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Genetic characterisation and heterologous expression of leucocin C, a class IIa bacteriocin from *Leuconostoc carnosum* 4010

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Tiivistelmä — Referat — Abstract

Class IIa (pediocin-like) bacteriocins are a major group of bacteriocins produced by lactic acid bacteria (LAB) characterised by their antilisterial activity. As a protective LAB strain for meat products, *Leuconostoc carnosum* 4010 kills *Listeria* by producing two class IIa bacteriocins, the well characterised leucocin A (LeuA) and the less studied leucocin C (LecC). Although the amino acid sequence of the secreted LecC has been published, the genes required for its production remain unknown. The aims of this study were to characterise the genes needed for LecC production and to express the *lecC* gene in *Lactococcus lactis*.

The *lecC* gene was localised by Southern blot in a large plasmid different from the one harbouring LeuA genes in *Ln. carnosum* 4010 genome. Five genes in two operons were identified mainly by PCR-based methods and sequencing, namely, the structural gene (*lecC*) with a 72-bp signal sequence, the immunity gene (*lecI*) encoding a 97-aa immunity protein, two genes *lecTS* for an ABC transporter and the gene *lecX* for an accessory protein. The immunity function of LecI was demonstrated by expressing the *lecI* gene in LecC sensitive *Listeria monocytogenes*. Compared to the wild type, LecI-producing *Listeria* was more tolerant to LecC, thus corroborating the immunity function of LecI. For heterologous expression of LecC, the *lecC* gene was fused to the lactococcal *usp45* signal sequence in the nisin-selectable and nisin-inducible food-grade secretion vector pLEB690. Consequently, bioactive LecC was secreted efficiently by the recombinant *Lc. lactis*.

In conclusion, novel genes for the production of LecC in *Ln. carnosum* 4010 were identified. The findings indicate that LecC is produced by a dedicated system independent of LeuA. The successful production of functional LecC in *Lc. lactis* offers an attractive approach for the future application of bacteriocins in food production.

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Leuconostoc, class IIa bacteriocin, leucocin C, gene expression, bacteriocin immunity

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PREFACE

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Xing Wan
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ABBREVIATIONS

2/3CS	two- or three-component regulatory system
aa	amino acid
ABC	ATP-binding cassette
ADP	adenosine diphosphate
AMP	antimicrobial peptide
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
BHI	brain heart infusion
bp	base pair(s)
CD	circular dichroism
CIP	calf intestinal alkaline phosphatase
C-terminal	carboxyterminal
Da	dalton, atomic mass unit
DIG	digoxigenin
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dUTP	deoxyuridine triphosphate
e.g.	*exempli gratia*, for example
Erm	erythromycin
et al.	*et alii*, and others
FAO	Food and Agriculture Organization of the United Nations
G^-	Gram-negative bacteria
G^+	Gram-positive bacteria
GG	double-glycine
GMO	genetically modified organism
GRAS	generally regarded as safe
HPK	histidine protein kinase
i.e.	*id est*, in other words
IF	induction factor
im	immunity, immunity protein(s) or immunity gene(s)
iPCR	inverse PCR
IU	international unit
JECFA	Joint FAO/WHO Expert Committee on Food Additives
kb	kilobase pair(s)
kDa	kilodalton
L.	*Listeria*
LAB	lactic acid bacteria
Lb.	*Lactobacillus*
LB	Luria-Bertani medium
Lc.	*Lactococcus*
Ln.	*Leuconostoc*
M17G	M17 medium + 0.5% glucose
M17GS	M17 medium + 0.5% glucose + 0.5 M sucrose
Nis	nisin

NMR	nuclear magnetic resonance
nt	nucleotide
N-terminal	aminoterminal
o/n	overnight
OD_{600}	optical density at 600 nm wavelength
ORF	open reading frame
PCR	polymerase chain reaction
PNK	T4 polynucleotide kinase
PTS	phosphotransferase system
RR	response regulator
SP	signal peptide
SS	signal sequence
WHO	World Health Organization

1 INTRODUCTION

Bacteriocins are ribosomally synthesised antimicrobial peptides (AMPs) produced by bacteria. Many lactic acid bacteria (LAB), for example *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Carnobacterium*, *Enterococcus*, and *Pediococcus*, have been reported to secrete AMPs against competing microorganisms. Because of their natural activity against foodborne pathogens and food spoilage bacteria, bacteriocins have been noticed as potential natural preservatives (Eijsink *et al.* 1998; Nes *et al.* 2001; Fimland *et al.* 2005). Since there is no agreement on the classification of LAB bacteriocins, common structural characteristics have been usually accepted to be the basis for sorting these AMPs (Klaenhammer 1993; Cotter *et al.* 2005b; Zouhir *et al.* 2010; Belguesmia *et al.* 2011). LAB bacteriocins which have been thoroughly studied and abundantly reported are those in class I (lantibiotics) and class II (unmodified bacteriocins). Bacteriocins of class II contain 20 to 70 amino acids and permeabilise cell membrane to kill target cells. Unlike lantibiotics which contain unusual modified residues like lanthionines, class II bacteriocins do not undergo post-translational modifications (Ennahar *et al.* 2000; Drider *et al.* 2006; Nissen-Meyer *et al.* 2009).

Class II bacteriocins have been further divided into four subgroups, of which class IIa (pediocin-like) is comprised of bacteriocins sharing an N-terminal highly conserved sequence YGNGV/L (Drider *et al.* 2006; Nissen-Meyer *et al.* 2009). These peptides are the best characterised, and their activity against various *Lactobacillus*, *Enterococcus*, *Leuconostoc*, *Clostridium*, and especially the pathogenic *Listeria* strains have caught broad attention (Eijsink *et al.* 1998; Nissen-Meyer *et al.* 2009). Class IIa bacteriocins permeabilise the target cells by forming pores in the cytoplasmic membrane that subsequently leads to the depletion of the intracellular ATP pool, disturbance of the proton motif force and eventually the cell death (Herranz *et al.* 2001; Suzuki *et al.* 2005). After contacting the receptor molecule mannose phosphotransferase system (PTS) permease EII_t^{Man} on cell membrane, the N-terminal halves of these bacteriocins form a three-stranded anti-parallel β-sheet-like structure which stabilises the binding to the receptor on the target cell membrane (Kjos *et al.* 2011). Simultaneously, the helical C-termini penetrate into the receptor protein (Johnsen *et al.* 2005a). A hinge structure connects the two domains of a class IIa bacteriocin, facilitates the movement of either domain towards each other, and therefore mediates the pore formation (Johnsen *et al.* 2005a; Nissen-Meyer *et al.* 2009). Pores on the cell membrane result in the membrane leakage. Since such receptors

commonly exist on bacterial cells, these bacteriocins may also form pores on its producer cell membrane. Therefore, bacteriocin producing bacteria produce immunity proteins which bind to the bacteriocin-receptor complex from the cytosolic side of the cell membrane, and thus prevent the leakage of own membrane (Nissen-Meyer *et al.* 2009).

Several *Leuconostoc* strains, such as *Ln. carnosum* LA54a, *Ln. mesenteroides* TA33a and *Ln. gelidum* UAL187, have been reported to produce different class II bacteriocins, namely leucocin A (class IIa), leucocin B (class IId) and leucocin C (class IIa) (Lewus *et al.* 1992; van Belkum and Stiles 1995; Papathanasopoulos *et al.* 1997). *Ln. carnosum* strain 4010 has been shown to produce both leucocin A and leucocin C; and because of its high antimicrobial activity and suitability in food, this strain is commercially available as a biopreservative culture in vacuum-packaged meat products (Budde *et al.* 2003). The strain is sold as preservative strain by the Danish starter company Chr. Hansen A/S.

Although many class IIa bacteriocins including leucocin A have been studied extensively, the knowledge of leucocin C is limited. Only the amino acid sequence of the secreted 43-aa leucocin C peptide and its activity against *Listeria* has been published (Fimland *et al.* 2002b). In addition, class IIa cross-immunity study showed that leucocin A immunity protein does not protect the cell from leucocin C, indicating that each leucocin has its own production-immunity system (Fimland *et al.* 2002a). None of the factors typically needed for class IIa bacteriocin production, i.e., dedicated translocators for secretion, a self-protection (immunity) protein, and a regulatory system, has been reported for leucocin C production. As bacteriocins may one day be developed into drugs for medical use or additives in food and feed, studies on these antimicrobial bacteria like *Ln. carnosum* 4010 provide knowledge about the weapons against pathogenic bacteria.

In this work, the main objective was to study and characterise the genes needed for the production of antimicrobial peptide leucocin C in the industrial food bacterium *Ln. carnosum* 4010. The scientific literature focuses on the antimicrobial properties, in terms of bacteriocins produced by LAB, particularly class IIa bacteriocins. Heterologous expression of these peptides is also reviewed. The experimental section shows the localisation and identification of the genes needed for leucocin C production, as well as their explicit functions. Expression of leucocin C in a heterologous LAB host is also demonstrated for the development of new strains with improved antilisterial activity.

2 LITERATURE REVIEW

2.1 Lactic acid bacteria

Lactic acid bacteria (LAB) constitute a group of Gram-positive bacteria, which are non-sporeforming, non-respiring cocci or rods, and produce lactic acid as the major end product from fermentation of carbohydrates (Axelsson 2004). LAB are widely used in food and feed fermentation and normally associated with (healthy) mucosal surfaces of humans and animals. Conventionally, the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus* form the core of the group. The more peripheral genera *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Oenococcus*, *Sporolactobacillus*, *Tetragenococcus*, *Vagococcus*, and *Weissella* belonging to the order Lactobacillales are also regarded as LAB (Stiles and Holzapfel 1997). *Leuconostocs* are cocci in chains. They are commonly used as flavour starters in dairy products, but in refridgerated vaccum meat products, *Leuconostoc* species may cause food spoilage (Säde 2011).

LAB are generally recognised as safe (GRAS) organisms. The use of LAB has been found throughout the history of food fermentation. Using fermentation in food preparation originally was to prevent food spoilage (Caplice and Fitzgerald 1999). In addition to giving foods desirable flavour, texture and nutrients, LAB also improve the safety of foods by inhibiting the competing spoilage and pathogenic bacteria (Daeschel 1989). LAB can grow in non-fermented foods as well, for example in vacuum-packaged meat products (Castellano *et al.* 2004). In this specific case, LAB such as *Carnobacterium* spp., *Lactobacillus* spp. and *Leuconostoc* spp. are the main spoilage microbes (Borch *et al.* 1996; Korkeala and Björkroth 1997; Settanni and Corsetti 2008). However, by co-culturing with other strains, the antimicrobial properties of these LAB can be promoted, while their spoilage effect can be minimised (Settanni and Corsetti 2008).

Nowadays, safe foods with a long shelf-life or minimally processed products without chemical preservatives are favoured by modern consumers. Thus, biopreservation is an attractive option for food processors. Biopreservation is the use of natural microbes and their antimicrobial metabolites to increase safety and extend shelf-life of food (Stiles 1996). Due to their native antimicrobial properties, LAB have been used as protective cultures in the biopreservation of different food products, such as dairy, meat, fish, fruits and vegetables. These bacteria have been observed to increase the shelf-life of food

significantly without causing the sensory and structural changes in food (Budde *et al.* 2003; Cotter *et al.* 2005a; Castellano *et al.* 2010). LAB inhibit undesirable flora mainly by producing organic acids (Daeschel 1989; Cintas 2001). They also produce other antimicrobial substances like acetaldehyde, carbon dioxide, diacetyl, D-isomers of amino acids, fatty acids, hydrogen peroxide, and bacteriocins (Holzapfel *et al.* 1995; Cintas 2001).

2.2 Bacteriocins from LAB

As GRAS organisms, LAB have made noticeable contributions to food and feed fermentation. Their health promoting traits and their potency of producing AMPs attract great attention by researchers. AMPs present in innate immune systems of organisms protecting the hosts against invading organisms. The AMPs produced by bacteria are termed bacteriocins. They play an important role in the self-defence system of bacterial cells (Cotter *et al.* 2005a; Nes *et al.* 2007). Unlike conventional peptide antibiotics, bacteriocins are ribosomally synthesised, have relatively narrow spectrums of targeted bacteria, and inhibit against susceptible bacteria at very low (nanomolar) concentrations (Nes 2011). Some can inhibit nosocomial pathogens that are normally resistant to various antibiotics (Nes 2011). Bacteriocins can be introduced into foods in different ways. In fermentation, bacteriocin-producing LAB can substitute for part or all of the starter cultures. Bacteriocins can be added directly to foods as purified or semi-purified preparations (Cotter *et al.* 2005a). They can also be immobilised for the development of bioactive food packaging. In recent years, using bacteriocins in hurdle technology offers a novel approach for food preservation (Gálvez *et al.* 2007).

The history of bacteriocins goes back to 1920s, and by that time a bacteriocin was referred to an AMP produced by Gram-positive bacteria (Jacob *et al.* 1953). In fact, Gram-positive and Gram-negative bacteria both produce bacteriocins (Riley and Gordon 1992; Klaenhammer 1993). The bacteriocins from G^+ bacteria seem to have a relatively broader spectrum. In addition to killing bacteria from the same species of the bacteriocin producer, many of G^+ bacteriocins also inhibit the growth of bacteria of other species and/or genera than the producer bacteria (Nes *et al.* 2007).

Since the bacteriocin colicin was identified from *Escherichia coli* in 1920s, numerous microorganisms have been screened for their production of bacteriocins (Konisky 1982). In the past 20 years, there have been many studies focused on those produced by G^+ bacteria,

particularly by LAB. According to their structures and targets, LAB bacteriocins may be sorted into two main groups (Cotter *et al.* 2005a). Other than the large heat-labile proteins (formerly class III), two major groups, the modified bacteriocins (class I) and the non-modified (less modified) heat-stable bacteriocins (class II), cover the vast majority of the well investigated LAB bacteriocins (Nes 2011).

A major group of class I bacteriocins is called lantibiotics. Lantibiotic peptides commonly produced by LAB are effective against G^+ bacteria. They are characterised by post-translational modifications, during which *meso*-lanthionine(s) or β-methyllanthionine(s) or both ring structures are formed (Kuipers *et al.* 2011). Lantibiotics kill bacteria mainly by forming voltage-dependent pores in cell membranes. The production of lantibiotics is usually regulated by a two-component regulatory system (2CS), though some lantibiotics like lactocin S have alternative regulatory systems (Kleerebezem *et al.* 2001; Rawlinson *et al.* 2005).

A successful example of class I bacteriocins that have been used as peptide additive in food industry is nisin. This bacteriocin was approved by Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1969 and became commercial. Later on, nisin as food preservative was also legally accepted in European Union, the United States and many other countries. As the best studied lantibiotic peptide, nisin was first reported to be produced by *Streptococcus lactis* (*Lc. lactis* (Schleifer *et al.* 1985)) in milk fermentation in 1920s (Rogers 1928; Rogers and Whittier 1928). It took about 50 years to determine the structure of nisin. Lantibiotic nisin contains five intermolecular lanthionine ring structures that are formed during post-translational modification (Gross and Morell 1971). Nisin has several variants, four of which are nisin A, F, Q, and Z differing by only a few amino acid residues produced by *Lc. lactis* strains (Twomey *et al.* 2002; Zendo *et al.* 2003; de Kwaadsteniet *et al.* 2008). *Streptococcus uberis* produces an additional variant, nisin U, which is 78% identical to nisin A and shorter in three amino acids than other nisin variants (Wirawan *et al.* 2006).

The pore-forming activity of nisin is mediated by the cell wall precursor lipid II (Breukink *et al.* 1999). A stable pore complex consists of 8 nisin and 4 lipid II molecules. Two N-terminal lanthionine rings of nisin allow the bacteriocin interact with the lipid II by forming a pyrophosphate cage, followed by the penetration of C-terminus into the membrane (van Heusden *et al.* 2002; Hsu *et al.* 2004). The interaction of lantibiotics which

share the pattern of the two N-terminal rings with nisin, for example epidermin, mutacins, and ericins, do not form pores on targeted cells but still possess high antibacterial activity by inhibiting the synthesis of the cell wall (Hasper *et al.* 2006). Some lantibiotics like nisin and subtilin also inhibit the outgrowth of *Bacillus* and *Clostridium* spores (Thomas *et al.* 2002).

Unlike lantibiotics, class II bacteriocins do not undergo post-translational modifications and thereby are structurally simpler. Generally, only genes encoding the bacteriocin, immunity protein and transporters are required for the production of class II bacteriocins. Synthesis of some bacteriocins is under the control of a two- or three-component regulatory system (2/3CS) which includes a sensor (histidine protein kinase, HPK), a response regulator (RR), and an induction factor (IF, peptide pheromone in 3CS) (Nes *et al.* 1996; Nes and Eijsink 1999). Most bacteriocins are synthesised as a pre-peptide including an N-terminal leader peptide which is removed by the transporter during the exportation of bacteriocin. The most common signal peptides are double-glycine (GG) type leaders. The GG-leader is recognised and cleaved in secretion of many class II bacteriocins by ABC transporters (Nes *et al.* 2007).

Class II bacteriocins can be further divided into four subgroups. Two conventional subgroups are the pediocin-like antilisterial class IIa and the two-peptide class IIb bacteriocins. The pediocin-like bacteriocins constitute a group of the most studied class II bacteriocins. They are characterised by their high activity against the food pathogen *L. monocytogenes*. Class IIa bacteriocins kill targeted cells by permeabilising the cell membrane, therefore disrupting the proton motive force of the cells and depleting the ATP pool. Biosynthesis of some class IIa bacteriocins, such as curvacin A, enterocin A, and sakacin P, is under the control of 3CS, while some others like divercin V41 are regulated by 2CS (Fimland *et al.* 2005; Drider *et al.* 2006).

A commercialised example of unmodified bacteriocins from LAB is pediocin (also known as pediocin PA-1/AcH). Currently, pediocin is available as a commercial product named ALTA^{TM} 2341 (Ennahar *et al.* 2000; Rodríguez 2002). The application of pediocin has been shown to reduce the levels of *L. monocytogenes* in various dairy products (Rodríguez 2002). In raw meats, this bacteriocin can significantly lower the counts of *L. monocytogenes* and *Clostridium perfringens* (Rodríguez 2002; Nieto Lozano 2006).

2.3 Classification of bacteriocins

The classification of bacteriocins is very controversial. The bacteriocins from LAB have been sorted based on their producers, molecular sizes, structures and/or mode of actions, etc. (Klaenhammer 1993; Jack *et al.* 1995; Cotter *et al.* 2005b; Zouhir *et al.* 2010). Klaenhammer (1993) divided LAB bacteriocins into four classes which are now commonly accepted. Class I bacteriocins (lantibiotics) were characterised as lanthionine-containing bacteriocins that undergo extensive post-translational modifications. Class II bacteriocins were heat-stable peptides and could be further divided into three subgroups. Class III was composed by large and heat-labile proteins, while class IV bacteriocins were defined as protein complexes composed of protein and chemical moieties (Klaenhammer 1993). This classification scheme was later modified. The fourth class was abolished, and criteria for dividing class II bacteriocins were changed on the basis of leader differences (Nes *et al.* 1996). Later, according to their specific secretion pathways, the number of subclasses of class II bacteriocins was expanded to six (van Belkum and Stiles 2000). Bacteriocins were once divided into only two main groups: class I (lanthionine-containing bacteriocins/lantibiotics) and class II (the non-lanthionine bacteriocins). In this proposal, the large heat-labile class III bacteriocins were renamed as bacteriolysins, i.e. non-bacteriocin lytic proteins (Cotter *et al.* 2005a). Based on this classification scheme, class I lately covers all post-translationally modified bacteriocins from G^+ bacteria, whereas the class II and bacteriolysins retained the same (Rea *et al.* 2011). In the latest review of bacteriocins produced by LAB, these AMPs were divided into four major groups. Unlike Klaenhammer's four-class system, in the latest classification, cyclic AMPs constitute the fourth group, and class II bacteriocins were grouped by their non-modified nature (Nes *et al.* 2007). Recently, a new structure-based classification was proposed. G^+ bacteriocins were classified into 12 groups based on their amino acid sequences (Zouhir *et al.* 2010).

