

***Leuconostoc* bacteriocins and their application in genome editing**

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List of original publications

- I** **Wan, X.**, Li, R., Saris, P.E.J. and Takala, T.M., 2013. Genetic characterisation and heterologous expression of leucocin C, a class IIa bacteriocin from *Leuconostoc carnosum* 4010. Applied Microbiology and Biotechnology, 97:3509-3518.
- II** **Wan, X.**, Saris, P.E.J. and Takala, T.M., 2015. Genetic characterization and expression of leucocin B, a class IIc bacteriocin from *Leuconostoc carnosum* 4010. Research in Microbiology, 166:494-503.
- III** **Wan, X.**, Usvalampi, A.M., Saris, P.E.J. and Takala, T.M., 2016. A counterselection method for *Lactococcus lactis* genome editing based on class IIa bacteriocin sensitivity. Applied Microbiology and Biotechnology, 100:9661-9669.

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- I** **Wan, X** participated in the design of the study, performed all the experiments together with the co-authors, participated in data analysis and result interpretation, and was responsible for writing the manuscript together with the co-authors.
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- III** **Wan, X** participated in the design of the study, performed majority of the experiments (except constructing H1-H2-pUCori fragments for integration plasmids), analysed the data, interpreted the results, and wrote the manuscript together with the co-authors.

The publications are referred to in the following text by their roman numerals.

Abbreviations

3CS	three-component regulatory system
aa	amino acid
ABC	ATP-binding cassette
AMP	antimicrobial peptide
bp	base pair(s)
C-terminal	carboxyterminal
Da	Dalton, atomic mass unit
DCO	double crossover
DNA	deoxyribonucleic acid
e.g.	<i>exempli gratia</i> , for example
Erm	erythromycin
et al.	<i>et alii</i> , and others
FOA	5'-fluoroorotic acid
Gram ⁻	Gram-negative bacteria
Gram ⁺	Gram-positive bacteria
GG	double-glycine
i.e.	<i>id est</i> , in other words
kb	kilobase pair(s)
kDa	kilodalton
LAB	lactic acid bacteria
Man-PTS	mannose-phosphotransferase system
MRSA	methicillin-resistant <i>Staphylococcus aureus</i>
N-terminal	aminoterminal
o/n	overnight
ORF	open reading frame
PCR	polymerase chain reaction
SCO	single crossover
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SS	signal sequence
ssp.	subspecies

ABSTRACT

Leuconostocs are lactic acid bacteria used as starters in food fermentations. They can also be spoilage bacteria, especially in vacuum packed meat products. Many Leuconostocs produce antimicrobial peptides, also known as bacteriocins. *Leuconostoc* bacteriocins are usually small, less than 10 kDa non-modified peptides, which are secreted by dedicated transporters. These bacteriocins kill many different bacteria, including strains of *Enterococcus*, *Listeria*, and *Staphylococcus* genera. Sensitivity to bacteriocins is usually dependent on a specific receptor on the target cell membrane. On *Listeria* cell membrane, mannose-phosphotransferase system Man-PTS has been shown to be the target for bacteriocins leucocins A and C from *Leuconostoc*. These class IIa bacteriocins bind to the subunit IIC (MptC) of Man-PTS and open the transporter, which leads to cell leakage and eventually cell death. Bacteriocin producers express specific immunity proteins, which protect the producer cells by blocking the transporter pore.

In this doctoral dissertation, the bacteriocins produced by *Leuconostoc carnosum* 4010 were studied. The strain 4010 is a commercial protective culture for vacuum packaged meat products. The strain produces two antilisterial bacteriocins, leucocins A and C. In this study, the strain was found to produce a third bacteriocin, leucocin B, which is active against some *Leuconostoc* and *Weissella* strains. Genes required for the production of the three leucocins were characterised. The leucocin genes were arranged in operons on two native plasmids. A single ABC-transporter was found in the strain 4010 and was believed to carry out the bacteriocin secretion for all three leucocins. Immediately downstream of the leucocin genes *lcnA*, *lebb*, and *lecC*, putative immunity genes *lcnB*, *lebl*, and *lecl* were found. The immunity mechanisms of leucocin C was studied by expressing the *lecl* gene in *L. monocytogenes*. LecI producing *Listeria* was less sensitive to the leucocin C, showing the immunity function of LecI. By cloning the leucocin genes in plasmid vectors, active bacteriocins were produced in heterologous hosts *Escherichia coli* and *Lactococcus lactis*. Finally, leucocins A and C were used in a *Lc. lactis* genome editing method developed in this study. It has been shown that expression of Listerial *mptC* gene in *Lc. lactis* renders the host sensitive to class IIa bacteriocins. In this doctoral study, a counterselection method based on bacteriocin sensitivity was developed to select the loss of a plasmid. Listerial *mptC* gene was cloned in an integration vector aiming at a chromosomal deletion, and the plasmid was transferred into *Lc. lactis*. When the bacteriocin sensitive integrants were cultured with leucocin A or C, only the cells which had lost the *mptC*-plasmid through a second homologous recombination could survive. The second recombination may also cause the desired deletion. With this bacteriocin counterselection method, fragments up to 35 kb were deleted from *Lc. lactis* chromosome.

The results obtained from this doctoral study provide further knowledge of *Leuconostoc* bacteriocins and their scientific use.

TIIVISTELMÄ (abstract in Finnish)

Leuconostoc-suvun bakteerit ovat elintarvikkeiden hapatteina käytettyjä maitohappobakteereita. Leukonostokit voivat olla myös pilaajabakteereita, erityisesti lihatuotteissa. Monet Leukonostokit tuottavat antimikrobisia peptidejä eli bakteriosiineja. Leukonostokkien tuottamat bakteriosiinit ovat lähes aina pieniä, alle 10 kDa kokoisia modifioimattomia peptidejä, jotka eritetään spesifisillä kuljettimilla ulos solusta. Ne tappavat monia erilaisia bakteereita, kuten *Enterococcus*, *Listeria*, ja *Staphylococcus* –sukujen kantoja. Bakteerien herkkyys bakteriosiineille perustuu yleensä spesifiseen reseptoriin solun pinnalla. Listerian solukalvolla olevan mannoosi-fosfotransferaasi Man-PTS -kuljettimen on osoitettu toimivan reseptorina mm leukonostokkien tuottamille leukosiini A:lle ja C:lle. Nämä luokan IIA bakteriosiinit tarttuvat Man-PTS –kuljettimen alayksikkö IIC:hen (MptC), ja aiheuttavat kuljettimen avautumisen. Avoin kuljetin toimii vuotavana reikänä solukalvolla, jolloin solu kuolee. Bakteriosiinien tuottajilla on itsellään erityinen immuniteetti-proteiini, joka suojaa tuottajasolua tukkimalla avoimen kuljettimen.

Tässä väitöstyössä tutkittiin *Leuconostoc carnosum* 4010 –bakteerikannan tuottamia bakteriosiineja. *Ln. carnosum* 4010 on vakuumpakattujen lihatuotteiden kaupallinen suojaviljelmäkanta, joka estää *L. monocytogenes* –bakteerin kasvua tuottamalla kahta *Listeria*-aktiivista bakteriosiinia, leukosiineja A ja C. Työssä osoitettiin 4010-kannan tuottavan myös kolmatta bakteriosiinia, leukosiini B:tä, joka tappaa mm *Leuconostoc*- ja *Weissella* -bakteerikantoja. Työssä karakterisoiittiin näiden kolmen bakteriosiinin tuottoon tarvittavat geenit 4010-kannassa. Leukosiinigeenien osoitettiin olevan järjestäytyneinä operoneiksi kahdessa eri plasmidissa. Bakteriosiinien erittämiseen löytyi vain yksi kuljetin, jonka uskotaan erittävän kaikkia kolmea leukosiinia. Kaikkien leukosiinigeenien, *lcnA*, *lebB*, ja *lecC*, perässä oli todennäköiset immuniteetti-geenit *lcnB*, *lebl*, ja *lecl*. Leukosiini C:n immuniteettimekanismia tutkittiin ilmentämällä *lecl*-geeniä *L. monocytogenes* –kannassa. *Lecl*:tä tuottava *Listeria* sietä paremmin bakteriosiinia, mikä osoitti *Lecl*:n olevan leukosiini C:n immuniteetti-proteiini. Aktiivisia leukosiineja tuotettiin heterologisissa *Escherichia coli* ja *Lactococcus lactis* –isäntäkannoissa kloonamalla bakteriosiinigeenit plasmidivektoreihin. Lopuksi leukosiineja A ja C käytettiin tässä työssä kehitetyssä *Lc. lactis* –bakteerin genominmuokkausmenetelmässä. Aiemmin on osoitettu, että Listerian *mptC*-geenin ilmentäminen *Lc. lactis* –bakteerissa tekee laktokokkisolusta bakteriosiineille herkän. Tätä ominaisuutta hyödynnettiin vastaselektiossa, jossa estetään plasmidin sisältävien solujen kasvu. Listerian *mptC*-geeni liitettiin kromosomaalista deleetiota varten rakennettuun integraatiovektoriin, joka siirrettiin *Lc. lactis* –soluihin. Vektorin integraatio kromosomiin tekee kannasta bakteriosiiniherkän. Kasvatettaessa integranttia leukosiini A:n tai C:n kanssa, vain ne solut, joista *mptC*-integraatiovektori on toisen homologisen rekombinaation kautta poistunut, pystyvät kasvamaan. Vektorin poistuessa tapahtuu mahdollisesti myös haluttu kromosomaalinen deleetio. Tämän bakteriosiini-vastaselektiomenetelmän avulla poistettiin 17-35 kb kokoisia paloja *Lc. lactis* –kannan kromosomista.

Tämän työn tulokset lisäävät tietoaamme maitohappobakteerien antimikrobisista ominaisuuksista ja bakteriosiinien käyttömahdollisuuksista tieteellisissä sovelluksissa.

INTRODUCTION

1 Lactic acid bacteria

Lactic acid bacteria (LAB) constitute a diverse group of Gram-positive (Gram⁺) bacteria which produce lactic acid from the fermentation of available carbon sources, e.g. glucose (Axelsson 2004). LAB can quickly lead to the acidification (lower than pH 4.0) of food materials due to the production lactic acid, thus they effectively prevent the outgrowth of almost all potential spoilage microorganisms. LAB can also give food characteristic textures, flavours and aromas. Therefore, LAB are widely used in fermentation industry all over the world, with the most applications of LAB in the dairy sector of the food industries.

Depending on the sugar fermentation patterns, LAB are divided into two broad metabolic categories: homofermentative and heterofermentative. Representative homofermentative LAB include *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Pediococcus* and *Streptococcus*. In the presence of excess glucose and limited oxygen, homofermentative LAB catabolise one mole of glucose into two moles of pyruvate, which in the end after oxidation of NADH are reduced merely to lactic acid. Whereas obligate heterofermentative LAB yield theoretically equimolar quantities of CO₂, ethanol and lactic acid from one mole of glucose. This category includes *Fructobacillus*, *Leuconostoc*, *Oenococcus*, *Weissella*, and certain *Lactobacilli* (Axelsson 2004).

Lactococcus lactis is a model LAB widely used for industrial production of fermented dairy products, such as milk, cheese and yoghurt. Extensive studies have been done to increase the efficiency of *Lc. lactis* for food industry. Genetically modified *Lc. lactis* has also been used alive to treat human inflammatory bowel disease (Braat et al. 2006). *Lc. lactis* genome editing tools are in good need for a better exploiting the microorganism.

1.1 The genus of *Leuconostoc*

Leuconostoc species are LAB that are commonly associated with foods and used as flavour forming culture in fermentations, for example, in Finnish traditional milk product Viili. Morphologically, leuconostocs are Gram⁺ cocci in chains. Leuconostocs are closely related to *Fructobacillus*, *Oenococcus* and *Weissella* species, and together they are commonly known as the *Leuconostocaceae* family of LAB. The *Leuconostoc sensu stricto* includes, at the time of writing, 13 validly published species names (**Table 1**) .

Table 1. *Leuconostoc* species and their sources of identification, according to LPSN bacterio.net (2015).

Species	Source	Reference
<i>Ln. carnosum</i>	chilled meat	Shaw and Harding (1989)
<i>Ln. citreum</i>	honey-dew of rye ear	Farrow et al. (1989)
<i>Ln. fallax</i>	sauerkraut	Martinez-Murcia and Collins (1991)
<i>Ln. gelidum</i> *	chilled meat	Shaw and Harding (1989)
<i>Ln. holzapfelii</i>	coffee fermentation	De Bruyne et al. (2007)

<i>Ln. inhae</i>	kimchi	Kim et al. (2003)
<i>Ln. kimchii</i>	kimchi	Kim et al. (2000)
<i>Ln. lactis</i>	milk	Garvie (1986)
<i>Ln. mesenteroides</i>	sugar beet solution	Garvie (1986)
<i>Ln. miyukkimchii</i>	brown algae kimchi	Lee et al. (2012)
<i>Ln. palmae</i>	palm wine	Ehrmann et al. (2009)
<i>Ln. pseudomesenteroides</i>	cane juice	Farrow et al. (1989)
<i>Ln. rapi</i>	sous-vide rutabaga	Lyhs et al. (2015)

* *Ln. gasicomitatum* is now reclassified as *Ln. gelidum* ssp. *gasicomitatum*. *Ln. oenos* is reclassified as *Oenococcus oeni*, hence no longer belongs to *Leuconostoc* (Dicks et al. 1995). Similarly, *Ln. paramesenteroides* is renamed as *Weissella paramesenteroides* (Collins et al. 1993).

1.2 Leuconostocs in food production

Like other LAB, leuconostocs also play important roles in food fermentation and preservation. In the 1990s, people started to realise that a buttery aroma in many dairy products was associated with leuconostocs (Dessart and Steenson 1995; Thunell 1995). For example, during cheese ripening, low concentration of citrate in cheese curd could be metabolised by leuconostocs together with lactococci to produce a number of volatile flavour compounds (McSweeney and Sousa 2000). For instance, *Ln. mesenteroides* is used as flavour forming culture in the Finnish fermented dairy product Viili, which has an iconic slimy texture from *Lc. lactis* (Kahala et al. 2008; Sundman 1953). Various *Ln. mesenteroides* and *Ln. citreum* strains have been found to improve fermentation of Korean pickled vegetables kimchi (Lee et al. 2015). *Ln. mesenteroides* is responsible for souring and leavening Indian fermented food idli or dosa (Mukherjee et al. 1965; Soni and Sandhu 1991). Leuconostocs are also used as protective culture as they can prolong the shelf life and increase safety of food products by producing antimicrobial compounds including hydrogen peroxide and antimicrobial peptides (AMPs) i.e., bacteriocins (Holzapfel et al. 1995; Säde 2011). Because of the bacteriocin production, *Ln. carnosum* 4010 was used as protective culture in refrigerated vacuum packaged meat products (Budde et al. 2003). More detailed information regarding bacteriocins produced by leuconostocs is presented in the introduction chapter **2.3 Leuconostoc bacteriocins**. Applications of *Leuconostoc* bacteriocins can be found in chapter **4 LAB bacteriocin applications**.

