Enhanced antibacterial action of bacteriocin producing cells by binding to the target pathogen

Shanna Liu

Division of Microbiology and Biotechnology
Department of Food and Environmental Sciences
Faculty of Agriculture and Forestry
University of Helsinki
Finland

ACADEMIC DISSERTATION IN MICROBIOLOGY

To be presented, with the permission of the Faculty of Agriculture and Forestry of the University of Helsinki, for public criticism in the auditorium 2402 of Biocenter 3, Viikinkaari 1, University of Helsinki, on 15th August 2014, at 12 o'clock noon.

Helsinki 2014

Supervisor
Professor Per Saris
Department of Food and Environmental Sciences, University of Helsinki, Finland

Reviewers
Professor Matti Karp
Department of Chemistry and Bioengineering, Tampere University of Technology, Finland

Docent Arto Pulliainen
Department of Biosciences, University of Helsinki, Finland

Opponent
Professor Heikki Ojamo
Department of Biotechnology and Chemical Technology, Aalto University, Finland

Custos
Professor Per Saris
Department of Food and Environmental Sciences, University of Helsinki, Finland

ISBN 978-952-10-9992-2 (paperback)
ISBN 978-952-10-9993-9 (PDF)
ISSN 1799-7372

Hansaprint
Helsinki 2014

To my family

TABLE OF CONTENTS

List of original publications

This thesis is based on the following articles, which are referred to in the text by their Roman numerals. In addition, some unpublished data are presented.

I **Liu, S**., Han, Y. and Zhou, Z. (2011). Fusion expression of *pedA* gene to obtain biologically active pediocin PA-1 in *Escherichia coli*. *J Zhejiang Univ-Sci B (Biomed & Biotechnol)* 12(1): 65-71.

II **Liu, S**., Kylä-Nikkilä, K. and Saris, P. E. J. (2011). Cell immobilization studies using a cellulose-binding domain fused to PrtP in *Lactococcus lactis*. *Bioeng Bugs* 2(3): 160-162.

III **Liu, S**., Takala, T. M., Wan, X., Reunanen, J. and Saris, P. E. J. (2013). Cell-mediated killing of *Listeria monocytogenes* by leucocin C producing *Escherichia coli*. *Microbiol Res* 168(5): 300-304.

IV **Liu, S**., Takala, T. M., Reunanen, J., Saris, O. and Saris, P. E. J. (2014). Attachment of *Escherichia coli* to *Listeria monocytogenes* for pediocin-mediated killing. Submitted manuscript.

The author's contribution in articles:

I The author participated in the design of the study, conducted all the experiments, analysed the data, interpreted the results and was responsible for writing the manuscript.

II The author participated in the design of the study, conducted the filter paper immobilization tests, analysed the data, interpreted the results and had the main responsibility for writing the publication under the supervision of Per Saris.

III The author planned the work together with Per Saris and Timo Takala. The author carried out all the laboratory work. Justus Reunanen and Xing Wan constructed/provided the vector and leucocin C gene parts used for the constructs in this study. The author analysed the data, interpreted the results and had the main responsibility for writing the publication under the supervision of Per Saris.

IV The author planned the work together with Per Saris and Timo Takala. The author carried out the major part of experimental work. Timo Takala and Ossian Saris conducted the construction of CBD500-YadA plasmids and whole-cell ELISA. The author analysed the data, interpreted the results and had the main responsibility for writing the publication under the supervision of Per Saris.

Abbreviations

aa	Amino acid(s)
Ab	Antibody
ABC	ATP-binding cassette
Amp	Ampicillin
AP	Alkaline phosphatase
ATP	Adenosine triphosphate
AU	Arbitrary Unit
BHI	Brain heart infusion
bp	Base pair(s)
CBD	Cell wall binding domain
CD	Circular dichroism
cfu	Colony forming unit
C-terminal	Carboxy-terminal
CW	Cell wall
DNA	Deoxyribonucleic acid
ds	Double-stranded
EAD	Enzymatically active domain
EEC	European Economic Community
e.g.	*exempli gratia*, for example
ELISA	Enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
Erm	Erythromycin
et al.	*et alia*, and others
FACS	Fluorescence-activated cell sorting
FAO	Food and Agriculture Organization of the United Nations
FDA	Food and Drug Administration
GFP	Green fluorescent protein
GRAS	Generally Recognized As Safe
GSH	Reduced glutathione
GSSH	Oxidized glutathione
h	Hour
INP	Ice nucleation protein
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IU	International Unit
Kan	Kanamycin
kb	Kilobase pairs
LAB	Lactic acid bacteria
LB	Luria Bertani medium
Lpp	Lipoprotein
man-PTS	Mannose phosphotransferase system
MIC	Minimum inhibitory concentration
MW	Molecular weight

M17G	M17 medium + 0.5 % glucose
NCBI	National Center for Biotechnology Information
Nis	Nisin
nsr	Nisin-resistance gene
N-terminal	Amino-terminal
OD	Optical density
OMP	Outer membrane protein
PCR	Polymerase chain reaction
PG	Peptidoglycan
RTE	Ready-to-eat
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SS	Signal sequence
WHO	World Health Organization
wt	Wild type
WTA	Wall teichoic acid

Abstract

Bacteriocins are natural weapons of bacterial inter-species competition in food preservation arsenal. Bacteriocins produced by lactic acid bacteria have gained particular attention owing to their potential application as the substitute of artificial chemical preservatives. This research made use of genetic engineering technologies to clone the class IIa bacteriocin genes and construct bacteriocin structural gene expression systems, aiming at solving the problem of low bacteriocin production in wild type lactic acid bacteria strains and achieving efficient killing of *Listeria monocytogenes*.

The total DNA of *Pediococcus acidilactici* PA003 was used as the template to amplify the structural gene *pedA*, which was inserted into pET32a(+) vector and transformed into *Escherichia coli*. The recombinant plasmid containing the *pedA* gene was verified by DNA sequencing. This recombinant strain was induced with IPTG and it efficiently expressed a 22 kDa thioredoxin-PedA fusion protein as inclusion bodies. One protein band corresponding to the predicted molecular mass of pediocin was obtained after renaturation and enterokinase treatment. The agar diffusion assay revealed that 512 arbitrary unit (AU) antilisterial activities were obtained from 1 ml culture of recombinant *E. coli*. The same strategy was adopted using pET20b(+) as the expression vector. The PelB signal peptide in this vector resulted in soluble expression of fusion protein both in the cytoplasmic and periplasmic spaces with totally 384 AU/ml production.

Lactococcus lactis cells were engineered to bind to cellulose by fusing cellulose-binding domain of *Cellvibrio japonicus* with PrtP, NisP and AcmA anchors for surface display. The CBD-PrtP showed the most efficient immobilization. Expression of sortase with the CBD-PrtP fusion did not improve binding of the anchor to the cell wall. Next, the surface display technique was aimed to be combined with secretion of antilisterial bacteriocins in order to construct an *E. coli* strain with capacity to bind and kill *L. monocytogenes* cells. Such cells could be used to test the hypothesis that antilisterial bacteriocin secreting cells kill listerial cells more efficiently if they also have the capacity to bind to listerial cells. Therefore, the CBD500 and CBDP35 from *Listeria* phage endolysins were used to engineer *E. coli* cells to bind to *L. monocytogenes* cells using different cell anchoring domains. First CBD500 was fused to the outer membrane anchor of *Yersinia enterocolitica* adhesin YadA for potential surface display. Whole-cell ELISA showed that CBD-YadA fusion was displayed on the cell surface. However, production of the fusion protein was detrimental to the growth of recombinant cells. Therefore, a fragment of the *E. coli* outer membrane protein OmpA was selected for fused expression of CBD500 in *E. coli*. Western blot revealed the OmpA-CBD was mainly localized on the external surface of recombinant cells. However, the accessibility of the CBD on the cell envelope to cells of *Listeria* could not be shown.

For an improved surface display, CBD was expressed as FliC::CBD chimeric protein in flagella. CBD500 and CBDP35 domain coding sequences were inserted into vector pBluescript/*fliC*Δ_{H7}. CBD insertion in flagella was confirmed by Western blot. The FliC::CBDP35 flagella were isolated and shown to bind to *L. monocytogenes* WSLC 1019 cells.

To test the hypothesis that bacteriocin-secreting cells kill target cells more efficiently by binding to the target cells, bacteriocin-secreting strains with binding ability to *Listeria* cells were constructed. Antilisterial *E. coli* was obtained either by transferring pediocin production from *Lactobacillus plantarum* WHE 92 or leucocin C production from *Leuconostoc carnosum* 4010. The *Listeria*-binding cells producing pediocin decreased approximately 40 % of the *Listeria* cells during three hours, whereas the cell-free medium with the corresponding amount of pediocin could only inhibit cell growth but did not decrease the number of viable *Listeria* cells after the three hours incubation. The cell-mediated leucocin C killing resulted in a two-log reduction of *Listeria*, whereas the corresponding amount of leucocin C in spent culture medium could only inhibit growth without bactericidal effect. These results indicate that close contact between *Listeria* and bacteriocin producing cells is beneficial for the killing effect by preventing its dilution in the environment and adsorption onto particles before taking effect to the target cells.

Introduction

1. Bacteriocins

Microorganisms are well documented for producing inhibiting compounds as part of their defense or immune systems for their survival in a competing niche (Roces et al. 2012). Organic acids, ethanol, hydrogen peroxide, bacteriocins and other antibacterial compounds are recognized antimicrobials synthesized by a variety of microbes. Bacteriocins are ribosomally synthesized antimicrobial peptides or precursor peptides produced by bacteria. Their activities are not restricted to the same species and the producer strains are immune to their own bacteriocins (Cotter et al. 2005). With the increasing outbreaks of food-borne diseases and changing of diet habits among consumers, bacteriocins are promising as natural preservatives.

The first report of the inhibitory effect of a bacteriocin, named colicin, was almost a century ago in antagonism studies with *Escherichia coli* strains (Gratia 1925). Then a second bacteriocin was described in 1928 (Rogers and Whittier 1928) and later named as nisin (group **N** *Streptococcus* **I**nhibitory **S**ubstance, **-in** ending indicating an antibiotic) (Mattick et al. 1947). This heat-stable, soluble and proteinaceous substance was accepted as a safe food additive by the FAO/WHO in 1969 (WHO 1969). Nisin was adopted for commercial use in several countries and later added to the EEC food additive list assigned as E234 (EEC 1983). FDA in the United States gave it a GRAS status in 1988 (FDA 1988). Until now, nisin is the only approved and available bacteriocin food preservative commercially used in the food industry. Since many LAB are GRAS, LAB bacteriocins are of great industrial importance as natural food preservatives and have potential as therapeutic agents for gastrointestinal and other infections. The most studied bacteriocins are those produced by LAB and increasing number of different bacteriocins have been identified and characterized from wt strains (Roces et al. 2012).

1.1 Classification of bacteriocins

Bacteriocins from Gram-positive bacteria are classified according to their primary structures, MWs, post-translational modifications and genetic properties. Although the classification system has evolved with the increasing knowledge and identification of new bacteriocins, bacteriocins are commonly divided into four groups as shown in Table 1. Class I bacteriocins, also called lantibiotics, are characterized by their post-translational modification with nisin as the representative. Class II including most of the LAB bacteriocins are small, heat-stable, and non-modified peptides. Among them, class IIa group contains pediocin-like *Listeria* active peptides, gaining particular attention for food preservation. To date, nearly 50 different kinds of class IIa bacteriocins have been identified from LAB including *Lactobacillus* spp., *Enterococcus* spp., *Pediococcus* spp., *Carnobacterium* spp., *Leuconostoc* spp., *Streptococcus* spp. and *Weissella* spp. (Cui et al. 2012). They have been isolated from fermented meat, fermented vegetable, dairy products, smoked salmon and human gastrointestinal tract. Class III bacteriocins contain large and heat-labile proteins, and class IV group consists of large complexes with other macromolecules.

Table 1. Classification of bacteriocins adapted from Beshkova and Frengova 2012.

Class	General features
I	Lantibiotics, small (< 5 kDa), cationic, hydrophobic peptides containing unusual and post-translationally modified aa (lanthionine, methyllanthionine, dehydrobutyrine, and dehydroalanine)
Type A	Elongated and positively charged molecules
Subtype A_1	Leader peptides are cleaved by a dedicated serine proteinase
Subtype A_2	Leader peptides are cleaved by a dedicated ABC ATP-binding cassette transporter
Type B	Globular and noncharged molecules
II	Nonmodified, heat-stable, small (< 10 kDa), cationic, hydrophobic peptides
Subclass II a	Pediocin-like bacteriocins, *Listeria*-active peptides
Subclass II b	Two-peptide bacteriocins
Subclass II c	Sec-dependent bacteriocins
Subclass II d	Other bacteriocins-leaderless bacteriocins; antimicrobial peptides derived from larger proteins
III	Large (> 30 kDa), hydrophilic, heat-labile proteins
IV	Cyclic, hydrophobic macromolecules

Class IIa bacteriocins generally consist of 36 to 48 aa with a 40-60 % aa sequence similarity when the corresponding sequences are aligned (Garsa et al. 2014). The peptides of this group have a highly conserved hydrophilic and charged N-terminal region and a hydrophobic and/or amphiphilic C-terminal region terminating in a hairpin structure. Structural analysis suggested that the N-terminal domain containing consensus YGNGV/L motif has a three-stranded β-sheet-like structure which is followed by a hinge, while the variable C-terminal domain is folded into an amphiphilic α-helical structure (Tominaga and Hatakeyama 2006). The hinge allows the N-terminal β-sheet-like structure and the C-terminal helix-containing region to move relative to each other. Therefore, when the more hydrophobic C-terminal part penetrates into the target cell membrane, the hydrophilic N-terminal parts can remain in the exterior of the cell (Haugen et al. 2011). The conserved YGNGV/L motif has been used as specific sequence in PCR-based detection method for identification of class IIa bacteriocin producing strains (Sood et al. 2013).

