGCAAACCTTGACAGATAAGC
mk156	GCTTATCTGTCAAGGTTTGCGTATCGCGATTCCAGCTGCAG
mk157	GTACTGTAAACCAGAAGACAGGATCACCAACACCAGCTAGA
mk158	TCTAGCTGGTGTTGGTGATCCTGTCTTCTGGTTTACAGTAC
mk159	CGAATTGTAAACCAGAATACTGGGTCACCGATACCGGCAAG
mk160	CTTGCCGGTATCGGTGACCCAGTATTCTGGTTTACAATTCG
mk165	GGACGGAATACCTGCTACCCCTTGTCCACCTAAAACCATCAAGATAGATGATGCG
mk166	TTAGGTGGACAAGGGGTAGCAGGTATTCCGTCCATCGTTCCTGCTGCTATCTTG
mk180	GCTACCCCAAATCCACCTAATAC
mk181	GTATTAGGTGGATTTGGGGTAGC
mk236	ACCCGGGAAATTAAAATAGGAGGTTTATTATG; XmaI
mk237	CCTCCTATTTTAATTTCCCGGGTTTATTGTGTCTTTAATTCGTG; XmaI
mk239	ACCCGGGAAATTAAAATAGGAGGTTTATTATGGAATACGGTGTTTTATCTG; XmaI
mk240	AGCTCGAGATAAAAAAACCAATCCAAAGATTG; XhoI
mk266	CCTGCTACATGTTGTCCACCTAATAC
mk267	GTATTAGGTGGACAACATGTAGCAGG
mk270	ATATTAGTATTAAGTGGACAAGGGG
mk271	CCCCTTGTCCACTTAATACTAATAT
mk272	AGTATTAGGTAATCAAGGGGTAG
mk273	CTACCCCTTGATTACCTAATACT
mk277	GGTGGACAAGCGGTAGCAGG
mk278	CCTGCTACCGCTTGTCCACC
mk279	GGGTAGCAGCTATTCCGTCC
mk280	GGACGGAATAGCTGCTACCC
mk281	CGATGCTGCCCTTGCCTCTGTAGCTTCAGCAATTATATTAGT
mk282	ACTAATATAATTGCTGAAGCTACAGAGGCAAGGGCAGCATCG
mk283	CTTAACAATGATCGTTCGTACACTTTCAGTTGTGCTCGTTCAC
mk284	GTGAACGAGCACAACTGAAAGTGTACGAACGATCATTGTTAAG

[a] Restriction sites in sequences are underlined.

ancy can be resolved, since it is likely that the heterologous MptC protein interacted with the endogenous PtnD protein in *L. lactis* and that the two together formed a receptor complex that resulted in sensitivity to class IIa bacteriocins.

A defined N-terminal region of IIC is involved in specific interactions with class IIa bacteriocins. MptC and PtnC are 268 amino acids (aa) and 270 aa long, respectively, and have significant sequence similarity over the entire lengths of the sequences (59% identity and 75% similarity). Both proteins are predicted to contain seven transmembrane segments (TMSs) and four extracellular loops. To determine the part(s) of MptC that are directly involved in the specific interaction with class IIa bacteriocins, two reciprocal chimeric IIC genes were constructed from *mptC* and *ptnC*. An N-terminal sequence of either MptC (151 aa) or PtnC (155 aa) was genetically fused with the remaining sequence of the counterpart (resulting in MptC-PtnC or PtnC-MptC). The adjoining region was thus selected to be in the middle of the protein, within transmembrane segment 5 (TMS5), so that the topologies of the loops or TMSs potentially involved in bacteriocin interaction were kept largely intact. The resulting hybrid IIC gene was coexpressed with either *mptD* or *ptnD*, giving rise to clones H5 to H8. As shown in Fig. 1B, only clones producing a class IIC protein with the N terminus from MptC (H5 and H7) became sensitive to class IIa bacteriocins, while the other clones producing the reciprocal hybrids (H6 and H8) did not. Although there were some variations found in comparisons of H5 and H7 with respect to receptor potency for some of the bacteriocins, e.g., H7 was less sensitive than H5 to sakacin P and avicin A, the source of the coexpressed IID gene in H5 and H7 (*mptD* or *ptnD*) did not have any major effect on the receptor specificity. Together, these findings indicate that the specificity for class IIa bacteriocins relies mainly on the N-terminal part of the IIC protein.

As the poor receptor activity exhibited by some of the man-PTS hybrids tested could have been due to poor expression of the cloned genes, the functionality of the resulting man-PTSs in terms of sugar import was examined. When cells were grown in a medium containing 2-deoxy-D-glucose, a nonmetabolizable glucose analogue, growth inhibition has provided evidence for the presence of a functional sugar transporter (36). Indeed, all clones expressing the hybrid man-PTS species (H1 to H8) were shown to be sensitive to 2-deoxy-D-glucose (data summarized in Fig. 1), which implies that all the hybrid genes were expressed and their gene products correctly structured into functional sugar permeases.

An extracellular loop of MptC determines the specificity for class IIa bacteriocins. Next, we wished to reveal the feature in

TABLE 3. Plasmids used to express different man-PTS hybrid genes

Plasmid	Description[a]	Reference or source
pNZ9530	Expressing nisin-regulatory genes *nisRK*, Cam^r	25
pNZ8037	Expression vector in lactococci, nisin-responsive promoter, Ery^r	8
pH1	pNZ8037 with *mptA*—*mptC*$_{1–268}$—*mptD*$_{1–303}$	This study
pH2	pNZ8037 with *mptA*—*ptnC*$_{1–270}$—*ptnD*$_{1–307}$	This study
pH3	pNZ8037 with *mptA*—*mptC*$_{1–268}$—*ptnD*$_{1–307}$	This study
pH4	pNZ8037 with *mptA*—*ptnC*$_{1–270}$—*mptD*$_{1–303}$	This study
pH5	pNZ8037 with *mptA*—*mptC*$_{1–151}$/*ptnC*$_{152–266}$—*ptnD*$_{1–307}$	This study
pH6	pNZ8037 with *mptA*—*ptnC*$_{1–155}$/*mptC*$_{156–272}$—*ptnD*$_{1–307}$	This study
pH7	pNZ8037 with *mptA*—*mptC*$_{1–151}$/*ptnC*$_{152–266}$—*mptD*$_{1–303}$	This study
pH8	pNZ8037 with *mptA*—*ptnC*$_{1–155}$/*mptC*$_{156–272}$—*mptD*$_{1–303}$	This study
pH9	pNZ8037 with *mptA*—*mptC*$_{1–268}$—*mptD*$_{1–113}$/*ptnD*$_{114–306}$	This study
pH10	pNZ8037 with *mptA*—*mptC*$_{1–268}$—*ptnD*$_{1–114}$/*mptD*$_{115–304}$	This study
pH11	pNZ8037 with *mptA*—*ptnC*$_{1–270}$—*mptD*$_{1–113}$/*ptnD*$_{114–306}$	This study
pH12	pNZ8037 with *mptA*—*ptnC*$_{1–270}$—*ptnD*$_{1–114}$/*mptD*$_{115–304}$	This study
pH13	pNZ8037 with *mptA*—*mptC*$_{1–151}$/*ptnC*$_{152–266}$—*mptD*$_{1–113}$/*ptnD*$_{114–306}$	This study
pH14	pNZ8037 with *mptA*—*mptC*$_{1–151}$/*ptnC*$_{152–266}$—*ptnD*$_{1–114}$/*mptD*$_{115–304}$	This study
pH15	pNZ8037 with *mptA*—*ptnC*$_{1–155}$/*mptC*$_{156–272}$—*mptD*$_{1–113}$/*ptnD*$_{114–306}$	This study
pH16	pNZ8037 with *mptA*—*ptnC*$_{1–155}$/*mptC*$_{156–272}$—*ptnD*$_{1–114}$/*mptD*$_{115–304}$	This study
pH1 (G86S)	pH1 with mutation G86S in *mptC*	This study
pH1 (G87N)	pH1 with mutation G87N in *mptC*	This study
pH1 (Q88F)	pH1 with mutation Q88F in *mptC*	This study
pH1 (G89H)	pH1 with mutation G89H in *mptC*	This study
pH1 (G89A)	pH1 with mutation G89A in *mptC*	This study
pH1 (G92A)	pH1 with mutation G92A in *mptC*	This study
pH2X1	pH2 (where L85 to S95 from *mptC* replaces Q87 to T99 in *ptnC*)	This study
pH2X2[b]	pH2 (where V77 to R151 from *mptC* replaces I79 to R155 in *ptnC*)	This study
pH2X3	pH2 (where V77 to T116 from *mptC* replaces I79 to M120 in *ptnC*)	This study

[a] Cam^r, chloramphenicol resistance; Ery^r, erythromycin resistance. Subscripts denote amino acid positions originating from the specific genes.
[b] pH2X2 is an intermediate for construction of pH2X3.

the N-terminal half of MptC that is involved in specific bacteriocin recognition. Amino acid sequence alignment of the N-terminal halves of PtnC and MptC revealed a region with a markedly higher heterogeneity than the rest of the sequence (Fig. 2A). This region of 14 to 16 amino acids, corresponding to residues 85 to 99 in MptC and 87 to 103 in PtnC, is predicted to constitute an extracellular loop. Such marked differences in this region could also be seen when the alignment was extended to include other IIC proteins from a set of man-PTSs previously shown to display various receptor activities for class IIa bacteriocins (e.g., no, poor, low, or high receptor potency) (24) (Fig. 2B). In general, the sequences of man-PTSs with medium or high receptor activity are more similar to each other in this region than to those of the man-PTSs with low or no receptor activity.

To investigate whether this region is important for specific interaction with the class IIa bacteriocins, six site-directed mutations in five residues situated in the predicted extracellular loop of MptC were performed (Fig. 2A and Table 3). The MptC residues were changed to the amino acids found at similar positions in PtnC (G86S, G87N, and Q88F) (Fig. 2A), with the exception of G92, which was mutated to alanine (G92A), and G89, which was mutated to two different amino acids: histidine (G89H), which is found in a similar position in PtnC, and alanine (G89A), which is a relatively conserved mutation. By and large, four of the mutations (G86S, G87N, G89A, and G92A) had little or no effect on receptor activity (Fig. 1C). The remaining two mutations (Q88F and G89H), on the other hand, caused a significant reduction in receptor activity for all the bacteriocins tested. Q88F caused MIC values to increase 6- to 500-fold, with avicin A being the most affected; most drastically, the G89H mutation totally disrupted the receptor function for all the bacteriocins tested. Surprisingly, three of the mutations (G86S, Q88F, and G89H) led to a compromised sugar transport function of the man-PTSs (Fig. 1C). The molecular nature of this adverse effect on sugar import is unknown, but it might indicate that these mutations somehow interfered with the substrate binding and/or caused major structural changes in the protein that impaired sugar uptake and possibly also bacteriocin interaction (for Q88F and G89H). Interestingly, G86, Q88, and G89 in MptC (together with G87) constitute a sequence (GGQG) which highly resembles the loop-located GGXG sequence motifs that are known to be important for the transport function of other transmembrane proteins (11). Residues in the extracellular loop might therefore have an important role in sugar uptake, in addition to being a potential target site for bacteriocins. It should be

TABLE 4. Bacteriocin-producing strains used in this study

Bacterial strain (plasmids)	Bacteriocin	Strain reference
Enterococcus avium LMGT 3465	Avicin A	3
Enterococcus faecium RC714	Bacteriocin RC714	7
Enterococcus faecium P13	Enterocin P	4
Enterococcus hirae DCH5	Hiracin JM79	35
Lactococcus lactis B190	Lacotococcin A	10
Pediococcus acidilactici LMG 2351	Pediocin PA-1	29
Lactobacillus sakei B316	Penocin A	9
Lactobacillus sakei Lb790 (pSAK20, pSPP2)	Sakacin P	1

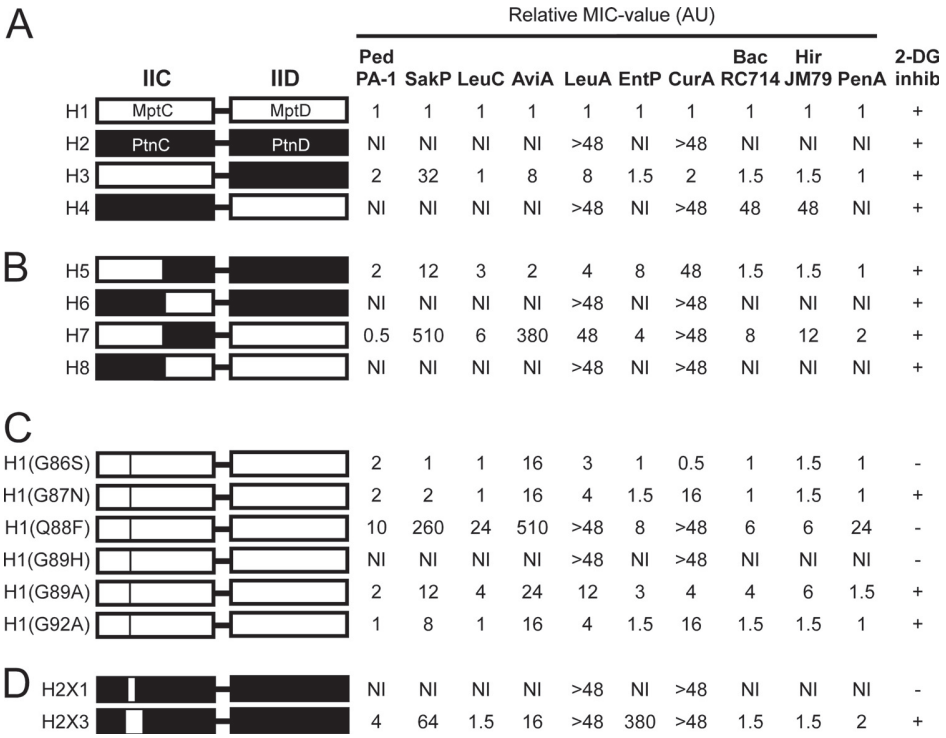

FIG. 1. Relative bacteriocin sensitivities of *L. lactis* B488 clones expressing different combinations of man-PTS genes of the listerial system (*mpt*) and the lactococcal system (*ptn*). (A) Wild-type IIC and IID genes (H1 and H2) and intergenic hybrids (H3 and H4); (B) intragenic IIC hybrids (H5 to H8); (C) six different clones with point mutations in *mptC* (G86S, G87N, Q88F, G89H, G89A, and G92A); (D) two clones with an exchange of one of the extracellular loops (H2X1 with 11 aa exchanged and H2X3 with 40 aa exchanged). Ptn proteins (PtnC and PtnD) are represented by black boxes and Mpt proteins (MptC and MptD) by white boxes. The different constructs were coexpressed with *mptA* (encoding a IIAB subunit in *Listeria monocytogenes*) to form a complete man-PTS complex (Table 3). Ten different class IIa bacteriocins were tested: pediocin PA-1 (Ped PA-1 [20, 29]), sakacin P (SakP [38]), leucocin C (LeuC [15]), avicin A (AviA [3]), leucocin A (LeuA [18]), enterocin P (EntP [4]), curvacin A (CurA [37]), bacteriocin RC714 (Bac RC714 [7]), hiracin JM79 (Hir JM79 [35]), and penocin A (PenA [9]). One arbitrary unit (AU) was defined as representing the MIC of clone H1, and the MICs of the other test clones were determined relative to this value. MIC values were determined at least three times. Clones were defined as not inhibited (NI) when the MIC values increased more than 600-fold (>600 AU). Growth inhibition by 2-deoxy-D-glucose (2-DG) is indicated with a plus sign; absence of inhibition is indicated with a minus sign.

noted that, since the G89H mutant is inactive both as a bacteriocin receptor and as a sugar transporter, whether the proteins encoded in this construct are stably expressed and correctly folded is unknown. Importantly, however, a functioning man-PTS in terms of sugar uptake is not a requirement for a potent bacteriocin receptor, as demonstrated with the G86S mutant. Furthermore, our previous study (10) also showed that heterologous coexpression of *mptC* and *mptD* without *mptAB* could confer bacteriocin sensitivity even though the man-PTS itself was not a functional sugar transporter.