Nevertheless, in addition to the numerous benefits leuconostocs have brought to the food industry, they can also be responsible for the spoilage of cold-stored foods, particularly meat products. Different *Leuconostoc* species can cause various defects in meat and vegetable products, such as blowing, off-odour, sour odour, slime, discolouration and exudation (Björkroth et al. 2000; Cai et al. 1998; Diez et al. 2008; Korkeala et al. 1988; Torriani et al. 1999). *Ln. mesenteroides* for example was found responsible for greening colour and slime of cooked bacon through sugar fermentations and degradation of nitrogen compounds (Comi et al. 2016). The blowing of packages is due to CO₂ accumulation caused by the *Ln. gelidum* catabolism of carbohydrates, while unwanted buttery off-odours were because of diacetyl/acetoin formation by *Ln. gelidum* (Jääskeläinen et al. 2015; Vihavainen and Björkroth 2007; Vihavainen et al. 2008).

2 LAB bacteriocins and how to find them

There have been several reports of antimicrobial activity of LAB, due to the presence of organic acids, hydrogen peroxide or other produced chemicals. In the past century, it has gradually become clear that LAB combat microorganisms by at least one other mechanism, namely by secretion of AMPs. These ribosomally synthesised AMPs produced by bacteria are called bacteriocins. Many LAB, for example *Carnobacterium*, *Enterococcus*, *Lactococcus*, *Lactobacillus*, *Leuconostoc*, and *Pediococcus* species have been shown to produce and secrete bacteriocins against competing microorganisms (Alvarez-Sieiro et al. 2016). There have been lots of fundamental and applied researches on LAB bacteriocins, due to their potential applications as biopreservatives in food and food products to inhibit the growth of food-borne pathogens and to prevent food spoilage. In general, LAB bacteriocins kill target cells by forming pores on the cell membrane, which leads to the cell leakage and eventually cell death (Cotter et al. 2013).

The inhibitory effect of lactococcal substance on the growth of other LAB can date back in the late 1920s (Rogers and Whittier 1928), shortly after the discovery of “colicin” from the Gram-negative (Gram⁻) *Escherichia coli* (Gratia 1925). The first LAB bacteriocin was named as nisin (group **N** *Streptococcus* **I**nhibitory **S**ubstance, **-in** ending referring to an antibiotic) (Mattick et al. 1947). In the following 40 some years, nisin has been accepted as a safe food additive by World Health Organisation (1969) and European Economic Community (1983), and affirmed as generally recognised as safe and used in cheese spreads in the United States (FDA 1988). Nisin is a great example in terms of the AMP developing pattern from antimicrobial activity to legal approval and to final commercial application. Yet, although tremendous amount of bacteriocins are discovered every year, not single one of them has been accepted like nisin (Cotter et al. 2013).

A study of bacteriocins from LAB has historically started with the discovery of the antimicrobial activity of LAB isolated from various sources, of which especially fermented food products have been the most common places for bacteriocinogenic LAB. This discovery pattern is still common nowadays. For example, the latest identified enterocin NKR-5-3 was purified and characterised from *Enterococcus faecium* NKR-5-3 isolated from Thai fermented fish (Ishibashi et al. 2012). From traditional Chinese fermented vegetables, *Lb. plantarum* 163 was isolated and was found to produce plantaricin 163 which was mainly active against *Staphylococcus aureus* and *Bacillus cereus* (Hu et al. 2013). In the discovery process, preliminary antimicrobial activity from a strain is tested on indicator lawn. Then the bioactive compound produced by this certain strain is purified from bacterial culture, and the protein nature of the compound is confirmed. Afterwards, the structures of the bacteriocin are then studied, e.g. through amino acid (aa) sequencing and mass spectrometry. Genetic studies of the bacteriocin are often conducted at the same time.

Nowadays, as the sequencing technology advances, more and more bacterial genomes have become available. The pace of bacteriocin discovery has also been accelerated. Though genome mining, significant amount of putative bacteriocin genes can be identified

together with their biosynthetic mechanisms (Wang et al. 2013; Zhao and Kuipers 2016). It is crucial to confirm the antimicrobial activity of a putative bacteriocin through various biochemical methods. Structural studies are normally followed. For example, the discovery of lichenicidin was a successful story through mining *Bacillus licheniformis* genome for LanM proteins (Begley et al. 2009). Many internet based mining tools have been developed for such purpose, such as BAGEL3 <http://bagel.molgenrug.nl/> (van Heel et al. 2013), which can analyse DNA sequences in FASTA format and give possible ORFs for both modified and non-modified putative bacteriocins.

Yet to find an optimal condition to isolate naturally expressed AMPs from any given microorganism can be very challenging. Therefore, with the help of nisin expression and modification system, van Heel et al. (2016) were able to overexpress putative lantibiotics obtained from genome mining. These obtained lantibiotics were shown to display high antimicrobial activity against various indicator strains including methicillin-resistant *S. aureus* (MRSA) (van Heel et al. 2016).

2.1 Classification of bacteriocins

According to Bactibase, the online database for bacteriocins (Hammami et al. 2007), there are 229 characterised bacteriocins by the time of writing. Moreover, with the use of BAGEL3, 238 complete LAB genomes were analysed and another 785 putative bacteriocin gene clusters were proposed (Alvarez-Sieiro et al. 2016). To date, many classification schemes have been proposed for the better understanding of bacteriocin basics.

Since the first classification system of LAB bacteriocins by Klaenhammer (1993), many modified schemes have been suggested. A structure-based classification for Gram⁺ bacteriocins can be as many as 12 groups (Zouhir et al. 2010). In general agreement, bacteriocins can be divided into three classes, excluding complex protein containing lipid or carbohydrate moieties (Cotter et al. 2013).

Class I bacteriocins are small (<10 kDa), heat-stable, membrane-active peptides, which have gone through post-translational modifications. These bacteriocins are ribosomally synthesised as inactive form. Only after post-translational enzymatic modifications to form unusual amino acids and structures, they can become biologically active (Alvarez-Sieiro et al. 2016). According to their structures, lantibiotics alone can be even divided into 11 subgroups (Cotter et al. 2005).

Class II bacteriocins are heat-stable small peptides (<10 kDa), which formation does not require any modification. There have been unsettled sub-divisions in this class due to many unique characteristics, especially, regarding the bacteriocins with cyclic structures. Some have seen circular bacteriocins as an individual class, e.g., class V in addition to Klaenhammer (1993)'s four-class scheme (Kemperman et al. 2003), or class IV with modifications based on Cotter et al. (2005)'s three-class system (Heng and Tagg 2006; Nes et al. 2007). In addition, Franz et al. (2007) even suggested a whole new classification

scheme based on the variety of enterocins, with cyclic peptides being class III in the enterocin scheme. Leaderless bacteriocins were listed in their own subgroup class IId by Nes et al. (2007). In their classification scheme, Nes et al. (2007) also separated the AMPs derived from larger proteins from the others and grouped them in class IIe.

Class III bacteriocins are generally considered to be large (>10 kDa) thermo-labile AMPs, including bacteriolysins and non-lytic bacteriocins (Alvarez-Sieiro et al. 2016), despite of the variations in the grouping of cyclic peptides (Alvarez-Sieiro et al. 2016; Cotter et al. 2013; Heng and Tagg 2006; Perez et al. 2014; Yang et al. 2014).

In this doctoral thesis, the classification of LAB bacteriocins is modified according to Alvarez-Sieiro et al. (2016), Arnison et al. (2013), and Nissen-Meyer et al. (2009) as shown in **Table 2**. Yet it is safe to say that, as more novel bacteriocins are expected to be discovered with the help of advancing technology, the classification scheme is inevitably to evolve accordingly.

Table 2. Proposed classification of LAB bacteriocins, modified according to the schemes of Alvarez-Sieiro et al. (2016), Arnison et al. (2013), and Nissen-Meyer et al. (2009).

Group	Descriptions	Examples	Producers	References
Class I Small (<10 kDa), heat-stable, modified bacteriocins				
Ia	Lantibiotics/ lantipeptides	Nisin A	<i>Lc. lactis</i>	Gross and Morell (1971)
		Lacticin 481	<i>Lc. lactis</i> CNRZ 481	Piard et al. (1993)
Ib	Sactibiotics	Subtilosin A	<i>Bacillus subtilis</i> 168	Babasaki et al. (1985)
Ic	Linear azol(in)e-containing peptides	Streptolysin S	<i>Streptococcus pyogenes</i>	Todd (1938)
Id	Glycocins	Sublancin 168	<i>Bacillus subtilis</i> 168	Paik et al. (1998)
Class II Small (<10 kDa), heat-stable, unmodified bacteriocins				
IIa	Pediocin-like bacteriocins	Leucocin C	<i>Ln. carnosum</i> 4010	Budde et al. (2003)
		Pediocin	<i>Pediococcus acidilactici</i> PAV-1.0	Henderson et al. (1992)
IIb	Two-peptide bacteriocins	Lactococcin G	<i>Lc. lactis</i> LMG 2081	Nissen-Meyer et al. (1992)
IIc	Circular bacteriocins	Lactocyclicin Q	<i>Lactococcus</i> sp. QU 12	Sawa et al. (2009)
IId	Non-pediocin-like single linear bacteriocins	Lacticin Q	<i>Lc. lactis</i> QU 5	Fujita et al. (2007)
		Lactococcin A	<i>Lc. lactis</i> ssp. <i>cremoris</i> LMG 2130	Holo et al. (1991)
Class III Large (>10 kDa), heat-labile bacteriocins				
IIIa	Bacteriolysin	Enterolysin A	<i>En. faecalis</i> LMG 2333	Nilsen et al. (2003)
IIIb	Non-lytic	Helveticin J	<i>Lb. helveticus</i> 481	Joerger and Klaenhammer (1986)

2.2 Class II: unmodified bacteriocins

This thesis focuses on *Leuconostoc* bacteriocins, all of which belong to the class II. For that reason, unmodified bacteriocins are discussed in more detail here.

Class II bacteriocins compose a vast amount of non-modified single linear heat-stable small AMPs with molecular mass less than 10 kDa. Class II bacteriocins often have a narrow antimicrobial spectrum, and are normally most active against closely related bacteria, which likely occur in the same ecological niche. It has been well established that class II bacteriocins kill target cell by forming pores or interfering with the integrity of the cell membrane (Eijsink et al. 2002).

Genes involved in class II bacteriocins synthesis are arranged in operons located in either the chromosome or the plasmids. Most class II bacteriocins are plasmid-borne. Yet there are a few examples which genetic determinants are found on bacterial chromosome, including enterocins A and B produced by *En. faecium* strains (Aymerich et al. 1996; Franz et al. 1999), divercin V41 from *Carnobacterium divergens* (Metivier et al. 1998), carnobacteriocin BM1 (Quadri et al. 1994), and lactacin B from *Lb. acidophilus* PA3 (Barefoot and Klaenhammer 1983). Lactacin F is chromosomally encoded on a conjugative transposon that is mobilized as a large extrachromosomal element (Muriana and Klaenhammer 1987). Most of class II bacteriocins possess N-terminal leader sequence, which is recognised by a dedicated transporter during the process of bacteriocin secretion. The most common leader sequence has two glycine residues (GG) at the positions -1 and -2 immediately before the cleavage site, and is cleaved off by ABC transporter when crossing the cytoplasmic membrane (Nes et al. 1996).

To date, at least 98 class II bacteriocins from LAB have been isolated and characterised, according to Bactibase, Nissen-Meyer et al. (2010), Maqueda et al. (2008), and Iwatani et al. (2011). Due to their unique features, such as structure, subgrouping is proposed according to various criteria (**Table 2** on page 11), although with increased knowledge it has become apparent that classification need to be modified when more bacteriocins are discovered. In this thesis, I followed the 4-subgroup scheme: IIa pediocin-like, IIb two-peptide, IIc circular, and IId single, linear, non pediocin-like bacteriocins.

2.2.1 Class IIa pediocin-like

Class IIa bacteriocins are often called pediocin-like bacteriocins, as the first well-studied member in this group is pediocin. These bacteriocins have common mode of action and are best known for their antimicrobial activity against food-borne pathogen *Listeria monocytogenes*. Bacteriocins in this group contain an N-terminal consensus sequence (YGNGV/L) and at least one C-terminal disulphide bridge formed by two cysteines. So far, there are 42 bacteriocins included in the class IIa, including new members like bacteriocin L-1077 from *Lb. salivarius* (Svetoch et al. 2011), and weissellin A from *Weissella paramesenteroides* DX (Papagianni and Papamichael 2011).

In general, bacteriocin genetic determinants are organised in one or a few operons and transcribed independently (Drider et al. 2006). For a functional production of class IIa bacteriocins, at least four genes are needed (Ennahar et al. 2000). These genes are: 1) the structural gene encoding bacteriocin precursor; 2) the immunity gene encoding immunity protein to protect producer from its own bacteriocin; 3) one or two genes encoding ATP-binding cassette (ABC) transporter for bacteriocin secretion; 4) a gene encoding an accessory protein necessary for bacteriocin secretion. Instead of using ABC transporter, a few class IIa bacteriocins such as bacteriocin 31 (Tomita et al. 1996), enterocin P (Cintas et al. 1997), and listeriocin743 (Kalmokoff et al. 2001), are secreted through *sec*-dependent pathway.

On the cell membrane of target bacteria, a receptor responsible for the bacteriocin binding has been identified to be the proteins of **mannose phosphotransferase system** (Man-PTS). These sugar permeases help the cells with intake and phosphorylation of mannose and glucose (Postma et al. 1993). In addition to the general PTS enzyme I (EI), Man-PTS also possesses a carbohydrate-specific protein complex, i.e., enzyme II (EII), composed of four subunits IIA, IIB, IIC and IID. The individual transmembrane proteins (IIC and IID) form a carbohydrate-specific translocation channel (Postma et al. 1993).