For most part of class IIa bacteriocins, a single intramolecular disulfide bond between cysteine^{9} and cysteine^{14} is common. The C-terminal hairpin-like structure is in several class IIa bacteriocins such as divercin V41, pediocin PA-1/AcH and enterocin A, stabilized by an additional disulfide bridge between a C-terminal cysteine residue and a cysteine residue in the middle of the α-helical structure. Research showed that the C-terminal disulfide bridge provides broad antimicrobial spectrum and less temperature-dependent activity (Fimland et al. 2000). However, the peptides lacking these two cysteine residues may contain a tryptophan residue near the C-terminal end for stabilization (Haugen et al. 2005).

Class IIa bacteriocins are biosynthesized as precursors, composed of a leader peptide and mature bacteriocin. The leader sequence usually has 15 to 30 residues of the double-glycine type which is cleaved off at the C-terminal side of two glycine residues. The

leader peptide facilitates the signal interaction during translocation. Additionally, it maintains the inactive state of bacteriocin before successful secretion (Fimland et al. 2005). Much of the interest in class IIa bacteriocins is due to their antimicrobial activity against food-borne pathogen *Listeria* and thus to their potential for use as food additives (Johnsen et al. 2000). Other pathogenic bacteria being sensitive to this group include *Clostridium*, *Enterococcus*, *Staphylococcus*, *Salmonella*, *Micrococcus*, *Yersinia*, *Aeromonas* (Papagianni and Anastasiadou 2009; Devi and Halami 2013).

Pediocin PA-1/AcH is a 44 aa peptide with the MW of around 4.6 kDa. It is produced mainly by genus *Pediococcus* without post-translational modification. Pediocin PA-1/AcH is the only class IIa bacteriocin for both cross-species and cross-genera production (Rodríguez et al. 2002). Pediococci are natural microflora in plant sources and used as starter cultures in variety of food fermentation (Ray and Miller 2000). CD spectroscopy analysis suggested that pediocin PA-1/AcH contains largely unordered sequence in aqueous buffer and undergoes a substantial conformational reorganization upon binding to the membrane (Watson et al. 2001). The disulfide bond between cysteine^{24} and cysteine^{44} is essential for showing antimicrobial effects. Pediocin PA-1/AcH has demonstrated antilisterial effect on food model systems, especially meat products in which nisin frequently fails, such as sliced cooked sausages, frankfurter sausages and Spanish dry-fermented sausages (Santiago-Silva et al. 2009; Nieto-Lozano et al. 2010). *In vivo* study on the effectiveness of pediocin PA-1 and pediocin producing *P. acidilactici* UL5 also showed positive results (Dabour et al. 2009). Besides, administration of pediocin PA-1 had no effect on the main composition of the mouse intestinal ecosystem and *in vitro* human terminal ileum fermentation model (Kheadr et al. 2010; Le Blay et al. 2012).

Leuconostoc species are heterofermentative LAB often used as components of mesophilic cultures to produce aroma and flavor compounds during milk fermentation (Alegría et al. 2013). The antimicrobial effects of *Leuconostoc* against pathogenic and spoilage microorganisms have been reported early due to the production of organic acid, peroxide as well as bacteriocin (Trias et al. 2008). Many *Leuconostoc* strains produce one or more bacteriocins. Leucocin A was the first class IIa bacteriocin to be sequenced (Hastings et al. 1991). It has been identified in *Leuconostoc gelidum* UAL-187 and *Leuconostoc pseudomesenteroides* QU15. Leucocin B-Ta11a and Leucocin B-KM432Bz were found in *Leuconostoc carnosum* Ta11a and *Ln. pseudomesenteroides* KM432Bz, respectively (Makhloufi et al. 2013). *Ln. carnosum* 4010 showing strong inhibition against *L. monocytogenes* was isolated from the surface of sliced vacuum-packed ham. It was proposed to produce two bacteriocins, leucocin A and leucocin C. Leucocin C was 100 % identical to the partial sequenced leucocin 7C and leucocin 10C from *Leuconostoc mesenteroides*, and shared similarity with leucocin C-TA33 (Budde et al. 2003). Leucocin C contains 43 aa with the theoretical MW of 4596 calculated from the sequence (Fimland et al. 2002).

1.2 Mode of action of class IIa bacteriocins

LAB bacteriocins exert activity against closely related Gram-positive microorganisms, whereas the bacteriocin producing cells are immune against their own bacteriocins (Cotter et al. 2005, De Vuyst and Leroy 2007). Briefly, the action mechanism of class IIa bacteriocins includes three main steps. Firstly, bacteriocins bind to the anionic

phospholipids of the target cell membrane by electrostatic interaction. Functional analysis confirmed that the N-terminal domain of class IIa bacteriocins is hydrophilic and cationic, which facilitates the initial binding of the bacteriocin to the target cell membranes. Secondly, the amphiphilic α-helical region belonging to the C-terminal domain penetrates into the membrane of target cells and induces the formation of hydrophilic pores (Lohans and Vederas 2012). It has been proved by hybrid gene analysis that the C-terminal part determines target specificity, which coincides with the variations of C-terminal domains among different class IIa bacteriocins. After membrane permeabilization, pore formation leads to the total or partial dissipation of the proton motive force, depletion of intracellular ATP and leakage of aa and ions (García et al. 2010). Different class IIa bacteriocins may result in different characteristics in the size, stability and the electrical conductivity of the pore.

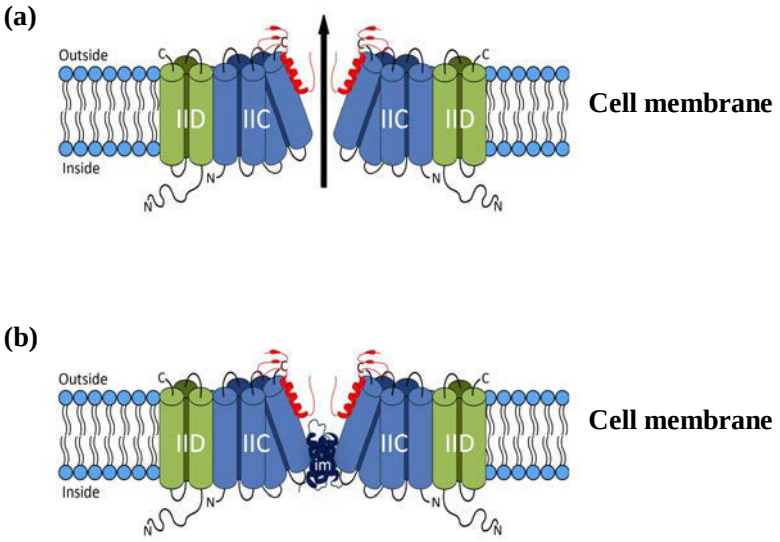

Figure 1. Proposed mode of action of class IIa bacteriocins (a) and immunity (b) adapted from Kjos et al. (2011a) and Wan 2012. Bacteriocin (red) binds to the extracellular loop of the EII_t^{Man} IIC domain (light blue), interacting with IIC and/or IID (light green), and causing pore formation. The immunity protein (dark blue) tightly binds to EII_t^{Man}-bacteriocin complex and block pore formation.

The class IIa bacteriocin receptor found in the membrane of susceptible cells was proposed to be man-PTS (Lohans and Vederas 2012). It is a sugar uptake system for mannose as well as glucose transporter. Studies showed a correlation between the man-PTS gene expression level and extent of sensitivity of target cells to class IIa bacteriocin (Kjos et al. 2009). The man-PTS contains four structural domains: IIA, IIB, IIC and IID. IIA and IIB are cytoplasmic domains which are fused as a single subunit complex in *L. monocytogenes* (Drider et al. 2006). IIC and IID together form a membrane-located complex involved as receptors for bacteriocins (Fig. 1a). Diep et al. (2007) found that presence of IIC and IID was sufficient for sensitivity of target cells to lactococcin A. Kjos et al. (2011a) later proved that a single extracellular loop in the IIC protein is responsible for the specificity of class IIa bacteriocins. Downregulation of man-PTS expression can be observed among naturally resistant strains or laboratory-induced resistant mutants (Kjos et al. 2011b). The immunity protein protecting class IIa bacteriocin producing cells from

being killed by their cognate bacteriocins is interacting with the same mannose permease indirectly by recognizing receptor-bacteriocin complex, binding with it and blocking the pore (Diep et al. 2007, Fig. 1b). The intracellular immunity protein acts via association with the internal side of the cell membrane. It is the C-terminal half of the immunity protein that contains a region being involved in the specific recognition of the bacteriocin to which it confers immunity (Johnsen et al. 2005).

1.3. Genes involved in the production of class IIa bacteriocins

The production of class IIa bacteriocins occurs during the exponential growth phase. As ribosomally synthesized peptides, bacteriocins are usually encoded by a plasmid- or chromosome-borne structural gene clustered with genes coding for immunity protein(s) and dedicated transport (García et al. 2010). There are at least four genes organized in one or two operons required for production of the class IIa bacteriocins (Fimland et al. 2005): (1) the structural gene encoding the bacteriocin precursor; (2) the immunity gene encoding the immunity protein that protects the producer from being killed; (3) the gene encoding a membrane-associated ABC transporter responsible for transportation of the bacteriocin across the membrane concomitantly with removal of the leader sequence; and (4) the gene encoding an accessory protein with unclear function.

The genetic determinants related to pediocin PA-1 production are located in a plasmid-borne operon governed by a promoter located directly upstream (Fig. 2). The high copy number plasmid could be transferred to plasmidless *P. acidilactici* strains (Ray et al. 1989). The plasmid-encoded properties are of great interest to biotechnology industry for genetic manipulations and improvement of strains for conventional starter cultures (Kumar et al. 2011). Pediocin PA-1 structural gene (*pedA*) of *P. acidilactici* PAC1.0 encodes 62 aa precursor including 18 N-terminal residues of leader sequence. The immunity gene (*pedB*) is located downstream to *pedA* and encodes a 112 aa protein. Also present in the operon are genes associated with membrane translocation (*pedC* and *pedD*) (Marugg et al. 1992; Motlagh et al. 1994). This two-component processing/export system is responsible for pediocin PA-1 maturation and secretion. Both *pedC* and *pedD* are necessary for translocation of pediocin across the cytoplasmic membrane (Venema et al. 1995). The accessory protein PedC has a similar structure of HlyD, a protein required for secretion of the *E. coli* hemolysin A, and LcnD, a protein involved in lactococcin A transport (Rodríguez et al. 2002). The *pedD* gene encodes a transmembrane translocator protein of the ABC superfamily whose proteolytic domains contain motifs thought to be part of the active site of the leader peptidase (Chikindas et al. 2010). The operon of pediocin AcH of *P. acidilactici* H has also been characterized as structural gene (*papA*), immunity gene (*papB*), ABC transportation genes (*papC* and *papD*) associated with translocation and processing of active pediocin AcH (Miller et al. 1998). Pediocin PA-1/AcH contains no modified aa residues and has been heterologously produced in *E. coli*, *Bifidobacterium longum*, *Lactobacillus reuteri*, *Streptococcus thermophilus*, *Lactococcus lactis* and *Lactobacillus casei* (Moon et al. 2005a; Moon et al. 2005b; Eom and Moon 2010; Renye Jr. and Somkuti 2010). Heterologous production of bacteriocins brings several advantages (Martín et al. 2007): (1) increasing bacteriocin production; (2) producing bacteriocins in safer hosts; (3) producing food ingredients with antimicrobial activity; (4) constructing multibacteriocinogenic strains with a wider antagonistic spectrum; (5) realizing better

adaptation of selected hosts to food environments; and (6) providing antagonistic properties of LAB used as starter, protective, or probiotic cultures. Improvements in heterologous systems have been made by using different promoters for enhanced expression, secretory proteins for fusion and peptide tags for facilitating purification (Kumar et al. 2011).

The gene clusters encoding leucocins have not been fully clarified. Leucocin A gene cluster containing structural gene *lcaA*, immunity gene *lcaB*, and ABC transporter genes *lcaECD*, has been studied previously. For leucocin C, Wan et al. (2013) revealed that leucocin C cluster in *Ln. carnosum* 4010 contains two operons, one includes *lecCI* and the other includes *lecXTS* (Fig. 2). The gene *lecC* encodes leucocin C precursor. The immunity gene *lecI* encoded 97 aa which shares 48 % similarity with the immunity genes of sakacin P and listeriocin. The genes of *lecXTS* are associated with the expression of ABC transporter and accessory protein, with 97 % homology with the leucocin A transporter operon *lcaECD* of *Ln. gelidum*.

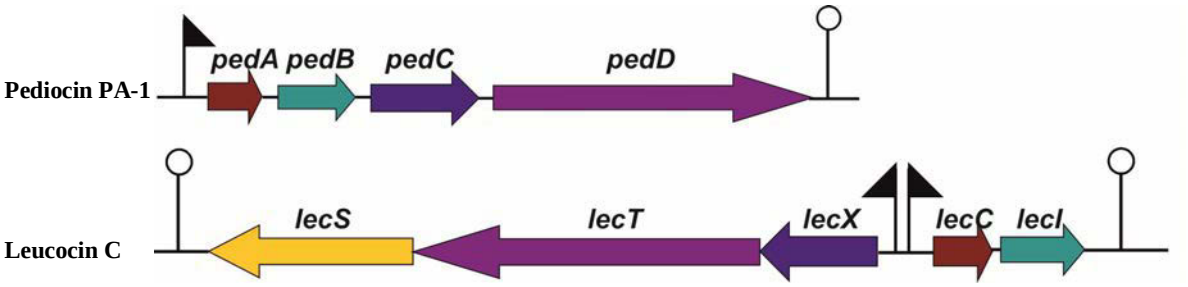

Figure 2. Organisation of the genes for production of pediocin PA-1 and leucocin C adapted from Wan 2012. Promoters are shown as black flags and transcriptional terminators as lollipop symbols.