As the site-directed mutagenesis results provided indications that some residues located in the extracellular loop of MptC are critical for the interaction with class IIa bacteriocins, we further investigated whether this region is entirely responsible for the specific interaction with class IIa bacteriocins by replacing 13 putative loop residues in PtnC (Q87 to T99) with the corresponding residues from MptC (L85 to S95). However, the resulting clone (H2X1) did not become sensitive to class IIa bacteriocins (Fig. 1D), and this chimeric system was also unable to transport sugars, suggesting that the exchange of these loop residues could have a severe impact on the total structuring of PtnC or that the construct could not be stably expressed. As flanking regions could play a role in the stability and/or structuring of the loop structure, a longer region of 40 aa (V77 to T116) from MptC, including the residues of the flanking transmembrane helices, was used to replace the corresponding region in PtnC. Remarkably, the resulting chimera became a highly potent receptor for most of the class IIa bacteriocins as well as being a functional sugar transporter (Fig. 1D).

Together, these results show that the predicted extracellular loop and the flanking regions involved in transmembrane segments (40 aa in total) are essential for the specific recognition by class IIa bacteriocins. We cannot exclude the possibility that other residues located elsewhere are important for the bacteriocin-receptor interaction; however, these interactions are not species-specific, because the bacteriocin-sensitive clone H2X3 contains both the lactococcal IIC and IID genes except for the cloned region (encoding 40 aa) derived from the listerial man-PTS.

Lactococcin A interacts with its receptor in a complex manner. The availability of the various *mpt-ptn* hybrid constructs allowed us to assess whether the *Lactococcus*-targeting bacteriocin lactococcin A also recognizes a specific region(s) on its man-PTS receptor. Overall, it can be seen that, unlike the class

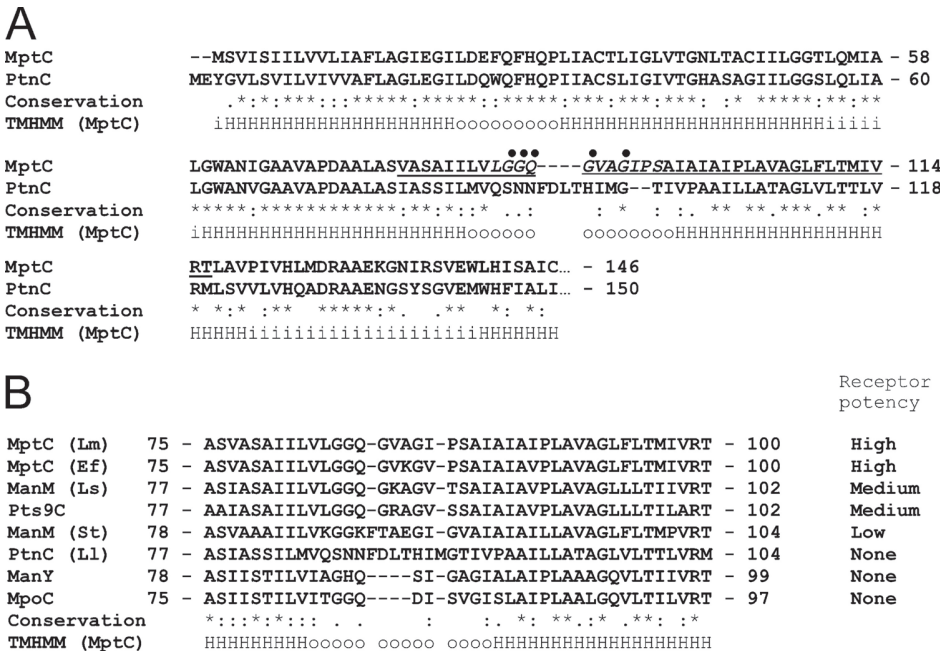

FIG. 2. (A) Alignment of MptC from *Listeria monocytogenes* and PtnC from *L. lactis*. Only the N-terminal part of the alignment is shown. An asterisk, two dots, and one dot (in that order) indicate decreasing degrees of conservation. Predicted transmembrane helix residues (H), intracellular loop residues (i), and extracellular loop residues (o) determined by the use of TMHMM version 2.0 software (27) are indicated for MptC. The bullets (●) designate residues subjected to site-directed mutagenesis. The cloned region in H2X3 is underlined; the cloned region in H2X1 is shown in italics. (B) Multiple sequence alignment of the region containing the predicted extracellular loop in eight different mannose-PTS IIC proteins: MptC (Lm) from *Listeria monocytogenes*, MptC (Ef) from *E. faecalis*, ManM (Ls) from *Lactobacillus sakei*, Pts9C from *Lactobacillus plantarum*, ManM (St) from *Streptococcus thermophilus*, PtnC (Ll) from *L. lactis*, ManY from *E. coli*, and MpoC from *Listeria monocytogenes*. The potencies of these proteins as receptors for class IIa bacteriocins were determined in a previous work (24). The alignments were constructed using MUSCLE version 3.7 software (12).

IIa bacteriocins, lactococcin A required both IIC and IID from the same source, namely, the lactococcal man-PTS PtnCD, to kill target cells (clone H2), as any interchange with a noncognate *ptn* component (clones H1, H3, and H4) had a detrimental effect on the receptor activity (Fig. 3A). To further examine whether lactococcin A recognizes discrete regions on IIC and/or IID clones, additional intragenic hybrid clones were constructed and analyzed for their sensitivity. Besides the clones producing intragenic hybrids of IIC (H5 to H8, H2X1, and H2X3; Fig. 1), four more clones containing intragenic hybrids of IID were made (MptD/PtnD and PtnD/MptD in H9 to H12; Table 3). Combinations of the two sets of hybrid genes gave rise to another four clones containing both IIC and IID hybrids (H13 to H16; Table 3). Bacteriocin sensitivity assays revealed that only three of them (H5, H11, and H2X3) could display some receptor activity (Fig. 3B), with clone H11 being the most sensitive (only a 4-fold increase in MIC compared to the wild-type receptor results). Clone H11 contains an intact PtnC and a hybrid IID composed of an N-terminal part (113 aa) from MptD and a C-terminal (193 aa) part from PtnD, indicating that the N-terminal part of PtnD is less important for specific lactococcin A interaction. Intriguingly, although the sequence of H2X1 is more similar to those of the wild-type genes (H2) than to that of H2X3, H2X1 exhibited much less receptor activity than H2X3. The nature of the factors behind these rather contrasting results is unknown; however, it is possible that a larger portion of MptC in H2X3 could have stabilizing effects on the structure that somehow allowed lactococcin A to form a stronger interaction with the receptor or that H2X1 was not stably expressed. It should be underlined here that the H2X3 hybrid and (to some extent) H5 are the first known (genetically modified) receptors being targeted by both lactococcin A and the class IIa bacteriocins and that such a receptor is yet to be found in nature.

Taken together, these results indicate a rather complex bacteriocin-receptor interaction for lactococcin A compared to what has been observed for class IIa bacteriocins; this might also explain why lactococcin A has an extremely narrow inhibitory spectrum, killing only lactococci (23).

Conclusions. Man-PTSs in both Gram-positive and Gram-negative bacteria function as receptors for several antibacterial macromolecules, including peptides and bacteriophages (2, 6, 10, 14, 19). Here we demonstrate that the members of one group of antimicrobial peptides, the class IIa bacteriocins, require a short region containing an extracellular loop in the N-terminal part of the IIC protein to specifically target sensitive cells. These results support the previously proposed idea (13, 32) that IIC is important for the specific interaction with bacteriocins whereas IID is essential for the stability and structuring of the protein complex. However, IIC is not the major specificity determinant for all man-PTS targeting bacteriocins, as we also demonstrated here that parts of both IIC and IID appeared to be essential for the specific recognition by the class IIc bacteriocin lactococcin A.

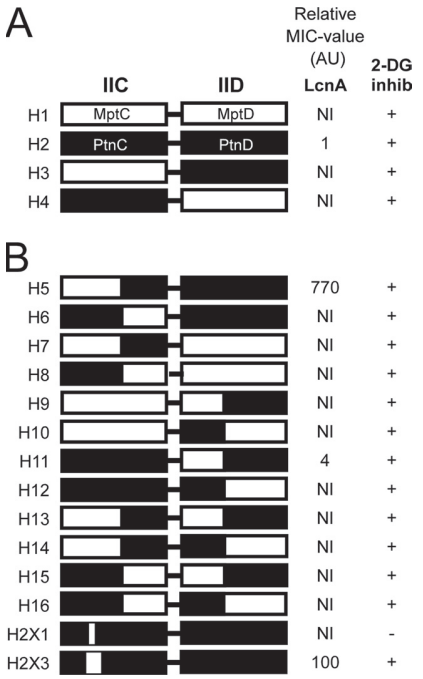

FIG. 3. Sensitivity of various *L. lactis* B488 clones to lactococcin A (LcnA [23]). (A) Wild-type IIC and IID genes (H1 and H2) and intergenic hybrids (H3 and H4); (B) intragenic hybrids H5 to H16, H2X1, and H2X3 (Table 3). For lactococcin A, 1 AU was defined as representing the MIC of clone H2; the MICs of the other test clones were determined relative to this value. MIC values were determined at least three times. Clones were defined as not inhibited (NI) when the corresponding MIC showed an increase of at least 4,000-fold. Growth inhibition and no growth inhibition induced by 2-deoxy-D-glucose (2-DG) are indicated with a plus sign and a minus sign, respectively.

Lactococcin A has several physiochemical properties different from those of class IIa bacteriocins: it is 54 aa in length whereas class IIa bacteriocins are in the range of 37 to 48 aa, and it shares little or no sequence similarity with class IIa bacteriocins whereas the latter share significant sequence similarity with each other (30). Such divergent features might suggest different mechanisms for targeting and/or killing sensitive cells, and this notion is in fact supported by the results of the present study, which show a much more complex receptor targeting for lactococcin A than for class IIa bacteriocins. Despite these marked differences, producer cells for both class IIa bacteriocins and lactococcin A somehow employ a common immunity mechanism. It has been shown for both types of bacteriocins that their cognate immunity proteins act by locking the bacteriocins on man-PTSs in a tripartite complex (bacteriocin–immunity protein–man-PTS), thereby preventing the bacteriocins from forming lethal pores (10). In terms of structural biology, it would have been interesting to discover how two such different classes of bacteriocins have evolved divergently in some aspects (different sequences and different degrees of species specificity) and at the same time have converged in some other aspects (use by both of a man-PTS as a receptor and a common mechanism of immunity). Future work aimed at resolving the structure of the tripartite complex is likely to provide detailed insights into these fascinating systems.

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PAPER III

Resistance mechanisms against bacteriocins targeting the mannose phosphotransferase system

Morten Kjos, Ingolf F. Nes and Dzung B. Diep*

Laboratory of Microbial Gene Technology and Food Microbiology, Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, P. O. Box 5003, 1432 Ås, Norway

*Corresponding author: Dzung B. Diep, Mailing address: Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, P. O. Box 5003, 1432 Ås, Norway. Phone: +47 6496 5910. Fax: +47 64 96 59 01. E-mail: dzung.diep@umb.no.

ABSTRACT

The membrane proteins IIC and IID of the mannose phosphotransferase system (man-PTS) together form a membrane-located complex that serves as receptor for several different bacteriocins, including the pediocin-like class IIa bacteriocins and the class IIc bacteriocin lactococcin A. Bacterial strains sensitive to class IIa bacteriocins readily give rise to resistant mutants upon bacteriocin exposure. In the present study we have therefore investigated lactococcin A resistant mutants of *Lactococcus lactis* as well as natural food isolates of *Listeria monocytogenes* with different susceptibilities to class IIa bacteriocins. We found two major mechanisms of resistance: (i) The first involves downregulation of man-PTS gene expression, which takes place both in spontaneous resistant mutants and in natural resistant isolates; (ii) the second involves normal expression of the man-PTS system but the underlying mechanism of resistance for these cells is unknown. In some cases, the resistant phenotype was linked to a shift in the metabolism, i. e., reduced growth on glucose due to reduction in man-PTS expression was accompanied by enhanced growth on another sugar, such as galactose. The implications of these findings in terms of metabolic heterogeneity are discussed.

INTRODUCTION

Bacteriocins are peptides or proteins with antimicrobial activity against bacteria (9, 33). Many bacteriocins are produced by food-grade lactic acid bacteria which are naturally present in vegetables, meat and dairy products, and since a number of these peptides can effectively kill food spoiling and pathogenic bacteria, they are often considered as promising agents for use in food preservation (9, 11). Most bacteriocins kill target cells by permeabilization of the cell membrane, and the activity is often very specific as they employ specific receptors on the target cell surfaces. The target receptors have been identified for a few bacteriocins. For example, nisin and a number of other lantibiotic bacteriocins (peptides containing post-translationally modified residues) use the cell-wall precursor lipid II as a docking molecule on target cells (5, 6). Furthermore, it has been shown in recent years that a set of bacteriocins produced by both Gram-positive and Gram-negative species can employ the membrane components of the mannose-phosphotransferase system (man-PTS) on sensitive cells as receptor molecules. These bacteriocins include the pediocin-like bacteriocins (12, 15, 22, 40), the lactococcal bacteriocins lactococcin A and B (15) as well as microcin E492 from *Klebsiella* which can target man-PTS in the inner membrane of *Escherichia coli* (4).