The expression of Man-PTS in *L. monocytogenes* and *En. faecalis* is regulated by the sigma factor σ^{54} encoded by the regulatory gene *rpoN* (Dalet et al. 2001; Robichon et al. 1997). Lack of σ^{54} and subsequently inactivation of *mpt* genes led to the bacteriocin resistance (Dalet et al. 2001; Héchard et al. 2001). By gene deletion and complementation, the IIC and IID of Man-PTS have been identified as the crucial anchor molecule for the class IIa bacteriocins to bind (Diep et al. 2007; Fimland et al. 2005). Later, an extracellular loop and its flanking regions (40 aa in total) located in the Man-PTS IIC were found essential for the specific recognition by class IIa bacteriocin (Kjos et al. 2010). Subunit IIC alone could already confer the sensitivity when listerial *mptC* was expressed in *Lactococcus* with an intact native IID encoded by *ptnD* (Wan et al. 2016). The function of IID was suspected to support the structure of Man-PTS and help IIC fold correctly in the membrane, facilitating the bacteriocin binding (Kjos et al. 2011a).

As in the N-terminal sequence of class IIa bacteriocins, there is a conserved motif (YGNGVxCxxxxCxVxWxxA) shared among all the group members, one might suspect that the N-terminal would be involved in target interaction. However, hybrid AMPs with N- and C-termini from different class IIa bacteriocins showed similar antimicrobial spectrum as that of the C-termini donors. The results indicated that the significantly varied C-termini is also responsible for the bacteriocin to specifically target the cells (Fimland et al. 1996; Johnsen et al. 2005). This obvious conflict leads to a possible explanation. The N-terminal β -sheet would first interact with the extracellular loop of the Man-PTS IIC protein, that enables the C-terminal α -helix engaging a helix-helix interaction with the Man-PTS regardless of the aa sequence (Kjos et al. 2011a). As the divergence of bacteriocin C-termini, it explains why immunity protein, various in sequence yet possesses a bundle of four antiparallel helix, could specifically bind to the cognate bacteriocin-receptor complex (**Figure 1** on page 15).

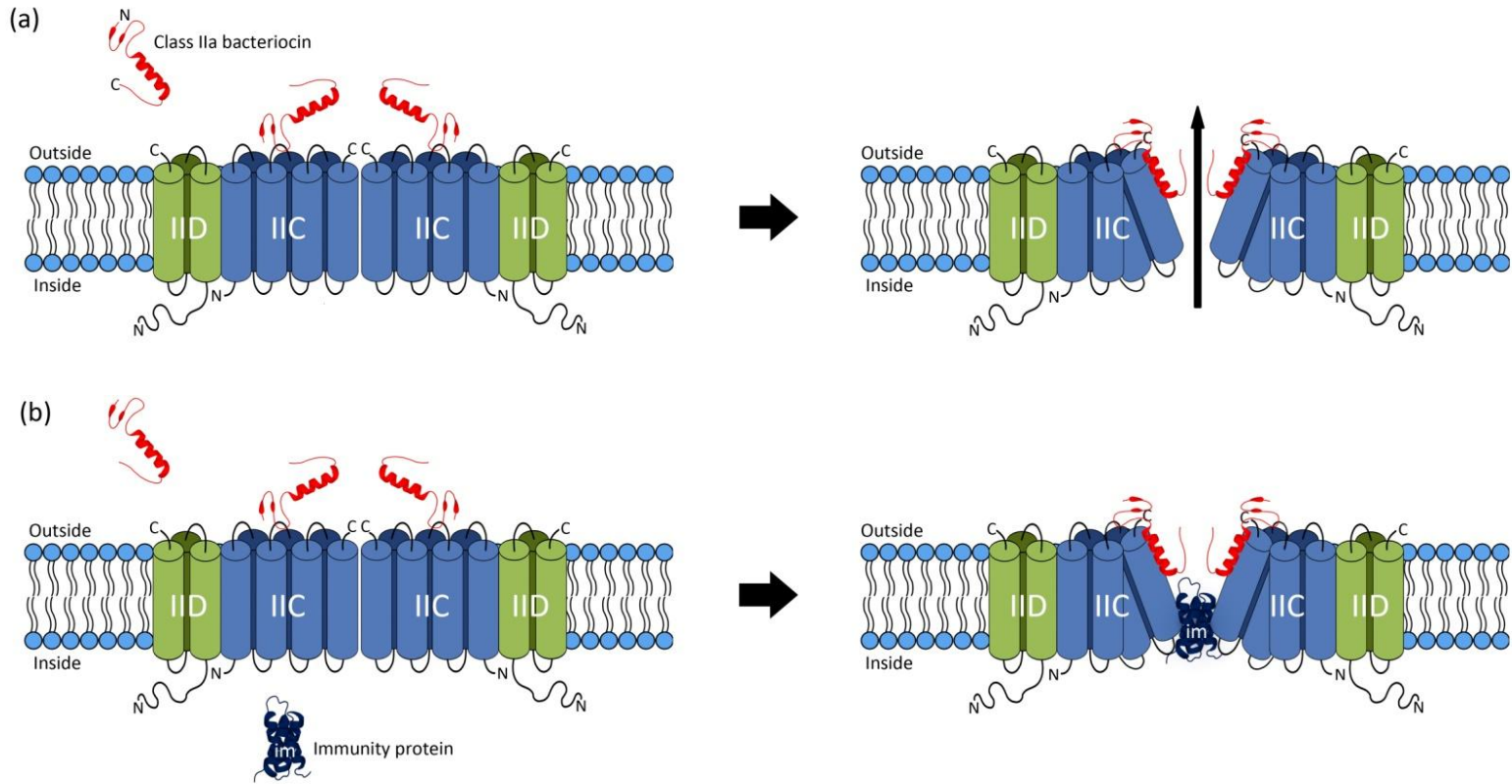


Figure 1. Proposed model of action (a) and immunity (b) of class IIa bacteriocins. The N-terminal β -sheet of the bacteriocin (red) binds to an extracellular loop (a) of the Man-PTS IIC domain (left). Then, the C-terminal helix interacts with transmembrane helices of the IIC (right) causing conformational 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 interacts with both IIC and IID of the receptor-bacteriocin complex. Adapted from Kjos et al. (2011a).

In order not to get killed by their own bacteriocins, bacteria have developed a self-protection system called immunity. In most bacteriocinogenic bacteria, self-immunity is accomplished by designated immunity protein encoded by the immunity gene located next to and co-expressed with the bacteriocin structural gene(s) (Cotter et al. 2005). Class IIa bacteriocins target the mannose phosphotransferase system on sensitive cells. Immunity protein protects the cells from Man-PTS targeting bacteriocins by blocking the pore formed by the bacteriocin from inside of the cells (Kjos et al. 2011a). Man-PTS IID has been shown to be indispensable, together with IIC, in the specific recognition between the bacteriocin immunity protein and the bacteriocin-receptor complex (Zhou et al. 2016). In comparative studies of immunity proteins, Fimland et al. (2002a) suggested three groups of immunity proteins to class IIa bacteriocins with clear similarities spanning along the sequence, which makes cross-immunity possible.

2.2.2 Class IIb two-peptide

Class IIb is a group of two-peptide bacteriocins, which optimal antimicrobial activity relies on the presence of both peptides at about equimolar amounts (Nissen-Meyer et al. 2010). The two peptides are normally named with consecutive two alphabetical letters, for example plantaricin E/F from *Lb. plantarum* C11 (Moll et al. 1999) or with additional α and β in the end of protein names, such as $W\alpha$ and $W\beta$ for enterocin W (Sawa et al. 2012). All two-peptide bacteriocins identified so far contain consensus motifs in their aa sequences, most commonly GxxxG (Nissen-Meyer et al. 2010), or similar AxxxA- and SxxxS- motifs in plantaricin $S\beta$ (Jiménez-Díaz et al. 1995) and plantaricin NC8 β (Maldonado et al. 2003), respectively. The genes encoding the two peptides are located next to each other in the same operon near the abovementioned crucial genes (Anderssen et al. 1998). Moreover, the production of some two-peptide bacteriocins is transcriptionally regulated through a three-component regulatory system (Oppegård et al. 2007), which will be discussed later in the introduction chapter **2.2.5 Regulation mechanism**.

All two-peptide bacteriocins have been shown to render cell membranes leakage of various small molecules, including a wide range of monovalent cations like Na^+ , K^+ , Li^+ , Cs^+ , Rb^+ etc., but not divalent cations (Oppegård et al. 2007). Plantaricins for example can form pores in the membranes of target cells and dissipate the transmembrane electrical potential and pH gradient (Moll et al. 1999). Circular dichroism studies of lactococcin G, plantaricin EF and plantaricin JK have found out that all these peptides are unstructured in aqueous face (Fimland et al. 2008; Rogne et al. 2008; Rogne et al. 2009). These results indicated that these bacteriocins would start structuring upon the contact with membrane-associated molecules.

A few targets for class IIb bacteriocin have been identified. By whole-genome sequencing, Kjos et al. (2014) identified mutation in or near the gene *uppP* in lactococcin G-resistant mutants. The **u**ndecaprenyl **p**yrrophosphate **p**hosphatase encoded by *uppP* is a membrane protein involved in peptidoglycan synthesis. Overexpression of lactococcal UppP in naturally resistant *Streptococcus pneumoniae* could convert the *Streptococcus* strain

sensitive to lactococcin G. The sensitivity to similar bacteriocins lactococcin Q (Zendo et al. 2006) or enterocin 1071 (Kjos et al. 2014) is also dependent on the presence of UppP. Lately, amino acid-polyamine-organocation (APC) transporters were suggested to be the target of two-peptide bacteriocin plantaricin JK based on sequence comparison studies (Oppegård et al. 2016). All identified two-peptide bacteriocins possess GxxxG-like motifs. They mediate the bacteriocins to form penetrating helix-helix structure in the target cell membrane (Nissen-Meyer et al. 2010; Oppegård et al. 2008). The recent structural study on plantaricin EF indicated that the G₅xxxG₉ motif in PlnE possibly interacts with the S₂₆xxxG₃₀ motif of PlnF in an antiparallel transmembrane orientation in a model lipid bilayer (Ekblad et al. 2016).

Alignment of existing immunity proteins of two-peptide bacteriocins also revealed high level of similarity (Oppegård et al. 2010). The function of immunity proteins is often confirmed by heterologous expression the immunity gene in naturally sensitive cells resulting in the cells resistant to corresponding bacteriocins. By cloning and expressing putative class IIb lactococcin G immunity protein LagC significantly increased the immunity in the host *Lactococcus*, suggesting the immunity function of LagC (Oppegård et al. 2010).

2.2.3 Class IIc circular

A class IIc cyclic bacteriocin has the structure of a closed head-to-tail circle. In general, the expression of circular bacteriocin structural genes must be combined with the activity of proteins involved in maturation (cleavage/circularization) and secretion via different transporter systems, as well as multifaceted immunity mechanisms to ensuring the cells' self-protection against own bacteriocin (Maqueda et al. 2008). Circular bacteriocins are expressed in the form of precursors with hugely variable N-terminal leader peptides (Gabrielsen et al. 2014b). The leader peptides are cleaved off from the precursors. Enterocin NKR-5-3B was found to be processed by proteins EnkB1234. These proteins are responsible for the leader and core peptide recognition, maturation and secretion of the circular bacteriocins (Perez et al. 2016).

Comparing with the other class II bacteriocins, class IIc bacteriocins have relatively broader antimicrobial spectra (Himeno et al. 2015; Maqueda et al. 2004). All the circular bacteriocins characterised so far from LAB, bacilli and clostridia are active against Gram⁺ bacteria of the phylum of *Firmicutes* (Acedo et al. 2015; Gabrielsen et al. 2014a; Perez et al. 2016). Most circular bacteriocins share a common three-dimensional structure including 4 or 5 α -helices and a hydrophilic core (Van Belkum et al. 2011). It is not clear whether circular bacteriocins require receptor molecules on the sensitive cells for target recognition. However, like other bacteriocins, circular bacteriocins permeabilize cell membrane resulting ion leakage, dissipation of the membrane potential, and eventually cell death (Gabrielsen et al. 2014a). Enterocin AS-48 (Gálvez et al. 1991), carnocyclin A (Gong et al. 2009), gassericin A (Kawai et al. 2004) and subtilosin A (Thennarasu et al. 2005) could permeabilize the liposomes and/or lipid bilayers, indicating that direct contacting to the target cell might be sufficient without any specific receptor. However, a

maltose ABC transporter complex in *Lc. lactis* was shown to be directly linked to the sensitivity to garvicin ML, as large deletion of *malEFG* encoding maltose transporter and its flanking region in chromosome was found in garvicin-resistant *Lactococcus* (Gabrielsen et al. 2012).

The specific ABC transporter can also protect the cells from exogenously produced bacteriocin. The transporter of cyclic bacteriocin enterocin AS-48 not only acts as a second immunity mechanism but also enhances the immunity to its bacteriocin (Diaz et al. 2003). DUF96 superfamily protein LcyD has also been shown to display immunity and secretion duo-function for the production of leucocyclicin Q (Mu et al. 2014). The protection mechanism is likely to be achieved by removing the bacteriocins from the cytoplasmic membrane. Additionally, leucocyclicin Q producer strain *Ln. mesenteroides* TK41401 can display cross-immunity against similar bacteriocin lactocyclicin Q, which is originally produced by *Lactococcus* sp. QU12 (Masuda et al. 2011).

2.2.4 Class IId single, linear, non pediocin-like

Class IId represents various heterogeneous one-peptide, linear, non-pediocin like bacteriocins according to the Cotter's classification scheme (2013). However, the range of this class covers broad and variable non-modified bacteriocins, comparing with the other three subclasses, and thus has been evolving along with the recognition of new bacteriocins. Because of the diverse nature of class IId bacteriocins, there is no consensus biosynthesis, mode of action, or immunity mechanism for all the members. According to the secretion pattern, Iwatani et al. (2011) consider there are three types of class IId bacteriocins: 1) *sec*-dependent bacteriocins; 2) leaderless bacteriocins; and 3) non-subgrouped bacteriocins.

The best characterised example of this group is lactococcin A from *Lc. lactis* ssp. *cremoris* LMG 2130. Like class IIa bacteriocins, lactococcin A is secreted through ABC transporter and leaks the target cell membrane by targeting to Man-PTS (Diep et al. 2007; Holo et al. 1991). The immunity protein LciA of lactococcin A was shown to have different conformation inside of the cells and to function differently with and without the presence of the associated bacteriocin. When the lactococcin A is present, LciA could tightly associate with the membrane located Man-PTS receptor and the bacteriocin (Diep et al. 2007).