In this chapter, LAB bacteriocins will be divided into 3 classes: class I (lantibiotics), class II (unmodified heat-stable bacteriocins), and class III (large heat-labile bacteriocins) based on recent classification schemes (Nes *et al.* 2007; Rea *et al.* 2011).

2.3.1 Class I: lantibiotics

Lantibiotics are small membrane-active peptides (<5 kDa) featured by post-translational modifications. These bacteriocins are ribosomally synthesised as inactive prepeptides.

After forming *meso*-lanthionine(s) and/or β-methyllanthionine(s), as well as other unusual amino acids and some dehydrated residues during post-translational modifications, they become biologically active (Kuipers *et al.* 2011). Nisin as the first identified lantibiotic has been the most studied. In the present classification, class I bacteriocins can be further divided into four subgroups on the basis of the modification pathway and antimicrobial activity (Rea *et al.* 2011).

Lantibiotics like nisin, epidermin and Pep5 are considered as **type I** lantibiotics which undergo modifications by two enzymes, LanB for aa dehydration and LanC for lanthionine ring formation (Willey and van der Donk 2007). On the other hand, **type II** lantibiotics, such as lacticin 481, mersacidin, and actagardin are modified by only one bifunctional enzyme, LanM (Siezen *et al.* 1996). Two-peptide lantibiotics like lacticin 3147 and haloduracin are also included in this class (Ryan *et al.* 1996; McClerren *et al.* 2006). **Type III** lantibiotics were described as lanthionine-containing peptides without antimicrobial activity. SapB, SapT and AmfS belong to type C, as their modification enzymes share homology to the C-terminal domain of LanM (Meindl *et al.* 2010; Kuipers *et al.* 2011; Rea *et al.* 2011). The **type IV** lantibiotics possess novel lanthionine synthetases called LanL. They have clear lantibiotic characteristics in their structures and biosynthetic means, but their antimicrobial activities are still unknown (Goto 2010; Rea *et al.* 2011).

2.3.2 Class II: unmodified bacteriocins

Class II bacteriocins are heat-stable bacteriocins with molecular masses less than 10 kDa. Unlike lantibiotics, these bacteriocins do not undergo post-translational modifications. Class II unmodified bacteriocins were originally subdivided into 3 groups: IIa pediocin-like, IIb two-peptide, and IIc thiol-activated bacteriocins (Klaenhammer 1993). Since then, there have been several alternative classifications about class II bacteriocins, but all of them agreed and identified the two major subclasses, i.e. the pediocin-like antilisterial bacteriocins (class IIa) and two-component bacteriocins (class IIb) (Nes *et al.* 1996; Eijsink *et al.* 2002; Drider *et al.* 2006). In addition to the two established subclasses, class IIc cyclic bacteriocins and class IId non-pediocin-like single linear peptides were suggested and retained in a review of class II bacteriocins (Cotter *et al.* 2005a; Nissen-Meyer *et al.* 2009). In this chapter, the four-subdivision classification will be present (Table 1).

At least 34 one-peptide antilisterial bacteriocins are included in class **IIa** and also known as pediocin-like bacteriocins (Table 2, p19). In this group, bacteriocins, such as leucocin A (LeuA) (Hastings *et al.* 1991), mesentericin Y105 (Hechard *et al.* 1992) and pediocin (Henderson *et al.* 1992; Marugg *et al.* 1992; Nieto Lozano *et al.* 1992) have very similar amino acid sequences and contain conserved YGNGV/L "pediocin box" motif and a C-terminal disulfide bridge formed by two cysteines. According to sequence similarities in the C-terminal region, IIa bacteriocins may be further sorted into three or four subgroups (Fimland *et al.* 2005; Nissen-Meyer *et al.* 2009). The class **IIb** currently includes 16 unmodified members (Nissen-Meyer *et al.* 2009). Class IIb bacteriocins such as lactococcin G, plantaricins, and lactocin 705 are composed of two different peptides, both of which are necessary for the full antimicrobial activity (Oppegård *et al.* 2007). Class **IIc** bacteriocins for example enterocin AS-48, gassericin A and reutericin 6 are cyclic bacteriocins. In their structures, the N- and C-termini are linked by a covalent bond (Cotter *et al.* 2005a). Class **IId** represents various heterogeneous one-peptide linear non-pediocin-like bacteriocins. They do not fit to any existing classification schemes. The best characterised example of this group is lactococcin A produced by *Lc. lactis* strains. Like class IIa bacteriocins, it permeablises targeted cell membrane by binding to the mannose phosphotransferase permease (Holo *et al.* 1991; Diep *et al.* 2007).

Table 1. Classification of class II bacteriocins produced by lactic acid bacteria.

Class	Description	Examples	Producer	Reference
IIa	pediocin-like bacteriocins, antilisterial activity	Leucocin A	*Ln. gelidum* UAL 187	Hastings *et al.* 1991
		Pediocin	*P. acidilactici* PAC-1.0	Henderson *et al.* 1992
IIb	two-peptide bacteriocins, pore-forming activity	Lactococcin G	*Lc. lactis* LMG 2081	Nissen-Meyer *et al.* 1992
		Lactacin F	*Lb. johnsonii* VPI 11088	van Belkum *et al.* 1991
IIc	circular bacteriocins	Lactococcin 972	*Lc. lactis* IPLA 972	Martinez *et al.* 1999
IId	non-pediocin-like one-peptide bacteriocins	Enterocin Q	*E. faecium* L50	Cintas *et al.* 2000
		Leucocin B	*Ln. mesenteroides* TA33a	Papathanasopoulos *et al.* 1998

2.3.3 Class III: large heat-labile bacteriocins

Klaenhammer (1993) defined class III LAB bacteriocins as large (>30 kDa) heat-labile proteins often with enzymatic activity. In their domain-type structures, different domains function differently for translocation, binding to receptor, and antimicrobial activity (Cotter *et al.* 2005a). The class has been suggested to have another name: bacteriolysin, since these proteins were considered to degrade the targeted cell wall leading to cytolysis. However, helveticin J does not kill bacteria by lytic effect (Joerger and Klaenhammer 1986).

Therefore, they should not be literally called bacteriolysins. Up to date, five class III bacteriocins produced by LAB have been identified and characterised. In addition to helveticin J from *Lb. helveticus*, they are zoocin A from *Streptococcus zooepidermicus* (Simmonds *et al.* 1997), enterolysin A from *Enterococcus faecalis* (Hickey *et al.* 2003), millericin B from *Streptococcus milleri* (Beukes *et al.* 2000) and linocin M18 produced by *Brevibacterium linens* (Valdes-Stauber and Scherer 1994).

2.4 Class IIa bacteriocins

Class IIa bacteriocins are probably the most important and well-studied bacteriocins among the unmodified AMPs. Many LAB strains produce class IIa bacteriocins. These bacteriocins are also called pediocin-like bacteriocins, which is derived from the first characterised bacteriocin in this class, pediocin (Biswas *et al.* 1991; Nieto Lozano *et al.* 1992). Class IIa bacteriocins are regarded to possess a narrow antimicrobial spectrum. They are particularly effective against the foodborne pathogen *Listeria* strains. Recently, some class IIa bacteriocins have been found to be effective against both G^+ and G^- bacteria, such as *Campylobacteri jejuni* and *Escherichia coli* O157:H7 (Drider *et al.* 2006; Stern *et al.* 2006; Line *et al.* 2008; Svetoch *et al.* 2008).

Class IIa bacteriocins are heat-stable, cationic, partly amphiphilic and/or hydrophobic and small (<10 kDa) peptides with amino acid residues ranging from 37 to 48. These bacteriocins contain a highly conserved hydrophilic part in the N-terminal region and a variable C-terminal half which is comparatively hydrophobic (Figure 1) (Nissen-Meyer *et al.* 2009). In this group, curvacin A (Tichaczek 1993), LeuA (Hastings *et al.* 1991), mesentericin Y105 (Hechard *et al.* 1992), pediocin (Henderson *et al.* 1992; Marugg *et al.* 1992; Nieto Lozano *et al.* 1992) and sakacin P (Tichaczek *et al.* 1994) are the first bacteriocins that have been found and characterised. So far, 34 bacteriocins have been identified in this group (Belguesmia *et al.* 2011).

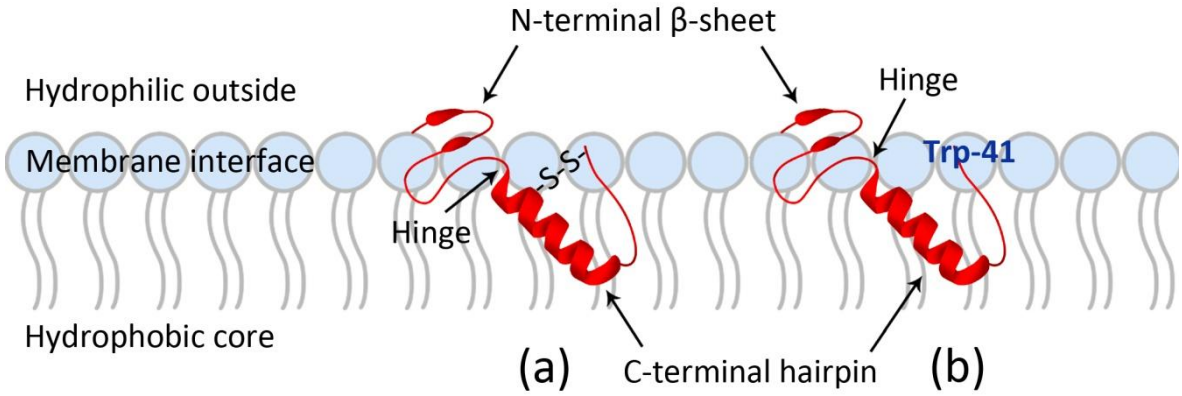

Figure 1. The structure and orientation of class IIa bacteriocins in membrane without receptor. The C-terminal hairpin structure is stabilised by (a) a disulfide bridge formed between two cysteines, e.g., pediocin, or (b) tryptophan near the C-end in the membrane interface, e.g., leucocin C. Trp, tryptophan. S-S, disulfide bridge. Hinge, Asp-17. Adapted from Nissen-Meyer *et al.* (2009).

Bacteriocins in this group possess the conserved "pediocin-box" motif (YGNGV/L) in their highly similar N-terminal region, but differ in the C-terminal halves. These two regions are connected by a hinge structure, which is at the highly conserved Asp-17 residue, providing structural flexibility for class IIa bacteriocins (Uteng *et al.* 2003). The diversity occurred in the hydrophobic C-termini offers a basis for further dividing these bacteriocins into four subclasses (Table 2) (Nissen-Meyer *et al.* 2009). The recently identified bacteriocin pentocin 31-1 has only been partly sequenced, but its incomplete amino acid sequence contains the YGNGV/L motif showing that this bacteriocin belongs to group IIa (Zhang *et al.* 2009). Bacteriocins in subgroup 2 are composed of fewer residues, because of shorter C-termini (Table 2). In the N-terminal region, there is a conserved disulfide bridge formed by two cysteines. In addition, another Cys-Cys disulfide bridge is also found in the C-termini of some class IIa bacteriocins, stabilising the hairpin structure. Peptides in subgroup 3 as well as enterocin SE-K4 and carnobacteriocin B2 in subgroup 4 exceptionally have neither the conserved tryptophan at the C-tails, nor the cysteine residues to form the C-terminal disulfide bridge (Table 2).

Table 2. Multiple sequence alignment of class IIa (pediocin-like) bacteriocins. Adapted from Nissen-Meyer *et al.* (2009).

	Class IIa bacteriocins	Amino acid sequence of mature peptide	Reference
Subgroup 1		. . . . .	
1	Divergicin M35	TKY**YGNGV**YCNSKKCWVDWGTAQGCID--VVIGQLGGGIPGKGKC	Tahiri *et al.* 2004
2	Coagulin	KY**YGNGV**TCGKHSCSVDWGKATTCIINNGAMAWATGGHQGTHKC	Le Marrec *et al.* 2000
3	Divercin V41	TKY**YGNGV**YCNSKKCWVDWGQASGCIGQTVVGGWLGGAIPG--KC	Metivier *et al.* 1998
4	Enterocin A	TTHSGKY**YGNGV**YCTKNKCTVDWAKATTCIAGMSIGGFLGGAIPG--KC	Aymerich *et al.* 1996
5	Pediocin	KY**YGNGV**TCGKHSCSVDWGKATTCIINNGAMAWATGGHQGNHKC	Henderson *et al.* 1992; Marugg *et al.* 1992
6	Sakacin P	KY**YGNGV**HCGKHSCTVDWGTAIGNIGNNAAANWATGGNAGWNK	Tichaczek *et al.* 1994
7	Listeriocin 743A	KS**YGNGV**QCNKKKCWVDWGSAISTIGNNSAANWATGGAAGWKS	Kalmokoff *et al.* 2001
8	Avicin A	TY**YGNGV**SCNKKGCSVDWGKAISIIGNNSAANLATGGAAGWKS	Birri *et al.* 2010
9	Mundticin L	KY**YGNGL**SCNKKGCSVDWGKAIGIIGNNSAANLATGGAAGWKS	Feng 2009
10	Mundticin KS	KY**YGNGV**SCNKKGCSVDWGKAIGIIGNNSAANLATGGAAGWKS	Kawamoto *et al.* 2002
11	Enterocin CRL35	KY**YGNGV**SCNKKGCSVDWGKAIGIIGNNSAANLATGGAAGWKS	Bennik *et al.* 1998
12	Piscicocin CS526	KY**YGNGL**S**X**NKKG**X**TVDWGTAIGIIGNNAAAN**X**ATGGAAG**X**NK	Yamazaki *et al.* 2005
13	Piscicolin 126	KY**YGNGV**SCNKNGCTVDWSKAIGIIGNNAAANLTTGGAAGWNKG	Bhugaloo-Vial *et al.* 1996; Jack *et al.* 1996
14	Sakacin 5X	KY**YGNGL**SCNKSGCSVDWSKAISIIGNNAVANLTTGGAAGWKS	Vaughan *et al.* 2001
15	**Leucocin C**	KN**YGNGV**HCTKKGCSVDWGYAWTNIANNSVMNGLTGGNAGWHN	Fimland *et al.* 2002b
16	Ubericin A	KTVN**YGNGL**Y**X**NQKK**X**WVNWSETATTIVNNSIMNGLTGGN	Heng *et al.* 2007
Subgroup 2			
17	Leucocin A	KY**YGNGV**HCTKSGCSVNWGEAFSAGVHRLANGGNGFW	Hastings *et al.* 1991
18	Mesentericin Y105	KY**YGNGV**HCTKSGCSVNWGEAASAGIHRLANGGNGFW	Fleury *et al.* 1996
19	Lactococcin MMFII	TS**YGNGV**HCNKSKCWID**V**SELETYKAGTVSNPKDILW	Ferchichi *et al.* 2001
20	Plantaricin 423	KY**YGNGV**TCGKHSCSVNWGQAFSCSVSHLANFGHGKC	van Reenen *et al.* 2003
21	Plantaricin C19	KY**YGNGL**SCSKKGCTVNWGQAFSCGVNRVATAGHGK**X**	Atrih *et al.* 2001
22	Sakacin G	KY**YGNGV**S**X**NSHGCSVNWGQAWT**X**GVNHLANGGHGVC	Simon *et al.* 2002
Subgroup 3			
23	Carnobacteriocin BM1	AIS**YGNGV**YCNKEKCWVNKAENKQAITGIVIGGWASSLAGMGH	Quadri *et al.* 1995
24	Curvacin A	ARS**YGNGV**YCNNKKCWVNRGEATQSIIGGMISGWASGLAGM	Tichaczek 1993
25	Enterocin P	ATRS**YGNGV**YCNNSKCWVNWGEAKENIAGIVISGWASGLAGMGH	Cintas *et al.* 1997
Subgroup 4			
26	Bacteriocin 31	ATY**YGNGL**YCNKQKCWVDWNKASREIGKIIVNGWVQHGPWAPR	Tomita *et al.* 1996
27	Bacteriocin RC714	ATY**YGNGL**YCNKEKCWVDWNQAKGEIGKIIVNGWVNHGPWAPR	Del Campo *et al.* 2001
28	Bacteriocin T8	ATY**YGNGL**YCNKEKCWVDWNQAKGEIGKIIVNGWVNHGPWAPRR	De Kwaadsteniet *et al.* 2006
29	Carnobacteriocin B2	VN**YGNGV**SCSKTKCSVNWGQAFQERYTAGINSFVSGVASGAGSIGRRP	Quadri *et al.* 1995
30	Enterocin SE-K4	ATY**YGNGV**YCNTQKCWVDWSRARSEIIDRGVKAYVNGFTKVLG	Eguchi *et al.* 2001
31	Penocin A	KY**YGNGV**HCGKKTCYVDWGQATASIGKIIVNGWTQHGPWAHR	Diep *et al.* 2006

Table 2. Continued

Unclassified			
32	Enterocin E50-52	TTKN**YGNGV**CNSVNWCQCGNVWASCNLATGCAAWLCKLA	Svetoch *et al.* 2008
33	Pentocin 31-1	VIAD**YGNGV**R**X**ATLL	Zhang *et al.* 2009
34	Bacteriocin OR-7	KTY**YG**T**NGV**HCTKNSLWGKVRLKNMKYDQNTTYMGRLQDILLGWATGAFGKTFH	Stern *et al.* 2006

The YGNGV/L 'pediocin box' is marked (bold), the leucine residue (L), odd, and uncertain amino acids (X) are marked as bold and red. Names of the six newly identified class IIa bacteriocins are in red (Rea *et al.* 2011). The sequence similarity (conserved residues) in the peptides of different subgroups are shaded with different colours. Shading patterns are listed as following: flexible hinge at the conserved Asp/Asn-17 (green), Trp and Met (black), Val (pink), Ile (violet), Thr and Arg (teal), Cys (grey).

2.4.1 Mode of action

Class IIa bacteriocins are active against various *Carnobacterium*, *Clostridium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Listeria*, and *Pediococcus* strains. These bacteriocins kill bacteria by forming pores in the cytoplasmic membrane. The targeted cell membrane is consequently permeabilised, leading to the depletion of the intracellular ATP pool, the disturbance of the proton motif force, and eventually the cell death (Chikindas *et al.* 1993; Herranz *et al.* 2001; Suzuki *et al.* 2005). On one hand, the highly conserved hydrophilic part in the N-terminal region facilitates the interaction between the bacteriocin and the negatively charged phospholipid-containing membrane and/or acidic bacterial cell wall. On the other hand, the variable C-terminal half which is comparatively hydrophobic permits the bacteriocin permeabilise the targeted cell membrane (Nissen-Meyer *et al.* 2009).

Circular dichroism (CD) spectroscopy and nuclear magnetic resonance (NMR) analysis on the structure of several class IIa bacteriocins showed that these bacteriocins only formed specific structures when they exposed to a membrane-mimicking matrix (Fregeau Gallagher *et al.* 1997; Watson *et al.* 2001; Uteng *et al.* 2003; Haugen *et al.* 2005). After contacting with the targeted cell membrane, the N-terminal half forms an S-shaped β-sheet structure stabilized by a conserved disulfide bridge, while the C-terminus concomitantly forms a hairpin structure. The C-terminal hairpin is comprised of an amphiphilic α-helix and an extended C-tail folding back to the central helix (Uteng *et al.* 2003; Johnsen *et al.* 2005a). In some cases, such as divercin V41, enterocin A and pediocin, the hairpin structure is stabilised by a disulfide bridge formed by two cysteine residues. However, most class IIa bacteriocins do not contain the two-cysteine frame, therefore the central tryptophan (Trp-18) and the C-terminal tryptophan stabilise the hairpin structure (Figure 1, p18) (Nissen-Meyer *et al.* 2009). The two domains of a class IIa bacteriocin are connected by a flexible hinge that permits the movement of either domain towards each other and the penetration of the C-terminal hairpin into the membrane central part (Uteng *et al.* 2003).

The N- and C-terminal domains may have independent functions in the mode of action of class IIa bacteriocins. By exchanging domains between several class IIa bacteriocins, hybrid bacteriocins were constructed and investigated (Johnsen *et al.* 2005a). The active hybrid bacteriocins displayed similar target-cell specificities with the parental bacteriocin which donated the C-terminal hairpin but quite different from N-terminal donator (Johnsen

et al. 2005a). The results showed that the conserved N-terminal β-sheet domain mediates the binding of the bacteriocin to the targeted cell membrane surface, but the C-terminal part is responsible in the determination of the target-cell specificity. The specificity seemed to rely on the interactions between the C-termini of the class IIa bacteriocins and the lipids and/or proteins in the hydrophobic core of the targeted cell membrane (Johnsen *et al.* 2005a).