1.4 Applications of class IIa bacteriocins in foods

Bacteriocins produced by food grade microorganisms have been consumed for centuries. They are usually heat stable, inhibitory to food pathogenic and spoilage organisms, and exhibiting desirable features in food application. Their properties for safe use depend on their inactivation by digestive proteases, having little influence on the gut microbiota, and showing no cross-resistance with antibiotics (Gálvez et al. 2007). The most famous commercial form of bacteriocins is Nisaplin, used as a preparation containing 2.5 % nisin with NaCl and non-fat dried milk. Applications of class IIa bacteriocins are mainly experimental to date. The one most close to industrial applications is pediocin PA-1/AcH. Since most cases of listeriosis in humans result from food-borne transmission, studies have been focused on antilisterial activity of pediocin PA-1/AcH as effective solution for *Listeria* contamination. Several patents cover the use of pediocin ALTA 2341 based on culture fermentates from pediocin producing strains (Rodríguez et al. 2002). Published results have shown that the incorporation of class IIa bacteriocins into foods is an attractive option to extend food shelf-life, control food-borne pathogens and provide effective antimicrobial hurdle together with other sublethal treatments in food systems (Table 2). The source of bacteriocins can be a purified or partially purified bacteriocin, a crude bacterial fermentate or bacteriocin producing culture (Stiles 1996).

Table 2. Applications of class IIa bacteriocins and their producing microorganisms to control the growth of food-borne pathogens and LAB. Adapted from Drider et al. 2006.

Bacteriocin	**Producer strain(s)**	**Food treated for targeting of the following organism(s)**			
		L. monocytogenes	***Clostridium botulinum***	***Staphylococcus aureus***	**LAB**
Divercin V41	*Carnobacterium divergens* V41	Smoked salmon			
Enterocin A	*Enterococcus faecium* CTC 492	Dry fermented sausage			
Enterocin CCM 4231	*Ec. faecium* CCM 4231	Dry fermented sausage, Soy milk		Soy milk	
Leucocin A	*Ln. gelidum* UAL187	Ground beef			Beef slices
Leucocin A-4010	*Ln. carnosum* 4010	Cooked sausage			
Mundticin	*Enterococcus mundtii* ATO6	Mung bean sprouts			
Pediocin PA-1/AcH	*P. acidilactici*	Cheese, Frankfurters, Chicken summer sausage, Red smear cheese	Chilled soup	Cheese	
Piscicolin 126	*Carnobacterium maltaromaticum* JG126	Ham paste, Camembert cheese			
Piscicosin CS526	*Carnobacterium pisciola* CS526	Surimi			
Plantaricin 423	*Lb. plantarum* 423	Ostrich meat salami			
Sakacin A or K or curvacin A	*Lactobacillus sakei* 790, *Lactobacillus curvatus* LTH 1174 and *Lb. sakei* CTC 494	Raw minced pork, Poultry, Cooked pork			Cooked pork
Sakacin P or bavaricin A	*Lb. sakei* 790 and *Lb. sakei* MI401	Cold smoked salmon			Brined shrimp

Concerns associated with class IIa bacteriocins are emerging such as low production and efficacy in food, negative sensory changes and risk of developing resistant strains. Considering the complex food matrix, various factors may influence the antimicrobial activities of bacteriocins added directed in the environment or secreted *in situ* by bacteriocin producing strains. For instance, inactivation by food enzymes, instability due

to pH changes, and interaction with food ingredients have been illustrated (Drider et al. 2006; Gálvez et al. 2007; Narsaiah et al. 2013).

2. *Listeria* phage endolysin cell wall binding domains

2.1 *Listeria*

Listeria is a ubiquitous genus which naturally inhabits soil, water, silage, animals, humans and diverse food materials and premises. Currently, there are eight recognized species belonging to this genus which are *L. monocytogenes*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. ivanovii*, *L. grayi*, *L. marthii* and *L. rocourtiae* (Kuenne et al. 2013). *L. monocytogenes* is a Gram positive, facultative anaerobic, motile, nonspore-forming rod, being tolerant to stresses such as low pH, low temperature and high concentration of NaCl, being persistent in foods and food processing equipment (Dabour et al. 2009; da Silva and De Martinis 2013). Therefore, the production environment is of important concern for contamination, considering the resistance and wide sources of this species. *L. monocytogenes* is the only pathogenic member within the *Listeria* genus, causing listeriosis with clinical severity and high mortality rate of up to 25-30 %, mainly targeting immunocompromised individuals, pregnant women, neonates and elderly persons (Winkelströter and De Martinis 2013). The infective dose varies among strains and depends on the susceptibility of people. After ingestion, *L. monocytogenes* transmits through the gastrointestinal tract. By expressing surface protein internalin A and internalin B, *Listeria* may attach to the surface of host cells and infect the epithelial cells. After cell invasion, listeriolysin O, a virulence factor for lysis of the phagosomal membrane helps *L. monocytogenes* escape the phagosome (Pamer 2004). *L. monocytogenes* moves through the cytoplasm and into neighbouring cell by polymerizing actin comet tails with ActA protein, contributing to its virulence (Portnoy et al. 1992; Pamer 2004).

There are four evolutionary lineages (I, II, III and IV) and 12 serotypes with distinct phylogenetic, ecologic and phenotypic characteristics (Kuenne et al. 2013). Lineage I strains are often related to the outbreaks of listeriosis in humans while lineage II strains are mostly found in contaminated food and animals with listeriosis. Lineages III and IV are rarely isolated from environmental and food samples or human clinical cases, but are usually isolated from animal sources (Orsi et al. 2011). Therefore, the common serotypes of *L. monocytogenes* in human clinical cases which are serotype 1/2a (lineage II), serotypes 1/2b and 4b (lineage I) belong to lineages I and II (da Silva and De Martinis 2013). Nowadays, *L. monocytogenes* is regarded as one of the leading emerging food pathogens causing food recalls especially in industrialized countries. It has been estimated that *Listeria* causes about 1600 people sick and 260 deaths every year in the United States (Centers for Disease Control and Prevention 2013). Many types of food products including seafood, vegetables, meat and dairy products have been contaminated with this pathogen (Wang et al. 2012). Particularly the RTE minimally processed and refrigerated foods turn out to be important public health risks due to extensive handling during process and absence of thermal treatment prior to consumption (Kovačević et al. 2013). The European Commission recommends a limit of 100 cfu/g *L. monocytogenes* throughout the shelf-life for RTE foods (Rosef et al. 2012). International standards for detection and enumeration of *L. monocytogenes* suffer of high time requirement of 7 days (ISO 11290-1

1996; ISO 10560 1999). Since *L. monocytogenes* is not detected by routine stool culture, symptoms like gastroenteritis and fever may not be diagnosed as the consequence of *Listeria* infection (Scallan et al. 2011).

2.2 *Listeria* phages

Bacteriophages are abundant self-replicating units in the environment and normal commensals of humans and animals (Carlton et al. 2005). These viruses specifically interact with their host bacteria by lytic or lysogenic propagation cycle. Lytic phages cause cell lysis to release progeny, while in lysogenic cycle, the phage DNA is included in the host chromosome and replicated along with the bacterial DNA (Griffiths et al. 1999). The host range is defined by phage recognition of receptors (e.g. certain proteins, sugars, and lipids) in the CW or appendages (e.g. flagellin and pili) to the bacterial cells (Smartt et al. 2012). Bacteriophages are natural means to identify bacteria since each bacteriophage has a host range to infect, which is the foundation of phage-typing methods used for decades. Nowadays, detection methods based on bacteriophage have been expanded to a broad and innovative range including labeled phage DNA, reporter phage, phage amplification assays, quantum dots, phage-mediated lysis of host cells, and conductance measurements (Smartt and Ripp 2011).

Bacteriophages have been regarded as biodegradable non-toxic food grade bactericidal agents with certain products approved as food additives and awarded GRAS status by the FDA and EPA (Arachchi et al. 2013). The use of bacteriophages as alternative of antibiotics provides some advantages such as: (1) abundance in source; (2) rapid replication and declining, along with bacterial growth not posing an ecological risk; (3) relatively narrow host range, having no influence on the useful bacteria and normal intestinal microflora; (4) decrease after killing the target bacteria being finally excreted, not posing any environmental risk; (5) a mixture of phages bringing synergistic effects can be applied; and (6) might be less expensive than using antibiotics (Oliveira et al. 2012).

L. monocytogenes phages were first reported in the last decade and have been isolated from more than 500 various environmental sources, featuring the long, non-contractile tails of the *Siphoviridae* family, or the complex contractile tail machines of the *Myoviridae* family (Klumpp and Loessner 2013). Most of the *Listeria* phages present circularly permuted genome structures. The genome is usually 30 to 65 kb in size and contains dsDNA encoding structural genes, functional genes for recombination, replication and repair, lysis genes encoding holin and endolysin, and a lysogeny control region for some temperate phages (Klumpp and Loessner 2013). *Listeria* phages have been used for detection, differentiation and biocontrol of *Listeria*. Construction of phage with reporter genes including *luxAB* and *celB* has been tested with high sensitivity (Loessner et al. 1997; Hagens et al. 2011). There are several commercial agents such as Listex P100 (phage P100) and ListShield^{TM} (LMP-102 phage preparation), based on *Listeria* phages to control *L. monocytogenes* contamination in a variety of food matrices (Carlton et al. 2005; Bren 2007).

2.3 *Listeria* phage endolysin cell wall binding domains

The mechanisms by which phages cause host cells lysis have been studied in detail, including endolysins involved in the release of the progeny. Bacteriophage endolysin

gained its name in 1958 when it was defined as probably a proteinaceous lytic substance acting on the CW from within the cell (Jacob and Fuerst 1958). Now, endolysin is described as bacteriophage-encoded murein enzyme produced near the end of the phage lytic multiplication cycle during infection process. Degradation of PG (a major structural component of the bacterial CW) leads to the destruction of intracellular osmotic pressure, hypotonic lysis of the bacterial cell ("lysis from within"), and release of phage progeny (Bernhardt et al. 2002; Borysowski et al. 2006). PG has a unique structure depending on bacteria with a sugar backbone of alternating units of N-acetyl glucosamine and N-acetyl muramic acid (van Heijenoort 1998; Vollmer et al. 2008). Endolysins form holes in the PG with the help of holins, hydrophobic proteins which oligomerize and form pores in the cell membrane, allowing endolysins to pass through (Young et al. 2000; Bernhardt et al. 2002). The holin gene is located immediately upstream of the endolysin gene and is clustered as a lysis cassette for regulation (Young et al. 2000; Young 2002). Owing to the unique feature of cleaving PG rapidly and specifically, endolysins gain much attention as alternative biocontrol agents with potential use in food industry, biotechnology, and medicine (Schmelcher et al. 2012). For potential use as antibacterials, endolysins possess a novel mode of action, a narrow antibacterial spectrum, rapid and potent antibacterial activity against bacteria regardless of their antibiotic sensitivity, apparent safety, a low probability of developing resistance, and relatively easy modifications by means of genetic engineering (Borysowski et al. 2006). Applications that have been explored in biological fields include: (1) elimination of bacterial colonization of mucous membranes; (2) treating bacterial infections; (3) biocontrol of bacteria in food and feed; and (4) protection of plants against phytopathogenic bacteria (Borysowski et al. 2006).

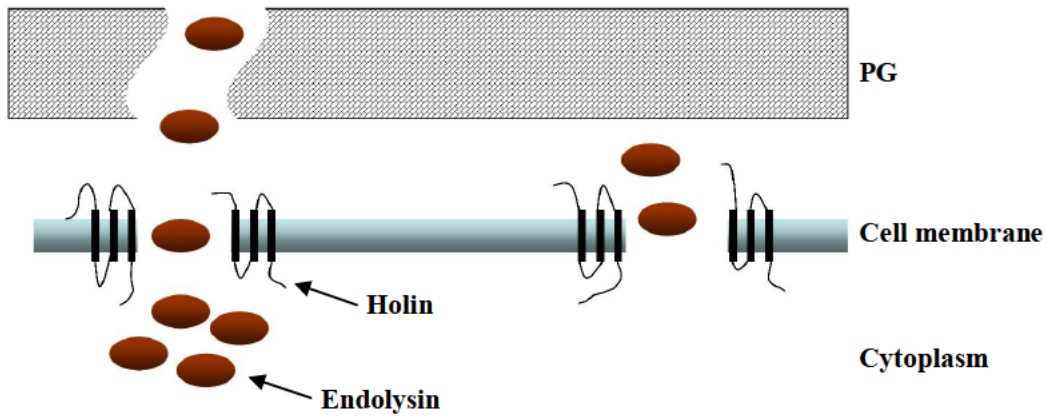

Figure 3. Schematic representation of mechanism of endolysin. Adapted from García et al. 2010.

Endolysins are classified into different categories according to the bonds targeted within the PG structure (Schmelcher et al. 2012): (1) glucosaminidases; (2) muramidases, also termed "lysozymes"; (3) transglycosylases cleaving within the aminosugar backbone; (4) *N*-acetylmuramoyl-L-alanine amidases; and (5) endopeptidases. One endolysin usually shows only one kind of muralytic activity with muramidases or amidases as common ones (Loessner 2005). As to *Listeria* phage endolysins, there are two different enzymatic

specificities which have been demonstrated: *N*-acetylmuramoyl-L-alanine amidase and L-alanoyl-D-glutamate peptidase (Schmelcher et al. 2012).

The modular structure of endolysin can be divided into two distinct parts: the N-terminal EAD and the C-terminal CBD. Sequence comparison of 12 different endolysins demonstrated high diversity reflected in various binding properties including specificity, number and distribution of ligands, and binding affinities (Schmelcher et al. 2010). Recombinant endolysins from *L. monocytogenes* phages resulted in rapid host cell lysis (Schmelcher et al. 2012). The CBD are necessary and sufficient to direct the enzymes to the substrate of the *Listeria* CW. Research has revealed that the interaction between CBD and carbohydrate-like ligands on the CW is a non-covalent binding in a rapid, saturation-dependent way (Loessner et al. 2002). Later Eugster et al. (2011) proved that CBD from phage P35 can recognize terminal GlcNAc residues in the *Listeria* WTA molecules. For CBD118, CBD511 and CBDP40, WTA polymers did not directly serve as binding ligands. Instead, PG backbone itself presents as binding sites (Eugster and Loessner 2012). Construction of fluorescent protein-CBD allows direct microscopic detection of *Listeria* strains among different bacteria. A new approach for rapid and easy detection and discrimination of *Listeria* has also been established based on fluorescent protein-CBD (Schmelcher et al. 2010).