Some class IId bacteriocins are synthesised without N-terminal leader sequence, that makes the bacteriocin recognition and secretion mysterious (Alvarez-Sieiro et al. 2016). For example, Lacticin Q is a leaderless bacteriocin from *Lactococcus* and has relatively wide antimicrobial spectrum. The presence of genes encoding ABC transporter suggests that the dedicated transporter possibly facilitates secretion and self-immunity of lacticin Q (Iwatani et al. 2012). Yet this particular bacteriocin seems not to require any specific docking molecule, but rather interact with the outer membrane to form "huge toroidal pores" coupled with lipid flip-flop (Yoneyama et al. 2009). Lacticin Q killing mechanism, however, is highly dependent on the physicochemical features of the outer membrane

components, i.e. only Gram⁺ bacteria would be possibly sensitive to the bacteriocin (Yoneyama et al. 2011). Li et al. (2013) also suggested that the strain-dependent level of hydroxyl radical accumulation by lacticin Q resulted in antimicrobial activity in specific strains. On the other hand, LsbB, another leaderless bacteriocin, appears to require a membrane-bound Zn-dependent metallopeptidase to bind to the target cell (Uzelac et al. 2013). In addition to being the common target molecule for class IIa bacteriocins, Man-PTS also serves as the receptor for the class IID bacteriocin lactococcin A. But both IIC and IID units from the same source are required to kill the target cells (Diep et al. 2007; Kjos et al. 2010).

Lactococcin 972 from *Lc. lactis* IPLA972 contains a 25-aa *sec*-dependent signal peptide, which is used in the *sec*-dependent pathway to secrete 66-aa mature bacteriocins without dedicated transporters (Martínez et al. 1999). Lactococcin 972 has been found to specifically interact with the cell wall precursor lipid II and inhibit the cell wall biosynthesis at the division septum. Furthermore, despite lipid II is a universal molecule for prokaryotes, this bacteriocin can kill only certain species of the *Lactococcus* family, suggesting other receptor might be needed to determine its specificity (Martínez et al. 2008). Similar activity against septum formation was also observed in transmission microscopy image of garvicin A from *Lc. garvieae* 21881 against other *Lc. garvieae* strains (Maldonado-Barragan et al. 2013).

2.2.5 Regulation mechanism

As briefly mentioned earlier, bacteriocin production can be tightly regulated. In some LAB, the production of class II bacteriocins is regulated by a quorum sensing-based **three-component regulatory system** (3CS) (Nes et al. 2007). The communication between cells depends on an induction factor, i.e. peptide pheromone, in the environment. When pheromone accumulates to a certain threshold concentration, the membrane-bound histidine protein kinase, is activated and triggers a series of autophosphorylation reactions. In the end, an intracellularly phosphorylated response regulator binds to the regulated promoters to activate a set of genes (**Figure 2** on page 20) (Nes and Eijsink 1999). In some instances, regulation is accomplished by a two-component system. The extracellular bacteriocin-like peptide functions as induction factor and is detected by the histidine kinase. The cytoplasmically located response regulator then mediates the response (Cotter et al. 2005).

The regulation system has been thoroughly studied with the synthesis of plantaricins in *Lb. plantarum* C11 (Diep et al. 1996; Diep and Nes 1995). Diep et al. (2003) has shown that the peptide pheromone plantaricin A induces the production of two-peptide bacteriocins plantaricin J/K and plantaricin E/F in a highly-regulated manner and fine timing. However, even though plantaricin A shared most physicochemical properties of other class II bacteriocins, the peptide itself did not show significant bacteriocin activity (Nes et al. 1996). Other examples which production is regulated by such system are class IIa bacteriocins carnobacteriocin B2 (Quadri et al. 1997) and sakacin A (Ennahar et al. 2000). Recently, it has been suggested that the full expression of leaderless bacteriocin

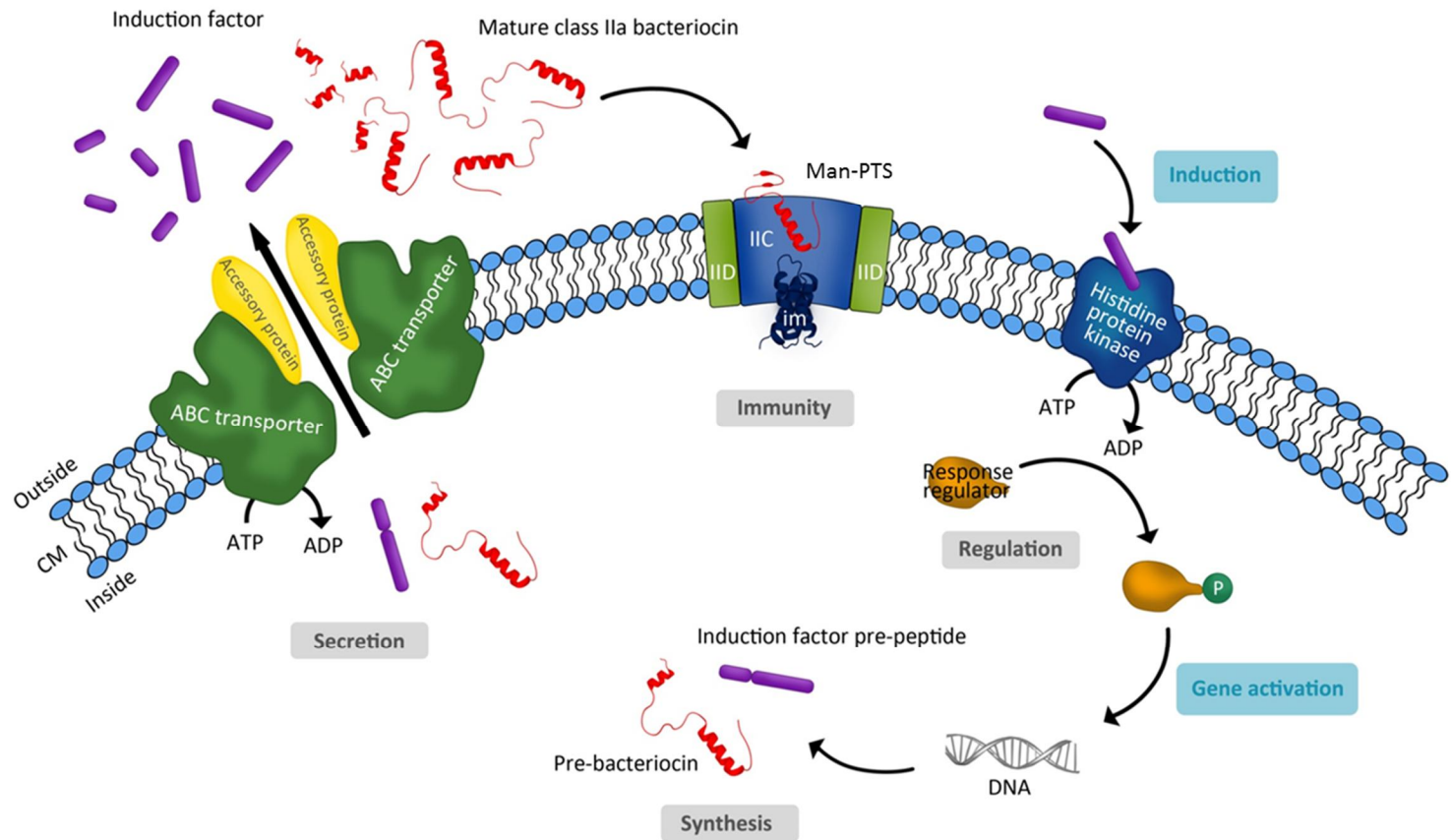


Figure 2. The production of class IIa bacteriocin: three-component regulatory system, biosynthesis, secretion, and immunity. CM, cell membrane. Im, immunity protein. Man-PTS, mannose phosphotransferase system, bacteriocin receptor IIC and IID. Adapted from Ennahar et al. (2000).

LsbB is regulated by a transcription terminator sequence located downstream of the structural gene (Uzelac et al. 2015). Moreover, the production of two-peptide bacteriocin plantaricin NC8 was induced only by co-culturing the producer *Lb. plantarum* NC8 with *Lc. lactis* MG1363 or *Pediococcus pentosaceus* FBB63 (Maldonado et al. 2003).

In addition, environmental factors also affect the bacteriocin biosynthesis, such as temperature, ionic strength and pH, etc. Most bacteriocinogenic bacteria tend to produce more bacteriocins at around 25 °C with slight acidic environment (Mataragas et al. 2003; Zhou et al. 2015). Many are reported to stop bacteriocin production at high temperature (above 35 °C). However, *P. acidilactici* manages to produce pediocin even at 45 °C (Turgis et al. 2016). Innate quorum sensing regulatory mechanism can be suppressed by environmental temperature. The production of sakacin A is regulated by 3CS at 25 - 30 °C, yet significantly reduced at higher temperature (33.5 - 35 °C) (Diep et al. 2000).

2.2.6 Bacteriocin spontaneous resistance

Resistance to bacteriocins may compromise their use as biopreservatives in food or as anti-infection agents. On the other hand, bacteriocin resistance inspires researchers to identify the bacteriocin target molecules thus understanding bacteriocins' mode of action.

For some class II bacteriocins, sensitive cells can develop resistance when exposed to bacteriocin. The frequency of resistance development varies depending on the bacteriocin and also the sensitive strains. *L. monocytogenes* can develop resistance to class IIa bacteriocins, with resistance frequency up to 10^{-4} (Gravesen et al. 2002b). As the targeting mechanism for class IIa bacteriocins has been well studied, their resistance mechanisms are investigated in detail. As more bacteriocin targets are identified, the view of how bacteria develop resistance to bacteriocins become clearer gradually.

For class IIa bacteriocins and class IId lactococcin A, the MptC and MptD of Man-PTS is responsible for bacteriocin targeting (Kjos et al. 2011a; Opsata et al. 2010). Down-regulation of Man-PTS expression not only leads to the reduction in cell growth on glucose, but also results in bacteriocin resistance in *L. monocytogenes*, *En. faecalis* and *Lc. lactis* (Gravesen et al. 2002b; Kjos et al. 2011b; Opsata et al. 2010; Tessema et al. 2009). Since the expression of Man-PTS in *L. monocytogenes* is regulated through several proteins (Vu-Khac and Miller 2009), it is possible that any mutation at these areas in the genome would interfere the Man-PTS expression and thus may cause the resistance.

In case of two-peptides, the sensitive *Weissella viridescens* developed stable mutation, resistant to the plantaricin JK upon bacteriocin challenge. Mutations were identified at genomic positions encoding the putative target of two-peptide bacteriocins, APC family transporter (Oppegård et al. 2016). As for lactococcin G, mutation in the bacteriocin target molecule UppP was shown to lead to the bacteriocin resistance (Kjos et al. 2014). The occurrence of resistance to class IId lactococcin 972 is linked to upregulation of *llmg2447* which encodes a putative extracytoplasmic function anti-sigma factor (Roces et al. 2012).

2.3 *Leuconostoc* bacteriocins

Bacteriocins produced by *Leuconostoc* species are often called leucocins. Some are named mesentericins, as they are produced by *Ln. mesenteroides* strains. There are at least 40 *Leuconostoc* bacteriocins identified so far (**Table 3** on page 23-24).

Although many *Leuconostoc* bacteriocins have been well characterised, nomenclature of these peptides seems confusing. As one of the most studied leucocins, leucocin A has several different names, most of which indicate the producer, but some created quite a confusion. For example, leucocin B Ta11a from *Ln. carnosum* Ta11a (Felix et al. 1994) and leucocin C LA7a from *Ln. paramesenteroides* La7a (Hastings et al. 1996) have identical aa sequence to leucocin A. The only differences between several leucocin As are found in the leader peptides (**Table 3**). As the producer strains are different for the leucocinAs, it might explain why the leader peptides vary. Considering all the different names for literally same compounds, it seems logical to use unified nomenclature in the future studies of leucocins.

Bacteriocins produced by *Ln. mesenteroides* species are confusingly given two names mesentericin or mesenterocin, among which mesenterocin 52A (Revol-Junelles et al. 1996) and mesenterocin E131 (Xiraphi et al. 2008) are identical to mesentericin Y105 (Hécharde et al. 1992). Some *Leuconostoc* bacteriocins are not given specific names, although their biologic features have been well studied (Mataragas et al. 2003). In terms of the aa sequence of bacteriocin core peptide, mesentericin/mesenterocin differs from leucocin A by only two aa. Meanwhile, not all the leucocin Cs identified so far shared exactly same sequence (**Table 3**).

Because of taxonomic changes, some previous *Leuconostoc* species are now included in the genus of *Weissella*, therefore the nomenclature of the corresponding bacteriocins are derived from the name of *Weissella*. For example, weissellin A produced by *Weissella paramesenteroides* (Papagianni and Papamichael 2011) is a class IIa bacteriocin and shares 73 % similarity with leucocin C. This particular *Weissella paramesenteroides* was firstly described as *Ln. paramesenteroides* (Garvie 1967), and later emended to *Weissella* genus (Collins et al. 1993; Padonou et al. 2010).

In the 1990's, the studies of *Leuconostoc* bacteriocins were mostly about the discovery of the compounds, their antimicrobial spectrum and the confirmation of bacteriocin nature (Daba et al. 1991; Janes et al. 1999). From later 1990's to the 2000's, more researchers started to reveal the bacteriocin sequences (Budde et al. 2003; Fimland et al. 2002b; Revol-Junelles et al. 1996). As time went on to the 2010's, the trend of bacteriocin studies went deeper to their mode of actions, and discovery through genome mining technique (Alvarez-Sieiro et al. 2016; Kjos et al. 2011a).

Table 3. Bacteriocins produced by *Leuconostoc* species.