The pediocin-like bacteriocins, also known as the class IIa bacteriocins, constitute a large group of peptides produced by lactic acid bacteria. Unlike lantibiotic bacteriocins, class IIa bacteriocins contain only non-modified residues except one or two disulphide bridges; they are 36 to 49 amino acids long, characterized by the presence of a conserved N-terminal motif (YGNGVxCxxxxCxVxWxxA, where x is any amino acids) and are known for their strong anti-listerial activity (reviewed by 36). Lactococcin A, produced by *Lactococcus lactis* (25), is an unrelated bacteriocin of 54 non-modified amino acids. This bacteriocin is a member of the class IIc which consists of linear, non-pediocin-like one-peptide bacteriocins (34).

Man-PTS, which is a major sugar uptake system in *Firmicutes* and γ-*Proteobacteria*, consists of four domains: IIA, IIB, IIC and IID (38). IIC and IID are membrane proteins which form reversible contacts with the cytosolic IIA and IIB (38). Only IIC and IID are involved as receptors for bacteriocins (15). It should be noted that lactococcin A and class IIa bacteriocins differ greatly in their inhibitory spectra: lactococcin A targets only man-PTS (*ptn*) from *Lactococcus* species, while the class IIa bacteriocins target the man-PTS from a wide range of genera including *Lactobacillus*, *Listeria* and *Enterococcus*, but somehow not the *ptn* system from *Lactococcus* (16, 25, 28). A recent study with reciprocal hybrid receptors

of the lactococcal (*ptn*) and listerial (*mpt*) man-PTSs has indeed revealed that lactococcin A differs from class IIa bacteriocins in the mode of receptor recognition: While lactococcin A appears to require several regions both on IIC (PtnC) and IID (PtnD) for species-specific targeting, the specificity of the class IIa bacteriocins is dependent on a single extracellular loop in the IIC (MptC) protein (29).

It is frequently observed that sensitive strains give rise to resistant mutants upon exposure to class IIa bacteriocins (19, 44). The resistance frequency varies from 10^{-4} to 10^{-9} depending on the species or genera tested, and in *Listeria monocytogenes* this phenotype has been linked to reduced expression of the man-PTS genes (21, 41, 44). Interestingly, although class II bacteriocins are known to have strong anti-listerial activity, natural isolates of *Li. monocytogenes* have been observed to vary greatly in their sensitivity to these bacteriocins (27); however the exact nature of these differences has not been investigated. Similarly, spontaneous resistant mutants to lactococcin A appear frequently but are poorly characterized. In the present study we have assessed the status of man-PTS in a collection of *Listeria* isolates with varying sensitivity to class IIa bacteriocins. Similar assessment was also performed on lactococcal mutants with varying sensitivity to the class IIc lactococcin A, in order to compare the mechanisms of resistance to these bacteriocins.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Unless otherwise stated, *Li. monocytogenes* was grown in brain heart infusion (BHI) medium (Oxoid) at 37 °C without shaking and *L. lactis* in M17 medium (Oxoid) supplemented with 0.4 % (w v^{-1}) glucose at 30 °C without shaking. When appropriate, 10 µg ml^{-1} chloramphenicol was added to the growth medium. The bacterial strains used in this study are listed in Table 1.

Bacteriocins, bacteriocin assays and growth analysis. Bacteriocins were concentrated from culture supernatants by precipitation with 30 % ammonium sulphate. The bacteriocin producers used were *Pediococcus acidilactici* LMGT2351 (35) for pediocin PA-1, *Enterococcus faecium* P13 (8) for enterocin P, *Lactobacillus sakei* Lb790 (pSAK20, pSPP2) (3) for sakacin P and *L. lactis* B190 (15) for lactococcin A.

Bacteriocin sensitivity was determined using microtiter plate assays where 100-fold dilutions of the test strains were exposed to two-fold serial dilution of bacteriocin (25). Alternatively, bacteriocin sensitivity was determined by a spot-on-lawn softagar assay, where 2 µl of the concentrated supernatants were spotted directly onto a softagar containing the test strain. Growth analysis was performed using Bioscreen C (Oy Growth Curves); overnight cultures were diluted 1000-fold, and OD_{600} was measured continuously.

DNA isolation and sequencing. Total DNA was isolated from *Li. monocytogenes* using FP120 FastPrep bead-beater (BIO101/Savent) and QiaPrep Miniprep kit (Qiagen) as described by Solheim et al. (42). The mannose-PTS genes (*mptACD*) were amplified using primers mk64 (5'-ACGTGCATGCGCAATAAATATAGCGGGTAGC-3') and mk65 (5'-ATCGCTCGAGTCGGTGAATATTGCACCAGC-3'), and the amplification product was sequenced using primers mk64, mk65, mk128 (5'-ATGTTTGCCCATCCAAGTGC-3') and mk129 (5'-TTATCGGTTTCGTAGTAGCAG-3'). The *mptA*-promoter region was amplified and sequenced using primers mk289 (5'-AAATGACTTTTTTAGAATTCCATCAA-3') and mk291 (5'-GATTGCTTTAACGTTTTCTTGC-3'). *rpoN* was amplified and sequenced using primers mk306 (5'-ATGAAGACAATAAATGGAATTTAG-3') and mk307(5'-AAAAGACGTTTTTTGTCCCACA-3'). *manR* was amplified using primers mk292 (5'-TAGTCATGCTAAGATAAATACA-3') and mk293 (5'-ATTATGAAAGTACTTCTGGTTGG-3'), and the amplification product was sequenced using primers mk292, mk293, mk294 (5'-GACTCTGGTACGTATAATAAACT-3'), mk295

(5'-TCAAGGTGTGGAAGATGATGA-3') and mk296 (5'-TCATCATCTTCCACACCTTGA-3'). *lmo0095*-homologs were amplified and sequenced using primers mk299 (5'-AAATGACTTTTTTAGAATTCCATCAA-3') and mk300 (5'-TCTATTTTAAGCACAAGATGCCT-3'), while *resD* were amplified and sequenced using primers mk301 (5'-TGAGTACTTATGAGTGAACAAGT-3') and mk302 (5'-CTTAGTCTGTTTTATTAATCTTCTG-3').

RNA-isolation, cDNA synthesis and RT-PCR. *Li. monocytogenes* cells were harvested by centrifugation of cultures in exponential growth (OD_{600} = 0.6), and RNA isolation, DNase treatment and cDNA synthesis were performed as described previously (28). RT-PCR was carried out using primers mk199 (5'-CAGCCATTAATCGCATGTACA-3') and mk200 (5'-CGAAGAACGGCCATACTTCT-3') targeting *mptC*, mk201 (5'-GTAGCATGGCGCTCTACGT-3') and mk202 (5' ACGAACATCCCGAGTATCGA-3') targeting *mptD* and 1F (5'-GAGTTTGATCCTGGCTCAG-3') and mk203 (5'-TTAGCCGTGGCTTTCTGGT-3') targeting the 16S housekeeping gene. Primers were designed based on the genome sequence of *Li. monocytogenes* EGD-e (18).

Isolation of lactococcin A resistant mutants. One bacteriocin unit (BU) was defined as the amount of lactococcin A required to produce 50 % growth inhibition in a 200 µl *L. lactis* IL1403 culture. In order to generate lactococcin A resistant mutants, *L. lactis* IL1403 and NZ9000 cultures were plated onto GM17 agar with a layer of softagar containing 25 BU ml^{-1}, 70 BU ml^{-1} or 220 BU ml^{-1}. Bacteriocin resistant colonies were cultivated in bacteriocin-free medium for at least 100 generations, before the bacteriocin sensitivity was assessed by microtiter plates assays.

Protein purification and SDS-PAGE. The plasmid p369 was transformed into *L. lactis* IL1403 wild type and four resistant clones for constitutive expression of the Flag-tagged lactococcin A immunity gene, *flciA*. Cells were grown to OD_{600} = 0.5, harvested by centrifugation at 7000 × *g*, and washed with ice-cold Tris-buffered saline (TBS). Cells were lysed mechanically using Fastprep Fp120 (Savant Instruments Inc, Holbrook, NY, USA). The Flag-tagged protein fLciA was then immunoprecipitated using M2 anti-flag agarose according to the manufacturer's protocol (Sigma Aldrich, St. Louis, MO, USA). The eluted proteins were analyzed by SDS-PAGE using 4 % stacking gel and 15 % separation gel, and visualized by silver-staining.

Transformation. Transformation of *L. lactis* was performed by electroporation as described by Holo and Nes (24).

RESULTS

Natural *Li. moncytogenes* isolates resistant to class IIa bacteriocins display reduced man-PTS gene expression. A distinctive feature of class IIa bacteriocins is their strong anti-listerial activity (16). However, it has previously been reported that a large collection of 200 food and food-industry isolates of *Listeria* which have not been exposed to class IIa bacteriocins prior to collection, display great variation in sensitivity when challenged with class IIa bacteriocins (27). Thirteen *Li. monocytogenes* isolates from this collection were selected to examine the molecular nature of these variations. Based on differences in MIC values for different class IIa bacteriocins (Fig. 1), the isolates were divided into three groups: (i) a highly sensitive group containing the isolates L31-H, L196-H, L228-H, L1036-H and L1207-H, (ii) an intermediately sensitive group containing the isolates L361-I, L852-I, L1283-I, L1310-I, L1401-I, L1485-I and L2462-I and a low sensitivity group with only one member (L1040-L). The difference in MIC values between the most and least sensitive strain was 43-, 16-, and 85-fold for pediocin PA-1, enterocin P and sakacin P, respectively, and in general, enterocin P displayed less strain variation than the other two bacteriocins.

In *Li. monocytogenes*, the man-PTS system is encoded by the genes *mptACD* genes, in which MptC and MptD constitute the membrane located receptor complex (IIC and IID). The *mptACD* genes were sequenced in five isolates with different bacteriocin susceptibilities (L31-H, L196-H, L361-I, L852-I and L1040-L), in order to investigate whether the observed bacteriocin susceptibility variations between the isolates could result from sequence variations in the receptor. Some nucleotide variations were observed, but the resulting amino acid sequences of MptA, MptC and MptD were identical in all isolates except for of a single polymorphism found in MptC (Ile-150 in L31-H, L196-H and L1040-L as opposed to Val-150 in L361-I and L852-I). However, this polymorphism is unlikely to have any significant effect with respect to receptor potency, as the same amino acid (Ile-150) was found both in the most (L196-H) and in the least (L1040-L) sensitive isolate.

It is known that some resistant mutants of *Li. monocytogenes* and *E. faecalis* arising from exposure to class IIa bacteriocins show reduced man-PTS gene expression compared to the levels seen in wild type sensitive cells (21, 37, 41, 44). Therefore, semi-quantitative RT-PCR with primers targeting *mptC* and *mptD* was performed to investigate their expression level in the five isolates (Fig. 2). These results clearly demonstrate that expression of receptor genes *mptC* and *mptD* was reduced in the isolate with low bacteriocin susceptibility (L1040-L) compared to the isolates with high and intermediate susceptibilities (L31-H, L196-H,

L361-I, L852-I), which all displayed similar expression levels. This result corresponds well with a previous study on *Lactobacillus sakei* strains which showed a correlation between man-PTS gene expression level and degree of sensitivity to class IIa bacteriocins (28).

MptACD is a major glucose uptake system in *Li. monocytogenes*, although glucose can also be transported by alternative PTS-systems (43). Growth analysis demonstrated that L1040-L grew considerably slower than L31-H, L196-H, L361-I and L852-I in both M17-medium supplemented with 0.4 % glucose and BHI-medium (containing glucose) (Fig. 3A and C). On the other hand, when the carbon source was substitute to cellobiose, which is transported by other PTS systems (43), the growth rates were similar for all five strains (Fig. 3B and D). Thus, the low susceptibility to class IIa bacteriocins for the *Li. monocytogenes* strain L1040-L is caused by reduced expression of man-PTS genes, which results in reduced growth on glucose. However, the smaller variation in sensitivity between the highly and intermediately susceptible isolates remains enigmatic, as their *mpt* expression levels were found to be very similar (Fig. 2).

Regulation of *mpt* gene expression in *Listeria* has been studied extensively, and several regulatory factors have been identified, including the σ^{54} factor RpoN (2, 12), the σ^{54}-associated activator ManR (12, 50), the response regulator ResD (31) and Lmo0095 (48, 49) whose function is unknown. Interestingly, a transversion mutation (Ala356Gly) in the ManR-homolog MptR of *E. faecalis* has been identified in several spontaneous class IIa bacteriocin resistant mutants, and downregulation of *mpt* gene expression has been attributed to this mutation (37). In order to find out whether similar polymorphisms in the regulatory genes could account for the low *mpt* expression in the strain L1040-L, four known regulatory genes (*rpoN*, *manR*, *resD* and *lmo0095*) as well as the *mpt* promoter region were sequenced in isolates L31-H, L196-H, L361-I, L852-I and L1040-L. Some differences in amino acid sequence between the strains were found (Table 2), however, most of these polymorphisms are unlikely to have any causative effect since similar amino acids were found in strains with high (L31-H, L196-H, L361-I, L852-) and low (L1040-L) *mpt*-expression. The exceptions are two polymorphic amino acid positions in the *manR* gene which were unique to L1040-L; Glu was replaced with Lys and Tyr with Cys in position 321 and 690, respectively. Whether these polymorphisms in the *manR* gene of the L1040-L strain are responsible for the reduced expression observed in this isolate awaits further investigation.

Reduced man-PTS expression is found in spontaneous lactococcin A resistant mutants. In order to compare class IIa bacteriocin resistance with the resistance mechanism against

another man-PTS targeting bacteriocin, lactococcin A resistant mutants were generated by exposing the sensitive strain *L. lactis* IL1403 to three different concentrations of lactococcin A on agar plates. The frequency of resistance was approximately $1.5 \cdot 10^{-5}$ for the lowest lactococcin A concentration (25 BU ml^{-1}) and about $5 \cdot 10^{-7}$ for the two higher concentrations (70 BU ml^{-1} and 220 BU ml^{-1}). The MIC of IL1403 resistant mutants (35 independent mutants tested) increased 16-67 times compared to the wild type, and the lactococcin A resistant phenotype was stably maintained after growth in bacteriocin-free medium for at least 100 generations.