3. Bacterial surface display

Cell surface proteins play important role in many biological functions such as signal transduction, cell-cell communication, and ion transportation. Microbial cell-surface display becomes a rapidly developing technology carried out by expressing the target protein on the surface of the host cells utilizing the natural surface proteins. Anchoring to the cells enables the displayed proteins to be more stable than their free states and accessible to the substrate(s) or ligands without membrane interference (Yim et al. 2013). To achieve this, the target protein (passenger) is often fused to a carrier protein (anchoring motif) which transports the passenger across the membrane for surface exposure. It can be a C-terminal fusion, N-terminal fusion or sandwich fusion depending upon the specific application. Carrier protein is crucial for correct anchoring and efficient expression of the target protein. Criteria for selecting a proper carrier protein are (1) efficient signal peptide or transporting signal to allow premature fusion protein to go through the inner membrane; (2) strong anchoring structure to prevent detachment of fusion proteins from the cell surface; (3) compatibility with different foreign sequences to be inserted or fused; and (4) resistance to proteases presenting in the periplasmic space or medium (Lee et al. 2003).

Heterologous surface display on bacteria was firstly reported nearly two decades ago when target protein was fused with *E. coli* outer membrane proteins such as LamB, OmpA and PhoE (Ståhl and Uhlén 1997). Nowadays, *E. coli* is still a common host for presenting various peptides and library screening. Gram-positive bacteria including LAB, *Bacillus* and *Staphylococcus* have been reported for successful display due to their rigid structure of CWs. Besides, yeast with the representative of *Saccharomyces cerevisiae* is also used for displaying foreign proteins especially mammalian proteins (Lee et al. 2003).

3.1 Surface display in *E. coli*

Different strategies for displaying both short peptides and large proteins have been investigated in *E. coli*, the most frequently used host in surface display systems. A display system has to cross both cytoplasmic and outer membranes of *E. coli* for a successful presenting. Diverse carrier proteins containing different anchor motifs have been described for many purposes depending on the type of carrier and its specific secretion pathway (Table 3). Basically, *E. coli* display systems can be divided into the following groups: (1) Most of the carrier proteins such as maltoporin LamB, OmpA and OmpC are based on the OMPs which span the outer membrane with antiparallel β-sheets. The insertion should be in a permissive extracellular loop of the outer membrane proteins with limited number of residues (Cornelis 2000). Since the target protein will be constrained when inserted into a permissive site, a successful display highly depends on the structure of the target proteins. (2) Lpps are usually anchored to the outer membrane covalently by virtue of their lipid-modified N-terminus. TraT, an oligomeric and plasmid-encoded Lpp allows C-terminal and internal insertion. The PG-associated Lpp (PAL) presents an N-terminally fused passenger (Samuelson et al. 2002). (3) Surface appendages referring to flagellin and fimbriae (or pili) have been used for displaying short peptides. The hypervariable region of *fliC* gene from *E. coli* flagella has been the target position for insertions. A commercial system based on *fliC* gene called FliTrx is available, suited for screening affinity peptides against various molecular target proteins (Samuelson et al. 2002). (4) Some virulence factors expressed on the pathogenic bacteria can be adopted as carrier proteins such as intimin protein EaeA and invasin. EaeA, a 102 kDa OMP originated from *E. coli* enables the display of C-terminal fusion proteins. Invasin is a 106 kDa OMP from *Yersinia pseudotuberculosis* which displays target protein fused to the C-terminus (van Bloois et al. 2011). Other types of carrier proteins which also gain much attention are autotransporter proteins and *Pesudomonas syringae* INP.

Table 3. Anchor proteins used in *E. coli* surface display adapted from van Bloois et al. 2011.

Scaffold	Anchor	Type of fusion	Passenger size (kDa)	Application
OMPs	eCPX derived from OmpX	Biterminal	0.8-1.6	Peptide library screening
	FhuA	Insertional	1.1-3.3	Peptide library screening
	Omp1	C-terminal	56	Biocatalysis
	OmpC	Insertional, C-terminal	18-52	Bioremediation, biocatalysis
	LamB	Insertional	1.2-25.5	Bioremediation, peptide library screening, vaccine development
	OmpA	Insertional	1-50	Peptide library screening, peptide display, vaccine development

Table 3. (Continued)

Scaffold	Anchor	Type of fusion	Passenger size (kDa)	Application
OMPs	OmpT		35	Directed evolution, substrate profiling
	OprF	C-terminal	50	Biocatalysis
	PgsA	C-terminal	34-77	Biocatalysis
	Wza-omp orf1/OmpU/Omp26La	C-terminal	27-50	Translocation studies
Surface appendages	F pillin	Insertional	1.6	Peptide antigen display
	Fimbriae (FimH and FimA)	Insertional	1-4	Peptide library screening, immunogenic peptide display, bioremediation
	Flagellin (FliC and FliD)	Insertional	1.2-33	Peptide display, peptide library screening, vaccines, bioremediation, exploring protein-protein interactions
Lpps	INP	C-terminal	7-119	Epitope mapping, biocatalysis, vaccines, protein library screening
	Lpp-OmpA	C-terminal	27-74	Bioremediation, biocatalysis, Ab library screening
	PAL	N-terminal	29	Display Ab fragments, bioremediation
	Tat-dependent lpp	C-terminal	27	Translocation studies
	TraT	Insertional, C-terminal	1.2-11	Antigen display
Virulence factors	AIDA-I	N-terminal	12-65	Biocatalysis, protein library screening, vaccine development
	EaeA	C-terminal	3.9-31.6	Translocation studies
	EspP	N-terminal	20	Protein library screening
	EstA	N-terminal	38-60	Biocatalysis, protein library screening
	Invasin	C-terminal	1.1	Peptide library screening
	MSP1a	N-terminal	4.6	Immunogenic peptide display

3.2 Surface display in lactic acid bacteria

LAB are being used as "cell factories" and good source of microbial-surface display systems by providing different anchoring motifs for functional expression of various proteins. Compared with *E. coli*, LAB have a much thicker CW and lack the outer membrane envelope. Basically, five different types of anchor proteins have been investigated for their translocation capacity of hybrid proteins to the cell surface of LAB (Leenhouts et al. 1999). (1) Transmembrane anchors allow insertion of foreign protein

with limited size in an exterior loop. For better presenting, at least 100 aa are needed to cross the CW. (2) Lpp anchors, such as PrtM and OppA, bind to the lipid bilayer covalently. (3) Most of the CW attached proteins contain a well-conserved pentapeptide LPXTG motif (where X denotes any aa) followed by a hydrophobic domain which is a membrane-spanning region, and then a short positively charged tail at the extreme C-terminus serving as a retention signal to prevent secretion of the polypeptide chain into the surrounding medium (Lee et al. 2003). After cleavage between the threonine and glycine by sortase (SrtA), anchor domains are attached to the peptide crossbridge in the PG of the CW by amide-linkage (Leenhouts et al. 1999). PrtP protein originated from *Lc. lactis* belongs to this group and allows fusion to the N-terminus for presenting the target proteins on the cell surface. (4) AcmA from the N-acetylglucosaminidase of *Lc. lactis* is the representative of LysM motif. AcmA contains three repeated LysM sequences of 44 aa coming from the C-terminal region (Buist et al. 1995). It has been reported that at least two repeat of AcmA is sufficient for CW binding, although the mode of action is still unknown in detail (Petrović et al. 2012). (5) Surface-layer-protein anchor is derived from surface proteins of Gram-positive bacteria and predicted to contain two α-helices flanking a β-strand.

3.3 Applications of cell surface display

Cell surface display has a wide range of applications in biotechnology and industry. Proteins being successfully displayed on the cell surface contain antigens, epitopes, enzymes, and single-chain antibodies (Kim and Yoo 1998). In vaccine development Gram-negative bacteria such as *E. coli* and *Salmonella* spp. were initially explored for presenting antigenic determinants on the surface for the live and oral delivery. Later, commensal or non-pathogenic Gram-positive bacteria were investigated (Ståhl and Uhlén 1997). Antigens for surface exposure include specific T- or B-cell epitopes, receptor-specific molecules, and colonization factors (Samuelson et al. 2002).

Surface display is a good source for biocatalysts especially for the immobilized ones which confer advantages including reduction in mass transfer limitations, facilitating cycling, improving efficiency of the enzymes, and elimination of enzyme purification. Potential use of such recombinant bacteria has been studied. For instance, β-lactamase from *E. coli* and cex exoglucanase from *Cellulomonas fimi* have been displayed on the surface of *E. coli* using Lpp-OmpA system (Ståhl and Uhlén 1997). Anchor proteins such as OprF from *Pseudomonas aeruginosa* and PgsA from *Bacillus subtilis* have also been used for lipase display on the surface of *E. coli* (Nagarajan 2012). Furthermore, bioremediation has been raised as an attractive solution to contamination of soil, sediments and groundwater. Recombinant biocatalysts displaying organophosphorus hydrolase (OPH) on the surface of *E. coli*, *Moraxella* sp. and *Pseudomonas* sp. resulted in improved hydrolysis of organophosphates (OP) (Shimazu et al. 2003).

Surface display technology is also used for construction of whole-cell bioadsorbents, applicable to removal of harmful chemicals and heavy metals, for instance, in waste water treatment. Compared with intracellular expression of metal bioadsorbents, surface expression of them eliminates the time-consuming and rate-limiting step of crossing the membrane, needs no interference with redox pathways in the cytosol. Displaying

metallothioneins and chitin binding domain on the surface of *E. coli* cells increased cadmium adsorption and cell immobilization (Tafakori et al. 2012).

Another important application of surface display is for the selection of peptides or recombinant Ab fragments from large libraries. High-throughput screening (HTS) can be realized by using this technology as an alternative to phage display for identification peptides or proteins of interest. Beneficial sides of using bacterial display can be summarized as (1) simpler by using only one bacterium for propagation of the library; (2) no need for reinfection to replicate the selected variants; (3) low occurring of affinity artifacts caused by avidity effects; and (4) possible for direct screening by FACS (Samuelson et al. 2002). Besides, microbial cells displaying the single chain antibodies can also be developed as biosensors or used in bioseparation (Bassi et al. 2000).

Aims of the Study

The main objectives of this study were to investigate the antilisterial activities of bacteriocin in heterologous expression systems, and to introduce *Listeria*-binding ability to bacteriocin producing strains. The detailed objectives of the research were to:

1. Construct bacteriocin producing strains in *E. coli* and *Lc. lactis* using different strategies for high level production of biologically active bacteriocins (I, unpublished).

2. Investigate the bacterial surface display systems for better presenting of *Listeria* phage endolysin CBDs (II, III, IV, unpublished).

3. Enhance the killing of *L. monocytogenes* by binding of recombinant bacteriocin producing *E. coli* cells to *Listeria* cells (III, IV).

Materials and Methods

1. Bacterial strains, plasmids and culture conditions

The plasmids used in this study are presented in Table 4, and bacterial strains in Table 5. *E. coli* host strains were cultured in LB medium at 37 °C. Strains with plasmids were grown in LB medium supplemented with 50 µg/ml kanamycin or 100 µg/ml ampicillin, when necessary. For production of flagella, *E. coli* JT1 strains were cultured at 28 °C for 72 h. *L. monocytogenes* strains were cultivated at 30 °C in BHI medium (Oxoid) or on *Listeria*-selective Oxford agar (Scharlab) plates at 30 °C. *Lc. lactis* host strains were grown overnight in M17G at 30 °C. Strains with plasmids were cultured in M17G medium supplemented with 50 IU nisin /ml (Nis^{50}) or 5 µg/ml erythromycin, when necessary.

Table 4. Plasmids used in this study.