Name	Producer	Description, sequence, antimicrobial spectrum	Reference
Leucocin A			
leucocin A-UAL 187	<i>Ln. gelidum</i> UAL 18710.....20.....30.....40.... MNNMKPTESYEQLDNSALEQVVGKKYYGNGVHCTKSGCSVNWGEAFSAGVHRLANGGNGFW-	Hastings et al. (1991)
leucocin 6A	<i>Ln. mesenteroides</i> 6 CFS	KYYGNGVHCTKSGCSVNWGEAFSAGVHRLANGGNGFW-	Vaughan et al. (2001)
leucocin A TA33a	<i>Ln. mesenteroides</i> TA33a	KYYGNGVHCTKSGCSVNWGEAFSAGVHRLANGGNGFW-	Papathanasopoulos et al. (1998)
leucocin A-QU 15	<i>Ln. pseudomesenteroides</i> QU 15	MNNMK SADNYQLDNNALEQVVGKKYYGNGVHCTKSGCSVNWGEAFSAGVHRLANGGNGFW-	Sawa et al. (2010)
leucocin A (Δ7)	<i>Ln. pseudomesenteroides</i> QU 15	KYYGNGVHCTKSGCSVNWGEAFSAGVHRLA-	Sawa et al. (2010)
leucocin A	<i>Ln. camosum</i> 4010	MNNMK SADNYQLDNNALEQVVGKKYYGNGVHCTKSGCSVNWGEAFSAGVHRLANGGNGFW-	Wan et al. (2013)
leucocin A-4010	<i>Ln. camosum</i> 4010	KYYGNGVHCTKSGCSVNWGEAFSAGVHRLA.....	Budde et al. (2003)
leucocin B TA11a	<i>Ln. camosum</i> TA11a	MNNMK SADNYQLDNNALEQVVGKKYYGNGVHCTKSGCSVNWGEAFSAGVHRLANGGNGFW-	Felix et al. (1994)
leucocin B-KM432Bz	<i>Ln. pseudomesenteroides</i> KM432Bz	MNNMK SADNYQLDNNALEQVVGKKYYGNGVHCTKSGCSVNWGEAFSAGVHRLANGGNGFW-	Makhloufi et al. (2013)
leucocin C-LA7a	<i>Ln. paramesenteroides</i> LA7a	MNNMK SADNYQLDNNALEQVVGKKYYGNGVHCTKSGCSVNWGEAFSAGVHRLANGGNGFW-	Hastings et al. (1996)
Leucocin B			
leucocin B TA33a	<i>Ln. mesenteroides</i> TA33a10.....20.....30.....40.... KGGKGFWSWASKATSWLTGPGQPGSPLLKKHR-	Papathanasopoulos et al. (1998)
leucocin B	<i>Ln. camosum</i> 4010	MLNKDMRDFKQLSDNDLQAI SGGKGGKGFWSWASKATSWLTGPGQPGSPLLKKHR-	Wan et al. (2015)
Leucocin C			
leucocin B-4010	<i>Ln. camosum</i> 401010.....20.....30.....40.... KNYNGVHCTKKGCSVDWGYAWTNIANN SVMNGLTGGNA...	Budde et al. (2003)
leucocin C	<i>Ln. mesenteroides</i> 6	KNYNGVHCTKKGCSVDWGYAWTNIANN SVMNGLTGGNAGWHN-	Fimland et al. (2002b)
leucocin C	<i>Ln. camosum</i> 4010	MMNMKPTESYEQLDNSALEQVIGGKKNYNGVHCTKKGCSVDWGYAWTNIANN SVMNGLTGGNAGWHN-	Wan et al. (2013)
leucocin 6C	<i>Ln. mesenteroides</i> 6 CFS	KNYNGVHCTKKGCSVDWGYAWTNIANN SVMNGLT.....	Vaughan et al. (2001)
leucocin 7C	<i>Ln. mesenteroides</i> 7 CFS	KNYNGVHCTKKGCSVDWGYAWTNIANN SV.....	Vaughan et al. (2001)
leucocin 10C	<i>Ln. mesenteroides</i> 10 CFS	KNYNGVHCTKKGCSVDWGYAWTNIANN SVMNGLT.....	Vaughan et al. (2001)
leucocin C TA33a	<i>Ln. mesenteroides</i> TA33a	KNYNGVHCTKKGCSVDWGYAWTNIANN SVMNGLTGT.....	Papathanasopoulos et al. (1998)
Other leucocins			
leucocin Q	<i>Ln. pseudomesenteroides</i> QU 1510.....20.....30.....40.... MKNQLMSFEVISEKELSTVQGGKGLGKLI GIDWLLGQAKDAVKQYKKDYKRWH-	Sawa et al. (2010)
leucocin N	<i>Ln. pseudomesenteroides</i> QU 15	MNKYEYNSISNFKKITNKDLQNINGGF IGRAIGDFVYFPAKGLRESGKLLNYYKHKH-	Sawa et al. (2010)
leucocin F10	<i>Ln. camosum</i> F10	antilisteria	Parente et al. (1998)
leucocin H	<i>Ln. MF215B</i>	two-peptide	Blom et al. (1999)
leucocin OZ	<i>Ln. camosum</i> OZ	antilisteria	Osmanağaoğlu (2007)
leucocin K	<i>Ln. sp. LAB145-3A</i>	against <i>Enterococcus</i> and <i>Listeria</i>	Choi and Ahn (1997)
leucocin K7	<i>Ln. mesenteroides</i> K7	antilisteria	Shi et al. (2016)
leucocin BC2	<i>Ln. mesenteroides</i> BC2	against <i>Listeria</i> , <i>Enterococcus</i> , <i>Bacillus</i>	Janes et al. (1999)
Leuconostoc bacteriocins not named as leucocin			
mesentericin Y105	<i>Ln. mesenteroides</i> Y10510.....20.....30.....40.... MNNMKSV EAYQLDNNQNLKQVVGKKYYGNGVHCTKSGCSVNWGEAA SAGVHRLANGGNGFW-	Hécharde et al. (1992)
mesentericin Y 105 ³⁷	<i>Ln. mesenteroides</i> Y105	KYYGNGVHCTKSGCSVNWGEAA SAGVHRLANGGNGFW-	Fleury et al. (1996)
mesenterocin E131	<i>Ln. mesenteroides</i> E131	KYYGNGVHCTKSGCSVNWGEAA SAGVHRLANGGNGFW-	Xiraphi et al. (2008)
mesenterocin 52A	<i>Ln. mesenteroides</i> ssp. <i>mesenteroides</i> FR52	KYYGNGVHCTKSGCSVNWGEAA SAGVHRLANGGNGFW-	Revol-Junelles et al. (1996)

mesenterocin 52B	<i>Ln. mesenteroides</i> ssp. <i>mesenteroides</i> FR52		KGVLGWLSMASSALTGPQQPNPWLAKIKNHK-	Revol-Junelles et al. (1996)
mesentericin ST 99	<i>Ln. mesenteroides</i> ssp. <i>dextranicum</i> ST99			Todorov and Dicks (2004)
mesenterocin 5	<i>Ln. mesenteroides</i> UL5	antilisteria		Daba et al. (1991)
mesenterocin Y	<i>Ln. mesenteroides</i> E131	against <i>Enterococcus</i> , <i>Listeria</i> , <i>Pseudomonas</i> , and <i>Salmonella</i> species		Zdolec et al. (2008)
mesenterocin	<i>Ln. mesenteroides</i> OZ	antilisteria		Osmanağaoğlu and Kiran (2011)
camocin 54	<i>Ln. camosum</i> LA54A	amylase-sensitive, against <i>Leuconostoc</i> , <i>Carnobacterium</i> , <i>Enterococcus</i> and <i>Listeria</i> species		Kepler et al. (1994)
dextranicin 24*	<i>Ln. mesenteroides</i> ssp. <i>dextranicum</i> J24		KGVLGWLSMASSALTGPQQ.....	Revol-Junelles and Lefebvre (1996)
kimchicin GJ7	<i>Ln. citreum</i> GJ7	enhanced bacteriocin production by growing with inducing factor from <i>Lb. plantarum</i>		Chang et al. (2007)
leuconocin J	<i>Ln. sp.</i> J2	against <i>S. aureus</i>		Choi et al. (1999)
leuconocin S	<i>Ln. paramesentroides</i> OX	amylase-sensitive, against <i>Lb. sake</i>		Lewus et al. (1992)
leucocyclin Q**	<i>Ln. mesenteroides</i> TK41401		MFLVNQLGISKSLANTILGAIAVGNLASWLLALVPGPGWATKAALATAETIVKHEGKAAAIAW-	Masuda et al. (2011)

* N-terminal sequence identical to mesentericin B105; ** cyclic bacteriocin; | cleavage site between the leader and core peptides; - indicates the sequence is complete; ... indicates incomplete sequences; x indicates unidentified amino acid; black shading, non-identical amino acids between leucocin As and mesentericins/mesenterocins; colour shading, non-identical amino acids between leucocin Cs.

3 Heterologous expression of class II bacteriocins from LAB

In many instances, the target bacteriocin we would like to study is produced by a bacterium which is difficult to handle, or at inconveniently low concentration, or co-produced with other active compounds in the native host. Heterologous expression thus enables the production of a certain peptide in an easier host and at higher yield, that facilitate the downstream researches and applications.

So far quite many class II bacteriocins have been overexpressed in heterologous hosts, either closely related, such as *Lactococcus*, or common engineer hosts like *E. coli* or yeast with the intention to increase bacteriocin yield. *Leuconostoc* bacteriocin leucocin C was produced under the control of P45 promoter in *E. coli*, and heterologous leucocin C also displayed antimicrobial activity against *L. monocytogenes* (Liu et al. 2013). A few yeast expression platforms have also been developed to produce heterologous peptides and proteins, including LAB bacteriocins such as enterocin A and bacteriocin E50-52 in *Pichia pastoris* and *Kluyveromyces lactis* (Borrero et al. 2012; Jiménez et al. 2014).

3.1 Expression of native bacteriocin genes

In the early studies of bacteriocins, genes required for the production of AMPs were conventionally cloned as a bundle in the heterologous hosts. The production of class IIa model bacteriocin pediocin requires the *ped* operon of four genes from *P. acidilactici* PAC1.0. Marugg et al. (1992) cloned all *pedABCD* in *E. coli* and the resultant heterologous pediocin showed activity against *L. monocytogenes*. Moreover, the whole operon about 1.2 kb encoding both of the two-peptides of lactacin F and the putative immunity protein from *Lb. johnsonii* was cloned and expressed in *Carnobacterium* species which is also the producer for carnobacteriocins (Allison et al. 1995). The native processing and exporting mechanism in *Carnobacterium* was shown to successfully produce bioactive lactacin F.

3.2 Optimisation of bacteriocin production

Most LAB bacteriocins require a dedicated processing and secretion system, i.e. ABC transporter and accessory proteins (Ennahar et al. 2000). The secretory mechanism can recognise bacteriocin's leader peptide, which can vary dramatically among different species and strains. To optimise the production in the other host, LAB bacteriocin's mature part can be fused with leader sequence which is recognised better in the host strain. Stronger promoters can be used to make the host produce heterologous peptides more. Inducible promoter such as P_{nisA} was used to produce pediocin at higher yield with negligible amount of nisin for induction. However, the optimal potency requires the accessory protein PedC (Back et al. 2016). Recently, a few strong constitutive promoters were identified from *Lc. lactis*, and nisin Z production could be doubled by using the newly discovered P8 promoter instead of conventional P45 (Zhu et al. 2015a). Additionally, Zhu et al. (2017) have found out that deleting non-essential genomic regions by only 2.83 % from *Lc. lactis* NZ9000 made the strain not only grow fast in both rich

media and chemically defined media, but also produce 2.5-fold more leucocin C. Bacteriocins have also been fused to an extra protein/peptide and co-expressed as one fusion protein in heterologous host. The protein extension, such as His-tag for plantaricin NC8 (Jiang et al. 2016) or thioredoxin plantaricin Pln1 (Meng et al. 2016), facilitates the purification procedure and is cut off by protease leaving bioactive bacteriocins. Furthermore, Enterocin E50-52 and pediocin hybrid bacteriocins have shown improved antimicrobial activities against *Micrococcus luteus*, *Salmonella enterica*, *E. coli* O157:H7 (Tiwari et al. 2015).

4 LAB bacteriocin applications

LAB have been associated with food fermentations for at least 4000 years. At the moment, when one searches “bacteriocin” and “application” as keywords, the majority of the researches focuses on using bacteriocins as bio-preservatives in food industry.

As LAB bacteriocins have strong antibacterial activity, being produced by harmless LAB and have not been shown to have any toxic effect on mammalian cells, they seem to be ideal for killing harmful and pathogenic bacteria in food and feed. Bacteriocins can be used in food as part of hurdle technology to prolong shelf life and improve food quality. For example, cider can be spoiled by the growth of exopolysaccharide producing bacteria, e.g. *Bacillus licheniformis*, forming slime and giving the drink a “ropy” appearance (Larpin et al. 2002). Enterocin AS-48 produced by *En. faecalis* A-48-32 has been found to make *B. licheniformis* more liable to heat-treatment, thus combining bacteriocin with heat-treatment would be more efficient to inactivate spore forming *Bacillus* in cider (Grande et al. 2006). The growth of *L. monocytogenes* is a major concern in dairy industry. Partially purified leucocin K7 from *Ln. mesenteroides* K7 was shown to inhibit *L. monocytogenes* in milk (Shi et al. 2016). Additionally, bacteriocin producing starter cultures have been found useful in preventing food spoilage and killing pathogenic bacteria in food (De Vuyst and Leroy 2007). Bacteriocin-containing freeze-dried LAB powder has been found to inhibit *Listeria* in hot dogs, simply by applying the powder onto the surface of the hot dogs (Ünlü et al. 2016).

In addition to being used directly, bacteriocins can also be incorporated to packaging films which are then used in food wrapping. Packaging films coated with solution containing nisin could inhibit the growth of *L. monocytogenes* on the surface of packed hot dogs (Franklin et al. 2004) and *M. luteus* spiked in milk (Mauriello et al. 2005). Ercolini et al. (2006) used bacteriocin 32Y from *Lb. curvatus* to coat regular polythene film, which in turn could reduce the superficial contaminant *Listeria* on the surface of food products packed in the film. Although the bacteriocin applications in food have been shown very successful in pilot studies, so far among hundreds of bacteriocins, nisin is the only commercial bacteriocin approved for application in e.g. certain cheese, cream and pudding products (EFSA 2006); ready-to-eat meat and poultry products, and finished sausage (FDA 2001).

MRSA and vancomycin-resistant enterococci have caused increasing number of infections harder to treat and become major medical problems in hospitals around the world. Okuda et al. (2013) reported that nisin A was especially effective against antibiotic-resistant staphylococci, followed by lactacin Q from *Lc. lactis* strains. As many staphylococci form biofilms which increase their resistance to chemotherapies making infections more difficult to treat. *In vitro*, combining nisin with other conventional antibiotics such as chloramphenicol, vancomycin or ciprofloxacin displayed enhanced inhibitory effects toward MRSA (Dosler and Gerceker 2012; Field et al. 2016). Geldart (2016) presented their results in the international antimicrobial peptide symposium in France, that a engineered *Lc. lactis* producing enterocin A, enterocin P and hiracin JM79 could significantly reduce the pathogenic vancomycin-resistant enterococci counts in murine model, and by combining these bacteriocins with traditional antibiotics may revive the activity against *Enterococcus*. Additionally, various bacteriocins have been reported to kill viruses. For example, enterocin CRL35 from *En. faecium* and subtilisin from *Bacillus amyloliquefaciens* display inhibitory activity towards human herpesvirus. These bacteriocins disturb the fusion of the viral envelope with the cellular membrane, therefore inhibit the viral replicative cycle (Quintana et al. 2014; Wachsman et al. 1999).