The man-PTS receptor for lactococcin A in *L. lactis* is encoded by the *ptnABCD* genes, which are homologs to *mptACD* in *Li. monocytogenes*. The *ptnABCD* genes were sequenced in the wild type strain IL1403 as well as in four resistant mutants (Rlac-A and Rlac-B isolated from the agar plate with 25 BU ml^{-1} lactococcin A and Rlac-C and Rlac-D isolated from 220 BU ml^{-1} lactococcin A), but no differences were found, demonstrating that lactococcin A resistance did not result from mutations in the receptor genes.

In a previous study, we have shown that in immune *L. lactis* which are exposed to lactococcin A, the immunity protein (LciA) specifically binds to the PtnABCD proteins to form a complex which prevents pore formation (15). By immunoprecipitation (using antibodies targeting a Flag-tagged version of the immunity protein, fLciA), the Ptn-proteins are thus readily co-purified with fLciA (15), and this method was used to assess the amounts of PtnABCD proteins in the four resistant mutants of *L. lactis* IL1403. As expected, high levels of the man-PTS proteins PtnAB, PtnC and PtnD co-purified with fLciA in the wild type strain (Fig. 4). In three of the four resistant mutants tested (Rlac-A, Rlac-C, Rlac-D), the PtnAB, PtnC and PtnD protein bands were absent or very weak, clearly demonstrating that the level of PtnABCD was downregulated in these cells. In the last resistant mutant (Rlac-B), amounts of precipitated man-PTS proteins were similar to those found in wild type cells, indicating that the man-PTS expression level in this mutant was not significantly reduced. These results corresponded well with the subsequent growth analysis (Fig. 5): The mutants Rlac-A, Rlac-C and Rlac-D, with markedly reduced expression of man-PTS genes, grew significantly slower than the wild type and the mutant Rlac-B in GM17 containing glucose as the major carbon source. On the other hand, when galactose, which is transported independently of man-PTS, was used as carbon source, the resistant clones with downregulated man-PTS displayed a notably higher growth rate than both the wild type strain and the Rlac-B mutant, suggesting that the resistant mutants Rlac-A, Rlac-C and Rlac-D have compensated the reduced glucose uptake by activating the galactose metabolism.

The results from protein isolation and growth analysis suggest that exposure of *L. lactis* to lactococcin A generates two different types of resistant cells: Type 1 mutants with downregulation of man-PTS expression, reduced growth on glucose and enhanced growth on galactose (like Rlac-A, Rlac-B, and Rlac-C), and Type 2 mutants with normal man-PTS expression and wild type-like growth profiles on glucose and galactose (like mutant Rlac-B). To determine the relative frequency of these two types of mutants, the glucose- and galactose growth profiles of 35 lactococcin A resistant *L. lactis* IL1403 mutants were monitored. Interestingly, all the mutants (12 out of 12 tested) obtained from the agar plates containing the higher concentrations of lactococcin A (70 and 220 BU ml^{-1}) belonged to Type 1, while among the mutants obtained from the agar plate with low lactococcin A concentration (25 BU ml^{-1}) 39 % (9 of 23) were Type 1 and 61 % (14 of 23) were Type 2. These findings indicate that downregulation of man-PTS expression is the main resistance mechanism arising from exposure to high bacteriocin concentrations while a second resistance mechanism (associated with normal man-PTS expression) can play an important role at lower bacteriocin concentrations.

Expression of cloned receptor genes in spontaneous resistant mutant restores sensitive phenotype. *L. lactis* NZ9000 is a strain that has been constructed to allow heterologous gene expression based on the nisin regulatory system (30). In order to examine whether expression of cloned receptor genes in lactococcin A resistant mutants can render the cells sensitive to lactococcin A, we took advantage of NZ9000 as expression host. In a similar fashion as IL1403, NZ9000 was exposed to lactococcin A (220 BU ml^{-1} in softagar) to generate resistant mutants. The resistance frequency for this strain was 1000-times higher compared to IL1403 ($5 \cdot 10^{-4}$ versus $5 \cdot 10^{-7}$), and MIC-values of five randomly selected mutants showed that they were 3-10 times less sensitive to lactococcin A compared to wild type NZ9000. All five mutants displayed a Type 1 resistant phenotype with reduced growth rate on glucose, suggesting that the expression of man-PTS was downregulated, and when *ptnABCD* were expressed from a plasmid in one of the resistant mutants (NZ9000-Rlac), bacteriocin sensitivity was indeed restored (Fig. 6).

DISCUSSION

Results presented in this study suggest that two different mechanisms confer resistance to man-PTS-targeting bacteriocins in *Listeria* and *Lactococcus*. The first and main mechanism involves downregulation of man-PTS gene expression leading to bacteriocin resistance due to limited amounts or absence of receptor proteins, and we demonstrate that downregulation of man-PTS expression is found both among naturally resistant isolates and in laboratory-induced resistant mutants. This resistance mechanism is often associated with highly resistant cells and has indeed been reported in previous studies dealing with class IIa bacteriocin resistance (21, 41, 44). The man-PTS expression level is, however, not the only factor determining the sensitivity to these bacteriocins (Fig. 2 and 4), because in the second mechanism which normally occurs in cells with intermediate resistance, we found relatively high man-PTS gene expression, at levels comparable to that found in wild-type and sensitive cells. Although the exact nature of the second resistance mechanism is yet unknown, some circumstances suggest that cell surface changes affecting the interaction between bacteriocin and its membrane-located receptor might be involved. For instance, previous works have shown that bacteriocin resistant *Li. monocytogenes* mutants display a variety of altered phenotypes compared to the sensitive wild type cells, e. g., being different in membrane composition and cell surface charge (44-46). In a preliminary work, we observed that lactococcin A resistant cells of *L. lactis* somehow attached better to glass slides submerged in bacterial culture compared to wild type cells (data not shown), indicating a change on the membrane surface that affected their affinity to the glass surface. It should also be noted that a number of genetic loci in *Li. monocytogenes* involved in resistance to the lantibiotic bacteriocin nisin, such as the cell wall synthesis gene *dltA* (1), the penicillin-binding protein *lmo2229* (20) and the transporter *anrB* (10), appear to have a direct role in cell envelope composition, and these genes might confer general bacteriocin resistance. Future studies to decipher the molecular nature underlying such bacteriocin resistance will therefore primarily focus on unraveling differences in the cell envelope between wild type and resistant cells.

During normal growth with glucose as primary carbon source, the expression of man-PTS is high while the metabolic pathways for alternative sugars are commonly repressed and only when glucose is no longer available, these alternative pathways are turned on. This regulatory phenomenon is generally referred to as carbon catabolite repression (14). In this context, it was interesting to observe that lactococcin A resistant mutants displayed reduced ability to grow on glucose, but enhanced growth on the alternative sugar galactose as

compared to wild type cells. Exposure to bacteriocins has thus generated resistant cells in which the alternative galactose pathway has been derepressed as a result of downregualted man-PTS expression.

The molecular switch that turns off or downregulates the man-PTS expression in resistant cells is a central but still poorly understood aspect of bacteriocin resistance. Most probably, the resistance phenotype is manifested in stable genetic changes as we and others have observed that the resistance phenotype is not lost after hundreds of generations in non-selective medium. Indeed, some mutations have been found in important regulatory genes involved in man-PTS expression. For instance, the gene activator MptR/ManR could represent such a genetically variable hot-spot, as polymorphism in this gene has been detected in resistant isolates of both *E. faecalis* (37) and *Li. monocytogenes* (present study). Nevertheless, given the high frequencies of bacteriocin resistance resulting from reduced man-PTS expression, as seen for several different bacteria (e. g., *L. lactis*, *Li. monocytogenes* and *E. faecalis*), it is tempting to speculate that downregulation of man-PTS expression is not primarily due to regular spontaneous mutations, but rather due to a process which causes metabolic variability in a bacterial culture. In recent years, it has been established that bacterial monocultures exhibit stochastic switching of gene expression in order to generate phenotypic heterogeneous populations, and bacteria can use this heterogeneity as a survival strategy to cope with stressful and fluctuating environments (26, 32, 39). Since the man-PTS is involved in global carbon catabolite control (2, 14, 37, 48), instability in man-PTS gene expression could be used as a mechanism to generate phenotypic heterogeneity with respect to carbon source utilization. Moreover, the man-PTS is a known vulnerable spot for biological attack, since it is used as a target for several antimicrobial agents, including different classes of bacteriocins as well as bacteriophages (4, 15, 17, 23). Stochastic man-PTS gene expression could thus be seen as a defense mechanism to ensure that at least a small subpopulation of cells in a bacterial culture could escape from such extracellular attacks. Further investigation may reveal whether population heterogeneity indeed contributes to the high resistance frequency observed for man-PTS targeting bacteriocins.

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Table 1. Bacterial strains and plasmids used in this study.

	Description[a]	Reference
Bacterial strain		
Lactococcus lactis		
IL1403	Lactococcin A sensitive strain	(7)
IL1403-Rlac-A	IL1403 clone resistant to lactococcin A (isolated from 70 BU ml^{-1})	This study
IL1403-Rlac-B	IL1403 clone resistant to lactococcin A (isolated from 70 BU ml^{-1})	This study
IL1403-Rlac-C	IL1403 clone resistant to lactococcin A (isolated from 220 BU ml^{-1})	This study
IL1403-Rlac-D	IL1403 clone resistant to lactococcin A (isolated from 220 BU ml^{-1})	This study
IL1403 (p369)	IL1403 with p369	This study
IL1403-Rlac-A (p369)	IL1403-Rlac-A with p369	This study
IL1403-Rlac-B (p369)	IL1403-Rlac-B with p369	This study
IL1403-Rlac-C (p369)	IL1403-Rlac-C with p369	This study
IL1403-Rlac-D (p369)	IL1403-Rlac-D with p369	This study
B100	IL1403 with pMG36e	(15)
NZ9000	*L. lactis* strain for nisin controlled gene expression (*nisRK* integrated in the genome). Lactococcin A sensitive.	(30)
NZ9000-Rlac	NZ9000 clone resistant to lactococcin A (220 BU ml^{-1})	This study
NZ9000 (pNZ8037)	NZ9000 carrying plasmid pNZ8037	This study
NZ9000-Rlac (pNZ8037)	NZ9000-Rlac carrying plasmid pNZ8037	This study
NZ9000 (p423)	NZ9000 carrying p423	This study
NZ9000-Rlac (p423)	NZ9000-Rlac carrying p423	This study
Listeria monocytogenes		
L31-H	Isolated from cheese, highly sensitive to class IIa bacteriocins	L. M. Rørvik
L196-H	Isolated from meat, highly sensitive to class IIa bacteriocins	L. M. Rørvik
L228-H	Isolated from meat, highly sensitive to class IIa bacteriocins	L. M. Rørvik
L361-I	Isolated from meat, intermediate sensitivity to class IIa bacteriocins	L. M. Rørvik
L852-I	Isolated from smoked salmon, intermediate sensitivity to class IIa bacteriocins	L. M. Rørvik
L1036-H	Isolated from sea water, highly sensitive to class IIa bacteriocins	L. M. Rørvik
L1040-L	Isolated at a fish processing plant, low sensitivity to class IIa bacteriocins	L. M. Rørvik
L1207-H	Isolated at a fish processing plant, highly sensitive to class IIa bacteriocins	L. M. Rørvik
L1283-I	Isolated from smoked salmon, intermediate sensitivity to class IIa bacteriocins	L. M. Rørvik
L1310-I	Isolated at a fish processing plant, intermediate sensitivity to class IIa bacteriocins	L. M. Rørvik
L1401-I	Isolated from chicken, intermediate sensitivity to class IIa bacteriocins	L. M. Rørvik
L1485-I	Isolated from chicken, intermediate sensitivity to class IIa bacteriocins	L. M. Rørvik
L2462-I	Isolated from chicken feces, intermediate sensitivity to class IIa bacteriocins	L. M. Rørvik
Plasmid		
pMG36e	Lactococcal expression vector with strong P32 promoter, EryR.	(47)
pNZ8037	Lactococcal expression vector containing nisin-responsive promoter, CamR	(13)
p369	pMG36e with *lcnA-flciA* downstream of the P32 promoter, EryR	(15)
p423	pNZ8037 with *ptnABCD* downstream of the nisin-responsive-promoter, CamR	(15)

[a]CamR; chloramphenicol resistance, EryR; erythromycin resistance

Table 2 Polymorphisms identified in *Li. monocytogenes* genes *lmo0095*, *manR*, *resD* and *rpoN*.

Gene	Amino acid postion	Amino acid in different *Li. monocytogenes* strains[a]				
		L31-H	L196-H	L361-I	L852-I	L1040-L
lmo0095	95	T	T	A	A	T
	96	D	D	E	E	D
	104	G	D	D	D	G
	114	E	E	Q	Q	E
	150	Q	Q	E	E	Q
manR	86	N	N	S	S	N
	91	D	D	E	E	D
	204	D	D	E	E	D
	321[b]	E	E	E	E	K
	690[b]	Y	Y	Y	Y	C
resD	174	R	R	K	K	R
rpoN	183	S	A	S	S	T
	284	N	N	S	S	N
	295	N	N	S	S	N
	363	T	T	I	I	T
	373	K	K	M	M	K

[a] High sensitivity to class IIa bacteriocin, L31-H, L196-H; intermediate sensitivity, L361-I, L852-I; low sensitivity L1040-L

[b] Positions with unique polymorphisms in the low sensitivity strain L1040-L

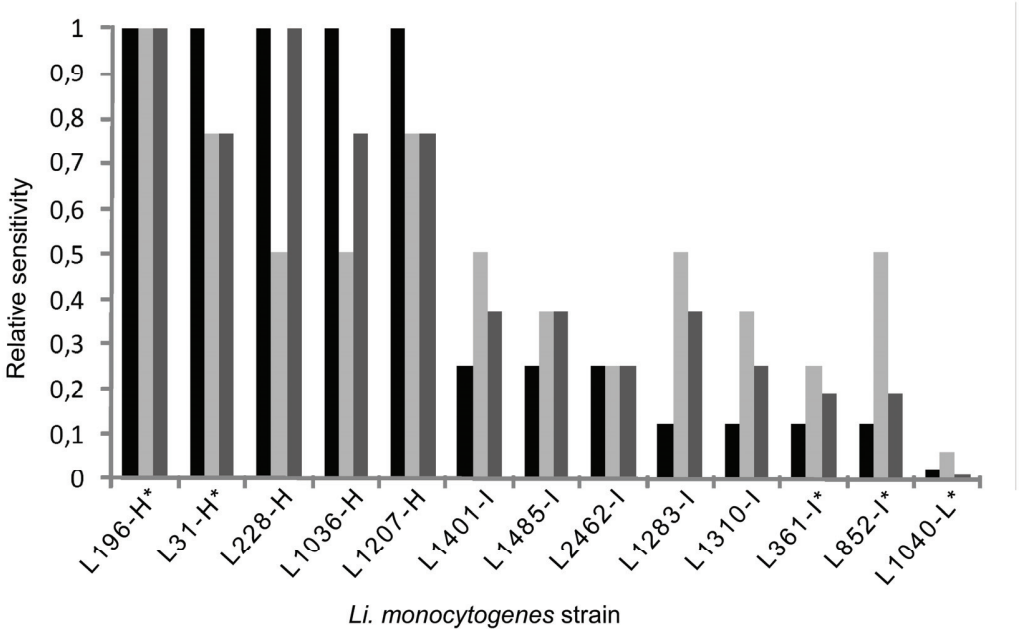

FIG. 1 Relative sensitivity of *Li. monocytogenes* isolates to bacteriocins pediocin PA-1 (black bars), enterocin P (light grey) and sakacin P (dark grey). One MIC was defined as the amount of bacteriocin required to produce 50 % growth inhibition in a 200 µl culture. The MIC of the most sensitive strain (L196-H) was defined as 1, and the MICs of the other strains were determined relative to this (relative sensitivity = 1 divided by MIC). The asterisk denotes strains chosen for further analysis.