In this interaction, one protein expressed by the targeted cell has been suggested to be the targeted molecule (bacteriocin receptor): the membrane-associated mannose phosphotransferase system (PTS) permease, named EII_t^{Man} (Hechard *et al.* 2001; Ramnath *et al.* 2004; Xue *et al.* 2005; Drider *et al.* 2006). In both G^+ and G^- bacteria, PTSs are involved in transport and phosphorylation of carbohydrates (Postma *et al.* 1993). In *L. monocytogenes*, EII_t^{Man} is one of the main glucose transporters (Stoll and Goebel 2010). The mannose PTS permeases are composed of IIA, IIB, IIC and IID four domains, arranged in two to four subunits. The IIA and IIB are cytoplasmic domains responsible for phosphorylation, while the IIC and IID membrane domains function in transport (Postma *et al.* 1993). In *L. monocytogenes* the IIA and IIB domains of the EII_t^{Man} are fused as a single subunit complex (Drider *et al.* 2006).

The EII_t^{Man} permease is known to be encoded by the *mptACD* operon. By inactivating this operon by directed mutagenesis in *L. monocytogenes* and *E. faecalis*, the mutant strains displayed high resistance to the class IIa bacteriocin mesentericin Y105 (Dalet *et al.* 2001; Hechard *et al.* 2001). Heterologous expression of *mptACD* operon of *L. monocytogenes* in an insensitive *Lc. lactis* rendered the sensitivity of the resultant *Lc. lactis* strain to divergent class IIa bacteriocins (Ramnath *et al.* 2004). Meanwhile, separate expression of each gene encoding fused IIAB, IIC and IID domains specified that the IIC itself is sufficient in the target recognition by class IIa bacteriocins (Ramnath *et al.* 2004). The membrane-located IIC and IID have both been shown to be necessary for the receptor (Diep *et al.* 2007). Later, co-expression of IIC and IID conferred strong and similar sensitivity to class IIa bacteriocins with the host which only produces IIC, confirming that IIC is the main determinant of specificity (Kjos *et al.* 2010). An N-terminal extracellular loop comprised of 40 aa in this IIC domain was then identified to be the major determinant of the species-specificity. The loop thus may function as an interaction site for the recognition of class IIa bacteriocins (Kjos *et al.* 2010). The function of IID is not yet known, but it might indirectly interact with the bacteriocin during the pore formation. In a

latest review, the IID was considered to have a structural function helping IIC to fold correctly in the membrane (Figure 2, p26) (Kjos *et al.* 2011).

The receptor recognition and the subsequent pore formation require serial steps and different regions of a class IIa bacteriocin. The N-terminal β-sheet of a class IIa bacteriocin firstly binds to the extracellular loop of the IIC domain of EII_t^{Man}. The C-terminal hairpin of the bacteriocin then inserts into the membrane resulting in the helix-helix interactions between the bacteriocin central α-helix and transmembrane IIC and/or IID of the mannose permease. Subsequently, the insertion of bacteriocin renders structural changes in the EII_t^{Man} contributing to the opening of the permease, i.e., the formation of pores (Figure 2, p26) (Kjos *et al.* 2011).

2.4.2 Bacteriocin immunity

In order to protect the producers from being killed by their own bacteriocins, bacteria have a self protective system termed immunity. The immunity of bacteriocins is accomplished by the protective proteins that are co-produced with the bacteriocins. According to BACTIBASE, among all 34 class IIa bacteriocins, more than 20 have been shown to have putative immunity proteins (Drider *et al.* 2006).

An immunity protein is encoded by an immunity gene which is located immediately downstream from its cognate class IIa bacteriocin structural gene usually in the same operon. Transcription of both bacteriocin and its cognate immunity genes is co-regulated in bacterial cells, as immunity can be shut down concomitantly (Eijsink *et al.* 1998). For example, sakacin P producer *Lb. sake* LTH673 has a stable bacteriocin-free phenotype. The strain has been shown to become sensitive to its own bacteriocin and several other class IIa bacteriocins (added in culture), when the cells stopped producing sakacin P (Eijsink *et al.* 1998).

The immunity proteins, which sizes range from 81 to 115 amino acids, share only a little similarities in their amino acid sequences, although class IIa bacteriocins are quite similar to each other in the sequences. However, common sequence motifs may still be identified based on comparison studies, and three subgroups have been consequently proposed (Fimland *et al.* 2002a). The subgroup B contains immunity proteins of bacteriocins like sakacin P, listeriocin 743A and piscicolin 126, which belong to the same subgroup of class

IIa bacteriocins without C-terminal disulfide bridges (Table 2, p19). In these immunity proteins, there is a noticeable motif (SNIRYGY) in the C-terminal end. Another two immunity proteins in this subgroup are orfβ3-im and divT2-im. They share some consensus motifs with the other immunity proteins but do not have corresponding AMPs (Fimland *et al.* 2005).

The NMR solution and crystal structures of immunity proteins for carnobacteriocin B2 (subgroup C), enterocin A and pediocin (subgroup A) showed that there is a bundle comprised of four antiparallel helix and a flexible C-tail in the structure of these proteins (Dalhus *et al.* 2003; Sprules *et al.* 2004; Johnsen *et al.* 2005b). Later, the structure of subgroup B immunity protein (piscicolin 126-im) was shown to have the conserved four-helix bundle but relatively shorter and less flexible C-terminal part (Martin-Visscher *et al.* 2008). Structural studies on hybrid immunity proteins proposed that the C-terminal parts of the immunity proteins determine the immunity and specific recognition of the C-terminal hairpin of class IIa bacteriocins (Johnsen *et al.* 2004; Johnsen *et al.* 2005a). Moreover, piscicolin 126 immunity protein contains a C-terminal hydrophobic pocket comprising residues that are highly conserved in subgroup B immunity proteins (Martin-Visscher *et al.* 2008). As hydrophobic interactions are normally involved in the interactions between proteins, this region may be crucial in the interaction between the bacteriocin and its immunity protein (Martin-Visscher *et al.* 2008).

Heterologous expression of an immunity gene in sensitive bacteria makes the bacteria less sensitive to the corresponding class IIa bacteriocin, confirming the function of the immunity protein (Fimland *et al.* 2005). The differences in the sequences among immunity proteins suggest these proteins are highly specific to their cognate bacteriocins. For example, LeuA immunity protein expressed in *E. faecalis* and *Lb. sake* provided maximum protection against its own bacteriocin but not against leucocin C (Fimland *et al.* 2002a). Nevertheless, expression of an immunity gene in a heterologous host may provide protection not only to its corresponding bacteriocin but also to some other similar class IIa bacteriocins (Eijsink *et al.* 1998). The concept of cross-immunity was also proposed based on the evidence that certain immunity proteins are most likely to protect against structurally related bacteriocins (Fimland *et al.* 2002a). For example, sakacin P immunity protein (sakP-im) from group B is able to protect cells against pediocin, leucocin C and its own bacteriocin sakacin P. All three belong to the same subclass (subgroup 1) of class IIa bacteriocins. However, sakP-im-producing bacteria were susceptive to enterocin A, which

is also in the subgroup 1, even though enterocin A immunity protein protected against sakacin P.

Class IIa bacteriocins are secreted to extracellular environment, but their immunity proteins retain inside of the cells. So the intracellular immunity proteins may hardly have direct interaction with their bacteriocins, especially because the C-terminal hairpin structure of class IIa bacteriocins is too small to penetrate through the targeted cell membrane (Figure 1, p18 and Figure 2) (Quadri *et al.* 1995). Besides, the function of immunity protein has been found to be strain-dependent (Johnsen *et al.* 2005a). Therefore, instead of direct binding to the bacteriocins, the immunity proteins may interact with a receptor protein which varies in different strains (Figure 2 and Figure 3, p30) (Fimland *et al.* 2002a; Johnsen *et al.* 2004).

As mentioned before, the EII_t^{Man} permease functions as the receptor for class IIa bacteriocins. This receptor was also suggested to mediate immunity mechanism of the cells. The immunity proteins have been shown to bind the mannose permease strongly only when the bacteriocin also connects the permease (Diep *et al.* 2007). The flexible C-terminal part of the immunity protein has been shown to recognise the C-terminal half of the cognate bacteriocin, but no direct physical contact has been found between the two proteins (Sprules *et al.* 2004; Johnsen *et al.* 2005b). The immunity proteins do not prevent the formation of bacteriocin-receptor complex by binding to the receptors competitively. They also do not interact directly with the bacteriocins or cause structural changes of the bacteriocins. Instead, these proteins interact with the cytoplasmic parts of the IIC and/or IID domains of EII_t^{Man}, thus blocking the pore formed by the receptor-bacteriocin complex and interfering the membrane leakage (Figure 2) (Kjos *et al.* 2011). As the C-termini of class IIa bacteriocins vary to a large extent, the pores formed by these bacteriocins may have different characteristics, such as acidity, size and hydrophobicity. The sequential variations in the immunity proteins, therefore, may allow the immunity proteins fit and block the pores formed by the cognate bacteriocins (Figure 2, 3 p30) (Kjos *et al.* 2011). As a side effect, glucose transport is also blocked, leading to the reduced growth on glucose.

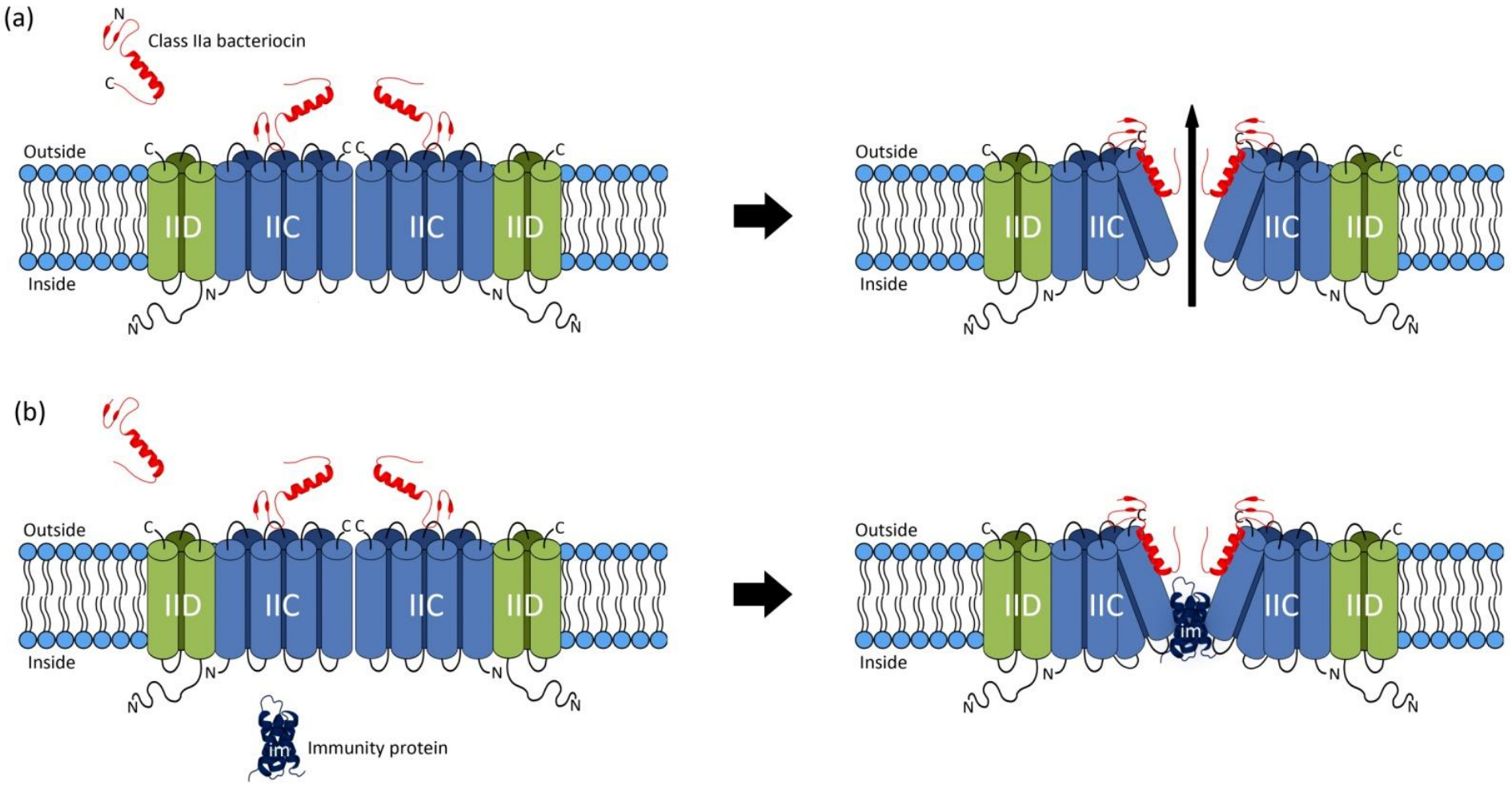

Figure 2. Proposed model of action of class IIa bacteriocins (a) and immunity (b). The N-terminal β-sheet of the bacteriocin (red) binds to an extracellular loop (a) of the EII_t^{Man} IIC domain (left). Then, the C-terminal helix interacts with transmembrane helices of the IIC and/or IID proteins (right) causing conformational changes, thereby resulting in the pore formation and eventually cell death. In immune cells (b), the bacteriocin mediates the same conformational changes, but the pore is blocked by a specific immunity protein (dark blue) which tightly binds to EII_t^{Man}-bacteriocin complex. Adapted from Kjos *et al*. (2011).

2.4.3 Biosynthesis

Class IIa bacteriocins are commonly found to be encoded by genes on plasmids, but there are a few exceptions. For example, genes for enterocin A (Aymerich *et al.* 1996), divercin V41 (Metivier *et al.* 1998), sakacin P (Huhne *et al.* 1996), and carnobacteriocin B2 and BM1 (Quadri *et al.* 1997b) are localised on bacterial chromosomes. Yet, despite of the different loci of the bacteriocin genes, a class IIa bacteriocin usually need 2-8 genes for its production. The genes: 1) a structural gene, 2) an immunity gene, 3) one or two genes for ATP-binding cassette (ABC) transporter, and 4) a gene encoding accessory protein, have been shown to involve in class IIa bacteriocin production, secretion of functional peptides, and protection of the producer cells. The structural gene encodes an inactive pre-bacteriocin containing an N-terminal leader peptide (Nes *et al.* 1996; Ennahar *et al.* 2000). The corresponding immunity gene, usually in the same operon, is co-transcribed along with the bacteriocin precursor. The ABC transporters and their accessory proteins that are responsible for the secretion of the bacteriocins are encoded by a cluster of genes usually localised in different operon than the bacteriocin/immunity operon (Klaenhammer 1993; Ennahar *et al.* 2000).

The structural gene and the genes at flanking regions are often organised in one or a few operons being transcribed independently (Fimland *et al.* 2005). Commonly, genes involved in the production of class IIa bacteriocins are arranged in three operons: one for the structural and immunity genes, the second for transporter genes, and the third for regulation of bacteriocin production. However, bacteriocins like pediocin, plantaricin 423 and coagulin only have one operon containing all four genes for the production (Ennahar *et al.* 2000). Due to particular DNA rearrangements, the divercin V41 operon has particular gene organisation (Metivier *et al.* 1998).

The leader peptides of most class IIa pre-bacteriocins are of GG-type. However, several class IIa bacteriocins, for example, listeriocin 743A (Kalmokoff *et al.* 2001), bacteriocin 31 (Tomita *et al.* 1996) and enterocin P (Cintas *et al.* 1997) have been found to have a *sec*-type leader. These bacteriocins are assumed to be secreted by *sec*-dependent exporting pathway.

The GG-type leaders can be recognised by the dedicated ABC transporters (Ennahar *et al.* 2000). The ABC transporters for production of class IIa bacteriocins have an extra N-

terminal cysteine protease domain that is used to cut off the leader after the GG-motif (Figure 3, p30) (Havarstein *et al.* 1995). The GG-leader is consequently removed by the ABC transporters during transmembrane translocation, and the mature bacteriocin is subsequently secreted (Havarstein *et al.* 1995). By exchanging the leader peptides, it is possible to heterologously express class IIa bacteriocin via another secretion system, which may be more efficient (Ennahar *et al.* 2000).

In addition, the existence of leader peptides in bacteriocin precursors also protects the producer at the cytoplasmic side of the cell membrane. Studies on pre-LeuA (Fregeau Gallagher *et al.* 1997), pre-mesentericin Y105 (Biet *et al.* 1998) and pre-carnobacteriocin B2 (Quadri *et al.* 1997a) revealed reduced activities to sensitive cells compared with their mature peptides. The pre-bacteriocin maintains inactive and interferes in the interactions between the bacteriocin and the target cell membrane (Drider *et al.* 2006). In the leader peptide of pre-bacteriocin, an amphiphilic α-helix structure containing 10 amino acids has been identified. The minor helix folds back onto the central α-helix of the bacteriocin without causing other structural changes. This helix-helix structure interferes in the interaction between the central part of bacteriocin and the receptor on targeted cell membrane, thereby diminishing its antimicrobial activity (Sprules *et al.* 2004).

2.4.4 Regulation of biosynthesis

The productions of some class IIa bacteriocins are regulated. Bacteriocins such as curvacin A, enterocin A, and sakacin P are transcriptionally regulated by quorum sensing, a mechanism for monitoring the population density. Quorum sensing mechanisms for regulating the production of class IIa bacteriocins are usually termed as three component regulatory systems (Figure 3, p30) (Fimland *et al.* 2005; Drider *et al.* 2006). The three components in 3CS are encoded by a set of genes encoding 1) peptide pheromone as an induction factor (IF), 2) a transmembrane histidine protein kinase (HPK), and 3) a cytosolic response regulator (RR) (Ennahar *et al.* 2000).

Like associated bacteriocins, the IF is also ribosomally synthesised at low level as a prepeptide with a GG-type leader peptide, which is subsequently processed and secreted by the dedicated ABC transporter (Nes and Eijsink 1999; Ennahar *et al.* 2000). The IF functions as a signal in the induction of the transcription of particular genes. In such regulatory mechanism, IF production requires low constitutive expression. Concomitant

with the increase of cell density, IF concentration gradually accumulates to a certain level that triggers the autoinduction loop. The excess IF then activates HPK. Consequently, the conserved His residue at the cytosolic side of HPK is autophosphorylated. The HPK-P is then able to transfer its phosphate group onto the conserved Asp residue of corresponding RR. The RR-P therefore can bind to specific promoters in order to stimulate the transcription of the bacteriocin genes (Nes and Eijsink 1999).

Some class IIa bacteriocins like divercin V41 from *Carnobacterium divergens* V41 are produced under the regulation of 2CS (Metivier *et al.* 1998). The 2CS regulatory system is composed of HPK and RR but not peptide pheromone. Additionally, it is worth mentioning that carnobacteriocins B2 and BM1 have both 2CS and 3CS for their regulations (Saucier *et al.* 1995; Quadri *et al.* 1997b).

In addition to the quorum sensing mediated regulation, the syntheses of several class IIa bacteriocins are liable to environmental factors, for example temperature, ionic strength and pH. The optimum temperature for bacteriocin synthesis has been reported to be near 20 ºC, and the bacteria generally tend to stop producing bacteriocins when the temperature is 37 ºC or higher (Cintas *et al.* 1997; Fimland *et al.* 2000). Most bacteriocins show decreased activity at 37 ºC or higher temperature. However, pediocin has been shown to remain active at higher temperature, because of its disulfide bridge at C-terminal region stabilising the bacteriocin structure (Fimland *et al.* 2000; Kaur *et al.* 2004). The influence of environmental temperature on the synthesis seems to overwhelm the innate quorum sensing regulatory mechanisms (Diep *et al.* 2000). For example, the production of sakacin A, which is naturally regulated by the quorum-sensing mechanism, ceases at about 37 ºC, even though the inducer peptide has been added into the growth medium (Nes and Eijsink 1999).