Plasmid	Relevant properties	Reference/source	Used in
pET32a(+)	*E. coli* cloning vector, Amp^R	Novagen	I
pET20b(+)	*E. coli* cloning vector, Amp^R	Novagen	I
pPA003PED1	*pedA* in pET32a(+), Amp^R	This work	I
pPA003PED2	*pedA* in pET20b(+), Amp^R	This work	I
pLEB688	*Lc. lactis* food-grade cloning vector, Nis^R	Li et al. 2011	unpublished
pLEB691	Pediocin food-grade expression in *Lc. lactis*, Nis^R	Li et al. 2011	unpublished
pLEB750	SS_{usp45}-*pedA* in pLEB688, Nis^R	This work	unpublished
pLEB795	SS_{usp45}-*pedAB* in pLEB688, Nis^R	This work	unpublished
pHGFP_CBD006-C	Vector containing CBD006 gene as template, Amp^R	Schmelcher et al. 2010	unpublished
pLEB597	*Lc. lactis* plasmid constructed for expression of CBD-PrtP 344 aa, Erm^R	Kylä-Nikkilä et al. 2010	II
pLEB595	*Lc. lactis* plasmid constructed for expression of CBD-NisP 121 aa, Erm^R	This work	II
pLEB607	*Lc. lactis* plasmid constructed for expression of CBD-PrtP-sortase, Erm^R	This work	II
pTF1	*E. coli-Lc. lactis* shuttle vector pLEB579 carrying multiple cloning site from pBluescript, Erm^R	Beasley et al. 2004 (pLEB579); Fieseler, ETH Zürich, CH, unpublished	III
pBluescript/$fliC\Delta_{H7}$	$fliC\Delta_{H7}$ display vector, derivative of pBluescript, Amp^R	Westerlund-Wikström et al. 1997	III, IV
pET-LOAvi	Expression vector, derivative of pET20b(+), Kan^R	Wang and Chao 2006	III, IV

Table 4. (Continued)

Plasmid	Relevant properties	Reference/source	Used in
pHPLP35	Vector containing CBDP35 gene as template, Amp^R	Schmelcher et al. 2012	III
pLEB728	*Lc. lactis* plasmid carrying leucocin C secretion cassette P45-SS_{usp45}-*lecC*, Nis^R	Wan 2012	III
pLEB731	P45-SS_{usp45}-*lecC* in pTF1, Erm^R	This work	III
pLEB746	P45-SS_{usp45}-*lecC* in pET-LOAvi, Kan^R	This work	III
pLEB747	Surface display of CBDP35, derivative of pBluescript/*fliC*Δ_{H7}, Amp^R	This work	III
pLEB748	P45-SS_{usp45}-*lecC* in pBluescript/*fliC*Δ_{H7}, Amp^R	This work	III
pLEB749	P45-SS_{usp45}-*lecC* in pLEB747, Amp^R	This work	III
pASG-IBA4	Vector for CBD500-YadA surface display, *tet*-promoter, SS_{ompA}, StrepTag, Amp^R	IBA GmbH, Göttingen, Germany	IV
pLEB588	pHELIX2 + *pepR* promoter of *Lactobacillus rhamnosus*, Amp^R	Takala et al. 2003	IV
pHPL500	Vector containing CBD500 gene as template, Amp^R	Loessner et al. 1996	IV
pLEB733	SS_{usp45}-*papA* in pLEB588, Amp^R	This work	IV
pLEB735	*CBD500-yadA* in pASG-IBA4, Amp^R	This work	IV
pLEB736	PpepR-SS_{usp45}-*papA* in pLEB735, Amp^R	This work	IV
pLEB737	Vector for display of CBD500, derivative of pET-LOAvi, Kan^R	This work	IV
pLEB738	Vector for display of CBD500, derivative of pBluescript/*fliC*Δ_{H7}, Amp^R	This work	IV
pLEB739	P45-SS_{usp45}-*papA* in pBluescript/*fliC*Δ_{H7}, Amp^R	This work	IV

Table 5. Bacterial strains used in this study.

Bacterial strain	Relevant properties	Reference/source	Used in
P. acidilactici PA003	wt pediocin producer	This work	I
E. coli DH5α	Transformation host	Hanahan 1983	I, IV
E. coli BL21(DE3)	Transformation host	Invitrogen	I, III, IV
E. coli TG1	Transformation host	Gibson 1984	III, IV
E. coli JT1	Transformation host	Westerlund-Wikström et al. 1997	III, IV
L. monocytogenes CVCC1595	Bacteriocin indicator strain	China Institute of Veterinary Drug Control	I
L. monocytogenes WSLC 1018	Bacteriocin indicator strain	Loessner et al. 2002	unpublished, IV
L. monocytogenes WSLC 1001	Bacteriocin indicator strain	Loessner et al. 2002	unpublished
L. monocytogenes WSLC 1019	Bacteriocin indicator strain	Loessner et al. 2002	III, IV
Lc. lactis NZ9000	Transformation host	Kuipers et al. 1998	unpublished
Lc. lactis MG1363	Transformation host	Gasson 1983	II
Lc. lactis IL1403 *htrA*	Transformation host, IL1403 derivative containing disrupted $htrA_{L1}$ gene	Poquet et al. 2000	II
Staphylococcus aureus NCTC 8530	Template for amplification of *srtA* gene	Cossart and Jonquières 2000	II
LAC413	*Lc. lactis* NZ9000/pLEB750	This work	unpublished
LAC242	*Lc. lactis* MG1363/pLEB606	Kylä-Nikkilä et al. 2010	II
LAC247	*Lc. lactis* MG1363/pLEB596	Kylä-Nikkilä et al. 2010	II
LAC248	*Lc. lactis* MG1363/pLEB597	Kylä-Nikkilä et al. 2010	II
LAC246	*Lc. lactis* MG1363/pLEB595	This work	II
LAC243	*Lc. lactis* MG1363/pLEB607	This work	II
ECO776	*E. coli* JT1/pLEB747	This work	III
ECO770	*E. coli* JT1/pBluescript/$fliC\Delta_{H7}$	Westerlund-Wikström et al. 1997	III, IV
ECO779	*E. coli* JT1/pLEB746	This work	III
ECO777	*E. coli* JT1/pLEB748	This work	III
ECO778	*E. coli* JT1/pLEB749	This work	III
ECO615	*E. coli* TG1/pLEB588	Takala et al. 2003	IV
ECO759	*E. coli* TG1/pLEB733	This work	IV
ECO760	*E. coli* TG1/pASG-IBA4	This work	IV
ECO762	*E. coli* TG1/pLEB735	This work	IV
ECO764	*E. coli* TG1/pLEB736	This work	IV

Table 5. (Continued)

Bacterial strain	Relevant properties	Reference/source	Used in
ECO767	*E. coli* BL21(DE3)/pLEB737	This work	IV
ECO768	*E. coli* JT1/pLEB738	This work	IV
ECO769	*E. coli* JT1/pLEB739	This work	IV
ECO766	*E. coli* BL21(DE3)/pET-LOAvi	This work	IV

2. PCR primer sequences

Sequences of the PCR primers are listed in Table 6.

Table 6. Sequences of the PCR primers used in this study. Relevant restriction sites added to the primers are shown underlined.

Primer name with restriction site	Sequence (5' - 3')	Used in
PedAF1, *Bgl*II	AACCCCAGATCTCGACGACGACGACAAGAAATACT ACGGTAATGGG	I
PedAR1, *Xho*I	CCCGGGCTCGAGTTATGATGCCAGCTCAGCATAATG CTA	I
PedAF2, *Hin*dIII	AACCCCAAGCTTATGAGCGATAAAATTATTCACCTG ACTGACG	I
PedAR2, *Xho*I	CCCGGGCTCGAGCTATTATCAGCATTTATGATTACC TTGATGTCCA	I
Usp45F, *Sma*I	AATCCCGGGATAAGAACTTAATGGGAGG	unpublished
Usp45R, *Nae*I	GAAGATCTGCCGGCGTAAACACCTGACAACG	unpublished
PedAF3	AAATACTACGGTAATGGG	unpublished
PedAR3, *Sph*I	CCGGCATGCCTAGCATTTATGATTAC	unpublished
PedBF, *Sph*I	ACGGCATGCCATTATGCTG	unpublished
PedBR, *Bam*HI	AACGGATCCCTATTGGCTAG	unpublished
P45F2, *Bss*HII	AAAGCGCGCGGATCCACATAACAGTCATAAAACC	unpublished
PrtPR	AAAACGCGTCTATTCACGTTGTTTCCGC	unpublished
NIS191, *Xba*I	TGTTCTAGAGGATCCGGGAAAAATAAAGCTTTTAGC	II
NIS192, *Apa*I	ATATGGGCCCTCAATTTTTAGTCTTCCTTTTC	II
NIS218, *Apa*I	GTGAGGGCCCTAAAAGGAGCCTTAACGTATG	II
NIS219, *Xma*I	GCGTCCCGGGTTATTTGACTTCTGTAGCTAC	II
FliCBDP35F, *Acc*I	ACGGTAGACCCACATTTTGAAGCA	III

Table 6. (Continued)

Primer name with restriction site	Sequence (5' - 3')	Used in
FliCBDP35R, *Acc*I	ATCGTCTACCTTATCGTCATCGTCTTTCTTGATGTCAAAC	III
P45F, *Eco*RI	ATTGCGGCCGCGAATTCCGTTAGGGGCTTGAAC	III
LecR	TTAGTTATGCCATCCAGCATTGC	III
NIS228	TTATTTTGGTTTGATGTTGCGAT	III
ErmR	TTATAGTTTTGGTCGTAGAGC	III
CBD500Forw, *Nco*I	ACGCCATGGCAAAACACTAATACAAAT	IV
CBD500Rev, *Nco*I	ACGCCATGGCAAAACACTAATACAAAT	IV
yadAForw, *Nco*I	ACGCCATGGCAAAACACTAATACAAAT	IV
yadARev, *Nco*I	ACGCCATGGCAAAACACTAATACAAAT	IV
PedF, *Xho*I	GAGCTCGAGTTTAAATACTACGGTAATGGGGTTAC	IV
PedRev, *Sal*I	GACGTCGACTAGCATTTATGATTACCT	IV
PedRev2	AGTCCACTACGTGCTAGCATTTATGATTACCT	IV
uspF, *Sal*I	GACGTCGACATGAAAAAAAAGATTATCTCAGC	IV
uspR, *Xho*I	GAGCTCGAGGCCGGCGTAAACACCTGACAACGG	IV
pepRF	ACTGCACATTGTGTGCTTTGATACTCACCAATG	IV
CBD500F, *Nco*I	ACGCCATGGCAAAACACTAATACAAAT	IV
CBD500R, *Bam*HI	AACGGATCCTTAGTGATGGTGATGGTGATGTTTTAAGAAGTATTC	IV
FliCBD500F, *Acc*I	ACGGTAGACCAAAACACTAATACAAATTC	IV
FliCBD500R, *Acc*I	ATCGTCTACCTTATCGTCATCGTCTTTTAAGAAGTATTC	IV
P45F	ATTGCGGCCGCGAATTCCGTTAGGGGCTTGAAC	IV
PedAR, *Sal*I	GACGTCGACTAGCATTTATGATTACCTTG	IV

3. Analysis methods

Main analysis methods are provided in Table 7 with detailed information as follows as well as in the Materials and Methods sections in the publications I-III and manuscript IV.

Table 7. Methods used in this study.

Method	Used in	Reference
Basic DNA techniques, including PCR, enzyme modifications, electrophoresis, plasmid isolation from *E. coli*	I-IV	Sambrook et al. 1989; Catalogues of enzyme suppliers
DNA transformation - *E. coli* - *Lc. lactis*	I-IV	 Sambrook et al. 1989 Holo and Nes 1989
SDS-PAGE	I-IV	Sambrook et al. 1989
Western blot	II-IV	Invitrogen WesternBreeze Chromogenic Kit
Whole-cell ELISA	II, IV	Kylä-Nikkilä et al. 2010
Antilisterial bioassay	III, IV	Wan et al. 2013
Aggregation and sedimentation of cells	III	Kos et al. 2003
Cell fractionation	IV	Yang et al. 2008

3.1 Agar diffusion assay (I)

Agar diffusion assay is used for quantification of antimicrobial activity. The prepared agar plate was overlaid with 10 ml of soft agar inoculated with 50 µl of an overnight *Listeria* culture as indicator. A total of 12.5 µl samples were added to a well and then in two-fold serial dilutions. Plates were incubated at 37 °C for 16 h. One arbitrary unit (AU) was defined as the reciprocal of the highest dilution yielding a visible inhibition zone. The total AUs per milliliter were calculated by 80×2^{n}, where n is the number of wells showing inhibition.

3.2 Purification of his-tag fusion protein (I)

Solution was filtered through a 0.45 µm filter membrane before loaded onto 10 ml nickel-iminodiacetic acid His-bind resin column. After washing with 100 ml washing buffer (50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 50 mM imidazole), fusion protein was eluted with elution solution (50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 400 mM imidazole).

3.3 Bioscreen C growth analysis (unpublished)

To determine antilisterial activity of pediocin produced by *Lc. lactis* construct, *Lc. lactis* cells were cultivated overnight in M17G medium. Two hundred µl *Lc. lactis* cell-free culture supernatant and 20 µl overnight *Listeria* cells were mixed in 3 ml BHI medium before added in microtiter plates. Five parallel plates were prepared for each growth condition. M17G medium was used as the control. The plates were grown in Bioscreen C (SORVALL MC 12V, Labsystems) at 30 °C with constant shaking for 20 h. The optical density was measured at 590 nm wavelength.

Results and Discussion

1. Antilisterial activities (I, III, IV, unpublished)

1.1 Expression of pediocin/leucocin C gene in *E. coli* (I, III, IV)

Heterologous expression of interested protein products in *E. coli* system has been widely used (Huang et al. 2012). LAB produce usually low yield of bacteriocins in wt strains and may lose capacity to produce bacteriocins upon cultivation (Rodríguez et al. 2003). Many LAB bacteriocin genes have been explored for expression in *E. coli* strains. However, there is no guarantee that the recombinant protein maintains the biological activity. Sometimes high levels of expression may cause misfolding and aggregation of the products (Austin 2003).

1.1.1 Fusion expression of *pedA*

Pediocin structural gene *pedA* (132 bp) was heterologously expressed in *E. coli* for functional analysis. The amplified sequence with the size of 198 bp was obtained by PCR using the total DNA of *P. acidilactici* PA003 as template. This fragment was sequenced and found to be 100 % identical to that of the *pedA* gene which has been published in the NCBI (GenBank No. AY083244). The resulting *pedA* gene product was treated with *Bgl*II and *Xho*I digestion before cloned into the *E. coli* vector pET32a(+). Thioredoxin was chosen as the fusion part to decrease protease hydrolysis of pediocin and to increase the solubility of the fusion protein. The fusion protein thioredoxin-PedA was expressed at 37 °C after 1 mM IPTG induction for 4 h. High yield led to the insoluble form of the fusion protein since the target protein was present mainly in the pellet after ultrasonic disruption of recombinant cells. It is reported that the inclusion body may protect cells from toxic effects of the recombinant peptides (Lee et al. 2000). Austin (2003) and Tian et al. (2007) have documented the expression of fusion proteins as inclusion bodies in the construction of pET32 vectors. It is possible the two disulfide bonds of pediocin are not formed, especially when in high concentration, as the cytoplasmic environment is not suitable for oxidation.