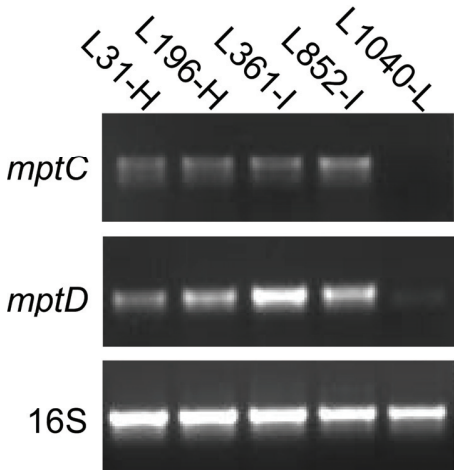

FIG. 2 RT-PCR with primers targeting *mptC*, *mptD* and housekeeping gene 16S (control) in five different *Li. monocytogenes* strains (L31-H, L196-H, L361-I, L852-I, L1040-L).

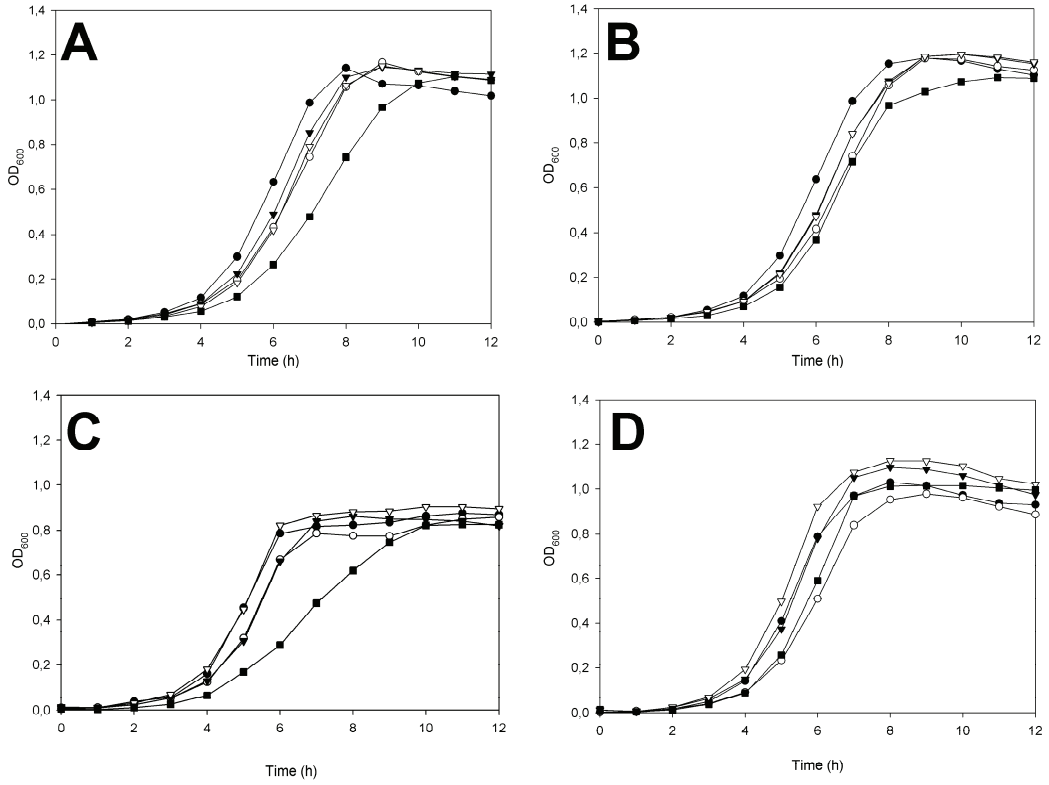

FIG. 3 Growth of *Li. monocytogenes* strains L31-H (●), L196-H (○), L361-I (▼), L852-I (▽) and L1040-L (■) in M17 supplemented with 0.4 % glucose (A), M17 supplemented with 0.4 % glucose and 0.4 % cellobiose (B), BHI (C) and BHI with 0.4 % cellobiose (D).

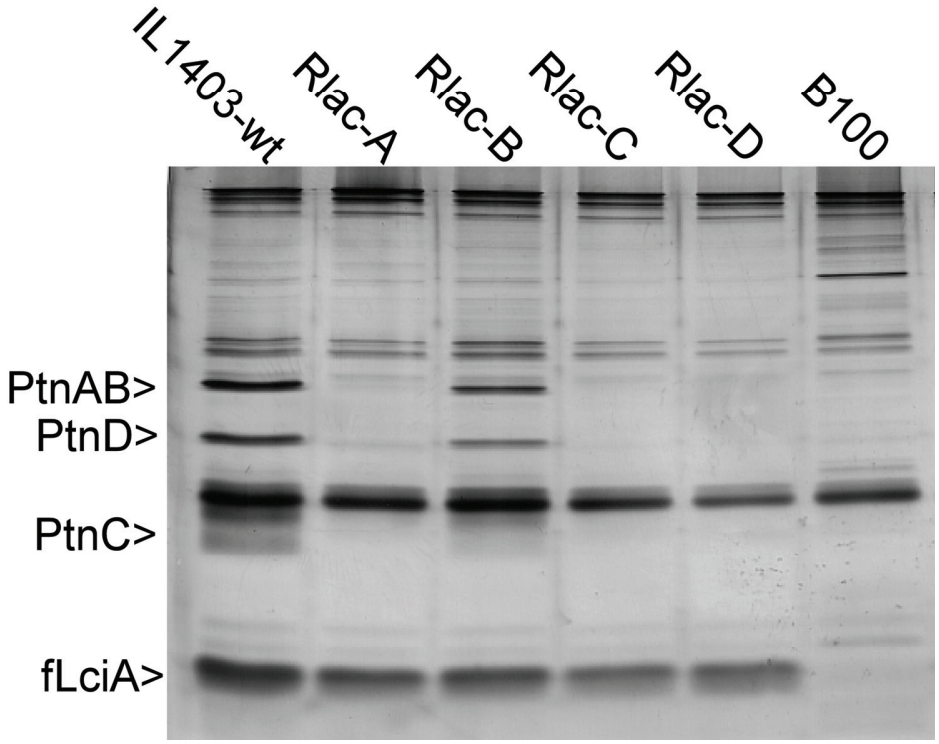

FIG. 4 Differential expression of PtnABCD. Silver-stained SDS-PAGE gel showing fLciA and its co-purified proteins in *L. lactis* IL1403 wild type and lactococcin A resistant mutants Rlac-A, Rlac-B, Rlac-C and Rlac-D. All clones contain plasmid p369 for expression of fLciA, except the negative control B100 (IL1403 with empty plasmid). The identities of the protein bands have previously been determined by mass spectrometry (15).

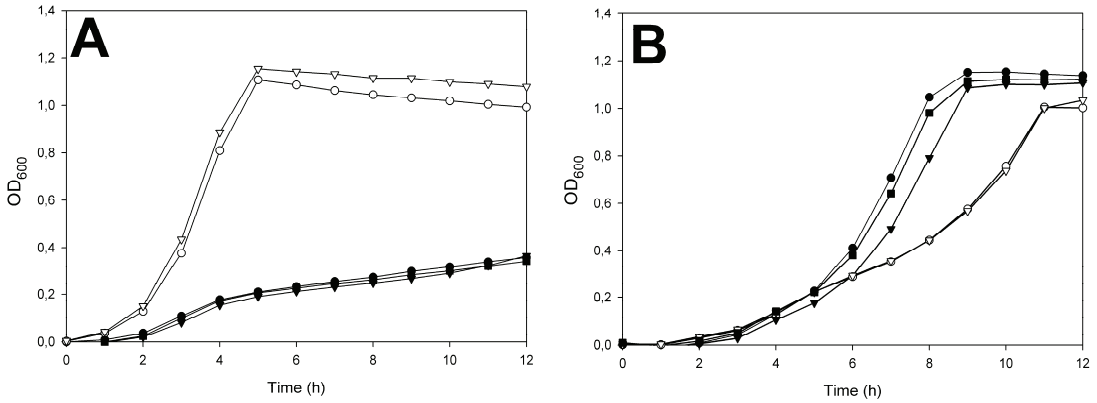

FIG.5 Growth of *L. lactis* IL1403 wild type (○) compared to the four lactococcin A resistant mutants Rlac-A (●), Rlac-B (▽), Rlac-C (▼), Rlac-D (■) in M17 supplemented with glucose (A) and galactose (B).

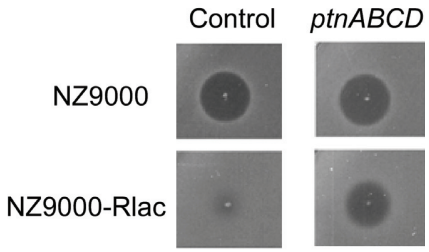

FIG. 6 Lactococcin A sensitivity of *L. lactis* NZ9000 wild type and resistant clone NZ9000-Rlac with empty plasmid (control) and with plasmid p432 expressing *ptnABCD*. Expression of *ptnABCD* was induced with addition of 1 ng ml^{-1} nisin to the softagar. Lactococcin A sensitivity is seen as clear zones. Expression of *ptnABCD* rendered the resistant clone sensitive, however, expression of *ptnABCD* in the wild-type control NZ9000 did not affect the bacteriocin sensitivity of this strain.

PAPER IV

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The Abi Proteins and Their Involvement in Bacteriocin Self-Immunity▽†

Morten Kjos,[1] Lars Snipen,[2] Zhian Salehian,[1] Ingolf F. Nes,[1] and Dzung B. Diep[1]*

Laboratory of Microbial Gene Technology and Food Microbiology[1] and Section for Biostatistics,[2] Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, N-1432 Ås, Norway

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The Abi protein family consists of putative membrane-bound metalloproteases. While they are involved in membrane anchoring of proteins in eukaryotes, little is known about their function in prokaryotes. In some known bacteriocin loci, Abi genes have been found downstream of bacteriocin structural genes (e.g., *pln* locus from *Lactobacillus plantarum* and *sag* locus from *Streptococcus pyogenes*), where they probably are involved in self-immunity. By modifying the profile hidden Markov model used to select Abi proteins in the Pfam protein family database, we show that this family is larger than presently recognized. Using bacteriocin-associated Abi genes as a means to search for novel bacteriocins in sequenced genomes, seven new bacteriocin-like loci were identified in Gram-positive bacteria. One such locus, from *Lactobacillus sakei* 23K, was selected for further experimental study, and it was confirmed that the bacteriocin-like genes (*skkAB*) exhibited antimicrobial activity when expressed in a heterologous host and that the associated Abi gene (*skkI*) conferred immunity against the cognate bacteriocin. Similar investigation of the Abi gene *plnI* and the Abi-like gene *plnL* from *L. plantarum* also confirmed their involvement in immunity to their cognate bacteriocins (PlnEF and PlnJK, respectively). Interestingly, the immunity genes from these three systems conferred a high degree of cross-immunity against each other's bacteriocins, suggesting the recognition of a common receptor. Site-directed mutagenesis demonstrated that the conserved motifs constituting the putative proteolytic active site of the Abi proteins are essential for the immunity function of SkkI, and to our knowledge, this represents a new concept in self-immunity.

Bacteriocins are ribosomally synthesized antimicrobial peptides and proteins produced by a wide variety of bacterial genera. The majority of bacteriocins from Gram-positive bacteria are classified into two groups: the class I lantibiotics, containing posttranslationally modified peptides with ring-forming lanthionine or methyllanthionine residues, and the nonmodified class II peptide bacteriocins (8, 33, 34). Class II bacteriocins are further subdivided into pediocin-like bacteriocins (class IIa), two-peptide bacteriocins (class IIb), and non-pediocin one-peptide bacteriocins (class IIc) (33). Bacteriocin-producing bacteria normally possess a mechanism of immunity to protect themselves from their own bacteriocins, and such self-immunity is often mediated by a dedicated protein (32). For a few bacteriocin systems, the mechanisms by which these proteins confer immunity have been elucidated. For instance, immunity to the lantibiotic nisin (class I) involves a combined action which includes (i) sequestering of bacteriocins on the bacterial cell membrane by a protein called NisI and (ii) removal of the bacteriocins from cells by a dedicated ABC transporter (NisFEG) (39, 44). On the other hand, proteins conferring immunity to pediocin-like bacteriocins (class IIa) as well as lactococcins A and B (class IIc) have been shown to bind directly to the bacteriocin receptor and thereby inhibit pore formation (13). Hitherto, no immunity mechanism is known for any class IIb two-peptide bacteriocins.

Recently, we reported that several bacteriocin loci encode proteins belonging to the Pfam Abi protein family (Pfam accession no. PF02517) (14). These loci include the plantaricin (*pln*) locus of *Lactobacillus plantarum*, encoding two two-peptide bacteriocins (12), the multibacteriocin *pnc* locus of *Streptococcus pneumoniae* (25), and the streptolysin S (*sag*) locus found in group A streptococci (35) (Fig. 1A). Some of the Abi proteins encoded in these loci (PlnI in *L. plantarum*, PncO in *S. pneumoniae*, and SagE in *Streptococcus pyogenes*) are probable bacteriocin self-immunity proteins on the basis of gene knockout studies (10, 25) and genetic organization (i.e., being closely associated with bacteriocin structural genes), while others (e.g., PlnP and PlnTUVW in *L. plantarum* and PncP in *S. pneumoniae*) have completely unknown functions.