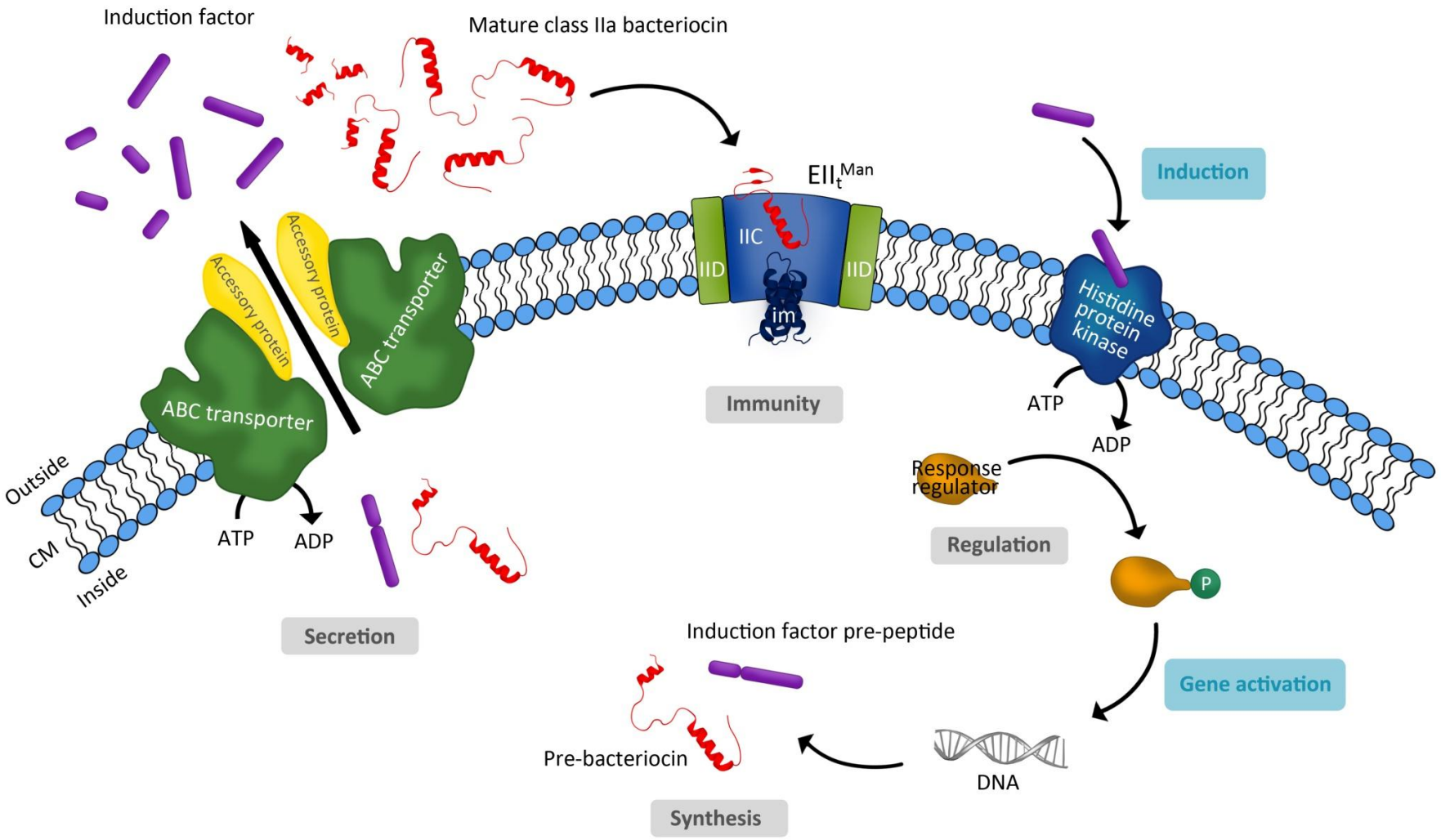

Figure 3. The production of class IIa bacteriocin: three-component regulatory system, biosynthesis, secretion, and immunity. CM, cell membrane. Im, immunity protein. EII$_t^{Man}$, mannose PTS permease, class IIa bacteriocin receptor. IIC and IID are the two EII$_t^{Man}$ domains that interact with class IIa bacteriocins. Adapted from Ennahar *et al.* (2000).

2.5 Heterologous expression of class IIa bacteriocins

Due to their potential for food biopreservation, bacteriocins produced by LAB have drawn broad attention, though natural defects of the producers limit the use of bacteriocins. First of all, most of bacteriocin producing LAB kill bacteria within narrow range, or the production of bacteriocins is not efficient. Further, the ability of producing bacteriocins by LAB can be spontaneously lost. Besides, LAB may not survive in certain food conditions, or they may cause unwanted flavours in foods. Therefore, heterologous expression of bacteriocins facilitates manipulation of bacteriocin gene expression, development of starter strains with increased antimicrobial properties, and functional studies on recombinant AMPs (Rodríguez *et al.* 2003).

In this chapter, the heterologous expression will focus on secretion of class IIa bacteriocins, which can be achieved by 1) cloning the entire bacteriocin operon into the desired strain or 2) exchanging leader peptides with either GG-type- or *sec*-dependent leader (Cintas *et al.* 2011).

Expression of native bacteriocin genes

Pediocin *ped* operon from *Pediococcus acidilactici* PAC1.0 has been cloned into *E. coli* and the sensitive strain *P. pentosaceus* PPE1.2. The results showed that pediocin was correctly produced and secreted by the hosts (Marugg *et al.* 1992). Usually, heterologous production of bacteriocins with this approach is at lower level than that of the native producer. By increasing the copy number of the recombinant pediocin plasmid and using the lactococcal constitutive promoter P32, the production of pediocin in *Lc. lactis* LL108 could be increased to the wild-type level (Chikindas *et al.* 1995). Nevertheless, abovementioned strains were not applicable in dairy industry. Therefore, *Lc. lactis* MM210 was then used as the production host, and the subsequent pediocin-producing strain together with other native cheese starters evidently retarded the growth of *Listeria* in experimental cheese (Buyong *et al.* 1998). In a later study, the intact pediocin operon was cloned into a nisin-inducible plasmid and functionally expressed in *S. thermophilus*, *Lc. lactis* and *Lb. casei (Renye and Somkuti 2010)*.

Enterocin A is naturally produced by *Enterococcus faecium* DPC1146 under the control of 3CS. Cloning of all genes and regulatory regions permitted the production of enterocin A and its induction factor by *E. faecalis* OG1X. However, bioactive enterocin A could also

be produced in *E. faecalis* OG1X and *L. lactis* IL1403, when only the structural (*entA*), immunity (*entI*), transporter and accessory genes (*entTD*) were expressed under the control of the promoter P32 (O'Keeffe *et al.* 1999).

Exchanging leader peptides

Most of the LAB bacteriocins require a dedicated processing and secretory system, i.e., ABC transporter and an accessory protein (Ennahar *et al.* 2000). The ABC transporter recognises its dedicated leader peptide and cut it off when the bacteriocin is secreted (Ennahar *et al.* 2000). The leaders and corresponding transporters are similar in most class II bacteriocins and some lantibiotics (Franke *et al.* 1999; Rodríguez *et al.* 2003). Therefore, exchanging leader peptides and/or dedicated transportation system allows the heterologous production of bacteriocins in LAB hosts.

Pediocin from *P. acidilactici* and lactococcin A from *Lc. lactis* WM4 both have GG-type leaders and their dedicated secretory systems. Gene encoding mature pediocin was cloned into the lactococcin translocation apparatus as a fusion with the lactococcin signal sequence, resulting in the secretion of functional pediocin (Horn *et al.* 1998). As *Lc. lactis* is naturally resistant to pediocin, the immunity gene *pedB* is not necessary for the production. However, co-expressing *pedB* increased the pediocin production to the level of the parental strain (Arques *et al.* 2008).

In a recent study, the *papA* gene encoding mature pediocin was fused to *usp45* signal sequence after a constitutive promoter P_{45} and a nisin promoter P_{nisZ} in a lactococcal expression vector. The resulting plasmid was then transferred into *Lc. lactis* NZ9000 with nisin regulatory genes *nisK* and *nisR* integrated into the chromosome. As a result, bioactive pediocin was produced by *Lc. lactis* with and without nisin induction, though the production level was higher with nisin induction (Li *et al.* 2011).

Active pediocin has also been secreted by *Bifidobacterium longum*, *E.coli* and *Lb. reuteri* with bifidobacterial α-amylase signal sequence (Moon *et al.* 2005; Eom *et al.* 2010). In addition, pediocin has been fused to the precursor of the maltose-binding protein (MBP) and secreted as a part of the resulting chimeric protein via *sec*-dependent secretory system. The signal peptide of MBP precursor is recognised by the *sec*-secretory system of *E. coli* and cut off during the secretion resulting in active MBP-pediocin protein (Miller *et al.* 1998).

The production of mesentericin Y105 (MesY105) in *Ln. mesenteroides* requires *mesYI* operon, encoding pre-MesY105 with GG-leader and the immunity protein, and the *mesCDE* operon for secretion. Cloning the two operons allowed the production of MesY105 in *E. coli* and *Ln. mesenteroides*. By replacing the gene encoding mature MesY105 with the gene for mature pediocin, the latter could be produced via MesY105 secretion mechanism in *Ln. mesenteroides* (Morisset and Frere 2002).

Another means that has been actively studied for heterologous production of bacteriocins is the general secretory pathway. In prokaryotes, *sec*-dependent pathway is a commonly existing universal protein export mechanism (Pugsley 1993).

Enterocin P (EntP) is a *sec*-dependent bacteriocin from *E. faecium* P13 (Herranz and Driessen 2005). The EntP gene along with its immunity gene were cloned and expressed via *sec*-dependent pathway in *E. coli*, but the production level was comparatively lower than that of the native producer (Gutierrez *et al.* 2005) However, the EntP signal peptide of *E. faecium* P13 could allow the efficient production of EntP in several *Lc. lactis* strains, including the nisin producer *Lc. lactis* DPC5598. Introduction of the EntP encoding plasmid in *Lc. lactis* DPC5598 allowed the co-production of both EntP and nisin (Gutierrez *et al.* 2006).

Divergicin A is another example of a bacteriocin exported by the general secretion pathway. By fusing divergicin A signal sequence with gene encoding mature carnobacteriocin B2, the bacteriocin could be secreted via the general secretory pathway by several heterologous hosts, like *C. piscicola* strains and *Lc. lactis* IL1403 (McCormick *et al.* 1996). Heterologous production of MesY105 was also achieved in *E. coli* DH5α by replacing its own leader with the divergicin A signal peptide, although the general secretory pathway seemed not to be as efficient as the bacteriocin's own secretion system (Biet *et al.* 1998).

In addition, yeast can also be used to produce bacteriocins. By inserting pediocin structural gene into an expression cassette in *Saccharomyces cerevisiae*, the bioactive bacteriocin was successfully produced to reduce spoilage bacteria in yeast-based fermentations. However, because of the attachment of bacteriocin on yeast cells, the concentration of free pediocin in the yeast culture supernatant was undesirably low (Schoeman *et al.* 1999).

2.6 Leucocin C and other *Leuconostoc* bacteriocins

Bacteriocins produced by *Leuconostoc* species are termed leucocins or mesentericins, usually characterised as heat-stable and class II bacteriocins. However, the nomenclature of leucocins lacks systematic agreement. For example LeuA has been called with at least 9 different names, including some confusing ones, such as leucocin B TA11a and leucocin C LA7a (Table 3). Several *Leuconostoc* strains like *Ln. carnosum* Ta11a, *Ln. gelidum* UAL 187 and *Ln. paramesenteroides* La7a seem to produce more than one bacteriocin, as they have been named differently, but these leucocins are actually either identical or nearly identical with LeuA (Hastings *et al.* 1991; Felix *et al.* 1994; Hastings 1996).

Because of their structural and functional disparities, leucocins are not sorted in one class or subclass according to the commonly accepted classification described in chapter 2.3 (Klaenhammer 1993). The most studied leucocin is the class IIa bacteriocin LeuA (Hastings *et al.* 1991). Leucocin S (previously called leuconocin S) belongs to class IV in Klaenhammer's classification (Lewus 1992; Klaenhammer 1993). Leucocin B-TA33a (31 aa) is a class IId bacteriocin sharing 62% identity with mesenterocin 52B, a 32 aa bacteriocin (Corbier *et al.* 2001; Nissen-Meyer *et al.* 2009). The name leucocin D-La54a is now abolished and replaced by carnocin 54 which classification has not been mentioned but which kills *Listeria* strongly dependent on pH (Schillinger *et al.* 1995).

Among leucocins, A-, B- and C-type bacteriocins are widely present. *Ln. carnosum* TA33a produces all three leucocins, whereas *Ln. carnosum* 4010 and *Ln. mesenteroides* 6 have not been shown to produce leucocin B but only A and C (Papathanasopoulos *et al.* 1998; Vaughan *et al.* 2001; Budde *et al.* 2003).

Table 3. Bacteriocins produced by *Leuconostoc* strains.

Name	Producer	Description	Reference
Leucocin A			
Leucocin 6A	*Ln. mesenteroides* 6	Nearly identical to leucocin A UAL 187 (S12X)	Vaughan *et al.* 2001
Leucocin A 4010	*Ln. carnosum* 4010	Partially sequenced, 33 aa	Budde *et al.* 2003
Leucocin A (ΔC7)	*Ln. pseudomesenteroides* QU 15	Deficient in seven C-terminal residues compared with leucocin A UAL 187	Sawa *et al.* 2010
Leucocin A QU 15	*Ln. pseudomesenteroides* QU 15	Identical to leucocin A UAL 187	Sawa *et al.* 2010
Leucocin A TA33a	*Ln. mesenteroides* TA33a	Identical to leucocin A UAL 187	Papathanasopoulos *et al.* 1998
Leucocin A UAL 187	*Ln. gelidum* UAL 187	24 aa leader and 37 aa mature peptide, mmnmkptesyeqldnsaleqvvggKYYGNGVHCTKSGCSVNWGEAFSAGVHRLANGGNGFW	Hastings *et al.* 1991
Leucocin B TA11a	*Ln. carnosum* TA11a	7 aa different from leucocin A UAL 187 in leader peptide, mature part identical to leucocin A	Felix *et al.* 1994
Leucocin C LA7a	*Ln./W. paramesenteroides* LA7a	Identical to leucocin B TA11a	Hastings 1996
Leucocin E TA33a	*Ln. mesenteroides* TA33a	Identical to leucocin A	Hastings 1996
Leucocin B			
Leucocin B TA33a	*Ln. mesenteroides* TA33a	31 aa, class IId	Papathanasopoulos *et al.* 1998
Leucocin C			
Leucocin C	*Ln. mesenteroides* 6	43 aa of mature peptide, unknown leader sequence, KNYGNGVHCTKKGCSVDWGYAWTNIANNSVMNGLTGGNAGWHN	Fimland *et al.* 2002b
Leucocin 6C	*Ln. mesenteroides* 6	Partially sequenced, 35 aa	Vaughan *et al.* 2001
Leucocin 7C	*Ln. mesenteroides* 7	Nearly identical to leucocin C TA33a (A22W), partially sequenced, 30 aa	Vaughan *et al.* 2001
Leucocin 10C	*Ln. mesenteroides* 10	Nearly identical to leucocin C TA33a (A22W), partially sequenced, 35 aa	Vaughan *et al.* 2001
Leucocin B 4010	*Ln. carnosum* 4010	Identical with leucocin C-Ta33a, partially sequenced, 39 aa	Budde *et al.* 2003
Leucocin C TA33a	*Ln. mesenteroides* TA33a	Partially sequenced, 36 aa, identical to leucocin C	Papathanasopoulos *et al.* 1998
Others			
Leucocin D La54a	*Ln. carnosum* La54a	Aka carnocin 54, antilisterial	Keppler *et al.* 1994
Leucocin F10	*Ln. carnosum* F10	pH and α- amylase sensitive	Parente *et al.* 1996
Leucocin Hα	*Ln.* MF215B	Two peptide bacteriocin	Blom 1999
Leucocin J	*Ln.* sp. J2	Aka leuconocin J	Choi 1999
Leucocin K	*Ln. paraplantarum* C7	52 aa	Accession number: AF420260
Leucocin N	*Ln. pseudomesenteroides* QU 15	Class IId, same operon as LeuQ	Sawa *et al.* 2010
Leucocin Q	*Ln. pseudomesenteroides* QU 15	Class IId, same operon as LeuN	Sawa *et al.* 2010
Leucocin S	*Ln. paramesenteroides* OX	Aka leuconocin S, α-amylase sensitive	Lewus 1992

Bacteriocins produced by *Leuconostoc* but not named as leucocin			
Mesentericin ST99	*Ln. mesenteroides* ST99		Todorov and Dicks 2004
Mesentericin Y105	*Ln. mesenteroides* Y105	Class IIa, 24 aa leader and 37 aa mature peptide, mtnmksveayqqldnqnlkkvvggKYYGNGVHCTKSGCSVNWGEAASAGIHRLANGGNGFW	Hechard *et al.* 1992
Mesenterocin 5	*Ln. mesenteroides* UL5		Daba *et al.* 1991
Mesenterocin 52A	*Ln. mesenteroides* FR52	Identical to mesentericin Y105	Revol-Junelles *et al.* 1996
Mesenterocin 52B	*Ln. mesenteroides* FR52	Class IId	Revol-Junelles *et al.* 1996
Mesenterocin E131	*Ln. mesenteroides* E131	Identical to mesentericin Y105	Xiraphi *et al.* 2008
Mesenterocin Y	*Ln. mesenteroides* E131	Partially purified peptide	Zdolec *et al.* 2008
Mesenterocin	*Ln. mesenteroides OZ.*	Chromosome-associated	Osmanagaoglu and Kiran 2011

Leucocin C (LecC), a member of class IIa bacteriocins, has been found to be produced by several *Leuconostoc* strains, such as *Ln. carnosum* 4010 and *Ln. mesenteroides* TA33a. Interestingly, *Ln. mesenteroides* 7 and 10 seemed to produce only one type peptide which amino acid sequence was nearly identical to LecC, but the C-terminal of this peptide could not be well identified (Vaughan *et al.* 2001). LecC shares high homology in the primary amino acid structure with bacteriocins in subgroup 1 of class IIa. Like other class IIa bacteriocins, it renders target cell death by permeabilising the cell membrane, and shows noticeably specific activity against several LAB and *Listeria* (Nes *et al.* 2001).

The earlier study published the incomplete LecC sequence consisting only 36 aa. However, the contradiction between the calculated molecular mass according to the sequence and the mass spectrometry results (4597-4598 Da) revealed that more residues were missing (Papathanasopoulos *et al.* 1997). Five years later, the complete amino acid sequence of mature LecC was determined, and its theoretical molecular weight (4596 Da) was closely consistent with the previous experimental value (Papathanasopoulos *et al.* 1997; Fimland *et al.* 2002b). Pediocin-like bacteriocins are translated as inactive precursors with N-terminal leader peptide mediating exportation of the bacteriocins (Fimland *et al.* 2005). However, since LecC was isolated as secreted peptide from culture supernatant, it was unable to identify the sequence of LecC leader peptide in the previous study (Fimland *et al.* 2002b). Since then, its leader sequence has never been identified.

As described in chapter 2.4.4 (P_{28}), immunity proteins of class IIa bacteriocins confer immunity against several other members in this group than its corresponding bacteriocin. However, it is not the case for leucocin producer. *Ln. mesenteroides* strains 6 and TA33a, and *Ln. carnosum* 4010 have been found to produce both leucocins A and C, but previous experimental results showed the LeuA-im producer was still killed by LecC (Papathanasopoulos *et al.* 1998; Vaughan *et al.* 2001; Fimland *et al.* 2002a; Budde *et al.* 2003). Therefore, there should be a LecC dedicated self-protective system in LecC native producers protecting against this bacteriocin. However, no such system has been published.

Up to now, the sequence of LecC gene has not been revealed, and there are still no hints for the other genes that are conventionally required for the production of IIa bacteriocins in any published studies.