5 LAB genome editing

Conventionally lactic acid bacterial genome editing has been accomplished through homologous recombination using an integration vector that carries two chromosomal fragments (*H1* and *H2*) flanking the area *X* to be deleted. This approach involves two crossover steps: 1) a plasmid carrying two adjacent chromosomal fragments *H1H2* integrates into the target strain's chromosome at either of the two homologous regions through single crossover (SCO). Selection of integrants typically relies on antibiotic resistance. 2) without antibiotic selection pressure, a second recombination occurs and the unnecessary plasmid is removed from bacterial chromosome. As a result, double crossover (DCO) cells have either deleted the target region *X*, or reversed to the wild-type genotype. The frequency of recombination is approximately 10^{-4} (Gravesen et al. 2002a) and only part of the DCO colonies carry the deletion, thus finding the desired strain without selection often requires extensive screening. So far, a few systems have been developed to increase DCO frequency or select the colonies in which DCO has happened.

Thermosensitive (TS) pG⁺Host vectors have been used to knock out chromosomal areas from *Lc. lactis* genome (Biswas et al. 1993). The TS vector is sensitive to temperature and only replicates at 28 °C. This feature facilitates the *Lc. lactis* to uptake the vector with high frequency and allow the vector to replicate at 28 °C. Once the temperature rises to 37 °C, vector stops replicating and can integrate into the chromosome through homologous recombination as designed. By shifting the temperature back to 28 °C, integrated plasmid starts to replicate and is removed from the chromosome through second recombination, resulting either deletion or wild-type reversion.

Another recombination system used in *Lc. lactis* is based on Cre-*lox* (Zhu et al. 2015b), which was originally used in multiple gene deletions *Lb. plantarum* (Lambert et al. 2007).

After SCO, integrant is first selected with erythromycin and chloramphenicol. Then part of the integrated vector including the erythromycin resistance gene is lost through DCO. As a result, the target region *X* is replaced by the chloramphenicol resistance gene *cat* flanked by *lox* recognition sites for the Cre recombinase. Finally, the *lox-cat-lox* cassette is removed via transient expression of the Cre recombinase, leaving short inactive *lox*-sites into the chromosome (Lambert et al. 2007). In this method, there is no actual selection, and the second crossover was still found by screening for the erythromycin sensitivity. Moreover, the Cre-*lox*-based gene deletion is not completely clean, as the *lox*-sites remain after the procedure.

In order to select the DCO colonies, a few systems incorporated with negative selection markers have been developed. The integration of the vector is selected with the positive marker (usually antibiotic resistance gene), whereas the loss of the plasmid is selected with the negative marker which causes the sensitivity towards certain selective agent. Viable strains will be the ones which have excised the negative marker through second homologous recombination. For *Lc. lactis*, a counterselectable vector based on 5'-fluoroorotic acid (FOA) sensitivity has been developed (Solem et al. 2008). The integration introduces the orotate transporter gene *oroP*, which makes the cells sensitive to FOA. Yet, *Lc. lactis* has to be cultured in chemically defined media supplemented with FOA to make DCO colonies selectable. Moreover, as *oroP* is commonly present in *Lc. lactis* strains (Siezen et al. 2011), unwanted homologous recombinations between the native and the introduced *oroP* genes may occur during the process. In *Lb. acidophilus*, *Lb. casei*, and *Lc. lactis*, a uracil phosphoribosyltransferase (*upp*) based counterselection method has been developed (Goh et al. 2009; Song et al. 2014). Similarly, expression of *upp* from integration vector causes sensitivity to 5-fluorouracil, which can be used to select the DCO colonies.

Interestingly, although the method using clustered regularly interspaced short palindromic repeats (CRISPR) and the associated DNA endonuclease Cas has gained most attention recently, the system has not yet been used in editing lactococcal genomes. CRISPR/Cas system was adapted and combined with the idea of recombination to select the desired gene deletion in Gram⁺ *Clostridium berjerinckii* (Wang et al. 2015). In terms of LAB, CRISPR/Cas was used to aid the screening of short gene replacement in *Lb. reuteri* genome (Oh and van Pijkeren 2014).

AIMS OF THE STUDY

The main objectives of this doctoral study were to characterise the genetics of bacteriocins produced by *Leuconostoc carnosum* 4010 (I, II), and to exploit the class IIa bacteriocin targeting mechanism in *Lc. lactis* genome editing (III). The detailed aims of the research were to:

1. study the genetics required for the production of leucocins B and C in *Ln. carnosum* 4010, and overexpress these leucocins in food-grade lactic acid bacteria *Lc. lactis*.
2. investigate the functions of the proteins related to leucocin production in the strain 4010.
3. utilise the class IIa bacteriocin targeting mechanism and develop a counterselection system for seamless gene deletions for *Lc. lactis*.

MATERIALS AND METHODS

Bacterial strains and plasmids used in this study are listed in **Table 4** and **Table 5** (on page 31), respectively. Experimental methods are summarised in **Table 6** (on page 32) and explicated in the articles I, II, and III present at the end of this book.

Table 4. Bacterial strains used in this study.

Bacterial strains	Relevant properties	Reference/source	Used in
<i>Leuconostoc carnosum</i> 4010	Wild-type leucocin producer, DMRICC 4010 ^a	Chr. Hansen A/S, Hørsholm, Denmark; Budde et al. (2003)	I, II
<i>Ln. mesenteroides</i> ATCC 8293	Leucocin C-sensitive indicator strain	ATCC	I
<i>Ln. pseudomesenteroides</i> CIP103316	Indicator strain sensitive to leucocins A, B, and C	A gift from Prof. Anne-Marie Revol-Junelles, University of Lorraine, Nancy, France	II
<i>Ln. pseudomesenteroides</i> AC ^R	Spontaneous mutant of CIP103316, resistant to leucocins A and C	This study	II
<i>Weissella paramesenteroides</i> LMA 19	Indicator strain sensitive to leucocins A, B, and C	A gift from Prof. Anne-Marie Revol-Junelles, University of Lorraine, Nancy, France	II
<i>Lactobacillus rhamnosus</i> 1/6	Wild type strain, <i>pepR</i> promoter	Valio Ltd., Helsinki, Finland; Varmanen et al. (1998)	I
<i>Lb. plantarum</i> WHE 92	Wild type pediocin producer	Ennahar et al. (1996)	III
<i>Lactococcus lactis</i> MG1614	Transformation host	Gasson (1983)	II
<i>Lc. lactis</i> MG1363	Transformation host	Gasson (1983)	I
<i>Lc. lactis</i> N8	Wild type nisin producer, target for chromosomal deletions	Valio Ltd. Helsinki, Finland	III
<i>Lc. lactis</i> NZ9000	Transformation host for nisin-induced gene expression, <i>nisK</i> and <i>nisR</i> integrated into the chromosome	Kuipers et al. (1998)	I, II
<i>Lc. lactis</i> LAC358	NZ9000 carrying expression vector pLEB688	Li et al. (2011)	I
<i>Lc. lactis</i> LAC360	NZ9000 carrying the secretion vector pLEB690	Li et al. (2011)	I, II, III
<i>Lc. lactis</i> LAC404	MG1614 carrying <i>lebB</i> -plasmid pLEB727	This study	II
<i>Lc. lactis</i> LAC405	MG1363 carrying <i>lecC</i> -plasmid pLEB728	This study	I
<i>Lc. lactis</i> LAC406	MG1363 carrying <i>lecCI</i> -plasmid pLEB729	This study	I
<i>Lc. lactis</i> LAC407	MG1363 carrying <i>lecI</i> -plasmid pLEB730	This study	I
<i>Lc. lactis</i> LAC408	NZ9000 carrying <i>lecCI</i> -plasmid pLEB729	This study	I
<i>Lc. lactis</i> LAC409	NZ9000 carrying <i>lecC</i> -plasmid pLEB728	This study	I, II, III
<i>Lc. lactis</i> LAC410	NZ9000 carrying <i>lebB</i> -plasmid pLEB727	This study	II

<i>Lc. lactis</i> LAC411	MG1363 carrying vector pLEB690	This study	I
<i>Lc. lactis</i> LAC420	MG1614 carrying <i>lcnA</i> -plasmid pLEB756	This study	II
<i>Lc. lactis</i> LAC428	NZ9000 carrying <i>lcnA</i> -plasmid pLEB756	This study	II, III
<i>Lc. lactis</i> MINL18	N8 carrying replicative <i>mptC</i> expression plasmid pWUST25	This study	III
<i>Lc. lactis</i> MINL20	N8 carrying integrated plasmid pWUST27	This study	III
<i>Lc. lactis</i> MINL21	N8 carrying integrated plasmid pWUST28	This study	III
<i>Lc. lactis</i> MINL25	N8 with 22-kb deletion in nisin-sucrose transposon Tn5481	This study	III
<i>Lc. lactis</i> MINL26	N8 with 33-kb deletion including <i>xyl</i> operon	This study	III
<i>Lc. lactis</i> MINL27	MINL25 carrying integrated plasmid pWUST28	This study	III
<i>Lc. lactis</i> MINL28	N8 with 22-kb deletion in Tn5481, and 33-kb deletion including <i>xyl</i> operon	This study	III
<i>Escherichia coli</i> TG1	Transformation host	Sambrook and Russell (2001)	I, III
<i>E. coli</i> ECO757	TG1 carrying <i>lecC</i> -plasmid pLEB731	This study	Liu et al. (2013)
<i>E. coli</i> ECO758	TG1 carrying <i>lecI</i> -plasmid pLEB732	This study	I
<i>E. coli</i> EMIN18	TG1 carrying replicative <i>mptC</i> plasmid pWUST25	This study	III
<i>E. coli</i> EMIN20	TG1 carrying integration vector pWUST27	This study	III
<i>E. coli</i> EMIN21	TG1 carrying integration vector pWUST28	This study	III
<i>E. coli</i> SAA594	TG1 carrying vector pTF1	This study	I
<i>Listeria monocytogenes</i> WSLC 1018	Indicator strain sensitive to leucocins A and C, ATCC 19118	Prof. Martin Loessner, ETH Zürich, Switzerland	I, II
<i>L. monocytogenes</i> WSLC 1033	ATCC 19117, Source of <i>mptC</i> gene	Prof. Martin Loessner, ETH Zürich, Switzerland	III
<i>L. monocytogenes</i> Scott A	Leucocin C-sensitive indicator strain, NRRL B-33013	Prof. Martin Loessner, ETH Zürich, Switzerland	I
<i>L. monocytogenes</i> MUU22	WSLC 1018 carrying vector pTF1	This study	I
<i>L. monocytogenes</i> MUU23	WSLC 1018 carrying <i>lecI</i> -plasmid pLEB732	This study	I
<i>Micrococcus luteus</i> ATCC10240	Indicator strain sensitive to nisin	Valio Ltd., Helsinki, Finland	this study

Table 5. Plasmid used in this study.

Plasmids	Relevant properties	Reference/source	Used in
pLEB579	<i>Lc. lactis</i> cloning vector containing pSH71 <i>repAC</i> and <i>ermC</i> , <i>Erm</i> ^R	Beasley et al. (2004)	III
pLEB688	<i>Lc. lactis</i> expression vector harbouring lactococcal promoter P45. 3466 bp, <i>Nis</i> ^R	Li et al. (2011)	I
pLEB690	<i>Lc. lactis</i> secretion vector harbouring lactococcal promoters P45 and <i>P_{nisZ}</i> , and signal sequence <i>SSusp45</i> . 3746 bp, <i>Nis</i> ^R	Li et al. (2011)	I, II

pLEB727	Leucocin B gene <i>lebB</i> fused to <i>SSusp45</i> in pLEB690 (<i>NaeI</i>)	This study	II
pLEB728	Leucocin C gene <i>lecC</i> fused to <i>SSusp45</i> in pLEB690 (<i>NaeI</i>)	This study	I, II
pLEB729	Leucocin C and the immunity gene <i>lecCI</i> fused to <i>SSusp45</i> in pLEB690 (<i>NaeI</i>)	This study	I
pLEB730	Leucocin C immunity gene <i>lecI</i> in pLEB688 (<i>NcoI/SmaI</i>)	This study	I
pLEB731	<i>lecC</i> fused with <i>SSusp45</i> and promoter P45 in pTF1 (<i>EcoRI/SmaI</i>)	This study	Liu et al. (2013)
pLEB732	<i>lecI</i> with <i>pepR</i> promoter from <i>Lb. rhamnosus</i> 1/6 in pTF1 (<i>EcoRI/SmaI</i>)	This study	I
pLEB756	Leucocin A gene <i>lcnA</i> fused to <i>SSusp45</i> in pLEB690 (<i>NaeI</i>)	This study	II
pTF1	Gram ⁻ /Gram ⁺ shuttle vector, 3043 bp, Erm ^R ; pLEB579 carrying multiple cloning site from pBluescript	Gift from Dr. Lars Fieseler, ETH Zürich, Switzerland. pLEB579, Beasley et al. (2004)	I
pWUST25	pLEB579 carrying nisin inducible promoter <i>P_{nisZ}</i> from <i>Lc. lactis</i> N8, <i>mptC</i> from <i>L. monocytogenes</i> WSLC 1033, Erm ^R	This study	III
pWUST27	Integration vector for <i>Lc. lactis</i> with pUC <i>ori</i> , <i>ermC</i> , <i>P_{nisZ}-mptC</i> , and two ~1-kb fragments for 22-kb chromosomal deletion from <i>Lc. lactis</i> N8	This study	III
pWUST28	Integration vector for <i>Lc. lactis</i> with pUC <i>ori</i> , <i>ermC</i> , <i>P_{nisZ}-mptC</i> , and two ~1-kb fragments for 33-kb chromosomal deletion from <i>Lc. lactis</i> N8	This study	III

Table 6. Methods used in this study.