The Abi family, also known as the CAAX amino-terminal protease family, consists of putative membrane-bound metalloproteases from both eukaryotes and prokaryotes (38), with the majority being bacterial proteins (90%). The Abi family is recognized by three highly conserved motifs (38): motif 1, consisting of two neighboring and invariant glutamate residues and a conserved arginine separated by three residues (EEXXXR, where X denotes any amino acid); motif 2, consisting of a conserved phenylalanine and a conserved histidine separated by three residues (FXXXH); and motif 3, with an invariant histidine. The three conserved motifs are thought to constitute the active site of the Abi protease, and their importance in proteolytic activity has been demonstrated by mutational analysis of the yeast Abi

* Corresponding author. Mailing address: Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, P.O. Box 5003, N-1432 Ås, Norway. Phone: 4764965910. Fax: 4764941465. E-mail: dzung.diep@umb.no.

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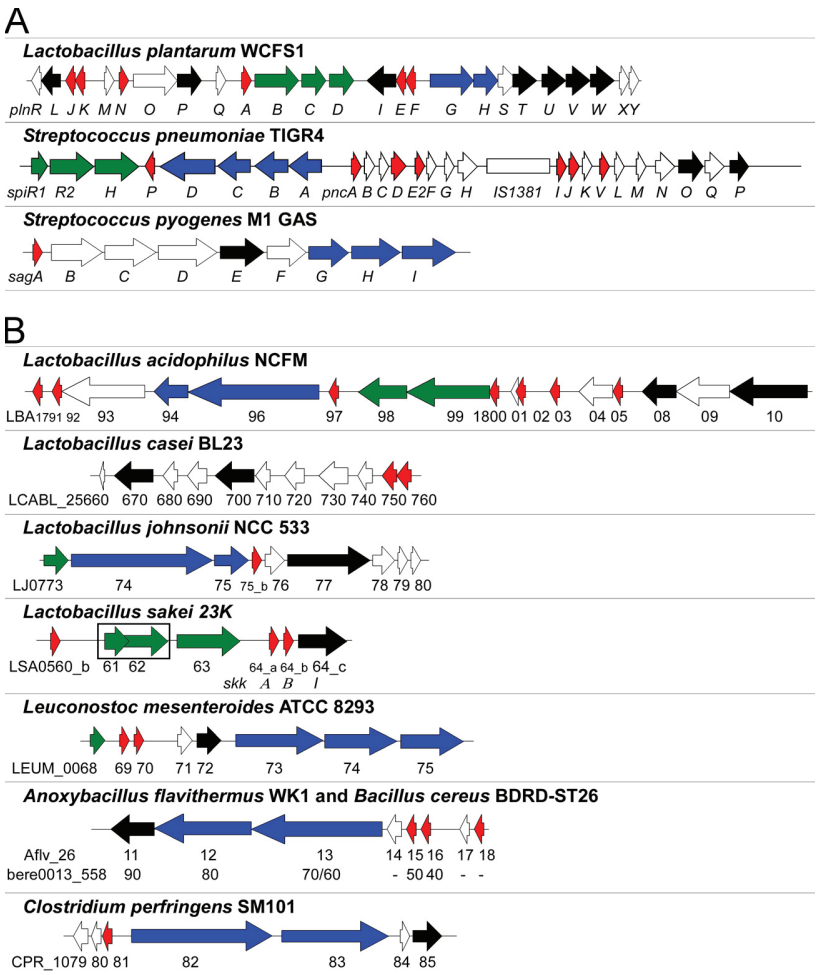

FIG. 1. Abi-associated bacteriocin and bacteriocin-like loci. (A) Three known bacteriocin loci containing Abi genes, including the *pln* locus of *L. plantarum* (14), the *pnc* locus of *S. pneumoniae* (25), and the *sag* locus of *S. pyogenes* (10). (B) Seven potential new bacteriocin loci identified by genome mining for bacteriocin-associated Abi genes. Abi genes are shown as black arrows, bacteriocin structural genes are shown in red, ABC transporter genes are shown in blue, regulatory genes are shown in green, and other genes are shown in white. Gene names or locus tags are shown below the arrows. The genes are drawn approximately to scale. The boxed arrows in the *L. sakei* 23K genome represent a disrupted histidine protein kinase gene.

protease RCE1 (15). In eukaryotic cells, Abi family proteins are involved in membrane targeting of proteins harboring the C-terminal sequence CAAX (C, cysteine; A, aliphatic amino acid; and X, any amino acid) via a process known as prenylation, which consists of the following three sequential reactions (23): (i) a geranylgeranyltransferase/farnesyltransferase attaches a prenyl group (lipid anchor) to the cysteine in the fourth-to-last position, (ii) a CAAX protease of the Abi family cleaves off the three C-terminal amino acids (-AAX), and (iii) an isoprenylcysteine carboxyl methyltransferase then attaches a methyl group to the C-terminal cysteine.

Despite being widespread in prokaryotic genomes, the function of Abi proteins has not been investigated much in bacteria, with the exception of gene knockout experiments on the Abi genes *sagE* and *pncO*, which implies their involvement in bacteriocin self-immunity in streptococci (10, 25). Another Abi-like gene, *prsW* in *Bacillus subtilis*, has been studied to some extent (16). PrsW does not belong to any Pfam family but contains the same three motifs as the Abi proteins, with the exception of the conserved histidine in motif 2, which has been replaced by a glutamate in this protein. PrsW is a protease involved in response to antimicrobial peptides via a process known as regulated intracellular proteolysis (16). In this process, PrsW together with another protease (YluC) cleaves an anti-σ factor (RsiW) to release σ^W, which subsequently regulates gene expression in a manner that protects the cells from antimicrobial peptides. However, the PrsW target protein RsiW in *B. subtilis* does not harbor the C-terminal consensus sequence CAAX found for Abi target proteins in eukaryotes.

Pfam (http://pfam.sanger.ac.uk/) is a comprehensive collection of protein families and domains. For each protein family in Pfam, a profile hidden Markov model (profile HMM) is built from an alignment of sequences from nonredundant representatives of the family (seed sequences), and this profile HMM is then used in an iterative fashion to find new members of the

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
L. plantarum 965	Strain sensitive to sakacin 23K	9
L. sakei Lb790(pSAK20)	Host strain for sakacin A (*sap*)-based expression system; carries pSAK20	4, 41
Plasmids		
pSAK20	Cam^r; contains all genes necessary for transcriptional activation and processing of peptides with *sap* leader	4
pLPV111	Ery^r; expression vector	4
pLG101	pLPV111 derivative containing genes for sakacin A (*sapA*) and sakacin A immunity (*saiA*) behind the *sapA* promoter	11
pSkkα	pLPV111 derivative containing genes for Skkα with *sapA* leader and for SkkI, behind the *sap* promoter	This study
pSkkβ	pLPV111 derivative containing genes for Skkβ with *sapA* leader and for SkkI, behind the *sap* promoter	This study
pMG36e	Ery^r; expression vector containing P32 promoter	48
pSkkI	pMG36e with *skkI* cloned downstream of P32	This study
$pSkkI_{E133A\ E135A}$	pSkkI with E133A E135A double mutation	This study
$pSkkI_{E133Q\ E134Q}$	pSkkI with E133Q E134Q double mutation	This study
$pSkkI_{H214D}$	pSkkI with H214D mutation	This study
pPlnI	pMG36e with *plnI* cloned downstream of P32	This study
pPlnLR	pMG36e with *plnLR* cloned downstream of P32	This study

[a] Cam^r, chloramphenicol resistance; Ery^r, erythromycin resistance.

protein family (18, 43). The Abi family in Pfam contains a large number of sequences (1,966 by September 2009). However, several proteins containing Abi or Abi-like motifs are somehow not detected by the current search tool. Examples of this include PrsW from *B. subtilis* and PlnL from *L. plantarum*, which apparently contain all three motifs but somehow do not fit into Pfam's Abi family. These observations suggest that the profile HMM for Abi may be based upon a slightly skewed sample of seed sequences, resulting in a low sensitivity. We provide here an updated profile HMM to detect Abi-like proteins in prokaryotes that are omitted in the present protein family. Furthermore, searches for novel Abi-associated bacteriocin loci were also performed *in silico*. Several potentially novel bacteriocin loci were identified, and one of them was assessed further by experimentation. The role of bacteriocin-associated Abi genes in self-immunity was also addressed by heterologous expression and site-directed mutagenesis.

MATERIALS AND METHODS

Construction of modified profile HMM for the Abi family. The profile HMM for the Abi family (PF02517) in Pfam is based on 46 seed sequences (September 2009). A modified profile HMM was constructed here for more sensitive detection of prokaryotic members of the Abi family, as some proteins containing typical Abi motifs are not identified by the current algorithms (see below). This modified profile HMM was based on 49 seed sequences, including the 46 existing seed sequences as well as the Abi-like region sequences from the proteins SkkI (GenBank accession no. YP_395178), PlnL (NP_784203), and PrsW (CAB14210). First, the original profile HMM for the Abi family was downloaded from Pfam and aligned with each of the three new protein sequences to detect the region most similar to the existing Abi pattern. These regions from the new sequences were then added to the existing 46 seed sequences, making a list of 49 new seed sequences. From this extended collection of seed sequences, a multiple alignment was constructed, and a profile HMM was estimated from this multiple alignment, using the same model length (115 match states) as in the original Abi profile HMM. All computations were done in Matlab (MathWorks Inc.), using the profile HMM tools in the Bioinformatics Toolbox, which is fully compatible with Pfam. The Matlab script file is available upon request.

Assigning proteins to the modified Abi family. To retrieve potential members of the Abi protein family, each of the 49 seed sequences was queried against the NR database of NCBI (ftp://ftp.ncbi.nih.gov/blast/db) by use of PSI-BLAST (2), with the maximum number of iterations set to 20 and with default parameters used otherwise. The hit sequences were then scored against the modified profile HMM. The score distribution showed a distinct bimodal shape, and a two-component Gaussian mixture model was fitted to separate high-scoring from low-scoring sequences. This was done by use of the mclust package (19) in R (www.r-project.org). Proteins with scores of >60 were assigned to the Abi family.

***In silico* identification of putative bacteriocin loci.** From the modified Abi family, we extracted the genes present in fully sequenced genomes (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). Given the fact that genes involved in bacteriocin synthesis normally are clustered (32), Abi flanking genes (5 upstream and 5 downstream of each Abi gene) were evaluated for their resemblance to bacteriocin structural genes or other genes involved in bacteriocin biosynthesis. A gene qualified as a potential bacteriocin structural gene when several of the following criteria were met: the encoded prepeptide should be relatively short (50 to 85 amino acids [aa]) and contain an N-terminal secretion signal (double-glycine or *sec*-dependent leader), and the predicted mature peptide (20 to 65 aa) should be cationic (having an isoelectric point above 7). Furthermore, bacteriocin-related genes involved in immunity, transport (e.g., an ABC transporter and its accessory protein), and/or regulation (e.g., a histidine protein kinase and a response regulator) should be located in the vicinity.

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. All lactobacilli were grown in de Man-Rogosa-Sharpe (MRS) medium (Oxoid) at 30°C without shaking. When appropriate, 5 μg ml^{-1} erythromycin and/or 5 μg ml^{-1} chloramphenicol was added to the growth medium for selection.

Heterologous expression of sakacin 23K genes. The genetic determinants for the putative two-peptide bacteriocin from *Lactobacillus sakei* 23K, here termed sakacin 23K (the individual peptides are called Skkα and Skkβ), were heterologously expressed in *L. sakei* Lb790 by use of the *sap* two-plasmid expression system (5). In this system, genes for transcriptional activation (*sapRK*) of *sap* promoters, as well as genes necessary for processing and export (*sapTE*) of peptides with the *sap* N-terminal double-glycine leader sequence, are located on one plasmid (pSAK20), while the other plasmid contains genes to be expressed downstream of a *sapRK*-dependent promoter (plasmid derived from pLPV111). The two genes encoding sakacin 23K were expressed separately as fusion genes, using the *sap* double-glycine leader sequence (4), and the fusion genes are called *sap-skkA* and *sap-skkB* (Fig. 2). To construct pSkkα, containing *sap-skkA*, the fragment containing the *sap* promoter and the *sap* leader was amplified from pLG101 (11), using primers dbd84F and mk189, and the fragment containing *skkI* was amplified using primers mk186 and mk187, with genomic DNA from *L. sakei* 23K as the template. The two overlapping primers mk189 and mk186 contain the *skkA*-encoding sequence (without a leader). The two PCR fragments

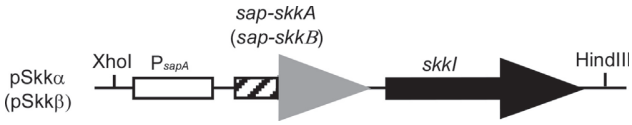

FIG. 2. *sap*-based expression system used for production of sakacin 23K. The *sapA* promoter (P_{sapA}) is located upstream of the bacteriocin fusion gene, *skkA* or *skkB* (gray arrow), and the immunity gene, *skkI*. Note that the fusion genes contain a sequence encoding the *sap* double-glycine leader (dashed bars) used for export. The restriction sites XhoI and HindIII were used for cloning.

were joined in a subsequent PCR step, using the outer primers dbd84F and mk187. The second plasmid, pSkkβ, containing *sap-skkB*, was constructed in a similar manner, using primers dbd84F and pr191 to amplify the fragment containing the *sap* promoter and the *sap* leader sequence and the primers mk194 and mk187 to amplify the fragment containing *skkB* (without a leader sequence) and *skkI*. Primer sequences can be found in Table S1 in the supplemental material. The final PCR products were cleaved with XhoI and HindIII and ligated into pLPV111 (4). The constructs were verified by DNA sequencing. Plasmids were transformed into *L. sakei* Lb790(pSAK20) by electroporation, as described previously (3).

Heterologous expression and site-directed mutagenesis of Abi immunity genes. The *skkI* gene (GenBank accession no. LSA0564_c) was amplified using the primers dbd376 and dbd377, with genomic DNA from *L. sakei* 23K as the DNA template. The *plnI* gene and the *plnLR* gene pair were amplified from *L. plantarum* C11 genomic DNA by use of primers dbd371 and dbd372 for *plnI* and primers mk241 and dbd374 for *plnLR*. The PCR products were cleaved with restriction endonucleases NcoI and XhoI (New England Biolabs, Ipswich, MA) and ligated into pMG36e downstream of a strong, constitutive promoter (P32).

Site-directed mutagenesis of the conserved motifs in SkkI was performed using a two-step PCR approach (20). Three mutations were introduced into *skkI*, using dbd376 and dbd377 as outer primers and mutagenic primers mk206 and mk207 for the E133A and E134A mutations, mk204 and mk205 for the E133Q and E134Q mutations, and mk210 and mk211 for the H214D mutation. The mutated genes were cleaved and ligated into pMG36e as described above. All plasmids used in this study are listed in Table 1. All plasmids were verified by sequencing and were transformed into *L. plantarum* 965 by electroporation as described previously (3).