3 AIMS OF THE STUDY

Since LeuA immunity protein does not provide immunity against LecC, *Ln. carnosum* 4010 needs a LecC-dedicated immunity system to survive from its own bacteriocin. The necessity for an immunity gene indicates that there would also be other LecC-dedicated genes, and that LecC is a bacteriocin with independent production system. The aims of this study were to localise the gene cluster for LecC production in *Ln. carnosum* 4010 genome, to characterise the genes and to demonstrate their explicit functions. In addition to the structural and immunity genes, LecC gene cluster could also contain a transporter gene(s) and/or regulatory genes. This study also aimed to produce LecC in a heterologous host *Lc. lactis* by exchanging *lecC* own signal sequence with lactococcal signal sequence of *usp45* for development of new strains with improved antilisterial activity.

4 MATERIALS AND METHODS

4.1 Bacterial strains, plasmids and culture conditions

Bacterial strains used in this study are listed in Table 4. BD Difco™ Agar (Granulated) (Difco, Becton Dickinson and Company Sparks, MD, USA) was used as solidifying agent for culture media, 1.5% for agar plates, and 0.75% for soft-agar. *Ln. carnosum* 4010 was grown on M17 (Oxoid Ltd. Basingstoke, UK) agar or in broth supplemented with 0.5% (w/v) glucose (M17G) at 30 °C, or on brain heart infusion (BHI; Lab M Ltd. Topley House, UK) agar at 30 °C. *Lc. lactis* strains were grown on M17G or BHI agar or broth at 30 °C. *E. coli* was grown in Luria-Bertani (LB; 10 g/l Bacto™ Tryptone, 5 g/l Bacto™ Yeast Extract, 10 g/l NaCl) agar or broth at 37 °C with shaking. *L. monocytogenes* was grown in BHI agar or broth at 30 °C with shaking. *Lb. rhamnosus* was grown on Man-Rogosa-Sharpe (MRS; Lab M Ltd. Topley House, UK) agar at 37 °C. *Lb. plantarum* was grown in MRS broth at 30 °C.

Plasmids used in this study are listed in Table 5. To maintain and select plasmids in *Lc. lactis*, media were supplemented with 50 IU nisin /ml (Nis^{50}). Erythromycin was used at final concentrations of 250 µg/ml for *E. coli* and 5 µg/ml for *Listeria*.

Table 4. Bacterial strains used in this study.

Bacterial strain	Relevant properties	Reference/source
Ln. carnosum 4010	Wild-type leucocin C producer	Budde *et al.* 2003
Lb. plantarum WHE 92	Wild-type pediocin producer	Ennahar *et al.* 1996
Lb. rhamnosus 1/6	Wild-type strain, *pepR* promoter	Varmanen *et al.* 1998,Valio Ltd., FI
E. coli TG1	Transformation host strain	Sambrook and Russell 2001
E. coli ECO758	TG1 carrying *lecI*-plasmid pLEB732	This study
E. coli SAA594	TG1 carrying vector pTF1	Takala, unpublished
Lc. lactis MG1363	Transformation host strain for *lecCI* cloning	Gasson 1983
Lc. lactis NZ9000	Transformation host, MG1363 with *nisK* and *nisR* integrated into the chromosome; nisin-inducible strain	Kuipers *et al.* 1998
Lc. lactis LAC360	NZ9000 carrying vector pLEB690	Li *et al.* 2011
Lc. lactis LAC361	NZ9000 carrying pediocin-plasmid pLEB691	Li *et al.* 2011
Lc. lactis LAC405	MG1363 carrying *lecC*-plasmid pLEB728	This study
Lc. lactis LAC406	MG1363 carrying *lecCI*-plasmid pLEB729	This study
Lc. lactis LAC407	MG1363 carrying *lecI*-plasmid pLEB730	This study
Lc. lactis LAC408	NZ9000 carrying *lecCI*-plasmid pLEB729	This study
Lc. lactis LAC409	NZ9000 carrying *lecC*-plasmid pLEB728	This study
Lc. lactis LAC411	MG1363 carrying vector pLEB690	This study
Lc. lactis LAC412	MG1363 carrying pediocin-plasmid pLEB691	This study
L. monocytogenes WSLC 1018	Transformation host strain for *lecI* cloning, indicator, LecC sensitive	A gift from prof. Martin Loessner, ETH Zürich, CH
L. monocytogenes MUU22	WSLC 1018 carrying vector pTF1	This study
L. monocytogenes MUU23	WSLC 1018 carrying *lecI*-plasmid pLEB732	This study

Table 5. Plasmids used and constructed in this study.

Plasmid	Relevant properties	Reference/source
pLEB688	*Lc. lactis* food-grade expression vector, P_{45} promoter, 3466 bp, Nis^R	Li *et al.* 2011; P_{45}, Koivula *et al.* 1991
pLEB690	*Lc. lactis* food-grade secretion vector harbouring lactococcal *SSusp45*, P_{45} and P_{nisZ} promoters, 3746 bp, Nis^R	Li *et al.* 2011; *SSusp45*, van Asseldonk *et al.* 1990
pLEB691	*papA* fused with *usp45* in pLEB690	Li *et al.* 2011
pLEB728	*lecC* without its own secretion signal in pLEB690 (*Nae*I)	This study
pLEB729	*lecCI* without *lecC* secretion signal in pLEB690 (*Nae*I)	This study
pLEB730	*lecI* in pLEB688 (*Nco*I/*Sma*I)	This study
pLEB732	*lecI* with *pepR* promoter from *Lb. rhamnosus* 1/6 in pTF1 (*Eco*RI/*Sma*I)	This study
pTF1	*E. coli*/*Listeria* shuttle vector, 3043 bp, Erm^R; pLEB579 carrying multiple cloning site from pBluescript	Takala and Fieseler, unpublished; pLEB579, Beasley *et al.* 2004
pTMT46	Leucocin A and its immunity genes	Takala, unpublished

4.2 DNA isolations

4.2.1 Isolation of plasmids from *Leuconostoc*

Ln. carnosum 4010 plasmid DNA was isolated according to Anderson and McKay rapid method of isolating large plasmids from *Lactococcus* with some modifications (Anderson and McKay 1983). The recipes of required solutions are listed in Appendix 1.

Ln. carnosum 4010 was grown overnight in 3 ml M17G at 30 °C. From the o/n culture, 1 ml was inoculated into 50 ml M17G and incubated at 30 °C for 14 h. The cells were collected by centrifugation (10000 rpm, 4 min, JA-14, Beckman Avanti™ J-251). Pelleted cells were resuspended in 3.8 ml buffer 1 and divided into two 15 ml Falcon tubes. Lysozyme was dissolved in buffer 2 with final concentration of 20 mg/ml. Then, for each tube, 485 µl of lysozyme solution and 25 µl of mutanolysin (5000 U/ml) were added in the cell suspension. Lysozyme and mutanolysin degrade the cell wall. The mixture was incubated at 37 °C for 1 h. After incubation, 240 µl of buffer 3 and 138 µl of buffer 4 were consecutively blended into each tube. SDS in buffer 4 dissolves cell membranes and therefore lyses the cells. If the cells have been lysed, the solution should become clear and viscous after adding buffer 4. The solution was then incubated at 37 °C for 10 min in order to achieve complete lysis. After cell lysis, the tubes were vortexed for 1 min. DNA was denatured with 138 µl of freshly prepared 3.0 N NaOH and the tubes were inverted gently for 10 min. The blend was neutralised by adding 250 µl of buffer 5 followed by 3 min gentle mixing. After mixing, 360 µl of 5.0 M NaCl was blended. In highly concentrated salt solution, chromosomal DNA and proteins are precipitated, while plasmid DNA retains in soluble form. Phenol (5 ml) was added into each tube and thoroughly mixed. After centrifugal separation (10000 rpm, 5 min), the upper aqueous layer was transferred into new Falcon tube. Phenol extraction was repeated if upper layer was still turbid. The clear aqueous layer was subsequently extracted with 5 ml of chloroform-isoamyl alcohol (24:1) and centrifuged. The resultant upper layer was transferred and precipitated with 1 volume of isopropanol in freezer for 1 h. The precipitated plasmid DNA was collected by centrifugation (10000 rpm, 25 min). Pelleted DNA was washed with 4 ml 70% ethanol. The DNA was collected by centrifugation (10000 rpm, 5 min) and thoroughly dried before dissolving in H_2O. RNA in the obtained DNA sample was degraded by RNase (0.1 mg/ml).

4.2.2 Isolation of plasmids from *E. coli* and *Lactococcus*

E. coli strains carrying plasmids were grown in 3 ml LB broth with appropriate antibiotic selection. Cells were collected by centrifugation. Vector and constructed plasmids were isolated from corresponding *E. coli* strains with EZNA plasmid mini kit (Omega Bio-tek, GA, USA) according to supplier's instructions.

Recombinant *Lactococcus* strains were grown in 5 ml M17G broth with appropriate antibiotic selection. Cells were collected by centrifugation. Plasmids in these strains were

isolated with EZNA plasmid mini kit with the following modification. After resuspending *Lactococcus* cells in solution I, lysozyme (20 mg/ml) was added. The mixture was incubated at 37 °C for 1 h. After incubation, the mixture was further processed according to supplier's instructions.

4.3 DNA manipulations

4.3.1 Enzymatic reactions

Restriction enzymes used in this study were from Fermentas (Vilnius, Lithuania), New England Biolabs (Ipswich, MA, USA), and Promega (Madison, WI, USA). Enzymes and DNA samples were mixed and incubated at the optimal reaction temperature in the buffers recommended by the suppliers.

For DNA insert ligation into vector DNA, reaction mixture was prepared as followed: 300 ng linear vector DNA, 5:1 molar ratio of insert DNA over vector, 3 µl 10 × T4 DNA ligase buffer, 0.5 µl T4 DNA ligase, and H_2O to total volume 30 µl. For linear DNA self-ligation, reaction was in the following condition: 50 ng linear DNA, 5 µl 10 × T4 DNA ligase buffer, 1 µl T4 DNA ligase, and H_2O to total volume 50 µl. Reactions were incubated at room temperature for 30 min, and the enzyme was heat inactivated at 65 °C for 10 min.

In order to prevent self-ligation of vectors, calf intestine alkaline phosphatase (CIP, New England Biolabs) was used to remove 5' phosphate from linear vector DNA. CIP treatment was performed at 37 °C for 30 min. Since CIP cannot be heat inactivated, CIP treated DNA was purified with the PCR clean-up system kit (Promega).

As primers are supplied non-phosphorylated, PCR fragments do not contain phosphate at their 5'-ends. Therefore, to ensure ligation of PCR products, T4 polynucleotide kinase (PNK, New England Biolabs) was used for phosphorylation. The PCR products were phosphorylated in T4 DNA ligase buffer (New England Biolabs) and incubated at 37 °C for 30 min. PNK treated DNA samples were directly used in ligation without kit purification.

Enzymes and their cognate buffers, and reaction conditions recommended by the manufacturers are listed in Appendix 2.

4.3.2 Polymerase Chain Reaction (PCR)

Fragments for screening, cloning or sequencing in this study were amplified by standard PCR (Sambrook and Russell 2001) with either *Taq* DyNAzyme™ II DNA polymerase or Phusion High-Fidelity DNA polymerase (Finnzymes, Espoo, Finland) in Eppendorf Mastercycler (Hamburg, Germany). *Taq* PCRs were carried out in reaction volume of 25 or 50 µl with the following conditions: 1 × DyNAzyme™ buffer, 0.2 mM dNTPs (Fermentas, Finnzymes), 0.5 µM forward primer, 0.5 µM reverse primer, 0.5 or 1 U DyNAzyme™ II DNA polymerase (Fermentas), and 0.5 or 1 µl template DNA. In order to ensure high fidelity of DNA amplification for cloning or sequencing, Phusion DNA polymerase with proofreading function was used. Phusion PCRs were performed in reaction volume of 50 µl with the following condition: 1 × Phusion HF buffer (Finnzymes), 0.2 mM dNTPs (Fermentas), 0.5 µM forward primer, 0.5 µM reverse primer, 1 U Phusion High-Fidelity DNA polymerase (Finnzymes), and 1 µl template DNA. PCR programmes used in this study are listed in Appendix 3. Primers used in PCR are listed in Table 6 (p45).

Inverse PCR (iPCR) was carried out like standard PCR. The DNA template for iPCR was firstly restricted with enzyme, purified with PCR clean-up kit (Promega), and self-ligated. The ligation mixture was used in iPCR without purification.

4.3.3 DNA kit purifications and concentration measurement

Linear DNA samples either extracted from agarose gel or directly from PCR or enzymatic treatments were purified with Wizard® SV Gel and PCR clean-up system kit (Promega).

The concentrations of DNA samples, including PCR products and plasmids were measured by NanoDrop-1000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

4.4 Transformations

The transfer of DNA into bacteria was carried out by electroporation with Bio-Rad GenePulser device (Bio-Rad Laboratories, Richmond, CA, USA). Buffers and media used for electroporation were listed in Appendix 4.

4.4.1 *Lc. lactis* transformation

Lc. lactis strains were grown overnight in M17G broth at 30 °C. From the o/n culture, 500 µl was inoculated into pre-warmed (at 30 °C) 50 ml M17GS supplemented with 1% glycine. When the cell wall is weakened by glycine, high concentration of sucrose protects the cells from lysis by stabilising the osmotic pressure. The cells were grown at 30 °C for 4-6 h until OD_{600} reached about 0.5. Culture was cooled down on ice before centrifugation (7000 rpm, 7 min, 4 °C) for collecting cells. Cells were washed twice with ice-cold electroporation buffer and resuspended in 1 ml buffer, and frozen in 100 µl aliquots. For electroporation, 100 µl competent cells were mixed with maximum 10 µl DNA samples in electroporation cuvette (gap 2 mm). Electric pulse was generated at 2 kV, 25 µFD and 400 Ω resulting in time constants about 7.5-8.5 ms. Cells were incubated in 1 ml of recovery media at 30 °C for 1 h. The cells were plated on M17G agar with Nis[50]. Plates were incubated at 30 °C for 1-2 d.

4.4.2 *L. monocytogenes* transformation

L. monocytogenes was grown overnight in BHI broth at 30 °C with shaking. To prepare competent *Listeria* cells, 500 µl of the o/n culture was inoculated in 10 ml BHI supplemented with 0.5 M sucrose. The cells were incubated at 30 °C with shaking for 2-3 h until OD_{600} reached about 0.2. Then, 10 µg/ml penicillin G was added into the culture and the cells were grown for 2 h. Cells were cooled down on ice and collected by centrifugation (7000 rpm, 5 min, 4 °C). Cells were washed 3 times with ice-cold electroporation buffer and resuspended in 80 µl of the same buffer. For electroporation, competent cells were kept on ice (< 1 min) and mixed with maximum 10 µl DNA samples in electroporation cuvette (gap 2 mm). Electric pulse was generated at 2.2 kV, 25 µFD and 200 Ω resulting in time constants about 4.2-4.8 ms. Cells were regenerated in 1 ml of recovery media at 30 °C for 2 h with shaking. The cells were plated on BHI agar with erythromycin. Plates were incubated at 30 °C for 2-3 d.

4.4.3 *E. coli* transformation

E. coli was grown overnight in LB broth at 37 °C with shaking. From the o/n culture, 2 ml was inoculated in 200 ml fresh LB. The culture was incubated at 37 °C with shaking for 2-3 h until OD_{600} reached about 0.5. The cells were cooled down on ice and collected by

centrifugation (7000 rpm, 5 min, 4 °C). Cells were washed 3 times with ice-cold 10% glycerol. After washing, the cells were resuspended in 2 ml of 10% glycerol, and frozen in 50 µl aliquots. For electroporation, 50 µl competent *E. coli* cells were mixed with maximum 5 µl DNA samples in electroporation cuvette (gap 2 mm). Electric pulse was generated at 2.5 kV, 25 µFD and 200 Ω resulting in time constants about 4.2-4.8 ms. Cells were incubated in 1 ml of recovery media SOC at 37 °C with shaking for 1 h. The cells were plated on LB agar with erythromycin. Plates were incubated at 37 °C for o/n.

4.5 Southern blot

DNA was transferred from gel to nylon membrane by vacuum blotting (LKB Bromma 2016 VacuGene Vacuum Blotting Pump). DNA on membrane was hybridised and detected according to Boehringer-Mannheim (Germany) non-radioactive digoxigenin (DIG) labelling kit (DNA Labeling and Detection Kit). DNA probes used in this study were made by PCR, in which DIG-labelled dUTP is randomly incorporated with *Taq* polymerase. In Southern blot, DNA was separated in agarose gel, denaturated in gel, and transferred to nylon membrane (Southern 1975). During o/n incubation at 68 °C, DIG-labelled DNA probes specifically hybridise with the complementary, single stranded DNA fragment(s) on the membrane. In immunological detection, the alkaline-phosphatase-conjugated DIG antibody binds to DIG-labelled probes. Then, the membrane is covered with NBT/X-phosphate solution. The phosphatase attached to the antibody degrades the substrate X-phosphate, resulting in the formation of the insoluble NBT diformazan with purple colour on the membrane. Buffers and solutions used in Southern blot and detection are listed in Appendix 5.

4.6 Leucocin C antilisterial activity bioassay

LecC antilisterial activity was determined by conventional spot-on-lawn method. The indicator strain *L. monocytogenes* WSLC 1018 was grown in BHI media for o/n. 200 µl of the cell culture was added to 5 ml of melted (about 50 °C) BHI soft agar, thoroughly mixed, and poured onto the top of BHI agar plate. A 5 µl droplet of either cell suspension or pasteurised culture supernatant (75 °C, 10 min) was spotted onto the surface of the BHI soft agar. The zone of inhibition was observed on *Listeria* plate after o/n incubation at 30 °C.

4.7 Leucocin C immunity assay

To determine *Listeria* sensitivity to LecC, 3 µl of o/n cultures were added to 300 µl BHI Erm[5] containing *Lc. lactis* LecC supernatant with different concentrations (0-100 µl/ml) in Bioscreen microtiter plates (100 wells). The plates were grown in Bioscreen C (Labsystems, Helsinki, Finland) at 30 °C with constant shaking for 24 h. The optical density was measured at 600 nm wavelength in every 2 h.

4.8 Primers and sequencing

PCR products and plasmid constructs were sequenced by out-sourced DNA sequencing service (Institute of Biotechnology, University of Helsinki). Primers used in this study are listed in Table 6. Primers were synthesised by Oligomer Oy (Helsinki, Finland), except *lecC* forward and reverse primers, *pepR* and *leuAI* primers, which were synthesised before this study. In order to obtain complete sequence of large unknown DNA fragments, primer walking method was used, which included serial sequencing, designing and synthesising new primers (Figure 9, p52).

Table 6. Primers used in this study. Relevant restriction sites added to the primers are underlined.

Primer	Use, restriction site	Sequence 5' → 3'
lecC forw	*lecC*	AAAAACTACGGTAACGGTGTTCACTG
lecC rev	*lecC*, 1600 bp, 2500 bp	ACTGCATGCTTACTTGTGCCAACCAGCGTTACC
lecC new rev	*lecC*	TTAGTTATGCCATCCAGCATTGC
lecC inverse forw	inverse PCR	GTTATGAATGGCCTAACCGGC
lecC inverse rev	inverse PCR	GTTTGCGATGTTGGTCCACGC
lecI forw	*lecI*, *Bsp*HI	ACTTCATGAAAATAAGATGGTTTTCTGGTG
lecI rev	*lecI*, *lecI* probe	TCAGTAGCCATATCTAATACTAGA
*lecI Apo*I forw	*lecI* probe	TCTGCTGTTCCCCTTATAGATG
leuAI forw	*leuAI*	AAGTATTATGGTAACGAGTTC
leuAI rev	*leuAI*	ACTGAATTCGCGGCCGCTATCTTTCAAAGATACTATAAAAC
P_{pepR} forw	P_{pepR}, *Eco*RI	ATGTGGAATTCTGCTTTGATACTCACCA
P_{pepR} rev	P_{pepR}	AGCCGGATCCTTAGGTCAGGATCGTTGTTC
Trans rev	1600 bp, 2500 bp	GTTGATTTCCCAGAACCACT
lecC upstr seq	primer walking	ATAGCCCCAATCAACTGAGC
Trans seq1	primer walking	GACATGGAAGGAACGACTGC
Trans seq2	primer walking	GCGTTGAGACGATTAAAGCG
Trans seq3	primer walking	CCCAATAGCTCATTAGTAGC
Trans seq4	primer walking	CCAACTCTGATTATTGTGCC
Trans seq5	primer walking	CTAAAGGCACAACCATTGCG
repA downstr forw	vector primer	TTATTTTGGTTTGATGTTGCGAT
Erm rev	vector primer	TTATAGTTTTGGTCGTAGAGC

4.9 Data analyses

Primers were designed manually according to the existing and/or obtained sequences. Oligo analyzer (http://eu.idtdna.com/analyzer/applications/oligoanalyzer/) was used to analyse designed PCR primers and to determine the melting temperatures for PCR or sequencing.