Method	Reference/source	Used in
Basic DNA techniques, incl. PCR, enzymatic modifications, electrophoresis	Sambrook et al. (1989); Catalogues of enzyme suppliers	I, II, III
Inverse PCR	Ochman et al. (1988)	I
Overlapping PCR	Higuchi (1990)	III
Two-step RT-PCR	Freeman et al. (1999)	II
Electroporation		
<i>E. coli</i>	Zabarovsky and Winberg (1990)	I, III
<i>Lc. lactis</i>	Holo and Nes (1989)	I, II, III
<i>L. monocytogenes</i>	Park and Stewart (1990)	I
DNA isolation		
<i>E. coli</i>	GeneJET Plasmid Miniprep Kit, Thermo	I, II, III
<i>Lc. lactis</i>	Fischer Scientific	II
<i>L. monocytogenes</i>		I
<i>Ln. carnosum</i>	Anderson and McKay (1983)	I
<i>In vitro</i> antimicrobial activity assay	Harris et al. (1989)	I, II, III
Southern blotting	Ausubel et al 1987	I
SDS-PAGE and gel overlay	Schägger and Von Jagow (1987)	I, II
MIC determination	This study	II, III
Bacteriocin ammonium sulfate precipitation	Green and Hughes (1955)	I, II, III
Protein concentration with resin	Agilent Genomics	I
RNA purification and RT-PCR	GeneJET RNA Purification Kit, Thermo Fisher Scientific	II

RESULTS AND DISCUSSION

1 Genetic characterisation of leucocins (I, II, unpublished)

By only knowing the aa sequences of mature leucocin C (Budde et al. 2003; Fimland et al. 2002b), primers were designed to amplify *lecC* gene from *Ln. carnosum* 4010 based on codon usage. *lecC* gene was amplified and sequenced. Primers to amplify leucocin A gene were designed according to the published DNA sequence. The plasmid DNA was isolated from the strain 4010, according to Anderson and McKay's method (1983) for isolating large plasmids from *Lactococcus* with proper modifications (I). Southern blots hybridised with digoxigenin-labelled leucocins A and C probes revealed that, like most of other class IIa bacteriocins (Ennahar et al. 2000), leucocins A and C genes were found on two large plasmids pLC-1 and pLC-2.

Ln. carnosum 4010 plasmids were digested with several enzymes. The restricted fragments were self-ligated forming circular templates for the following inverse PCR. Primers in the inverse PCR direct away from the core region of the known sequence, and amplify the unknown areas up- and downstream of the known sequence in the circular template. Sequence analysis of the amplified inverse PCR fragment showed that the 8-kb *AccII* restriction fragment contains not only the genetic determinants for leucocin C, its immunity protein and ABC transporter, but also 165-bp *lebB* and 486-bp *lebI* encoding a class II d bacteriocin leucocin B and its immunity protein, respectively (II, **Figure 3**).

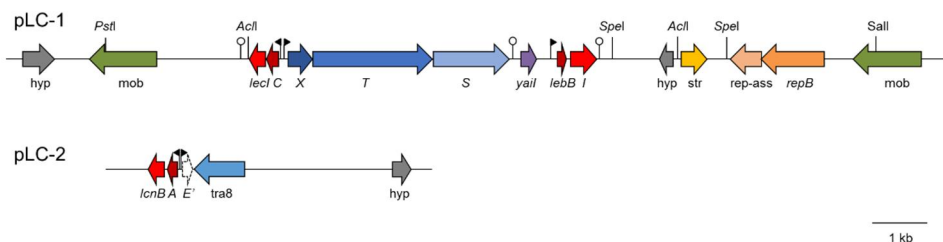


Figure 3. Gene clusters leucocins A, B, and C located in native plasmids of *Ln. carnosum* 4010. Promoters are shown as black flags. Transcriptional terminators are shown as lollipop symbols. *hyp*, hypothetical gene; *mob*, plasmid mobilisation gene; *str*, gene encoding stress induced DNA binding protein; *rep-ass*, replication assistant gene; *repB*, plasmid replication gene; *E'*, truncated accessory gene *lcaE*; *tra8*, transposase gene. Dark red, bacteriocin genes; red, immunity genes; X, accessory gene *lecX*; T, translocator gene *lecT*; S secretion gene *lecS*.

Through several inverse PCR and sequencing approaches, leucocin C was found to be encoded as a precursor by 204-bp *lecC* gene. The precursor possesses a GG-type leader peptide with 24 aa in length, which is typical for many class IIa bacteriocins. A 294-bp *lecI* encoding immunity protein share 48 % identity and 73 % similarity with immunity protein of other class IIa bacteriocins, listeriocin and sakacin P. Next to the *lecCI* operon, another operon with opposite direction contains 450-bp *lecX* encoding an accessory protein for ABC-transporter, 2169-bp *lecT* and 1374-bp *lecS* needed for the ABC transporter (I). Immediately after the *lecXTS* operon was a possible accessory gene *yail* and then the operon of *lebBI*. Leucocin B precursor also contains a GG-type leader, which

could be recognised by ABC transporter when crossing the cytoplasmic membrane (II). The signal peptide of leucocin A in the strain 4010 was identical to that of leucocin A in *Ln. carnosum* Ta11a, but different from leucocin A leader in *Ln. gelidum* UAL-187 by 7 aa (Felix et al. 1994). Despite the significant differences between leucocin and mesentericin sequences, the organisation of leucocin operons (*lecICXTS*, *yaiI* and *lebBI*) in *Ln. carnosum* 4010 resembles the mesentericins Y and B operons (*mesIYCDEFHB(G)*) from *Ln. mesenteroides* FR52 and Y105, respectively (Aucher et al. 2004). The *mesF*, located between the ABC transporter *mesCDE* and *mesHB*, encodes a putative induction factor for the bacteriocin production (Aucher et al. 2004). Likewise, the possibly secreted YaiI may also play a role in the leucocin production in *Ln. carnosum* 4010.

Interestingly, further sequence analysis revealed that on the leucocin A plasmid pLC-2 there was no transporter gene anywhere close to the *lcnAB* genes, but only a truncated gene *tra8* encoding transposase (unpublished, **Figure 3**). Strain 4010 was shown to produce leucocins A, B, and C, all of which kills *Ln. pseudomesenteroides* CIP103316 (II). Resistant colonies were obtained from the inhibition zone of leucocin C on a *Ln. pseudomesenteroides* CIP103316 indicator plate. The leucocin C resistant mutant was shown to be resistant also to leucocin A, but still sensitive to leucocin B, therefore named AC^R. Via plasmid curing, ΔpLC-1 and ΔpLC-1&2 strains were generated from *Ln. carnosum* 4010. Neither of the plasmid cured strain killed the indicators. Without the pLC-1 plasmid, the strain seemed to have stopped producing leucocin A, even though the genetic determinants of LcnA were intact in the strain (unpublished, **Figures 4 & 5**). These observations indicated that LecXTS might be the solo transporter for all three leucocins in *Ln. carnosum* 4010. There are a few other examples of ABC transporter, which can secrete multiple peptides. For instance, in *Lb. plantarum* at the *pln* loci, there is only one operon for the ABC transporter, but the strain can produce the four bacteriocin peptides of class IIb bacteriocins plantaricin EF and JK (Diep et al. 2009). Similarly in *E. faecium* NKR-5-3, a wide-range ABC transporter EnkT was found to secrete four different enterocins (Ishibashi et al. 2014). In the case of strain 4010, complementation of the ABC transporter LecXTS and corresponding recovery of the leucocin production would support the theory.

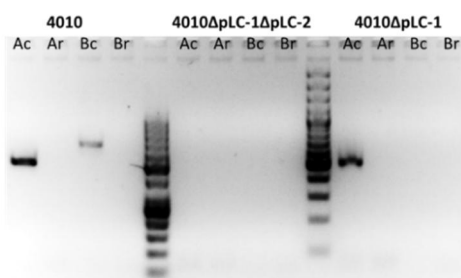


Figure 4. Transcription of *lcnA* and *lebBI* in wild type *Ln. carnosum* 4010, and plasmid-cured strains 4010ΔpLC-1 and 4010ΔpLC-1&2. A, PCR fragment of *lcnA*; B, PCR fragment of *lebBI*; c, cDNA as PCR template; r, isolated RNA as PCR template.

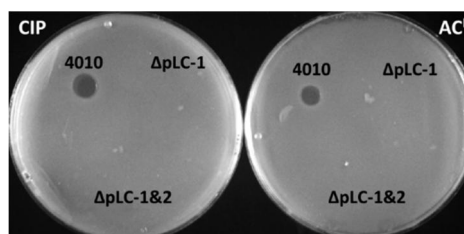


Figure 5. Bacteriocin production in native *Ln. carnosum* 4010 and two plasmid-cured derivatives *Ln. carnosum* ΔpLC-1 and *Ln. carnosum* ΔpLC-1&2. Five μl of pasteurised cell free supernatant from each o/n culture were spotted. *Ln. pseudomesenteroides* CIP103316 and *Ln. pseudomesenteroides* AC^R were used as indicators.

2 Heterologous expression of functional proteins (I, II, III, unpublished)

Recombinant production of protein could be regarded as one of the most powerful techniques in life sciences. The technique could provide pure desired protein, sometime even at significantly higher yield than by the native producer. With a careful selection of the efficiency of heterologous host cell, optimisation of transformation and production (promoter, vector, codon usage), consideration of cell's protein synthesis mechanism, theoretically any proteins can be expressed in a heterologous host. Yet in practice heterologous expression of functional proteins can be very challenging, as numerous factors may affect the production process. For example, proteins require specific folding manner to deliver their activities, and choosing a suitable host may facilitate this process. Nevertheless functional protein may be harmful to the host, for instance when producing transmembrane proteins, one has to ponder whether the host's cell membrane can handle the products.

2.1 Overexpression of leucocins (I, II, unpublished)

Many bacteriocins have their own leader peptides, which are functional in their native producers. However, these native signal sequences may not necessarily function well in a heterologous host. Therefore when dealing with heterologous production of bacteriocins, mature bacteriocins are often fused with optimal leaders for the heterologous hosts. In this study, the structural genes for leucocins A, B, and C were amplified from *Ln. carnosum* 4010 plasmids and individually fused with lactococcal signal sequence of unidentified secreted 45-kDa protein (Usp45) in pLEB690 plasmid. Usp45 is the major *sec*-dependent protein secreted by *Lc. lactis*. The signal peptide of Usp45 has been previously shown to be an effective signal peptide for heterologous production of bacteriocins in *Lactococcus* (van Asseldonk et al. 1990). For instance, class IIa bacteriocins enterocins A and P, pediocin, sakacin A, enterocin A and class IId bacteriocin hiracin JM79 have been functionally cloned as *SSusp45* fusions in LAB (Borrero et al. 2011; Jiménez et al. 2014; Li et al. 2011).

The expression of leucocins is under the control of both constitutive promoter P45 and nisin-inducible promoter P_{nisZ} (**Figure 6a** on page 36). Leucocins plasmids pLEB756, pLEB727, and pLEB728 were transferred in either *Lc. lactis* MG1363 (LecC) or *Lc. lactis* MG1614 (LcnA and LebB) for constitutive expression, and *Lc. lactis* NZ9000 (all three leucocins) for nisin-inducible expression. The immunity protein LecI was also co-expressed with leucocin C in the same manner (**Figure 6b**). With nisin induction, the *Lactococcus* strains could produce noticeably more leucocins than plain constitutive production; however, co-expression of the immunity protein had no significant effect on bacteriocin production (I, II). The P45- P_{nisZ} -*SSusp45*-lecC fragment was amplified by PCR from pLEB728 digested with *EcoRI*, and inserted into *EcoRI/SmaI* sites of pTF1 with erythromycin selection resulting in pLEB731 (**Figure 6c**), which is an expression shuttle plasmid in both *Lactococcus* and *Escherichia*. Both Gram⁺ *Lc. lactis* and Gram⁻ *E. coli* could produce functional leucocin against indicator strain *L. monocytogenes* (unpublished, **Figure 7** on page 36). Since *SSusp45* is not function in *E. coli*, the supernatant of ECO797

does not display inhibitory effect against *Listeria*. However, as the bacteriocin can leak out, ECO797 cells culture is active against the indicator. This efficient leucocin-producing system could be used as the source of the bacteriocin in further studies of the immunity proteins or utilisation of the bacteriocin and its heterologous producers.

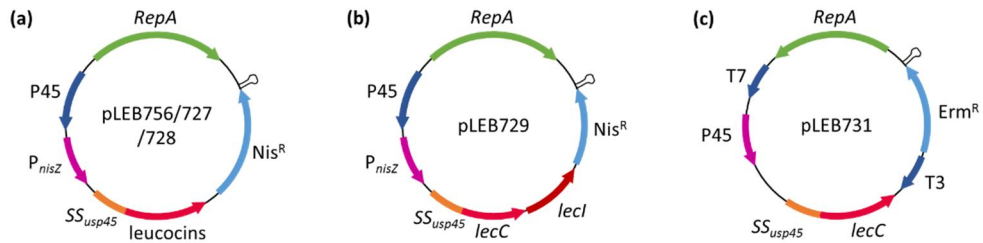


Figure 6. Leucocin expression plasmids. The leucocin genes *lcnA*, *lebB* and *lecC* were fused to signal sequence of *usp45*, and the expression is under the control of (a, b) nisin-inducible promoter P_{nisZ} with nisin selection marker Nis^R , or (c) constitutive promoter P45 with erythromycin selection marker Erm^R . Loop indicates transcriptional terminator.

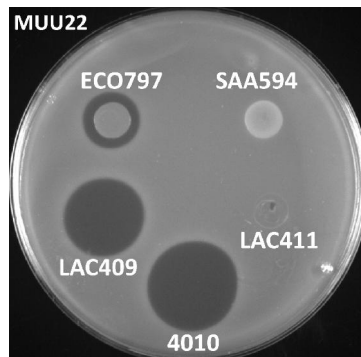


Figure 7. Inhibition of leucocin C produced by *Lc. lactis* LAC409 and *E. coli* ECO797, compared with wild type *Ln. carnosum* 4010 on *L. monocytogenes* MUU22 lawn. SAA594, *E. coli* TGI1 carrying vector pTF1. LAC411, *Lc. lactis* MG1363 carrying vector pLEB690. Five μ l concentrated bacteriocin preparations or o/n cultures were spotted.

Lactococci are naturally resistant to class IIa bacteriocins, because these bacteriocins do not bind to lactococcal Man-PTS, which in sensitive cells serves as the target (Kjos et al. 2009). In case of class IIa bacteriocin pediocin, co-expression of pediocin immunity gene has significantly increased the yields of heterologous pediocin in host *Lc. lactis* (Arqués et al. 2008). However, in this doctoral study, the protective effect of cognate immunity proteins LecI and LcnB could not be seen in host strains which are naturally resistant to leucocins (data not shown).

For constitutive gene expression, the phosphopentomutase promoter P8 has been shown to be even stronger than the nisin promoter (Zhu et al. 2015a). Replacing P45 and P_{nisZ} by P8 would logically result in higher production level. Nevertheless, high expression of bacteriocin may cause suicidal effect on the producer cells without immunity protein. Thus suitability of a promoter and co-expression of immunity protein should be tested for the strains to be used.