Bacteriocin assay. The individual peptides of sakacin 23K were concentrated from culture supernatants by precipitation with 40% ammonium sulfate as described previously (13). The plantaricin peptides, PlnE, PlnF, PlnJ, and PlnK, were synthesized by GenScript (Piscataway, NJ). Bacteriocin activity was assessed using an agar diffusion assay in which the bacteriocin samples were spotted directly onto MRS soft agar containing the appropriate indicator strain. The plates were incubated overnight at 30°C, and bacteriocin activity was seen as clear inhibition zones. Alternatively, the bacteriocin activity was analyzed using a microtiter plate assay, where 1,000-fold-diluted overnight cultures of the indicator strain were exposed to 2-fold dilution series of the bacteriocins (1:1 molar ratio of each peptide) in a microtiter plate, with a total volume of 200 μl in each well. The plates were incubated for 12 h at 30°C before the inhibition was scored spectrophotometrically at 600 nm.

RESULTS

Reanalysis of the Abi protein family. Each protein family in Pfam is based on a profile HMM which is constructed from a number of representative sequences called seed sequences, and a protein must score above a certain threshold value in order to be selected for the protein family (18, 43). By multiple sequence alignments, we observed that certain prokaryotic Abi-like proteins did not score above the threshold, indicating that the current profile HMM may be based on a skewed sample of seed sequences. To improve its sensitivity, we constructed a modified profile HMM, which was based on the Abi-like regions from PrsW (GenBank accession no. P50738), PlnL (CAA64196), and SkkI (YP_395178) in addition to the 46 seed sequences used for the Abi family in Pfam as of September 2009. By PSI-BLAST searches against NCBI's NR database, we obtained a total of 3,213 potential Abi proteins. These sequences were then scored against our modified profile HMM. The distribution of scores (histogram) is depicted in Fig. 3, where the curves illustrate a two-component Gaussian mixture model fitted to the histogram data. The optimal separation between low-scoring (green) and high-scoring (red) sequences is at a score of 48.5, but in order to reduce the number of false-positive results, we used a score of 60 as the threshold; hence, only proteins giving a score higher than the threshold value of 60 were qualified as members of the Abi family. With the modified algorithm, a total of 2,706 sequences were assigned to the Abi family, which is 740 more than the number found for Pfam's Abi family as of September 2009. After completion of this work, the Abi family in Pfam was revised (November 2009), using an updated profile HMM based on 155 seed sequences, and the size of this family has now increased from 1,966 to 4,443 members. Interestingly, although a large portion (95%) of the 740 new Abi sequences identified by our approach is included in the updated Abi family, a small but significant portion (37 sequences), including PlnL, SkkI, and PrsW, is still missing, demonstrating that the current profile HMM used for the Abi family is not sufficient.

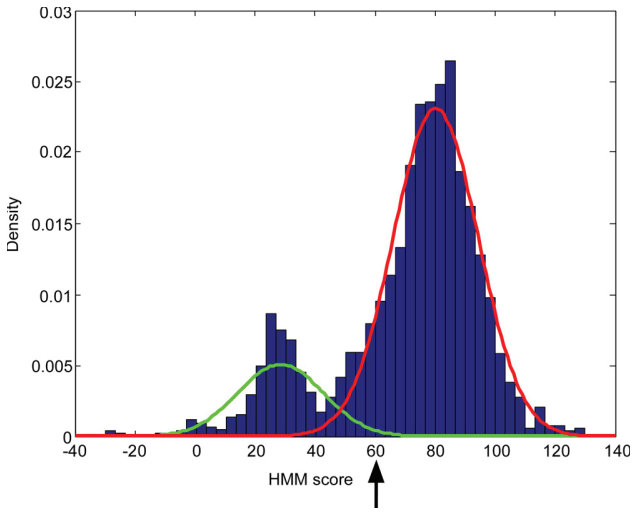

FIG. 3. Histogram of the bimodal score distribution obtained by scoring the 3,213 potential Abi proteins against the modified profile HMM. The overlaid curves illustrate a two-component Gaussian mixture model fitted to the histogram data to separate the low-scoring (green line) and high-scoring (red line) sequences. The threshold score for selecting proteins to the Abi family was set to 60 (black arrow).

***In silico* analysis of Abi flanking genes reveals seven novel bacteriocin-like loci.** Among the 2,706 Abi genes, 1,356 were found in 475 completely sequenced prokaryotic genomes. The number of Abi genes per genome was highly variable, e.g., with 15 in *Bacillus anthracis* but only a single or no Abi gene in many other genomes. In general, the number of Abi genes was much higher in the genomes of Gram-positive bacteria than in those of Gram-negative ones.

By examining Abi flanking genes in sequenced genomes, seven new bacteriocin-like loci were identified (Fig. 1B) in addition to the three known Abi-associated bacteriocin loci depicted in Fig. 1A. (A list of 1,356 Abi genes in sequenced

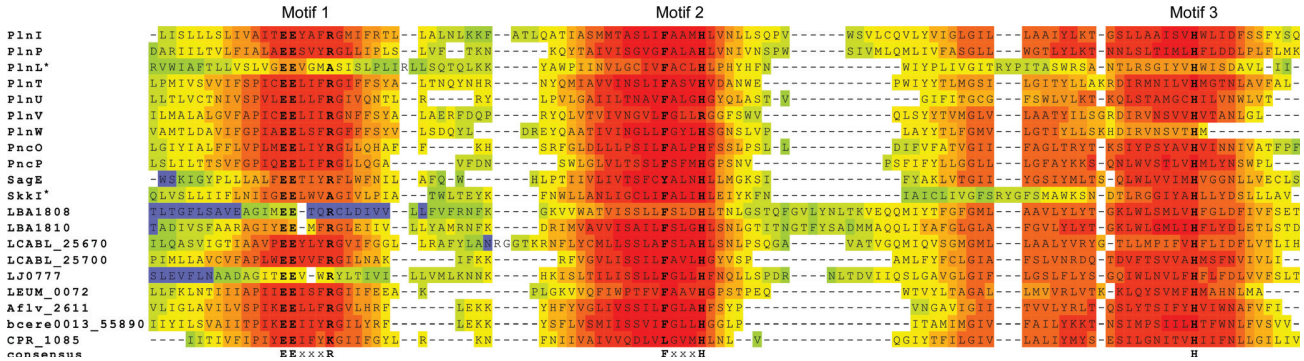

FIG. 4. Multiple sequence alignment of Abi proteins from the different bacteriocin loci shown in Fig. 1. Only the part containing the Abi motifs is shown, and the conserved residues in the three motifs are highlighted in bold. The alignment was constructed using T-COFFEE (www.tcoffee.org). The asterisks denote proteins (PlnL and SkkI) not classified as Abi proteins in the Pfam database.

genomes, as well as the functional annotation of 5 genes upstream and 5 genes downstream for each Abi gene, can be found in Table S2 in the supplemental material.) The vast majority of Abi genes were found in association with genes of various functions, and no predominant genetic organization involving the Abi genes was found, although some genetic links were often observed in a number of different bacterial species. For example, some Abi genes were often closely associated with ABC transporter genes (data not shown), suggesting that Abi proteins play an important but still unidentified role in many ABC transporter systems.

The seven new bacteriocin loci (Fig. 1B) were found in Gram-positive bacteria classified as *Firmicutes*, including five lactic acid bacteria which are either probiotic or used in food production. Four of the loci are from different species of *Lactobacillus*, one is from *Leuconostoc mesenteroides*, one is from *Clostridium perfringens*, and lastly, one locus is found in both *Anoxybacillus flavithermus* and *Bacillus cereus*. The seven new loci are diverse with respect to genetic organization, and they are also different from the three known bacteriocin loci containing Abi genes (*pln*, *pnc*, and *sag*) (Fig. 1A). A multiple sequence alignment of all bacteriocin-associated Abi proteins is shown in Fig. 4. Several of the putative bacteriocins encoded in the new loci display similarities with previously identified antimicrobial peptides (Table 2). The amino acid sequences and physiochemical properties of the putative bacteriocins are presented in Table 2.

Sakacin 23K, a novel bacteriocin from *L. sakei* 23K. In the putative bacteriocin operon of *L. sakei* 23K, the Abi gene is located immediately downstream of two bacteriocin-like genes (Fig. 1B), in a genetic organization identical to that of some known two-peptide bacteriocins, such as plantaricin JK and plantaricin EF in the *pln* system (Fig. 1A). To examine whether the two bacteriocin-like genes (called *skkA* and *skkB*) and the associated Abi gene (called *skkI*) in *L. sakei* 23K represent a novel bacteriocin system, these genes were selected for experimental analysis. *L. sakei* 23K has not been reported to produce any bacteriocins, and we could not detect any bacteriocin activity in the culture supernatant (data not shown). The regulatory histidine protein kinase gene in this locus appears to be disrupted by frameshift mutations, resulting in the formation of two open reading frames encoding different parts of the protein (Fig. 1B). Abolished bacteriocin production due to defects in the regulatory genes has been reported for *Pediococcus pentosaceus* ATCC 25745, and when this bacterium was complemented with an intact gene by cloning, normal bacteriocin production was restored (11). Similarly, to investigate whether the lack of active bacteriocins could result from no or low gene expression, the bacteriocin locus (GenBank accession no. LSA0564_abc) was cloned into a plasmid and overexpressed in the original host, *L. sakei* 23K. However, antimicrobial activity still could not be detected in the culture supernatant (data not shown), suggesting that a posttranslational process is probably defective.

To assess whether the two bacteriocin-like genes constitute a bacteriocin unit of class IIb, these two genes were then expressed separately as fusion genes in *L. sakei* Lb790, using the *sap* double-glycine leader for secretion (5). This strategy has previously proven successful for heterologous production of both one- and two-peptide bacteriocins (5, 11, 29, 36). The concentrated culture supernatants from the resulting clones were found to inhibit the growth of several *Lactobacillus* strains (*Lactobacillus cellobiosus* NCDO 928, *Lactobacillus curvatus* NCDO 2739, *L. plantarum* 965, *L. plantarum* LMGT 2379, *L. plantarum* LMGT 2389, *L. sakei* LMGT 2345, and *L. sakei* NCDO 2714) as well as a few strains of *Listeria* and *Staphylococcus*; the result for *L. plantarum* 965 is shown in Fig. 5A. Interestingly, both peptides (Skkα and Skkβ) displayed bacteriocin activity when assessed individually, and no major synergistic effect was seen when the two peptides were combined in different ratios. The antimicrobial activities of the two peptides were proteinase K sensitive and heat stable (intact activity after 10 min at 90°C).

Abi proteins confer bacteriocin immunity. To examine whether the *skkI* gene, which is located just downstream of the two bacteriocin genes (*skkAB*) (Fig. 1B), could serve as an immunity determinant, this gene was cloned and expressed in the sakacin 23K-sensitive strain *L. plantarum* 965. As expected, expression of this gene rendered strain 965 immune to both peptides individually and in combination, confirming that *skkI* is an immunity gene (Fig. 5B and Table 3). In a similar manner, we also demonstrated that two other bacteriocin-associated Abi genes, *plnI* and *plnL*, could confer immunity to their cognate bacteriocins, plantaricins EF and JK, respectively (Table

TABLE 2. Amino acid sequences of putative bacteriocins found in new loci

Peptide locus tag	Amino acid sequence[a]	Length (aa); pI[b]
Anoxybacillus flavithermus WK1 peptides		
Aflv_2616[d]	MRMFQELNHVELQGIDGG-SWKSHVVNLVGVVSGFGTTGAVIGGSFGGPLGAAAGGFVGAHYGAVAYAIGVLLDSSNRRK	61; 9.91
Aflv_2615[d]	MEGVVFTMELMLEKNGSISFLSEEELKEIDGG-RGSWTNAVIGAGTLSPIVASAVRGAQQGVRFGRLGGPWGVVAGAVVGAVVG	58; 10.83
Aflv_2618	MKELKADELVSIDGG-ISACGKVSVAAFAHTAAFTGLMTFAGVSGPVGWVLGTFVGGAWLGASAAAGCLK	54; 8.90
Bacillus cereus BDRD-ST26 peptides		
Bcere0013_55840	MEELKEFELENIDGG-SWKSHTINVVGNVATYGGIGTAICGPACGVVGAHYGAVAYGVGYLLDNK	49; 7.79
Bcere0013_55850[d]	MKLETNLNVVDLTNEELEINGG-GSWANASVGAGTGASVAVGALKGAQKGASWGSRVGPWGLAAGAVAGAAIGGYLAYD	56; 9.53
Clostridium perfringens SM101 peptide		
CPR_1081[d]	MENLNLNQLENINGG-YWKTIWAVGPGLYQRDTETGKYRWIQTQDNLSYTTNVIANGWAGSAAGGYFSGR	54; 9.23
L. acidophilus NCFM peptides		
LBA1791	MNKFKDLNELELSNIAGG-SNNIFWTRVGVGWAAEARCMIKPSLGNWTTKAVSCGAKGLYAAVRG	46; 10.06
LBA1797[c]	MEKLMVLNEEKLSYVIGG-GNPKVAHCASQIGRSTAWGAVSGAATGTAVGQAVGALGGALFGGSMGVIKGSAACVSYLTRHRHH	65; 10.32
LBA1800	MKKKVVKKTVLKEKELTKVVGG-KKAPISGYVGRGLWENLSNIFKHHK	25; 10.17
LBA1802	MKLRQEQLNRKELSQVIGG-RRDMILVALPHAVGPDGMPGSGRGGGAQMRAIGSIPPWRPNWWK	40; 8.33
LBA1803	MQEWKKTTLSDNELIDVIGG-SAKSYIRRLGPDGGYGGRESKLIAMADMIRRRI	33; 10.90
LBA1805	MKKLKVMNNGELEKVIGG-SLYEMKNSVPRLLGPDGMEGSMGGSTGGIQSFRHFPGFGR	44; 11.83
L. casei BL23 peptides		
LCABL_25750	MISKEVGITLKQHDLVLIQGG-AKRRNKPSGCIVSTIGGAVAGAAGLNPFTTVAGAAIGLSLCLSTNYIHPA	50; 9.85
LCABL_25760[d]	MSYNYRQLDDFQLSGVSGG-KKKFDCAATFVTGITAGIGSGTITGLAGGPFGIIGGAVVGGNLGAVGSAIKCLGDGMQ	58; 8.82
L. johnsonii NCC 533 peptide		
LJ0775b[d]	MSKFQQLTPEDLMETKGG-KIYHATPWQICNSKTHKCWADNAAIARTCGRVIVNGWLQHGPWGAR	46; 9.75
L. sakei 23K peptides		
LSA0564_a[d]	MERISEYKVLNNNVLAGVQGG-KKKKGGFFWHYFGDPIVSFGKGFIGY	26; 9.88
LSA0564_b[d]	MNKKLDSFSSIEDDKLGLVIGG-RNNLAYGLGKLVRAGVDIGIAIGSKGRYKPRH	32; 11.03
Leuconostoc mesenteroides ATCC 8293 peptides		
LEUM_0069[d]	MEKLSEQELAKVSGG-FPLLPIVVPIIAGGATYVAKDAWNHLDQIRSGWRKAGNSKW	41; 9.99
LEUM_0070	MDFKTQRNVLNSEKLMMISGG-STDDSWEGFGSGLHKTVNTVVYAGTTVARAHTRSHQRCFTGNKW	44; 9.18

[a] The dash indicates the predicted proteolytic cleavage site immediately after the double-glycine leader sequence.