The search for homology of DNA and amino acid sequences was performed with BLAST (blastn and blastp, http://blast.ncbi.nlm.nih.gov/Blast.cgi). Another online tool, ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/), along with BLAST, was also used to generate sequence alignments for DNA or proteins for sequence comparison.

NEBcutter V2.0, an online programme available to the public (http://tools.neb.com/NEBcutter2/), was used to determine the cleavage sites of DNA with restriction enzymes.

A translate tool in ExPASy (SIB Bioinformatics resource portal, http://web.expasy.org/translate/) was introduced to predict possible ORFs and to translate nucleotide DNA sequences to protein sequences.

5 RESULTS

5.1 Localisation of leucocin A and C genes

As most class IIa bacteriocin genes are plasmid-associated, the first aim in this study was to find out the loci of the genes needed for LeuA and LecC production in *Ln. carnosum* 4010 genome with *leuA* and *lecC* gene probes by Southern blot. The probes were produced and labelled by PCR. The *leuA* gene was amplified with its immunity gene (*leuAI*) with PCR primers designed according to previously published gene sequences. Because of the unknown nucleotide sequence of *lecC*, the PCR primers for amplifying the *lecC* gene were designed according to LecC amino acid sequence. The amplicons were confirmed to be *leuA* and *lecC* by sequencing (Takala, unpublished result).

The *Ln. carnosum* 4010 plasmid DNA was isolated, separated in gel electrophoresis, transferred to nylon membrane and hybridised with DIG-labelled *leuA* and *lecC* DNA

probes. *Ln. carnosum* 4010 was found to carry several plasmids (Figure 4 (a)). In the Anderson and McKay plasmid isolation method, some chromosomal DNA is also obtained resulting in an extra band of about 14 kb. Two plasmid bands were below the chromosomal band, and two plasmid bands above. It is not known whether those bands are all different plasmids or same plasmids of different supercoiling degrees. However, as the smallest and the largest bands migrated very differently in gel, at least these two bands should represent different plasmids. The *lecC* probe hybridised with two bands, the largest plasmid band and the chromosomal band, while *leuA* probe hybridised with other plasmid bands (Figure 4 (b)). Coincidentally, the *leuA* positive control plasmid pTMT46 produced bands with nearly the same size as those from the native *leuA* plasmid of 4010. During plasmid isolation, large plasmids might undergo partial degradation, which resulted in the migration of plasmid fragments along with the chromosomal band in agarose gel. Therefore, the *lecC* signal observed from the chromosomal band was presumably due to the presence of broken plasmids. In conclusion, the genes for the productions of the two bacteriocins were shown to be both plasmid-associated but located in different plasmids of *Ln. carnosum* 4010.

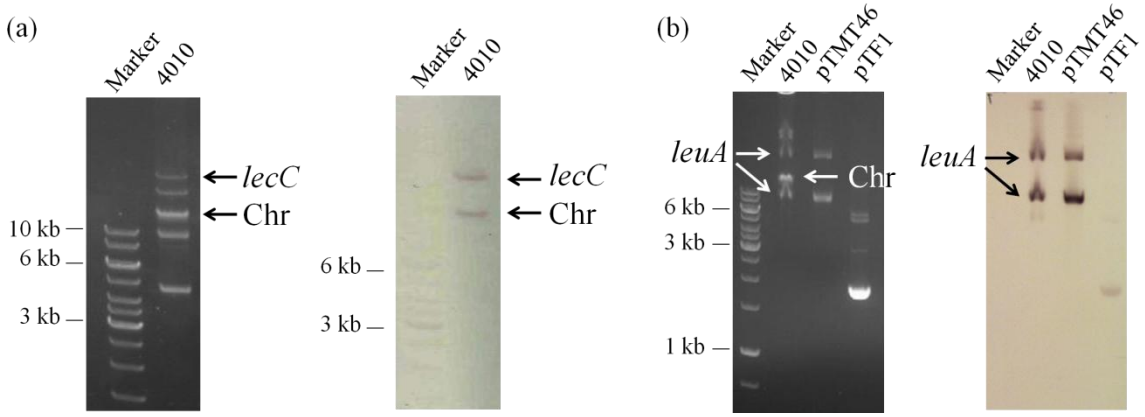

Figure 4. Agarose gel electrophoresis of *Ln. carnosum* 4010 total DNA and Southern blot membranes hybridised with *lecC* (a) or *leuA* (b) probes. Chr, chromosomal band. pTMT46, previously constructed plasmid carrying *leuA*, about 8 kb. pTF1, negative control plasmid. Marker, GeneRuler™ 1 kb DNA ladder (Fermentas).

5.2 Characterisation of leucocin C operons

Since *lecC* gene was shown to be located in a different plasmid than the one carrying *leuA*, it seemed that the two bacteriocins would have independent systems for their productions, and the large plasmid probably would also carry other LecC related genes. Therefore, the next aim was to identify the possible genes needed for LecC production by sequencing the flanking regions of *lecC*. Different approaches, including iPCR and restriction-Southern strategy, were used to obtain *lecC*-flanking fragments for sequencing. As both upstream

and downstream regions of *lecC* were searched simultaneously, and as it is more convenient to present them separately, the following paragraphs are not in an entirely chronological order.

5.2.1 The immunity gene

The immunity gene of a class IIa bacteriocin is usually found in the same operon downstream of the corresponding bacteriocin gene. In the subclassification of immunity proteins for class IIa bacteriocins, subgroup B immunity proteins share a C-terminal conserved motif (SNIRYGY) (Figure 5). The corresponding bacteriocins for all of these structurally similar immunity proteins belong to the subgroup 1 (Table 2, p19 and Figure 5). Since LeuC is also considered to belong to subgroup 1, its immunity protein is likely to contain the same C-terminal motif (SNIRYGY). Therefore, a consensus PCR primer (*lecI* reverse) was designed according to gene sequences of the conserved C-terminal regions of subgroup B immunity proteins and used in PCR with the *lecC* forward primer in order to obtain the fragment containing *lecC* and its putative immunity gene.

The amplicon with the size of 432 bp was obtained and confirmed to contain *lecC* and a novel ORF by sequencing. Between 132 bp *lecC* and the new 294 bp gene there were six non-coding bases (Figure 6). The mature *lecC* and its downstream ORF, named *lecI* in this study, were found to be unique genes. The nucleotide sequences of *lecC* and *lecI* shared no significant sequence similarity with any known genes in GenBank. However, the protein encoded by *lecI* was homologous with several class IIa immunity proteins, with the best matches to listeriocin 743A and sakacin P immunity proteins (48% identity, 73% similarity) (Figure 5). Though, no homology was found between LecC and LeuA immunity proteins.

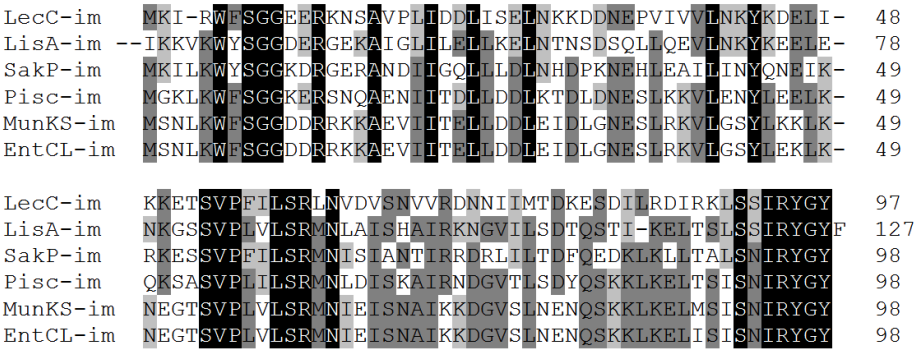

Figure 5. Sequence alignment of leucocin C immunity protein and its similar immunity proteins of other class IIa bacteriocins. The immunity proteins for the following class IIa bacteriocins belong to subgroup B: leucocin C (LecC-im), listeriocin 743A (LisA-im), sakacin P (SakP-im), piscicolin 126 (Pisc-im), mundticin KS (MunKS-im) and enterocin CRL35 (EntCL-im). Black, grey and light grey boxes indicate regions of sequence similarity. Except LecC-im, the figure was adapted from Fimland *et al.* (2005).

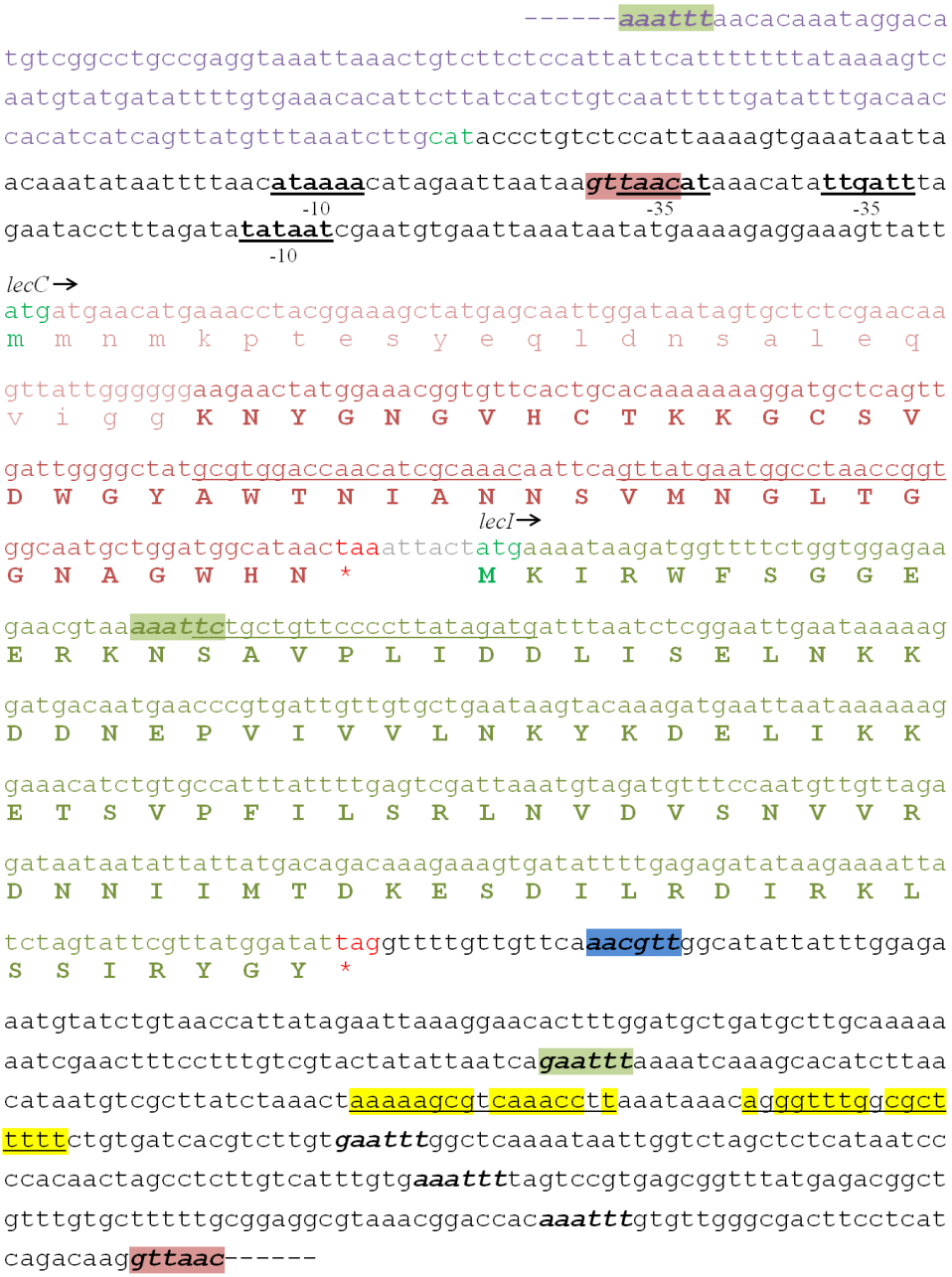

Figure 6. Nucleotide sequence of the *lecCI* operon and their flanking regions. Restriction sites are in bold and italic with different highlight colours: relevant *Apo*I light green, other *Apo*I sites bold and italic, *Hpa*I light red, *Acl*I light blue. Sequence in purple refers to the accessory gene nearly identical with that of leucocin A. PCR primers are underlined: inverse primers in *lecC* gene, *lecI Apo* forward primer in *lecI* gene. Terminator of *lecCI* operon is underlined and palindromic sequences are highlighted with yellow.

5.2.2 The downstream of *lecI*

As the gene sequences of *lecI* only confirmed the existence of LecC immunity gene, the actual 3'-end of *lecI* and its downstream sequence still remained unknown. Thus, in order to identify the downstream sequence of *lecI*, restriction-Southern method was used. The *lecI* probe for Southern blot was produced and labelled by PCR. The *Ln. carnosum* 4010 plasmid DNA was digested with *Apo*I, separated in gel, transferred to nylon membrane,

and hybridised with *lecI* probe. The *lecI* gene was cloned into pLEB688 and used as the positive control (detailed information in chapter 5.3, p52).

The quality of plasmid DNA preparation was good enough to get clear bands after *Apo*I restriction (Figure 7 (a)). Two bands with approximate size of 400 bp and 300 bp were detected on nylon membrane (Figure 7 (b)). Detected fragments were extracted from gel, and ligated with pTF1 (*Eco*RI, compatible with *Apo*I). Using *lecI Apo*I forward and vector Erm reverse primers and the ligation mixture (400 bp *Apo*+pTF1) as template, fragments with the size of about 500 bp, 600 bp and 1 kb were amplified by PCR (Figure 7 (c)). As the expected size of amplicons from *lecI Apo*I forward primer and the vector primer was about 570 bp, the 600 bp band should carry the right insert, and the other two bands might come from unspecific binding of primers. The 600 bp fragment was then extracted from gel and sequenced. A 392 bp fragment comprised by *lecI* and its downstream sequence (136 bp) was identified. However, the existence of a transcriptional terminator or a new ORF was not found (Figure 6, p49).

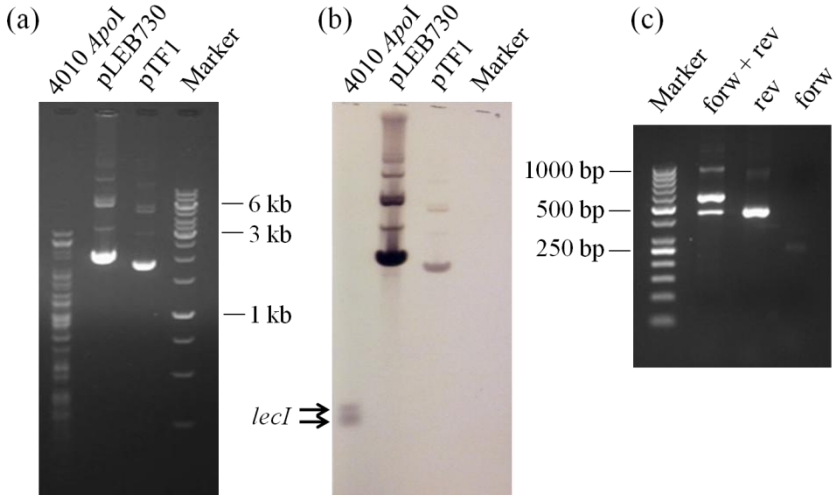

Figure 7. Agarose gel electrophoresis of *Ln. carnosum* 4010 total DNA restricted with *Apo*I, plasmid pLEB730 carrying cloned *lecI*, empty vector pTF1 (a) and its corresponding hybridised membrane with *lecI* probe after Southern blot (b); agarose gel of PCR amplicons from ligation mixture (400 bp *Apo*+pTF1) with *lecI Apo*I forward (forw) and Erm reverse (rev) primers (c). Markers in (a, b), GeneRuler™ 1 kb DNA ladder (Fermentas). Marker in (c), GeneRuler™ 50 bp DNA ladder (Fermentas).

To acquire more genetic information of the downstream region of *lecI*, the *Ln. carnosum* genomic DNA was restricted with *Hpa*I, self-ligated and used as the template in iPCR. A 1 kb band containing *lecCI* and its downstream sequence was obtained by iPCR and confirmed by sequencing with *lecI Apo*I forward primer. From 178 bp downstream from the stop codon of *lecI*, there started a nearly perfect palindromic sequence, which might function as a Rho-independent transcription terminator (Figure 6, p49).

5.2.3 The upstream of *lecC*

The presence of the *lecCI* terminator indicated the end of the operon. However, the start point, namely the upstream region of this operon remained unknown. *Ln. carnosum* 4010 plasmid DNA restricted by *Apo*I was self-ligated and used as the template in iPCR. A segment with the size of about 600 bp was amplified. This fragment harbouring *lecC* and its upstream region was confirmed by sequencing. The upstream region of *lecC* was found to be 99% identical to that of *leuA* operon from *Ln. gelidum*, and seemed to contain a gene encoding accessory protein for bacteriocin secretion (Figure 6, p49). In the sequence, a 72 bp signal sequence encoding a GG-type leader was identified. The *SSlecC* was different from the *SSleuA* in *Ln. gelidium* UAL187 by 5 nt, whereas the leader peptide differed by only 1 aa residue (Figure 8).

SSlecC-4010 MMNMKPTESYEQLDNSALEQVIGG
SSleuA-UAL187 MMNMKPTESYEQLDNSALEQVVGG

Figure 8. Sequence comparison between the signal peptides of leucocin A and leucocin C. Grey boxes indicate the difference.

5.2.4 The transporters

As there seemed to be an accessory gene for ABC transporter in the *lecC* upstream sequence, LecC is most likely secreted via the same kind of transporter as other class IIa bacteriocins. Thus, the next task of genetic characterisation was to identify the transporter genes for LecC secretion. For that, like designing *lecI* reverse primer, transporter sequences of several class IIa bacteriocins were compared and a conserved region was found in the middle of the translocator gene (Figure 9 and Appendix 6). Then, a consensus primer (Trans reverse) was designed and used in PCR with *lecC* reverse primer. Two bands (2500 bp and 1600 bp) were amplified, both of which were shown to carry *lecC* by sequencing. However, the obtained sequences were short, probably because of the quality of sequencing templates. Only 183 bp was sequenced from the *lecC* and its upstream region, but no further sequence was identified (results not shown).

The next attempt was to amplify *lecC* upstream fragments by iPCR. *Ln. carnosum* plasmid DNA was cut by *Acl*I, self-ligated and subject to PCR with inverse primers. A large fragment (~8 kb) was amplified and sequenced by primer walking strategy (Figure 9). The fragment was found to carry another operon in the opposite direction to *lecCI*. The operon

was comprised of two genes (secretion gene *lecS* and translocator gene *lecT*) for ABC transporter, and an accessory gene (*lecX*). Interestingly, the accessory gene and the transporter genes were practically identical with those of *leuA* operon (97% identity) in *Ln. gelidum* UAL187. The 8 kb fragment was entirely sequenced, but no other *lecC*-related genes/ORFs were found. The sequence of the 4920 bp LecC gene cluster is in Appendix 6.

In conclusion, the genes required for the production of LecC were characterised (Figure 9). Five genes required for LecC production were identified: the structural gene (*lecC*), the immunity gene (*lecI*), a gene (*lecX*) for an accessory protein and two genes (*lecTS*) for an ABC transporter. The structures of the two *lecC* operons were actually the same as *leuA* operons in *Ln. gelidum*. The five genes in two operons were identified to be class IIa associated genes by sequence homologies. The *lecXTS* genes and *lecC* secretion signal sequence were found highly identical to those of *leuA*, while the *lecCI* were novel. The novel gene *lecI* encodes a putative immunity protein for LecC, but experimental evidence would be needed to confirm its function.