2.2 Heterologous expression of other functional proteins (I, III, unpublished)

LecI shares 90 % identity with an immunity protein in *Ln. citreum* LBAE C10. Even though the genes *lebI* and *lecI* encoding leucocins B and C immunity protein share great similarity and consensus motifs with other bacteriocin immunity proteins, complementation of the gene expression causing phenotype change would be a firmer evidence. *L. monocytogenes* is naturally sensitive to class IIa bacteriocins including leucocins A and C. In this study, leucocin C immunity protein LecI was fused with *pepR* promoter from *Lb. rhamnosus* 1/6, cloned to a shuttle plasmid pTF1 and transferred into *L. monocytogenes*. LecI was expressed in *L. monocytogenes* MUU23, which was significantly more tolerant to the bacteriocin LecC than the vector carrying strain (I). Yet the immunity capacity of heterologous LecI in *Listeria* was quite limited, as when exposed to higher leucocin C concentration, LecI producing *Listeria* was as vulnerable as the non-LecI counterpart (data not shown). The expression level of LecI was not determined, however, theoretically stronger expression of the immunity protein could result in higher resistance.

Interestingly, cloning and expressing leucocin B putative immunity protein LebI in *Lc. lactis* was not successful in this study, despite several attempts. All obtained clones contained wrong constructs (II). LebI matches 55 % with the mesentericin B105 immunity protein MesH (Morisset and Frère 2002), and contains conserved regions identical to GtrA superfamily of integral membrane proteins involved in synthesis of cell surface polysaccharides. Unlike leucocin C immunity protein, but similarly to other GtrA superfamily members, LebI contains 5 transmembrane spans as predicted by Phobius (2017), a combined transmembrane topology and signal peptide prediction (**Figure 8** on page 38), or 3 spans by PHDhtm transmembrane helices prediction (PRABI 2016). The structure of LebI might explain the difficulty of heterologous expression of this protein. Attempts of expressing at lower level or using inducible promoter might be feasible to provide useful hints about the putative immunity protein.

An extracellular loop of the Man-PTS IIC component has been found to be responsible for specific targeting by class IIa bacteriocins (Kjos et al. 2010). In this doctoral study, listerial MptC was cloned and expressed under the control of P_{nisZ} promoter in *Lc. lactis* N8 (III) and *Lc. lactis* NZ9000 (data not shown). MptC was cloned in the form of both single copy chromosomal integration and plasmid-borne multiple replication. Both versions of transformants displayed sensitivity to several class IIa bacteriocins, including pediocin and leucocins A and C (III). As listerial MptC can form a functional Man-PTS complex with lactococcal PtnD, expressing only the IIC component of *L. monocytogenes* was sufficient to make *Lc. lactis* sensitive to class IIa bacteriocins.

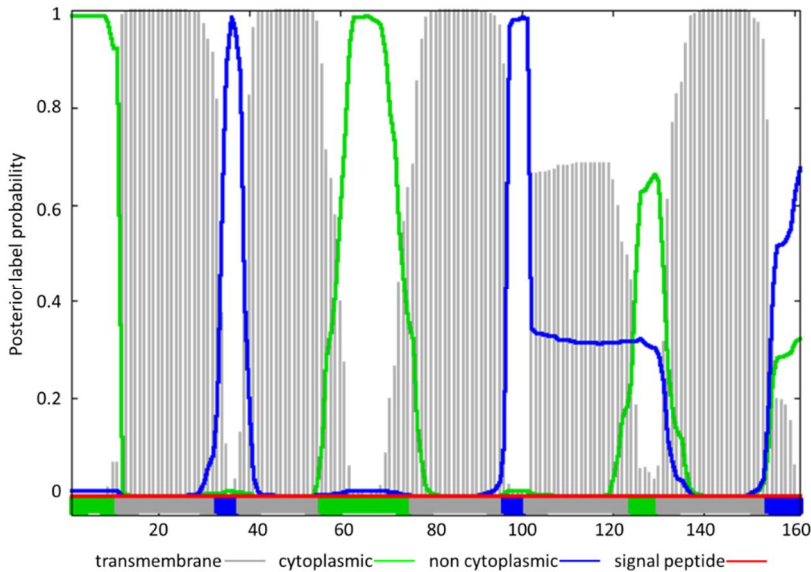


Figure 8. Transmembrane prediction of putative leucocin B immunity protein LeBI by Phobius.

On both *Listeria* and MptC producing *Lactococcus* plates, small colonies grew inside the bacteriocin halos, indicating that *Lc. lactis* can develop bacteriocin resistance same as *L. monocytogenes* (III). The resistance mechanism to class IIa bacteriocins and lactococcin A has been shown to be related to downregulation of Man-PTS expression (type 1) (Kjos et al. 2011b). Yet in some cases, Man-PTS level maintained the same in bacteriocin-resistant *L. monocytogenes* mutants (type 2). Changes in membrane composition and cell surface charge have been regarded as the cause to the type 2 resistance mutation (Tessema et al. 2009; Vadyvaloo et al. 2004; Vadyvaloo et al. 2002). Similarly in our study, the bacteriocin resistant mutant *Ln. pseudomesenteroides* AC^R displayed resistance to leucocins A and C, and pediocin, but not to sakacin A, all of which are from the same class IIa family (data not shown). Trinetta et al. (2012) suggested that sakacin A could break down specific peptide bonds in the cell wall structure, which might explain the sustained sensitivity of the strain AC^R to this bacteriocin.

3 Using bacteriocin targeting mechanism in genome editing (III, unpublished)

Heterologous expression of *mptC* in *Lc. lactis* N8 made the naturally resistant strain sensitive to class IIa bacteriocins, including leucocins A and C and pediocin. In this study, *mptC* was fused with P_{nisZ} promoter and cloned to an *E. coli* plasmid harbouring two homologous regions H1 and H2 to *Lc. lactis* N8 genome. The recombinant plasmid DNA was integrated in *Lc. lactis* N8 genome, leaving two sets of homologous areas H1H2, and *in vivo* expression of MptC made the strain vulnerable to the bacteriocins (III). When the MptC-producing strain is challenged with bacteriocins in its living environment, cell surviving mechanism is triggered to reverse the lethal effect. Since two sets of H1H2s are left after integration, the most feasible way to eliminate *mptC* from the cell would be going through another homologous recombination, during which the genomic area in between

the two homologous areas is deleted (III, **Figure 9**). Hence, DCO cells could be counterselected with class IIa bacteriocins. In this study, we succeeded to delete 17-, 22-, 33- and 35-kb chromosomal fragments from the wild-type nisin producing *Lc. lactis* N8 with this method. The deletion frequencies varied from about 8 to 40 % of all obtained DCO colonies. However, the large genome deletions, including multiple deletions of non-essential genes up to 55 kb in total neither affected the cell growth nor significantly increased the cell's production level of nisin (unpublished, **Figure 10**).

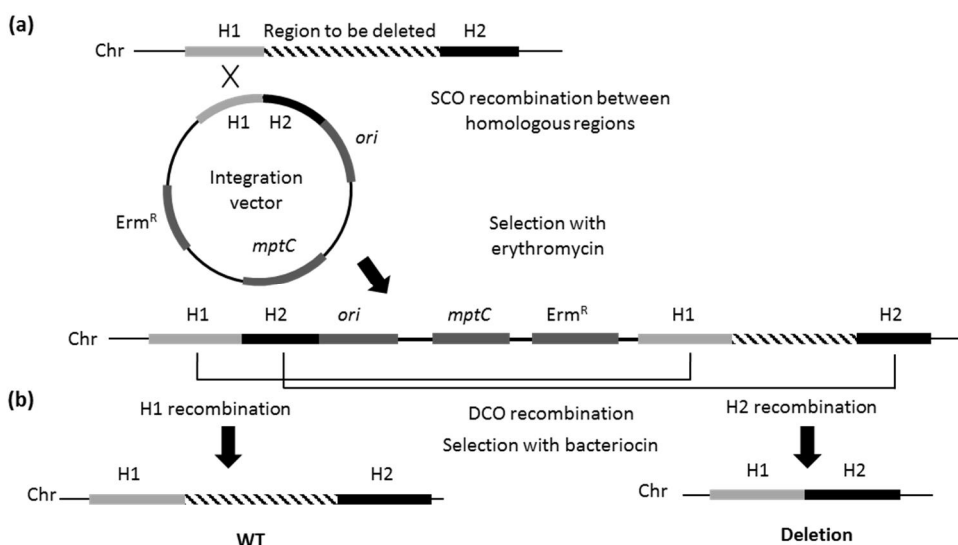


Figure 9. Scheme of using *mptC*-based selection/counterselection integration vector for constructing gene knockout in *Lc. lactis* (not in scale). (a) Plasmid carrying two homologous regions (H1, H2) flanking the region to be deleted in *Lc. lactis* N8 genome is integrated into the chromosome at either of the homologous regions, and the integrant is selected by erythromycin resistance. The resulting integrant becomes sensitive to class IIa bacteriocins. (b) A second recombination event at H1 or H2 leads to the excision of the integrated plasmid. Only the cells which have lost the integration vector can survive in the presence of class IIa bacteriocin. The second recombination at the same homologous region (H1) as in the integration event reverts the integrant to the wild type. If the second recombination takes place at H2, the event results in a chromosomal deletion between H1 and H2. Chr, chromosomal DNA; diagonal stripes the region to be deleted.

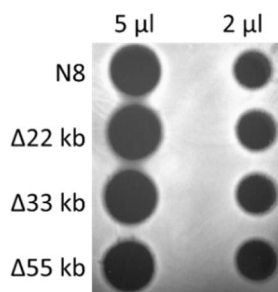


Figure 10. Nisin production in wild type *Lc. lactis* N8 and strains with 22-kb, 33-kb, and 55-kb genome deletions. Five μl or 2 μl cell free culture supernatants were spotted on *M. luteus* indicator lawn.

Two vital conditions are required for using this method in gene deletion: 1) the target strain should be naturally resistant to the bacteriocin, hence a conditionally harmful MptC is dispensable in its genome. Once the strain has taken recombinant MptC, bacteriocins function as counterselecting agent to select DCO colonies, as the SCO cells are susceptible to class IIa bacteriocins. As a result, the only survivor bacteria would be non-MptC mutants. Because deletion of genomic area between *HIH2* may happen at the same time when the MptC is removed through DCO, seamless deletion can be made in the genome. 2) the strain should probably be equipped with native Man-PTS to form functional complex with heterologous MptC. Otherwise, cloning of both MptC and MptD would be required to make the target strain sensitive. Yet depending on which bacteriocin is used, either/both IIC or/and IID would be needed. For example, lactococcin A requires both regions to conduct its activity to target cell (Kjos et al. 2010).

At present, the CRISPR/Cas system, popular for genomic deletions in bacteria, has not been reported in the use of *Lactococcus* genome editing. Instead, chromosomal deletions in *Lc. lactis* have often been made using TS pG⁺ Host vectors (Biswas et al. 1993). However, culturing *Lc. lactis* at a high temperature (37.5 °C) can result in stable heat resistant mutation (Smith et al. 2012), which makes this method less suitable for consecutive multiple genome deletions in one strain. In addition to the pG⁺ Host vectors, the only reported counterselectable markers for gene deletions of LAB are the *oroP* gene encoding the orotate transporter in *Lc. lactis* (Solem et al. 2008) and the *upp* gene encoding uracil phosphoribosyltransferase in *Lb. acidophilus*, *Lb. casei*, and *Lc. lactis* (Goh et al. 2009; Song et al. 2014). However, as the *upp* gene is generally existing and expressed in LAB, deleting *upp* from the host is obligatory if this counterselectable marker is used in gene deletion. Moreover, the selecting agent 5-fluorouracil may be metabolised and thus be toxic to the cells (Martinussen and Hammer 1994). Comparing with these two counterselection methods, selection of the DCO colonies with bacteriocin does not require chemically defined or semi-defined media. Commercial M17 supplemented with glucose and class IIa bacteriocin from cell free supernatant is sufficient for obtaining a deletion frequency of 10 to 40 % of DCO colonies.

CONCLUSIONS AND FUTURE PROSPECTS

In this thesis, the main targets were to genetically characterise and to overexpress *Leuconostoc* bacteriocins leucocins A, B, and C, and to apply the bacteriocins in genome editing. The genes required for the production of the three leucocins from *Ln. carnosum* 4010 were shown to be plasmid-borne, with leucocin A genetic determinants *lcnAB* on one plasmid and leucocins B and C determinants *lebBI* and *lecCI* on another. LecI was shown to protect the strain from its cognate bacteriocin leucocin C. According to the RT-PCR analysis, *lebI* is co-expressed with the bacteriocin gene *lebB* in *Ln. carnosum* 4010. However, heterologous expression of LebI was not successful, possibly due to its transmembrane spans. Therefore, optimisation of expression would be needed to produce the protein in a leucocin B sensitive host. In addition to the bacteriocin and the immunity genes, *lecXTS* genes encoding the ABC transporter were also identified. Since only one ABC transporter was found, this transporter may be responsible for secreting all three leucocins in *Ln. carnosum* 4010. Inactivation of transporter and restoration of the bacteriocin production by complementation would confirm the function of LecXTS. Bacteriocins have the potential to prolong the shelf life of food products by eliminating spoilage and pathogenic bacteria. Hence, overexpression of bacteriocins in different hosts would provide a good tool for food biopreservation. Here, LcnA, LebB and LecC were produced by heterologous hosts *E. coli* and *Lc. lactis*. The latter was shown to be an excellent host for leucocin production; the production levels were comparable to the native producer. These leucocin producing *Lc. lactis* strains could be used for food and research applications.

The killing mechanism of class IIa bacteriocins relies on Man-PTS on the target cell membrane. Man-PTS is not only responsible for transporting and catalysing phosphorylation of incoming sugar, but also providing a target for the bacteriocins to bind. Expressing the Man-PTS component IIC (MptC) from *L. monocytogenes* in *Lc. lactis* rendered the latter sensitive to class IIa bacteriocins. Using this acquired sensitivity, a counterselection system was developed for chromosomal deletions in *Lc. lactis*. A plasmid carrying *mptC* was constructed and integrated into *Lc. lactis* genome. With bacteriocin selection, the cells which had lost the *mptC* plasmid through DCO could easily be found. In addition to the loss of the plasmid, some DCO cells had also deleted the target region in the chromosome. However, in certain cases the vast majority of the obtained DCO colonies can be wild-type revertants, which leads to extensive screening for the wanted deletion. Therefore, a method selecting only the deletion would be even better. Still, the counterselection method developed in this study makes *Lc. lactis* genome editing much easier. Several large deletions, up to 35 kb, were created using leucocin selection. By deleting, inserting, or mutating genes in bacterial chromosome, it is possible to generate new strains with different properties, e.g. increased production of desired compounds.

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