Various culture conditions were tested to evaluate the stability and expression form of the recombinant protein. There was no visible difference of production level among the selected culture temperatures (24-37 °C), initial culture densities (OD_{600} = 0.2-1.0), induction times (2-5 h), and IPTG concentrations (20 μM-1 mM) (data not shown). The inclusion bodies were washed with 2 M urea to eliminate the cell debris as well as impurity proteins before solubilized in 8 M urea. Renaturation process was performed slowly in GSH/GSSH system with mild agitation. The combination of GSH with GSSH provided an appropriate redox potential for both the formation and reshuffling of disulfide bonds. Considering the precipitation amount of protein and the final dilution volume, 15 times dilution of recombinant protein with renaturation buffer was determined to be optimal. After purification with the aid of the his-tag in the fusion protein, single protein band with the size of around 22 kDa could be detected by SDS-PAGE. Enterokinase cleavage site between PedA and thioredoxin was designed to enable separation of the fusion part and recombinant pediocin. Recombinant pediocin revealed 10240 AU/ml

antibacterial activity (Fig. 4, I). Finally, pediocin production was 512 AU from 1 ml culture medium of *E. coli* BL21 (DE3) recombinant cells containing the pET32a(+) construct (One ml culture of recombinant cells resulted in 0.05 ml recombinant pediocin solution). High yield was achieved compared with the wt strain production (6.2 AU/ml).

A same fusion strategy was adopted using the pET20b(+) vector. The signal sequence *pelB* located upstream of the fusion part may help the target protein transport into periplasmic space, providing more oxidative environment and less protease activity than the cytoplasm (Makrides 1996). The result coincided with the expectation that the thioredoxin-PedA was observed as soluble protein both in the cytoplasm and periplasmic space. After purification by Ni resin column and enterokinase cleavage, antilisterial activity of recombinant pediocin in pET20b(+) was evaluated as 5120 AU/ml (Fig. 4, I). Finally, pediocin production was 384 AU from 1 ml culture medium of recombinant *E. coli* BL21 (DE3) cells (One ml culture of recombinant cells resulted in 0.075 ml recombinant pediocin solution). Comparison between pET32a(+) and pET20b(+) recombinants showed that the signal peptide designed in pET20b(+) vector may relieve high concentration of recombinant proteins accumulated in cytoplasm and contribute to correct folding process.

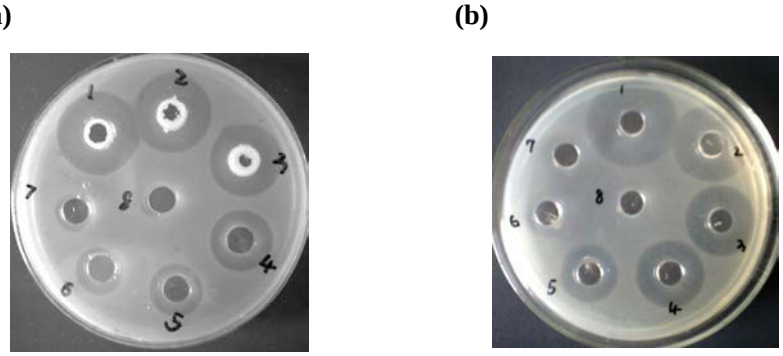

Figure 4. Agar diffusion assay of recombinant pediocin from *E. coli* BL21 (DE3) strains using *L. monocytogenes* CVCC1595 as the indicator strain. (a) *E. coli* harboring pET32a(+) recombinant plasmid (n = 7); (b) *E. coli* harboring pET20b(+) recombinant plasmid (n = 6). Recombinant pediocin was serially diluted 1:1. Numbers on the plates refer to dilution times.

1.1.2 Secretion of pediocin/leucocin C in *E. coli*

E. coli cells could produce bacteriocin by transferring P45-P*nisZ*-*SS*$_{usp45}$-bacteriocin structural gene (*papA/lecC*) cluster into pBluescript/*fliC*Δ_{H7} vector (Fig. 5, III and IV). *E. coli* JT1 was chosen as the host strain for bacteriocin production and further *Listeria*-binding constructs in cell-mediated killing study. Biologically active recombinant bacteriocins were analysed by antilisterial bioassay. Without nisin addition, P*nisZ* promoter is not functional and P45 promoter is in charge of the constitutive expression. Both recombinant pediocin and leucocin C were successfully secreted in *E. coli* systems. Hence, lactococcal promoter P45 and signal peptide of Usp45 lead to the transcription and translocation of target bacteriocins across the membrane. Interestingly, even though both pediocin PA-1/AcH and leucocin C belong to the class IIa bacteriocins, recombinant leucocin C performed a better expression, indicating a more efficient secreting system in the same genetic background (Fig. 6, III and IV). Heterologous expression of bacteriocins

may facilitate the development of starter or protective strains with increased antimicrobial properties. In this study, *Listeria*-binding proteins would be displayed on the bacteriocin producing cell surface by means of different anchors translocation to investigate their killing efficiencies.

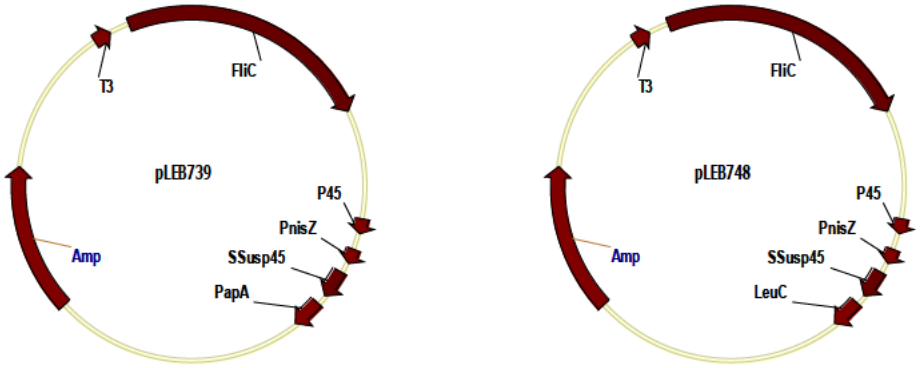

Figure 5. Schematic presentation of constructed plasmids pLEB739 and pLEB748.

Figure 6. Antilisterial bioassay of *E. coli* strains expressing *papA* or *lecC* using *L. monocytogenes* WSLC 1019 as the indicator strain. 1, ECO770 (= JT1/vector); 2, ECO769 (= JT1/*fliC*+*papA*); 3, ECO770 (= JT1/vector); 4, ECO777 (= JT1/*fliC*+ *lecC*).

1.2 Secretion of pediocin in *Lc. lactis* (unpublished)

To acquire active pediocin from LAB expression system, structural gene *pedA* was amplified and inserted into food-grade lactococcal vector pLEB688 together with SS_{usp45} under the control of P45 promoter (Fig. 7, unpublished). The constructed plasmid was then electroporated into *Lc. lactis* NZ9000 host strain, a derivative strain of *Lc. lactis* MG1363. As a result, pediocin secreted by *Lc. lactis* NZ9000 recombinant cells killed *Listeria* (Fig. 8, unpublished). Usp45 is a major extracellular protein secreted by *Lc. lactis* and its signal sequence has been used for heterologous secretion of different interested proteins including bacteriocins. Fusion expression of the mature bacteriocin enterocin P (EntP) or hiracin JM79 (HirJM79) to the SS_{usp45} permits the production and secretion of EntP or HirJM79 by *Lc. lactis* in the absence of specific immunity and secretion proteins (Borrero et al. 2011). However, the secretion efficiency may vary greatly as Borrero et al. (2011) demonstrated the antimicrobial activity of the recombinant EntP was slightly increased, while that of the HirJM79 was decreased compared with the wt strain. The production of recombinant pediocin in our experiments did not show high production level. Firstly, the *Lc. lactis* is probably sensitive to pediocin to some extent. Secondly, pediocin is secreted with the aid of dedicated ABC transporters and accessory protein in wt strain, which may not be compatible for Usp45 secretion system.

To optimize the expression system, pediocin immunity gene *pedB* was introduced into the previous construct and transformed *Lc. lactis* NZ9000. The *pedB* gene is a 339 bp

sequence (Genbank AJ242489.1) located in the same operon downstream of *pedA* gene. It provides immunity to the native pediocin producing cells. Co-expression of *pedB* did not improve the pediocin production from antilisterial bioassay (Fig. 8, unpublished).

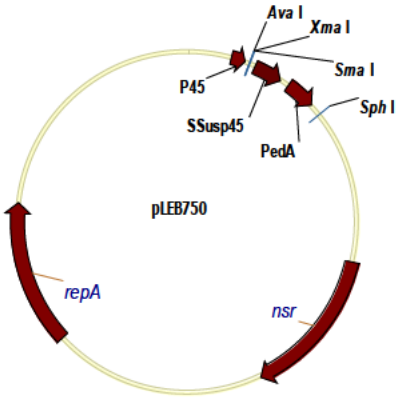

Figure 7. Schematic presentation of constructed vector pLEB750.

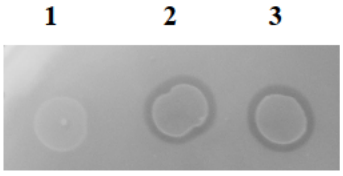

Figure 8. Antilisterial bioassay of *Lc. lactis* strains using *L. monocytogenes* WSLC 1018 as the indicator strain. 1, *Lc. lactis* strain harboring vector plasmid (pLEB688); 2, *Lc. lactis* strain harboring *pedA* expressing plasmid (pLEB750); 3, *Lc. lactis* strain harboring *pedAB* expressing plasmid (pLEB795).

Dynamic inhibitory effects of the biologically active recombinant pediocin in various *Listeria* culture conditions were investigated. *Listeria* cells mixed with M17G medium grew well in BHI medium at pH 7.5. When the pH decreased, more time was needed to reach the stationary state and lower concentration of *Listeria* cells was maintained after 20 h incubation. Addition of the cell-free culture supernatant of *Lc. lactis* NZ9000 (harboring pLEB750) inhibited the growth of *Listeria* at different pH values (Fig. 9, unpublished). The synergistic effect of acidity with bacteriocin has been reported by Benkerroum et al. (2002), promisingly being beneficial to fermented food in biopreservation.

It has been documented that *L. monocytogenes* may tolerate high salt concentration and survive in the processed food such as salted mushrooms and cheese (Junttila and Brander 1989; Ferreira et al. 2003). In this study, the logarithmic stage was lagged with the increasing concentration of NaCl. However, the final populations of *Listeria* cells in BHI medium mixed with M17G were at similar levels revealing high tolerance of this species to salt (Fig. 10, unpublished). For those samples containing cell-free culture supernatant of *Lc. lactis* NZ9000 (harboring pLEB750), final numbers of *Listeria* cells were reduced. Especially when the NaCl concentration reached 6 %, inhibitory effect attained the lowest amount of *Listeria* cells after 20 h cultivation at pH 7.5.

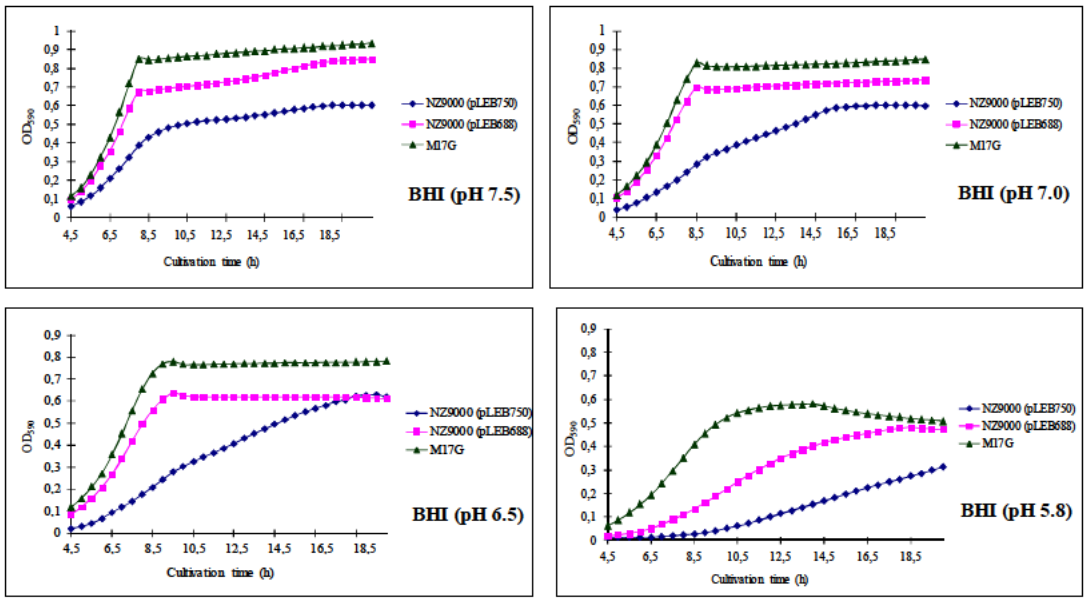

Figure 9. Growth curves of *L. monocytogenes* WSLC 1018 in BHI medium added with culture supernatant of *Lc. lactis* NZ9000 (harboring pLEB750), culture supernatant of *Lc. lactis* NZ9000 (harboring pLEB688), and M17G medium at different pH values.