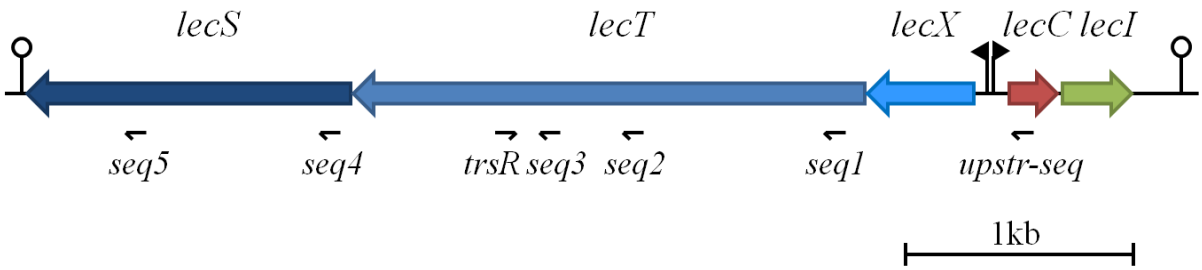

Figure 9. Organisation of the genes needed for the production of leucocin C and the loci of primers for primer walking. *trsR*, Trans reverse primer. Promoters are shown as black flags. Transcriptional terminators are shown as lollipop symbols.

5.3 Heterologous expression of leucocin C

In order to study the function of LecI, the bacteriocin LecC would be needed. As the wild-type *Ln. carnosum* 4010 also produces LeuA, which kills *Listeria* as well, the LecC peptide should be purified from the culture supernatant. Therefore, an easier way to get pure LecC would be to produce the bacteriocin in a heterologous non-bacteriocinogenic host, for example *Lc. lactis*. The mature *lecC* gene with and without the immunity gene *lecI* was amplified by PCR and cloned into the plasmid pLEB690 at *Nae*I site (Figure 10-I). Cloning at *Nae*I site allowed blunt-end fusion with the *SSusp45* in pLEB690 and the cloned *lecC* with no extra N-terminal aa residues in the mature secreted bacteriocin. The *lecI* gene (*Bsp*HI, compatible with *Nco*I) was also cloned alone into pLEB688 (*Nco*I, *Sma*I)

as a control (Figure 10-II). Adding *Nco*I in the *lecI* forward primer would have changed the second aa in LecI from Lys to Glu, therefore *Bsp*HI was used.

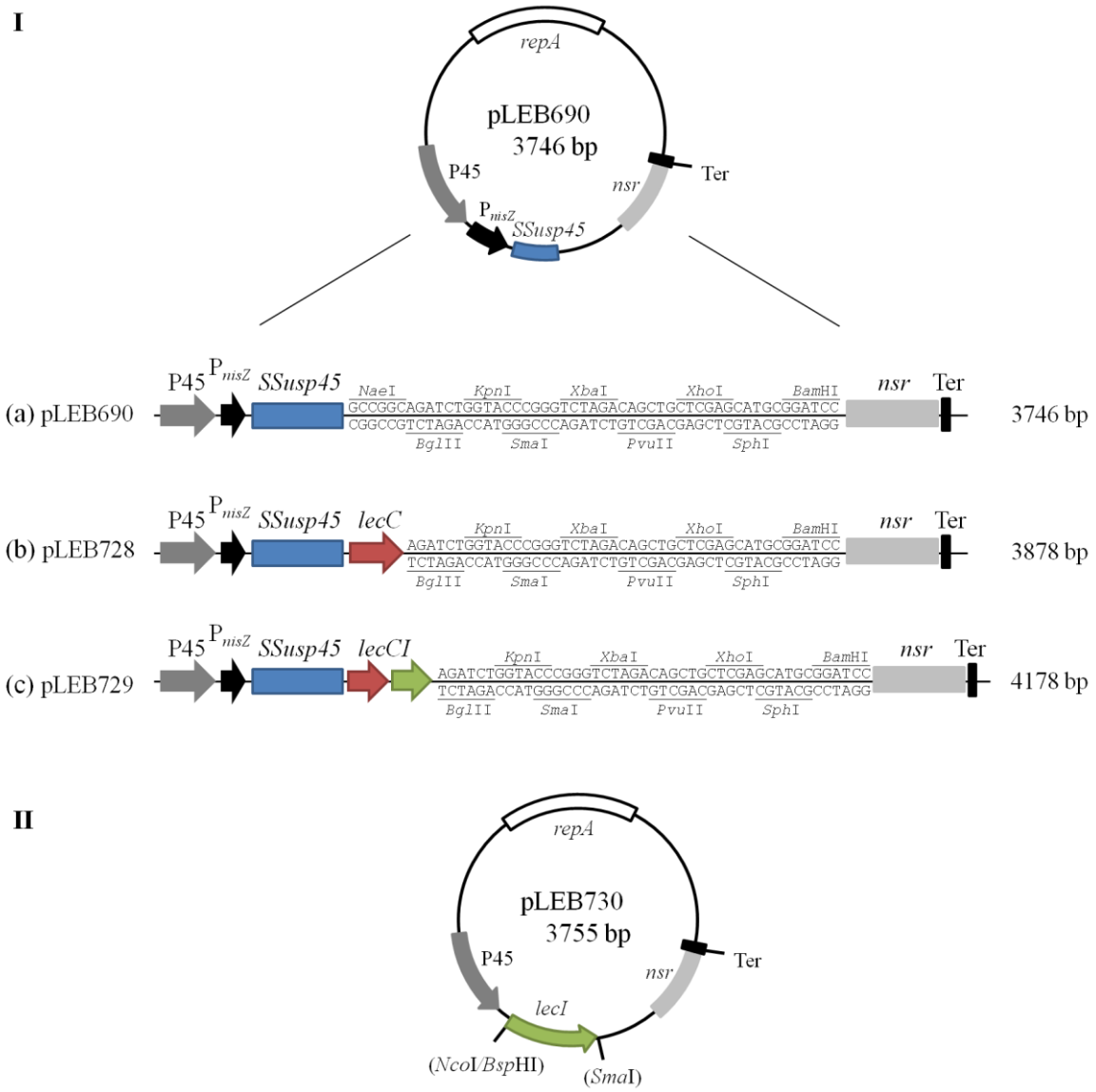

Figure 10. Construction of plasmids derived from the food-grade vector pLEB690 (I) and plasmid pLEB730 carrying *lecI* gene (II). pLEB690 was derived from pLEB688 with the insertion of nisin inducible promotor P_{nisZ} and the lactococcal *SSusp45* (Li *et al.* 2011). The vector plasmid pLEB690 (Ia) was restricted with *Nae*I and ligated with PCR fragments of *lecC* (Ib) or *lecCI* (Ic) without *lecC* signal sequence. The vector pLEB688 was digested with *Nco*I and *Sma*I, and ligated with the PCR fragment of *lecI* digested with *Bsp*HI (II).nsr, nisin resistance gene. P45, constitutive lactococcal promoter.

The constructed plasmids were introduced into *Lc. lactis* MG1363 for constitutive expression and into NZ9000 for nisin-inducible expression. The production of LecC was determined by spot-on-a-lawn antilisterial assay. Functional LecC was efficiently secreted by *Lc. lactis* strains. However, the co-expressed *lecI* did not improve the production (Figure 11). Previously *papA* encoding mature pediocin was cloned into the same plasmid in the same way (Li *et al.* 2011). In this study, that plasmid was used to compare the production and antilisterial activity of pediocin and LecC. Interestingly, even though

pediocin and LecC belong to the same subgroup (subgroup 1) of class IIa bacteriocins, *Lc. lactis* was capable to produce LecC far more effectively than pediocin with or without the immunity protein. Moreover, nisin induction could improve the production of pediocin to some extent, whereas the expression level of LecC was highly increased by nisin induction. Hence, LecC producing *Lc. lactis* could be used as LecC source for the following study on the function of the putative immunity gene *lecI*.

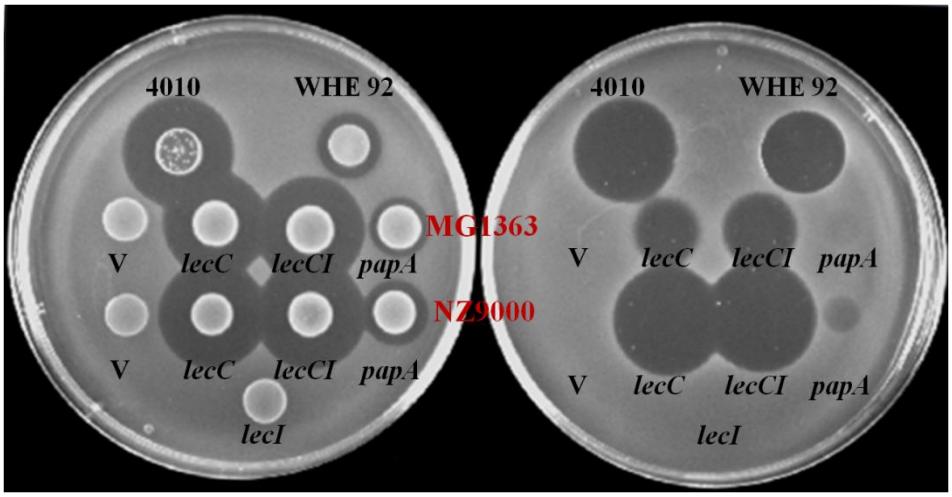

Figure 11. Antilisterial bioassay of *Lc. lactis* expressing *lecC*, *lecCI*, *papA* (pediocin) or *lecI*, compared to the wild-type bacteriocin producers *Ln. carnosum* 4010 (LecC) and *Lb. plantarum* WHE 92 (pediocin). Onto *Listeria* lawn, 5 µl of cell suspension (left) and corresponding supernatants (right) were spotted. All host strains were cultured with nisin (0.5 µg/ml) as selective agent for the plasmids and/or for nisin induction. Second row from top, *Lc. lactis* MG1363 as the host strain. Third row from top, nisin-inducible *Lc. lactis* NZ9000 as host. V, the vector plasmid pLEB690.

5.4 Demonstration of the immunity function of LecI

The last aim of this study was to confirm whether the putative immunity gene *lecI* really conferred the immunity to LecC. Since the previously constructed lactococcal *lecI*-plasmid pLEB730 is not selectable in *Listeria*, the *lecI* gene was cloned under the control of the lactobacillar *pepR* promoter into pTF1 (Figure 12).

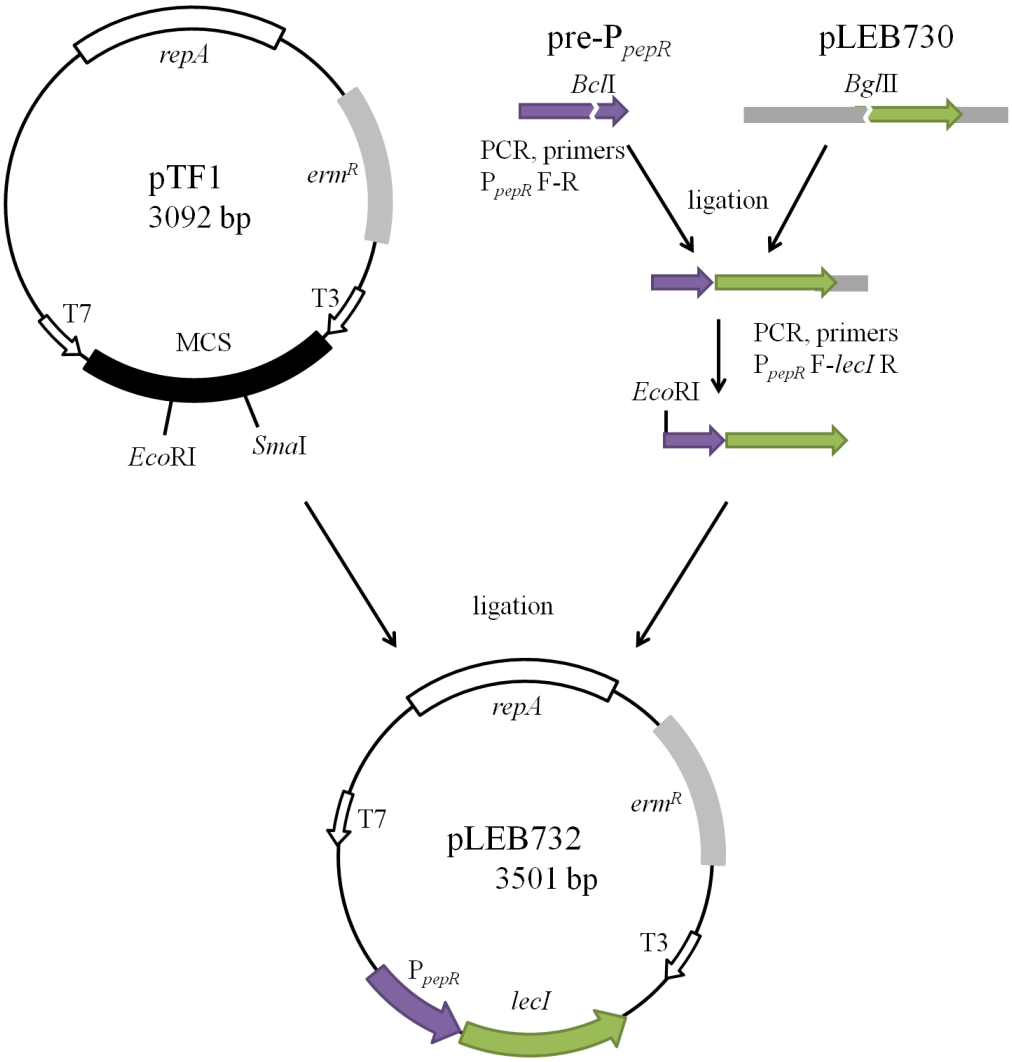

Figure 12. Construction of plasmid pLEB732 for expression of *lecI* in *Listeria*. The *pepR* promoter and *lecI*-containing plasmid pLEB730 were restricted with *Bcl*I and *Bgl*II, respectively. PCR fragment containing P_{pepR} and *lecI* was digested with *Eco*RI and inserted into the shuttle vector pTF1 (*Eco*RI, *Sma*I).

Ligated pTF1-*lecI* was first transferred into *E. coli* TG1 resulting in the plasmid pLEB732. The constructed plasmid pLEB732 and the vector pTF1 were then electroporated into *L. monocytogenes* WSLC 1018. Subsequently, the *Listeria* transformants were grown with serial concentrations of LecC-producing *Lc. lactis* supernatant. The growth was monitored with Bioscreen C plate reader. The growth curves are shown in Figure 13. The vector and LecI strains grew equally in LecC-free media. The growth of both strains was retarded when LecC supernatant was added, but the retardation degree of the vector strain was considerably higher than the LecI-producing strain (Figure 13). In media with higher LecC concentrations, the difference in growth between the two strains was less pronounced (data not shown), showing that the immunity capacity of LecI was limited. Nevertheless, production of LecI did protect *Listeria* cells against LecC, corroborating the immunity function of *lecI* gene.

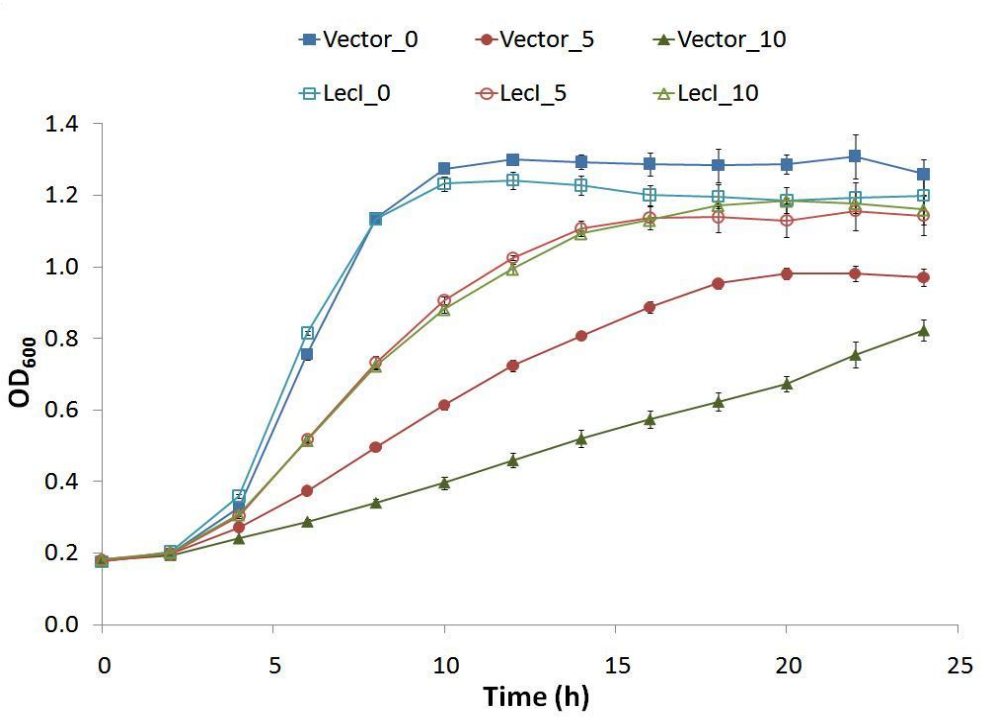

Figure 13. Growth of LecI-producing *Listeria* in different LecC concentrations (0, 5, 10 µl of LecC-producing *Lc. lactis* supernatant/ml). Vector, *Listeria* carrying pTF1.

6 DISCUSSION

Bacteriocins of LAB have been studied, characterised, and classified into three classes. The unmodified class IIa bacteriocins are characterised by their antilisterial activity. *Ln. carnosum* 4010 as a protective culture in meat products has been shown to produce two class IIa bacteriocins, LeuA and LecC (Budde *et al.* 2003). LeuA has been well studied and all the genes needed for its production are known, whereas the knowledge of LecC had been fairly limited. Its amino acid sequence has been revealed, and it is known to kill pathogenic *Listeria*. In this study, the genes involved in the production of LecC were identified and characterised. In addition, bioactive LecC was efficiently produced by the heterologous host *Lc. lactis*.

Previously, the LecC as a secreted peptide from *Ln. mesenteroides* 6 was purified and the amino acid sequence of mature LecC was determined (Fimland *et al.* 2002b). Nevertheless, the identity and location of LecC related genes were not known. Most class IIa bacteriocins are encoded by plasmid-associated genes (Ennahar *et al.* 2000). A few exceptions are enterocin A (Aymerich *et al.* 1996), divercin V41 (Metivier *et al.* 1998), sakacin P (Huhne *et al.* 1996), and carnobacteriocin B2 and BM1 (Quadri *et al.* 1997b). Genes needed for the productions of those bacteriocins are localised on the bacterial chromosomes. The results

in this study showed that like most class IIa bacteriocins, LecC genes were also plasmid-associated, and the genetic determinants of LecC co-resided in an individual plasmid in *Ln. carnosum* 4010. Since there have not been other genetic studies on LecC, it cannot be excluded that in some other LecC producing strains the bacteriocin associated genes could be on the bacterial chromosomes.