[b] Lengths and pIs of the mature peptides (after removal of the leader sequence) were calculated using the Expasy Compute pI/Mw tool (http://au.expasy.org/tools/pi_tool.html).

[c] The underlined sequence shows 100% identity to the bacteriocin acidocin J1132αβ, a two-peptide bacteriocin from *L. acidophilus* (46).

[d] Peptides displaying similarities with previously identified bacteriocins. Aflv_2615 and Aflv_2616 display 43.1% and 21.3% identity to peptides LF221B and LF221A of the bacteriocin acidocin LF221 from *Lactobacillus gasseri* (26), while Bcere0013_55850 displays 46.4% identity to the LF221B peptide. CPR_1081 is 33.3% identical to the class IIc lactococcal bacteriocin lactococcin A (21). LCABL_25760 displays 32.8% identity to thermophilin 13 from *Streptococcus thermophilus* (28). LJ0775_b is 32.6% identical with the class II *sec*-dependent bacteriocin hiracin JM79 from *Enterococcus hirae* (40). LSA0564_a and LSA0564_b are 50% and 25% identical to the β-peptide and α-peptide, respectively, of the two-peptide bacteriocin plantaricin S from *L. plantarum* (45). LEUM_0069 displays 34.1% identity with Orf4, a putative bacteriocin found in the plantaricin locus of *L. plantarum* J51 (31).

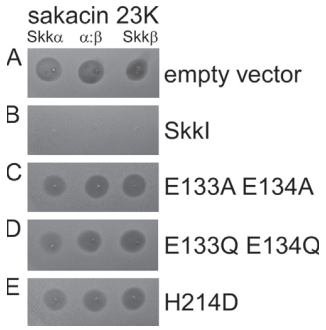

FIG. 5. Bacteriocin soft agar assay using *L. plantarum* 965 as the indicator bacterium. Antimicrobial activities of the peptides were assessed individually (Skkα or Skkβ) and in combination, at a ratio of 1:1 (Skkα and -β). *L. plantarum* 965 containing the empty expression vector was sensitive to both the individual and the combined peptides (A), but expression of the immunity protein SkkI rendered the cells immune (B). Site-directed mutations in the conserved Abi motifs, namely, E133A and E134A (C), E133Q and E134Q (D), and H214D (E), abolished the immunity function of SkkI. Bacteriocin sensitivity is seen as clear zones on the lawn of indicator cells.

3). These bacteriocin systems belong to the *pln* locus, in which *plnI* is located just downstream of the *plnEF* bacteriocin genes, while *plnL* is just downstream of the *plnJK* bacteriocin genes (Fig. 1A). Interestingly, while *plnI* functions alone as an immunity gene, *plnL* requires coexpression of the flanking gene *plnR* to confer immunity (data not shown). *plnR* encodes a putative protein of unknown function.

Most known immunity genes investigated hitherto show strong specificity toward their cognate bacteriocins. For instance, the lactococcal immunity proteins LciA and LciB confer immunity only toward the cognate bacteriocins lactococcins A and B (class IIc), respectively, and no cross-immunity is found between them (47). Similar strong specificity has also been observed for most pediocin-like bacteriocins (class IIa) and their cognate immunity proteins (17). Surprisingly, we observed a relatively high degree of cross-immunity between the Abi bacteriocin systems (Table 3). The indicator strain *L. plantarum* 965 heterologously expressing *skkI* showed cross-immunity toward plantaricins EF and JK (>2- and 256-fold increased protection, respectively). Similarly, expression of *plnI* also protected against sakacin 23K (16-fold increased protection) and plantaricin JK (64-fold increased protection), in addition to the cognate, plantaricin EF. On the other hand, expression of *plnLR* showed less cross-immunity, as it gave no or very little protection against plantaricin EF and sakacin 23K.

Conserved motifs in the Abi proteins are essential for immunity function. The immunity protein SkkI contains the three conserved motifs characteristic of the Abi protein family (Fig. 4). To our knowledge, the importance of these conserved motifs in the function of Abi proteins has not been studied in bacteria. By site-directed mutagenesis, we demonstrated that these motifs are indeed important for the immunity function. We found that the SkkI mutants in which the two invariant glutamate residues in motif 1 were changed to two alanine residues (E133A and E134A) (Fig. 5C) or two glutamine residues (E133Q and E134Q) (Fig. 5D) totally lost the ability to confer immunity. Likewise, replacement of the invariant histidine in motif 3 with an aspartate (H214D) (Fig. 5E) also had a detrimental effect on the immunity function of SkkI.

DISCUSSION

New bacteriocins have traditionally been identified by screening a large number of strains for bacteriocin production. However, the wealth of sequence information present today offers an invaluable source for genome mining of new antimicrobial peptides, and indeed, such *in silico* approaches have already proven successful for discovery of bacteriocin systems (6, 11). For instance, a novel two-peptide lantibiotic, lichenicidin, was identified following rational genome mining for LanM proteins, which are enzymes involved in posttranslational modification of lantibiotics (6). In the present work, we identified seven novel bacteriocin-like loci by genome mining for the bacteriocin-associated Abi genes, which encode putative metalloproteases also known as CAAX proteases.

Based on the observation that some clearly Abi-like proteins were left out of the Abi family in the Pfam database, we suspected a slightly skewed training set for this model. Extending the seed set from 46 to 49 sequences by including three Abi-like proteins, we re-estimated the HMM probabilities. By first using PSI-BLAST to search for Abi-like proteins and then filtering these through the new HMM, we ended up with 2,706 Abi proteins, which is over 700 more proteins than the number registered in the Pfam database for the Abi family by September 2009. We chose to use a conservative threshold value for assigning proteins to the Abi family, emphasizing specificity more than sensitivity. Based on the fitted mixture model, the threshold of 60 corresponds to an expected specificity of >95%, i.e., <5% false-positively detected Abi proteins (Fig. 3). Many of the new Abi proteins we found were recently assigned to the Abi family after an update of the Pfam database in November 2009. However, a number of Abi-like proteins (e.g., PrsW, PlnL, and SkkI) are still omitted from the family, suggesting that there are probably many more Abi proteins in prokaryotes than presently annotated in the databases. We suggest that our rationale should be taken into consideration in future updates of the Abi protein family.

Members of the Abi protein family are ubiquitous in the bacterial world, but surprisingly very little is known about their biological function(s) and how they take part in different pathways. *In silico* screening of Abi-containing genetic loci revealed that Abi genes were colocated with a wide variety of different genes, and this adds further to the notion that the Abi family

TABLE 3. Immunity and cross-immunity by bacteriocin-associated Abi genes

Bacteriocin	MIC (AU) for strain[a]			
	965(pMG36e)	965(pSkkI)	965(pPlnI)	965(pPlnLR)
Sakacin 23K	1	>256	16	2
Plantaricin EF	1	>2	>2	1
Plantaricin JK	1	256	64	>1,024

[a] One arbitrary unit (AU) was defined as the minimum amount of bacteriocin required to produce 50% growth inhibition of *L. plantarum* 965(pMG36e) in a 200-μl microtiter plate. For the synthesized peptides plantaricins EF and JK, 1 AU corresponds to concentrations of 0.625 ng μl^{-1} and 1.22 pg μl^{-1}, respectively.

appears to be a relatively promiscuous protein family involved in a diverse group of pathways. A small fraction of the Abi genes appear to be associated with bacteriocins, and among these, seven new bacteriocin-like loci were identified. All seven contain open reading frames encoding cationic peptides with an N-terminal double-glycine leader, features typical of bacteriocins (32). Like the case for the *pln* locus, some of these Abi genes are found just downstream of the bacteriocin structural genes (e.g., in *L. sakei* 23K), a genetic location resembling that of an immunity determinant, while others are found closely associated with genes involved in transport (e.g., in *Leuconostoc mesenteroides* ATCC 8293, *A. flavithermus* WK1, and *B. cereus* BDRD-ST26) or other functions (e.g., in *Lactobacillus casei* BL23). To our knowledge, none of the strains carrying the seven new bacteriocin-like gene clusters are known as bacteriocin producers, but this may be due to a lack of or suboptimal testing for bacteriocin production, or the bacteriocins may be nonactive, transcriptionally repressed, or possibly depleted of the machinery required for processing and export. Remnants of inactive bacteriocin loci are indeed relatively common in bacterial genomes (11, 30), and they represent an important source for discovery of novel bacteriocins. For instance, the genome of *P. pentosaceus* ATCC 25745 contains a bacteriocin locus which is inactive due to a naturally occurring frameshift mutation in the pheromone-encoding gene. When this bacterium was complemented with an intact pheromone gene by means of cloning, normal bacteriocin production was restored (11). The active bacteriocin itself (penocin A) was also produced when its gene was expressed in a suitable heterologous host (11). Similarly, we confirmed here that the bacteriocin structural genes *skkA* and *skkB* from the non-bacteriocin-producer *L. sakei* 23K were bacteriocinogenic, as they produced antimicrobial activity when expressed heterologously in a host that contains a complete regulatory network and a functional transport system. The two consecutive structural genes in the sakacin 23K locus suggest the presence of a two-peptide bacteriocin of class IIb whose optimal activity is, by definition, constituted by two different peptides (37). However, the Skkα and Skkβ peptides were active individually, and we could not detect any synergistic effects by combining them in different ratios, demonstrating that the sakacin 23K locus might contain two one-peptide bacteriocins. Still, we cannot exclude the possibility that these two peptides might act synergistically toward other bacterial strains. Moreover, potent antimicrobial activities of individual peptides in loci with a similar organization to that of sakacin 23K is not a unique feature, since such activity is found, for example, for the α-peptide from plantaricin S (22) and for LafA from the two-peptide bacteriocin lactacin F (1).

It has been shown that the Abi genes *sagE* and *pncO* are involved in self-immunity toward their cognate bacteriocins in streptococci (10, 25), and in the present work, three other Abi genes (*skkI*, *plnI*, and *plnLR*) were found to serve a similar immunity function. Most immunity determinants act specifically toward their cognate bacteriocins, and cross-immunity is a relatively rare phenomenon (8). Intriguingly, two of the three Abi immunity genes examined in the present study showed extensive cross-immunity. This fact might suggest that the encoded immunity proteins recognize and protect the same receptor(s) or pathway(s) in producer cells. Since these Abi genes encode putative metalloproteases, it is therefore tempting to speculate that Abi-mediated immunity arises from a proteolytic mechanism. This view is supported further by the fact that the conserved Abi motifs (Fig. 4), which are believed to constitute the active site of the enzyme (38), were found to be critical for the immunity function (Fig. 5). Based on these observations, it is reasonable to believe that the Abi proteases confer immunity by direct degradation of the bacteriocin, in a manner similar to that described for the extracellular metalloprotease gelatinase in *Enterococcus faecalis* (27, 42), or by modifying a receptor protein that can no longer be recognized by the cognate bacteriocin. It is also possible that Abi proteases confer immunity via complex transcriptional remodeling, such as occurs in the PrsW-mediated resistance to antimicrobial peptides in *B. subtilis* (7, 16). From some preliminary results, we could not observe any significant degradation of the bacteriocin when it was exposed to cell extracts from Abi-immune cells or to whole cells, thus disfavoring a bacteriocin degradation mechanism.

The critical role of the conserved motifs in the immunity function suggests that the mode of self-immunity mediated by Abi proteins is completely different from other known bacteriocin immunity mechanisms (e.g., immunity to nisin [44] or lactococcin A [13]). In several bacteriocin systems, self-immunity appears to be linked physically to the mechanism of receptor recognition. For example, class IIa and some class IIc bacteriocins employ membrane components of the mannose phosphotransferase system as target sites to form pores on sensitive cells, but in immune cells the immunity protein binds tightly to the target proteins to prevent the subsequent lethal pore formation by the bacteriocin (13, 24). Thus, studies of the immunity mechanism(s) and the protein(s) targeted by the Abi proteases could be a crucial step toward understanding target recognition and how the Abi-associated bacteriocins kill sensitive cells.

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Supplemental table 1 Primers used in this study

Primer name	Sequence (5' → 3'); restriction site [a]
dbd84F	GATCCTCGAGAATTCCTCGGAAAGCGAGCG; XhoI
dbd371	ATTTTAACCATGGCATTAATAAAAGTAATAATCACAGC; NcoI
dbd372	TCAGACTCGAGGATTACTCGTCTATTTTATCTTC; XhoI
dbd374	AACTTACTCGAGTTTTTCTTTCTAGCTTTGAAT; XhoI
dbd376	GTAAGACCATGGATACGTTAGAAGTGATTAGCC; NcoI
dbd377	TTCATTCTCGAGTAGACGCTAATCTTTTTTATAATAGT; XhoI
mk186	TTATTTTGGAGATCCTATTGTTAGTTTTGGTAAAGGATTTATTGGATATTAAAATCATAGGTAAGATAAATGAATAC
mk187	ACGTAAGCTTATCTTTTTTATAATAGTATATTCACA; HindIII
mk189	CAATAGGATCTCCAAAATAATGCCAAAAGAAGCCACCCTTTTTCTTTTTACCGCCGGTAATTGTTTGTAA
mk191	ACCGCCGGTAATTGTTTGTAA
mk204	ACATAATAGGTCAACAATTATGGGTCGC
mk205	GCGACCCATAATTGTTGACCTATTATGT
mk206	ACATAATAGGTGCAGCATTATGGGTCGC
mk207	GCGACCCATAATGCTGCACCTATTATGT
mk210	GTTTAATTGCTGCATTAATCGATTTAGAG
mk211	CTCTAAATCGATTAATGCAGCAATTAAAC
mk241	ATCGCCATGGGTGCGATCTTTCTTAATA; NcoI

[a] Restriction sites are underlined in the sequences