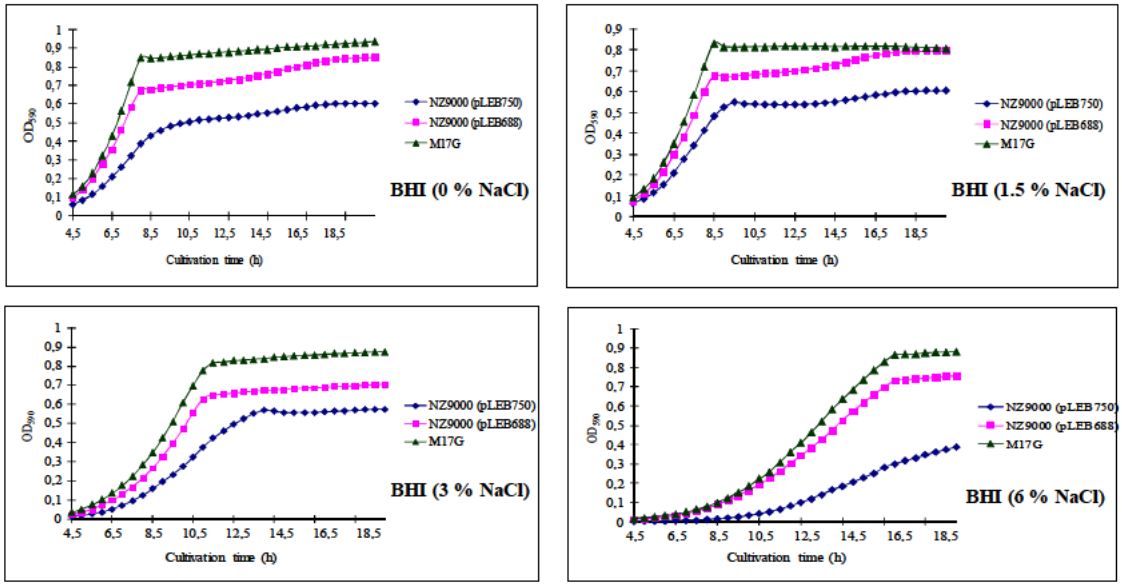

Figure 10. Growth curves of *L. monocytogenes* WSLC 1018 in BHI medium at pH 7.5 added with culture supernatant of *Lc. lactis* NZ9000 (harboring pLEB750), culture supernatant of *Lc. lactis* NZ9000 (harboring pLEB688), and M17G medium with different concentrations of NaCl.

The direct utilization of pediocin producing strain *P. acidilactici* in dairy industry either as starter culture or bacteriocin producing adjunct culture is an obstacle because of its lack of or slow lactose fermentation (Somkuti and Steinberg 2010). Studies are ongoing in transferring pediocin gene to other LAB which are constituents of normal starter cultures used in the production of fermented dairy products. Genetically improved *Lc. lactis* has great potential in the application as industrial cell factory (Maischberger et al.

2010). However, presently the legislation especially in Europe does not easily give permission to use genetically modified organisms in food products.

Hartmann et al. (2011) have explored the possibility of utilization of cell-free culture supernatants containing class IIa bacteriocins such as leucocin A, pediocin PA-1 and sakacin A to control *L. monocytogenes* in food. In our study, the supernatant of *Lc. lactis* NZ9000 (harboring pLEB750) suppressed the *Listeria* inoculated in artificially contaminated whole-fat (1.5 %) milk to some extent although *Listeria* population increased during storage at 4 °C for 6 days (data not shown).

In conclusion, functional gene *pedA* has been expressed both in *E. coli* and food-grade *Lc. lactis* host strains. High level production was achieved by pET vectors with high copy number and strong promoter T7. The lactococcal promoter P45 and SS_{usp45} are able to secret both pediocin and leucocin C in *E. coli* strains. Compared with heterologous leucocin C secreted in *E. coli* cells, recombinant pediocin was secreted at low level in the same expression system.

2. Surface display (II, III, IV, unpublished)

2.1 Surface display of the cellulose-binding domain (II)

Cellulose-binding domain is a specific catalytic module capable of binding cellulose (Hildén and Johansson 2004). In order to study the surface display property in *Lc. lactis*, the cellulose-binding domain (112 aa) of xylanase A from *Cellvibrio japonicus* was selected as the target protein. Cells presenting cellulose-binding domain on the surface could be adsorbed on the cellulose matrix stably and used for immobilization. Different anchors (PrtP 344 aa, PrtP 153 aa and AcmA 242 aa) derived from *Lc. lactis* were investigated for an efficient immobilization. Both whole-cell ELISA and filter paper assay for cell binding capacity revealed that the best surface display was achieved with the strain presenting cellulose-binding domain fused with PrtP 344 aa anchor (Fig. 11, Kylä-Nikkilä et al. 2010).

Further research demonstrated large portion of fusion protein was degraded in the culture supernatant which may be caused by HtrA protease. When the constructed plasmid was transferred into HtrA-protease deficient *Lc. lactis htrA*, protein degradation was alleviated. But still, part of the fusion protein was maintained in the culture instead of cells. To test whether the immobilization could be further improved, another LPXTG anchor, NisP (121 aa), derived from a protease needed for activation of nisin was fused with cellulose-binding domain and transformed into *Lc. lactis* MG1363 cells for expression. Poor surface presenting was observed by whole-cell ELISA possibly due to short length or wrong conformation (Fig. 12, II). It has been estimated at least 90-100 aa residues are required for the extended loop in the LPXTG anchor (between the target protein and LPXTG box) for proper surface display of the target protein (Fischetti et al. 1990; Strauss and Götz 1996). Therefore, shorter anchor such as NisP 121 aa and PrtP 153 aa are possibly inefficient for presenting. Attempt was then made to improve the production of sortase (206 aa) which is involved in translocation of LPXTG type anchors. Sortase is responsible for cleavage of polypeptides between threonine and glycine of the LPXTG motif, promoting covalent binding to the CW (Navarre and Schneewind 1994; Steidler et al. 1998; Mazmanian et al. 1999). The *srtA* gene was introduced into PrtP 344

aa construct. Both whole-cell ELISA (Fig. 12, II) and filter paper assay (Fig. 13, II) showed non-significant difference. Since the sortase production is hard to verify due to lack of specific antibodies, the reasons for inefficient immobilization can be explained as either the production of sortase is not increased enough or the staphylococcal sortase was not compatible with the LPXTG anchoring system.

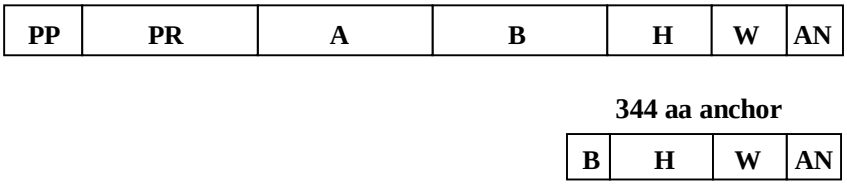

Figure 11. Schematic representation of the predicted domains in PrtP proteases and constructed PrtP anchor. PP, pre-pro domain; PR, protease domain; A, A-domain; B, B-domain; H, helical domain; W, CW domain; AN, anchor domain. Adapted from Kylä-Nikkilä et al. 2010.

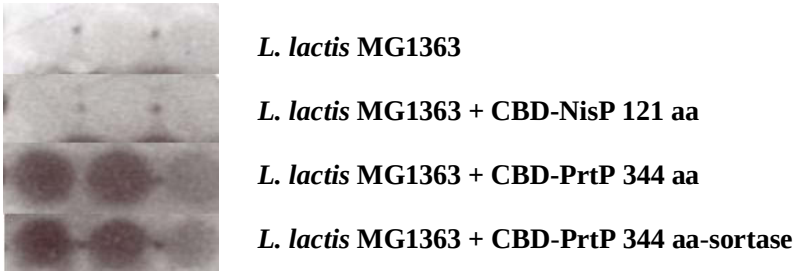

Figure 12. Whole-cell ELISA with CBD-specific rabbit Ab using *Lc. lactis* MG1363 as control; *Lc. lactis* MG1363 expressing CBD-NisP 121 aa fusion protein; *Lc. lactis* MG1363 expressing CBD-PrtP 344 aa fusion protein and *Lc. lactis* MG1363 expressing CBD-PrtP 344 aa fusion protein together with sortase. The volumes of the cell suspension used in the detection from right to left were 1, 5 and 20 µl.

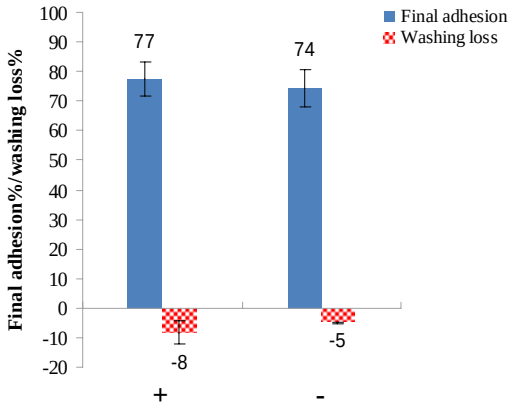

Figure 13. Filter paper immobilization test. +, *Lc. lactis* MG1363 expressing CBD-PrtP 344 aa fusion protein together with sortase; -, *Lc. lactis* MG1363 expressing CBD-PrtP 344 aa fusion protein.

In summary, *Lc. lactis* strains carrying surface-exposed cellulose-binding domain within chimeric surface anchor proteins were generated. Among them, PrtP 344 aa anchor provided efficient and stable attachment of cellulose-binding domain to the cellulosic matrix. As a cheap material, cellulose is available in many different forms indicating a

broad area in application. The representative of LPXTG anchor, PrtP 344 aa, was chosen as a promising candidate for presenting *Listeria*-binding proteins on the surface of *Lc. lactis*.

2.2 Surface display of the cell wall binding domain (III, IV, unpublished)

Previously, CBDs have been expressed as fusions with the GFP and studied for their binding properties to *Listeria* cells (Loessner et al. 2002). However, there are no reports on cell-cell binding using CBD on the cell surface. Studies on whether the CBDs function similarly when being displayed on cells instead of as a part of a fusion protein were conducted. It is the preliminary step in the characterization of the CBD binding effect *in vivo*.

2.2.1 *E. coli* display (III, IV)

Due to the absence of an outer membrane in Gram-positive bacteria, *Listeria* phage endolysins can function when added externally, which turns out to be a candidate of antimicrobials. Particularly the approval for the use of *L. monocytogenes* phages for food safety purpose promotes research field in phage application (Mahony et al. 2011). The unique binding properties of CBD provide an attractive tool in differentiation and localization of pathogenic *Listeria* cells in food safety and also therapeutic use. To gain more insight in bacteriophage-host interactions and characteristics of CBD, we investigated the applicability of presenting CBD500 (GenBank: X85009.1) and CBDP35 (GenBank: NC_009814.1) in *E. coli* system by fusing CBD with different anchor proteins. The resulting recombinant *E. coli* strains were obtained and tested for their expression and binding effects to *Listeria* cells.

Display of CBD with outer membrane anchor. CBD has specificity to bind to the surface of *Listeria* cells (Loessner et al. 2002). The first aim was to express CBD500 on the surface of *E. coli* by using the outer membrane anchor of *Yersinia enterocolitica* adhesin YadA, a non-fimbrial adhesion mediating adherence to hosts (Wollmann et al. 2006). YadA is composed of a C-terminal β-barrel domain in the outer membrane for translocation and an N-terminal passenger domain containing stalk, neck and globular head on the surface (Leo et al. 2008). Previous studies published by Ackermann et al. (2008) have demonstrated the constructed chimeric YadA proteins translocated the YadA passenger domain across the outer membrane in *Y. enterocolitica*, which provides a clue for potential utilization of YadA anchor domain for cell-surface display. In this study, CBD500 was inserted together with YadA anchor into *E. coli* vector. Whole-cell ELISA showed the CBD500-YadA fusion was displayed on the cell surface of ECO762 strain (Supplementary Fig. 1b, IV). However, ECO762 cells showed reduced growth rate and cell lysis after tetracycline (1 - 200 ng/ml) induction, presumably owing to the detrimental effect of fusion protein to the host cells.

Effort was further taken to test native *E. coli* outer membrane protein OmpA (Isoda et al. 2007) for the surface display of CBD500. The plasmid pET-LOAvi encodes the signal sequence, first nine N-terminal aa of *E. coli* major outer membrane Lpp and 46-159 aa of the OmpA. CBD500 was fused to the C-terminus of OmpA and supposed to be translocated out of the membrane together with the exposed loop. The CBD500 fusion protein, estimated to be around 30 kDa, was expressed at reasonable level after IPTG

induction (Fig. 14a, IV). The localization of the CBD500 on the external surface of *E. coli* was determined by Western blot using anti-His Ab. Strong band could be observed in the outer membrane fraction after IPTG induction, indicating that the CBD500 fusion was translocated to the outer layer of the cell membrane (Fig. 14b, IV).

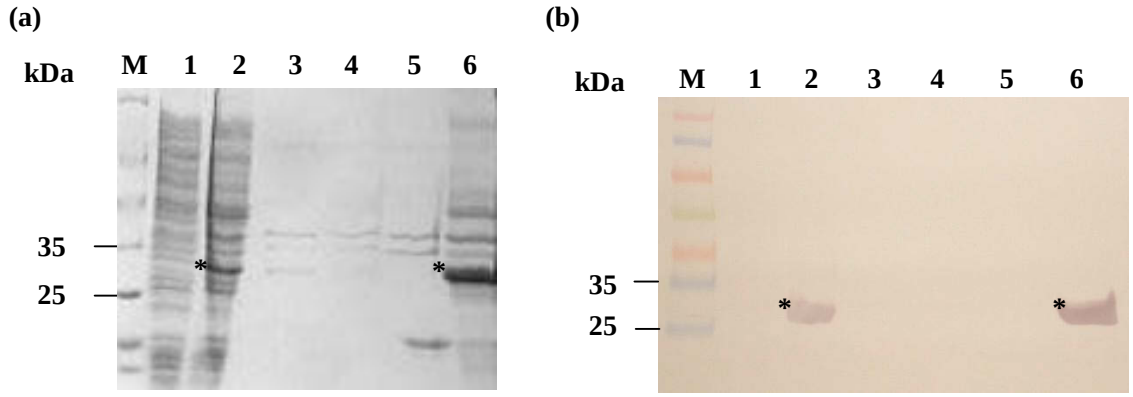

Figure 14. (a) SDS-PAGE analysis of the OmpA-CBD500 fusion protein. (b) Western blot analysis of the OmpA-CBD500 fusion protein. M, prestained MW marker; Lane 1, intracellular proteins without IPTG induction; Lane 2, intracellular proteins with 1 mM IPTG induction; Lane 3, inner membrane proteins without IPTG induction; Lane 4, inner membrane proteins with 1 mM IPTG induction; Lane 5, outer membrane proteins without IPTG induction; Lane 6, outer membrane proteins with 1 mM IPTG induction. The primary Ab used in the experiment was mouse anti-His/AP monoclonal Ab (Invitrogen). The OmpA-CBD500 fusion protein is marked with an asterisk.