In this study, two LecC operons containing five genes were characterised. Recently, LecC was classified into the subgroup 1 of class IIa bacteriocins along with other closely related bacteriocins, such as sakacin P, listeriocin 743A, and enterocin CRL35, which share high sequence similarity with LecC (Nissen-Meyer *et al.* 2009). According to the same subclassification scheme, LeuA belonged to the subgroup 2. However, the organisations of the genes involved in the production of class IIa subgroup 1 bacteriocins are diverse (Figure 14). Pediocin genes were all found in a single operon, with the secretion genes (*papCD*) following the structural and immunity gene (*papAB*) (Marugg *et al.* 1992; Miller *et al.* 2005). In most cases, for example LeuA and sakacin P, the bacteriocin structural genes are followed by its cognate immunity gene in the same operon, and another operon(s) contains genes for secretion proteins. The organisation of *lecCI* operon in *Ln. carnosum* 4010 agreed with previous findings, i.e., the immunity gene *lecI* was found immediately downstream of the structural gene *lecC*. Interestingly, the organisations of LeuA and LecC operons were the same. Moreover, the *lecXTS* genes encoding an accessory protein, translocator and secretion protein for LecC were nearly identical to those of LeuA (van Belkum and Stiles 1995). Although LecC shares high sequence similarity and belongs to the same subgroup with listeriocin 734A and sakacin P, the organisations of these bacteriocins' operons are different (Figure 14). Unlike listeriocin 734A, which does not possess own transporter genes and is secreted by the *sec*-pathway, LecC is secreted by its cognate ABC-transporter system encoded by *lecXTS*. Besides, in the case of sakacin P, two genes *sppK* and *sppR* encode the histidine protein kinase and the response regulator of a 2CS (Tichaczek *et al.* 1994), while in this study, regulatory genes were not found in the *lecC* flanking regions. On the other hand, divercin V41 regulatory genes are located 910 nt downstream from its immunity gene, though they share the same operon (Metivier *et al.* 1998). Since only 410 nt after the *lecCI* operon was sequenced in this study, it is not impossible that LecC biosynthesis is regulated and these regulatory genes locate in the same plasmid further downstream of *lecI* or at other genetic loci.

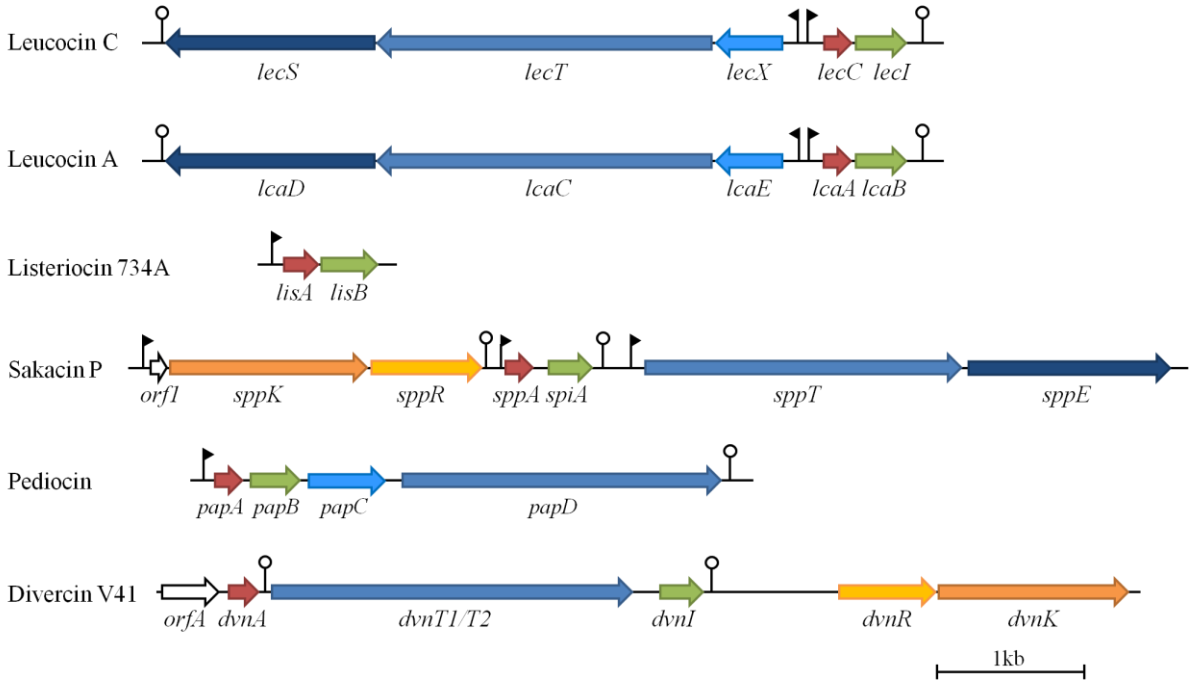

Figure 14. Organisations of the genes for the production of some class IIa bacteriocins. Genes encoding following proteins with shading patterns: bacteriocin (red), immunity protein (green), ATP-dependent translocator (blue), accessory protein (light blue), secretion protein (dark blue), histidine protein kinase (orange), respose regulator (yellow), undefined orf (white). Promoters (black flag), terminators (lollipop symbol). Sequence for divercin V41 is not complete; here is its putative schematic organisation.

In order to secrete antilisterial bacteriocins in *Lc. lactis* strains, the *usp45* lactococcal secretion signal has been used to replace the original signal sequences of these bacteriocins (Borrero *et al.* 2011; Li *et al.* 2011). In previous study, mature enterocin P gene was fused with *SSusp45* into a lactococcal expression vector. The constructed plasmid was transferred into *Lc. lactis* strain resulting in heterologous production of bioactive enterocin P against *L. monocytogenes* 4032 and *E. faecium* T136 (Borrero *et al.* 2011). Similar principle was applied for producing pediocin by *Lc. lactis* using a food-grade secretion vector with nisin selection (Li *et al.* 2011). The *papA* gene encoding mature pediocin was fused to *usp45* signal sequence carried by the food-grade vector pLEB690. As a result, pediocin secreted by *Lc. lactis* host strain killed *Listeria*. Here, in our study, production of LecC in *Lc. lactis* confirmed the applicability of *SSusp45* and pLEB690 in secreting other class IIa bacteriocin than pediocin. Surprisingly, LecC-producing *Lc. lactis* strains killed *Listeria* better than pediocin producer constructed by Li *et al.*(2011). With nisin induction, the difference between the inhibitions of *Listeria* by the two bacteriocins was even more pronounced (Figure 11, p54). As the yields of bacteriocins expressed by host strains were not determined in this study, it remains possible that the indicator strain *L. monocytogenes* WSLC 1018 is more sensitive to LecC than to pediocin. However, it is more likely that for some reason *Lc. lactis* could produce LecC more efficiently than pediocin. Previously, co-expression of pediocin immunity gene was shown to increase the yields of pediocin by host

Lc. lactis (Arques *et al.* 2008). Yet, there was no significant improvement in the production of LecC by the co-expression of *lecI* (Figure 11, p45). Although *Lc. lactis* is resistant to both LecC and pediocin, the strain seems to be somewhat sensitive to pediocin, and therefore the expression of LecC was more efficient.

Even though it seems that LecI helped to produce more LecC, the production of LecC was actually not limited due to the immunity problem, because with nisin induction *Lactococcus* was able to produce even more bacteriocins. The results indicated that the production of LecC by *Lc. lactis* MG1363 was constrained not because of the absence of its immunity protein but mainly because of the strength of the promoter. Nisin promoter is known to be stronger than the P45 promoter (Li *et al.* 2011). That explains why the production of LecC under nisin promoter was more effective than that under the P45 promoter (Figure 11, p54).

Earlier, nisin-induction in *Lc. lactis* NZ9000 increased the production of pediocin by the host (Li *et al.* 2011). In our study, nisin-induced LecC production was even more prominent increasing *Listeria* inhibition up to the wild-type level (Figure 11, p54). Though, it is not known how much exactly *Ln. carnosum* 4010 produces LecC, because the strain also produces LeuA, which is active against *Listeria* (Budde *et al.* 2003). Therefore, since the inhibition zones were about the same in size, the heterologous host *Lc. lactis* probably produced more LecC than the native producer strain. In conclusion, this study shows that *Lc. lactis* as a heterologous host is able to produce large amount of class IIa bacteriocin for potential use in food industry.

A common approach to demonstrate the function of an immunity protein is to express the putative im-gene in a bacteriocin-sensitive host, and to examine the increase of bacteriocin resistance. Previously, the functions of bacteriocin immunity genes of e.g., enterocin A, LeuA, sakacin P, and carnobacteriocin B2 have been shown by expressing them in heterologous hosts (Axelsson and Holck 1995; Huhne *et al.* 1996; Quadri *et al.* 1997b; Fimland *et al.* 2002a). The gene *lecI* identified in this study was shown to confer immunity by expressing *lecI* in LecC sensitive *Listeria* (Figure 13, p56). The sensitivity of LecI-producing *Listeria* towards LecC decreased, and this strain could grow in LecC containing medium. It is worth noticing that this study presents the first expression of an immunity gene in *Listeria*.

As the class IIa immunity proteins share some similarity in their amino acid sequence, it is possible to identify several common motifs in sequence comparison (Fimland *et al.* 2002a). Based on these common motifs, three subgroups have been suggested. In the subgroup B, all class IIa bacteriocin immunity proteins and two orphan immunity proteins (orfβ3-im and orf285-im) contain a C-terminal consensus motif (SN/SIRYGY) (Fimland *et al.* 2005; Drider *et al.* 2006). The amino acid sequence of LecI has the SSIRYGY region in its C-terminus, showing LecI homology with immunity proteins in this class (Figure 5, p48). Therefore, LecI is now suggested to be included in subgroup B immunity proteins of class IIa bacteriocins. Interestingly, bacteriocins pediocin and LecC are sorted in the same class IIa subgroup 1 (Nissen-Meyer *et al.* 2009), but their associated immunity proteins are quite different and belong to different subgroups (pediocin-im in subgroup A). However, even though they are so different, pediocin-im protects the host against LecC (Fimland *et al.* 2002a).

Earlier, the amino acid sequence of LecC was published, yet the sequence only covered the section of mature peptide (Fimland *et al.* 2002b). As revealed in this study, the DNA sequence of *lecC* gene also contains a conserved signal sequence region encoding a polypeptide with two consecutive glycine codons in its terminus, the GG-type leader peptide. The GG-leader is known to be cleaved off during the transportation of mature peptide to the outside of the producer cells. Though, secreted LecC and LeuA are different, homology comparisons of the GG-type leader with that of LeuA precursor showed high similarity between the two, with only one amino acid difference (Figure 8, p51). A GG-type leader is associated with the function of ATP-binding cassette (ABC) transporter for secretion of the peptide (Nissen-Meyer *et al.* 2009). Because of the high identity of transporter genes *lecXTS* with LeuA transporters (*lcaECD*) and also *mesCDE* for the secretion of mesentericin Y105 in *Ln. mesenteroides* FR52 (van Belkum and Stiles 1995; Aucher *et al.* 2004), it seems that these bacteriocins are secreted via similar transporters. Recently, in *Ln. carnosum* 4010, LeuA transporter accessory gene was recently shown to be interrupted with a putative transposase, therefore there is no LeuA dedicated transporter in this strain (Takala, unpublished). Thus, in this strain LeuA might be exported either by LecC secretion system or by other ABC-type pathways. Several *Leuconostoc* strains have been reported to produce both LeuA and LecC (Papathanasopoulos *et al.* 1998; Vaughan *et al.* 2001; Sawa *et al.* 2010). However, no one has showed the productions of these two bacteriocins would correlate with each other. It is still unknown whether LeuA and LecC

in the strain 4010 are secreted by the same system. Further studies on LeuA and LecC correlation would be required.

7 CONCLUSIONS

In this study, genes needed for the production of a class IIa bacteriocin LecC in *Ln. carnosum* 4010 were localised and characterised. The *lecC* gene encoding LecC precursor with a GG-type leader peptide was localised in a large plasmid of *Ln. carnosum* 4010. Five genes arranged in two opposite operons were identified for LecC production. One operon contains two novel genes with no matches to known genes in Genbank: the structural gene encoding LecC precursor and the immunity gene *lecI*. The other operon includes genes *lecXTS* encoding an accessory protein, an ATP-dependent translocator and a secretion protein, respectively. By exchanging *lecC* original signal sequence with a lactococcal signal sequence of *usp45*, bioactive LecC was efficiently produced by *Lc. lactis*. The immune function of the *lecI* gene was shown by expressing *lecI* in LecC sensitive *Listeria*. LecI-producing *Listeria* was less sensitive to LecC.

The organisation of LecC operons was the same as that of LeuA in *Ln. gelidum* UAL187. The upstream region of LecC including the *lecXTS* genes and non-coding sequences was 97% identical with known *leuA* (*Ln. gelidum* UAL187) sequences. The GG-type leader of LecC was different with LeuA leader only by 1 aa residue. Since in *Ln. carnosum* 4010 there is no dedicated transporter for LeuA secretion, both LeuA and LecC in this strain could be secreted with LecC transporter. Therefore, it would be interesting to study the exportation of LeuA in this strain and/or parallel strains producing both leucocins and to demonstrate the in/dependency of bacteriocins produced by the same strain.

Bioactive LecC was efficiently produced using the food-grade vector pLEB690 both constitutively and with nisin induction. The positive effects of nisin induction on the production of LecC overcame that of the immunity protein LecI. *Lc. lactis* could produce large amount of LecC, regardless of the presence of LecI. Thus, like in previous study (Li *et al.* 2011), the vector pLEB690 was shown to be a good tool for the production of functional bacteriocins in food industry.

The food pathogen *L. monocytogenes* has not been used before as a heterologous host for the expression of a bacteriocin immunity gene. In this study, *Listeria* was able to

functionally express the *lecI* gene thus tolerating higher LecC concentration. The aim was not to create *Listeria* strain resistant to class IIa bacteriocin, but to demonstrate the function of the immunity protein. As the strain became less sensitive, *lecI* was confirmed to be the factor mediating the bacteriocin immunity. According to the sequence comparison, LecI was suggested to be a subgroup B immunity protein.

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APPENDICES

Appendix 1. Buffers used in Anderson & Mckay (1983) rapid plasmid isolation method.

Name	Ingredients	Concentration	pH
Buffer 1	Sucrose Tris-HCl EDTA	6.7% 50 mM 1 mM	8.0
Buffer 2	Tris-HCl	25 mM	8.0
Buffer 3	EDTA Tris-HCl	250 mM 50 mM	8.0
Buffer 4	SDS Tris-HCl EDTA	20% 50 mM 20 mM	8.0
Buffer 5	Tris-HCl	2.0 M	7.0

Appendix 2. Enzymatic reactions and buffers.

Restriction enzymes and their corresponding buffers

Enzyme	Restriction site 5' → 3'	Optimal buffer	Optimal temperature	Company
FastDigest® *Acl*I	AA^CGTT	1 × FastDigest® Green Buffer	37 °C	Fermentas
*Apo*I (*Xap*I)	R^AATTY*	1 × Tango	37 °C	Fermentas
*Bcl*I	T^GATCA	1 × NEBuffer 3	50 °C	New England Biolabs
*Bgl*II	A^GATCT	1 × NEBuffer 3	37 °C	New England Biolabs
*Bsp*HI	T^CATGA	1 × NEBuffer 4	37 °C	New England Biolabs
*Eco*RI	G^AATTC	1 × Buffer EcoRI	37 °C	Fermentas
*Hpa*I	GTT^AAC	1 × NEBuffer 4	37 °C	New England Biolabs
*Nae*I	GCC^GGC	1 × Buffer A	37 °C	Promega
*Nco*I	C^CATGG	1 × NEBuffer 3	37 °C	New England Biolabs
*Sma*I	CCC^GGG	1 × NEBuffer 4	25 °C	New England Biolabs

* R = A or G, Y = C or T

Recommended protocols for enzymatic reaction

Enzymatic digestion		
H_20	to 50 µl	Incubate in supplied buffer at optimal temperature for 1-16 hour
10 × restriction buffer	5 µl	
DNA	1 µg	
Restriction enzyme	2 µl	
Total volume	50 µl	
Phosphorylation		
H_20	to 50 µl	Incubate at 37 °C for 30 minutes
10 × T4 DNA ligase buffer	5 µl	
10 mM ATP	5 µl	
DNA	1 µg	
T4 PNK (10000 U/ml)	1 µl	
Total volume	50 µl	
Dephosphorylation		
H_20	to 20 µl	Incubate at 37 °C for 30 minutes
DNA	1 µg	
10 × NEBuffer 3	2 µl	
CIP (10000 U/ml)	0.5 µl	
Total volume	20 µl	

Appendix 3. PCR programmes.

Enzyme		**Phusion HF polymerase**		***Taq* DNA polymerase**	
Cycle step	**Cycles**	**Temperature**	**Time**	**Temperature**	**Time**
Initial denaturation	1	98 °C	1 min 30 s	94 °C	2 min
Denaturation Annealing Extension	30	98 °C x °C 72 °C	7 s 20 s 15-30 s/kb	94 °C x °C 72 °C	30 s 30 s 1 min/kb
Final extension	1	72 °C	5 min	72 °C	5 min
Hold	1	4 °C	hold	4 °C	hold

x optimal annealing temperature. As a basic rule, for primers > 20nt, anneal for 10–30 seconds at a Tm +3°C of the lower Tm primer. For primers ≤ 20nt, use an annealing temperature equal to the Tm of the lower Tm primer.

Appendix 4. Buffers and media used in electroporation

***Lc. lactis* transformation**	
Electroporation buffer	0.5 M sucrose 15% glycerol
Recovery medium	1 ml M17GS 2 mM $CaCl_2$ 20 mM $MgCl_2$
***L. monocytogenes* transformation**	
Electroporation buffer	0.5 M sucrose 1 mM Hepes
Recovery medium	1 × BHI 0.5 M sucrose
***E. coli* transformation**	
SOC	20 g/L Bacto™ Tryptone 5 g/L Bacto™ Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose

Appendix 5. Southern blot solutions and buffers

Name	Ingredients	Concentration	pH
Preparation of Gel			
Depurination	HCl	0.25 M	-
Denaturation	NaOH NaCl	0.4 N 0.6 M	-
Neutralisation	NaCl Tris-HCl	1.5 M 0.5 M	7.5
Transfer			
Transfer buffer, 10 × SSC	NaCl Trisodium citrate	1.5 M 0.15 M	7.0-7.2
Hybridisation			
Hybridization buffer	SSC Blocking reagent N-Lauroylsarcosine SDS	5 × 1% 0.1% 0.02%	-
Wash buffer 1	SSC SDS	2 × 0.1%	-
Wash buffer 2	SSC SDS	1 × 0.1%	-
Detection			
Buffer 1	Tris-HCl NaCl	100 mM 150 mM	7.5
Buffer 2	Blocking reagent Buffer 1	1% 1 ×	7.5
Buffer 3	Tris-HCl NaCl $MgCl_2$	100 mM 100 mM 50 mM	9.5
Colour development buffer	NBT X-phosphate Buffer 3	4.5 µl/ml buffer 3 3.5 µl/ml buffer 3 1 ×	-
TE buffer	Tris-HCl EDTA	10 mM 1 mM	8.0

Appendix 6. Gene sequence of leucocin C operons: *lecXTS* (complementary) and *lecCI*.

lecS *lecT* *lecX* *lecC* *lecI*

sequence primers terminator trans R primer inverse primer

start codon stop codon aa different to that of leucocin A genes

Restriction site

AAACAGGTAAACACATTTTAAAATTATGTTTACCTGTTTAAATTAATTATTTCACCTAAGACAATGTCCTTA
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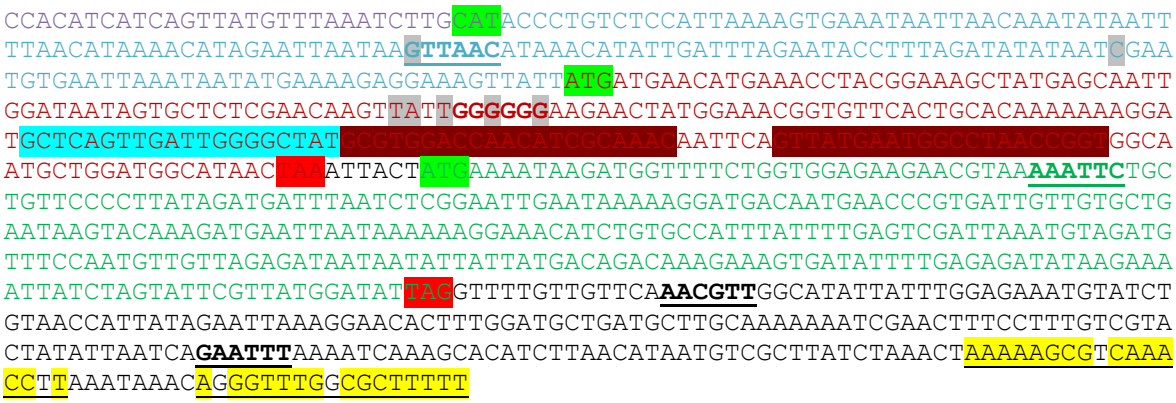
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