The availability of the CBD500 domain on the surface of intact cells for binding by antibodies was analysed with whole-cell ELISA. However, the availability of the CBD domain on the cell envelope was shown to be compromised (Supplementary Fig. 2b, IV). It is speculated that the presented CBD500 is not far enough away from the lipopolysaccharide layer, which may prevent the interaction as barrier between CBD500 and *Listeria* CW ligands. Or the CBD500 has penetrated into outer membrane after translocation leading to few accessible CBD500 for binding. Lack of positive signal in whole-cell ELISA could be a result of incorrect conformation of CBD500 and may cause inefficient interaction of CBD with *Listeria* cell ligands. Stathopoulos et al. (1996) has pointed out this display system is probably not compatible to extensive secondary and tertiary structures of the passenger protein. The bacterial enzyme alkaline phosphatase (PhoA) was failed for surface display using this motif (Stathopoulos et al. 1996).

Display of CBD along the filament of flagella. Flagellum is the bacterial motility organelle and its display system can be achieved by insertion of foreign DNA gene into variable region of *fliC*. FliC is responsible for prolonging flagella by assembling to approximately 20,000 copies per filament (Aizawa 1996). The conserved N and C-terminus form an inner core of the hollow filament by exposing variable central domain on the cell surface (Majander et al. 2005a). The highly variable region of FliC can be subjected to large deletions and insertions without suspending flagellar polymerization (Kuwajima 1988). Abundance of FliC in flagella provides an attractive way for bacteria surface display purposes. Majander et al. (2005b) have developed a multihybrid display system by presenting three foreign peptides using either FliC or FliD as carrier proteins.

However, there are insufficient data on presenting other peptides in flagellum display system.

In this study, pBluescript/*fliC*Δ_{H7} was selected as a vector which the *fliC*Δ_{H7} gene was inserted into commercial vector pBluescript II KS with a deletion of an *Acc*I fragment in the variable region. The CBD encoding amplified fragments were inserted at *Acc*I site before transformation into *E. coli* JT1 strain, in which simultaneous in *trans* complementation of *fliC::Tn10* occurs. Cells were collected and analysed by SDS-PAGE and Western blot (Fig. 15, IV and Fig. 16, III). According to Westerlund-Wikström et al. (1997) previous results, the strong reaction band represents FliC monomers, and the polypeptides of smaller apparent size reacted bands are most likely flagellar minor proteins and FlgE forming the flagellar hook. A protein band larger than FliC was detected in recombinant construct of CBD500 or CBDP35. It is controversial for the maximum size of interested protein that the flagella-based system can harbor. Georgiou et al. (1997) concluded 60 aa on the basis of available reports. However, Majander et al. (2005a) claimed expression of SlpA (96-245 aa) and YadA (84-385 aa) along the filament. The present results indicate that expression of CBD500 (120 aa) and CBDP35 (162 aa) can be achieved. The tandem form of CBD500 and CBDP35 can also be expressed with low production yield probably due to its large size (data not shown).

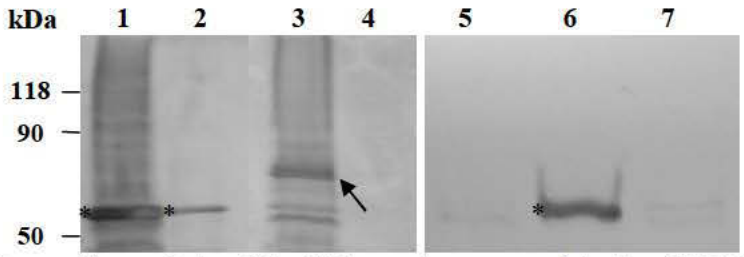

Figure 15. Western blot analysis of the FliC proteins expressed in *E. coli* ECO770 (= JT1/vector) and ECO768 (= JT1/*fliC*::*CBD500*). 1, *E. coli* ECO770 cells; 2, *E. coli* ECO770 cells after enterokinase digestion; 3, *E. coli* ECO768 cells; 4, *E. coli* ECO768 cells after enterokinase digestion; 5, flagella extract of *E. coli* JT1 cells; 6, flagella extract of ECO770 cells; 7, flagella extract of *E. coli* ECO768 cells. The primary Ab used in the experiment was polyclonal anti-H7 flagella antiserum (kindly provided by Prof. Benita Westerlund-Wikström as a gift). The FliC protein band is marked with an asterisk and the FliC::CBD500 protein band with an arrow.

An enterokinase site was designed at the C-terminus of CBD for each fusion, as a tool to check the correct display of chimeric flagella. Fusion protein band was disappeared after digestion proving again CBD was inserted in frame (Fig. 15, IV and Fig.16, III). Flagellar crude extract were later isolated by shaking the cells whereby the flagella are sheared off and collecting supernatant after centrifugation (Westerlund-Wikström et al. 1997). Fig. 15 (IV) and Fig. 17 (III) showed that sufficient amount of flagella can be isolated from recombinant CBDP35, while few can be obtained from recombinant CBD500 strain. Therefore, binding of recombinant CBD500 strain to *Listeria* could not be determined.

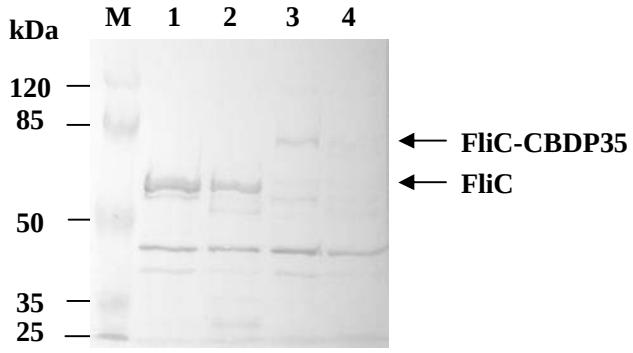

Figure 16. Western blot analysis of the FliC proteins expressed in *E. coli* ECO770 (= JT1/vector) and ECO776 (= JT1/*fliC*::*CBDP35*). M, prestained MW marker; 1, *E. coli* ECO770 cells; 2, *E. coli* ECO770 cells after enterokinase digestion; 3, *E. coli* ECO776 cells; 4, *E. coli* ECO776 cells after enterokinase digestion. The primary Ab used in the experiment was polyclonal anti-H7 flagella antiserum.

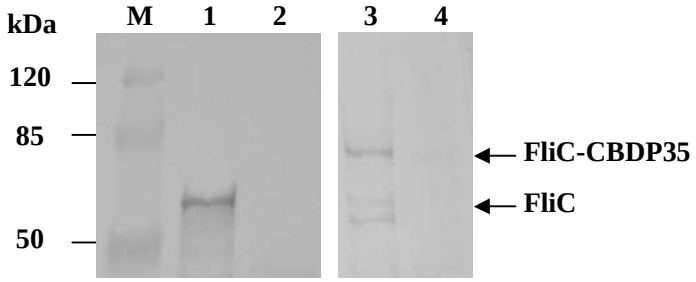

Figure 17. Western blot analysis of flagella extracts from *E. coli* JT1 cells. M, prestained MW marker; 1, flagella extract of ECO770 (= JT1/vector) cells; 2, pellet of ECO770 cells after flagella isolation; 3, flagella extract of ECO776 (= JT1/*fliC*::*CBDP35*) cells; 4, pellet of ECO776 cells after flagella isolation. The primary Ab used in the experiment was polyclonal anti-H7 flagella antiserum.

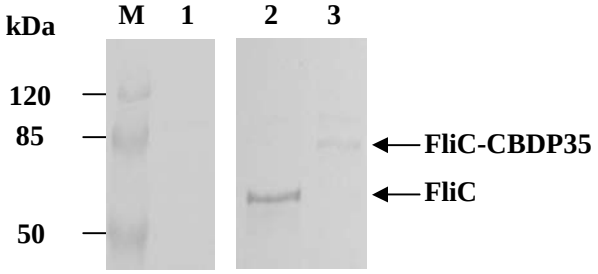

Figure 18. Western blot analysis of FliC and FliC::CBDP35 flagella binding to *Listeria* cells. M, prestained MW marker; 1, *L. monocytogenes* WSLC 1019 cells; 2, *L. monocytogenes* WSLC 1019 cells after mixing with flagella extract from ECO770 (= JT1/vector) cells; 3, *L. monocytogenes* WSLC 1019 cells after mixing with flagella extract from ECO776 (= JT1/*fliC*::*CBDP35*) cells. The primary Ab used in the experiment was polyclonal anti-H7 flagella antiserum.

To test if the FliC::CBDP35 flagella could bind to *Listeria* cells, flagella extracts were isolated, mixed with *Listeria* cells, washed and analysed. FliC::CBDP35 chimeric protein band could be detected in the pellet of *Listeria* cells (Fig. 18, III). Also the wt FliC flagella

from ECO770 cells were co-purified with the *Listeria* cells. Interestingly, there was no need to express the *Listeria*-binding CBD on the surface of *E. coli* as the flagella without CBD could bind to *Listeria* cells. Binding among cells leads to aggregation and rapid sedimentation of cells (Kos et al. 2003). The flagellated *E. coli* cells (ECO770 and ECO776) auto-aggregated whereas the non-flagellated *E. coli* BL21 (DE3) cells and the *Listeria* cells did not. Besides, both ECO770 and ECO776 resulted in more co-aggregation with *Listeria* cells compared to *E. coli* BL21 (DE3) strain.

In conclusion, YadA, OmpA and FliC have been used as carrier proteins for CBD display. Their displaying characteristics are diverse and many factors may influence presenting efficiencies including size of passenger protein, fusion strategy according to carrier protein, host toxicity and stability of fusion protein. Expression of the *Listeria* binding domain CBDP35 as a protein chimera with the flagella subunit FliC was achieved. The protein chimera was located on the surface of the cells and tested by enterokinase treatment. Both the chimeric flagella containing CBDP35 and wt flagella of the *E. coli* JT1 strain are capable of binding to *Listeria* cells, which were used in the further *Listeria* killing assay.

2.2.2 *Lc. lactis* display (unpublished)

In *Lc. lactis* system, PrtP 344 aa which has been proved previously for successful presenting cellulose-binding domain was considered for surface display of CBD (Results and Discussion section, chapter 2.1). CBD500 and CBD006 (GenBank: NC_009815.1) were fused to the N-terminus of this carrier protein and planned to be translocated across the cell membrane. P45-CBD-PrtP fragment was amplified using primers P45F2 and PrtpR, and then inserted into the bacteriocin-secretion plasmids before transformation into MG1614 strain. Hence, CBDs were designed to be displayed on the lactococcal cell surface for interaction with the *Listeria* ligands. Schmelcher et al. (2010) have proved CBDs feature unique binding patterns to CW of different serovar groups. CBD500 bound exclusively to cells belonging to serovar 4, 5, and 6, while CBD006 directed to the CW of *Listeria* strains belonging to serovar 1/2 and 3. Here, *Lc. lactis* cells were washed and mixed with *Listeria* cells for sedimentation assay. Both CBD500 and CBD006 constructs resulted in co-aggregation with corresponding *Listeria* cells. (Fig. 19 and Fig. 20, unpublished).

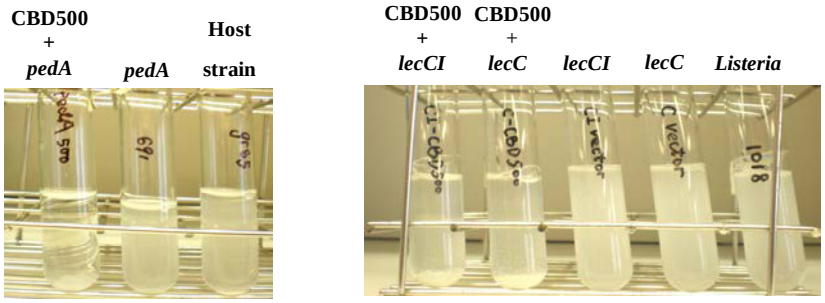

Figure 19. Sedimentation assay of *Lc. lactis* CBD500 constructs mixed with *L. monocytogenes* WSLC 1018 (serovar 4e).

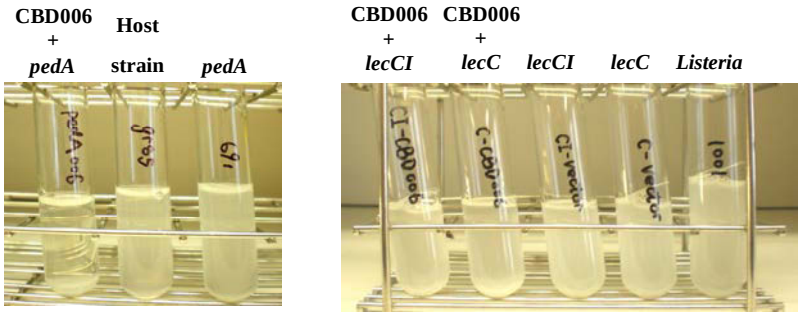

Figure 20. Sedimentation assay of *Lc. lactis* CBD006 constructs mixed with *L. monocytogenes* WSLC 1001 (serovar 1/2c).

3. Effects of antilisterial activities of *E. coli* cells binding to *Listeria* cells (III, IV)

Leucocin C-secreting *E. coli* cells binding to *Listeria* via presenting *Listeria* CW binding domain CBDP35 as chimeric flagella were constructed to test the killing efficiency upon cell to cell contact. The aim was to evaluate if *E. coli* cells could kill *Listeria* more efficiently by binding to *Listeria* cells and expressing leucocin C than by secreted leucocin C in the growth medium. The results demonstrated the cells of ECO777 (= JT1/*fliC+lecC*) and ECO778 (= JT1/*fliC::CBDP35+lecC*) strains secreting leucocin C and having flagella binding to *Listeria* cells both killed (two-log decrease) more *Listeria* cells in one hour than incubation in spent growth media for one hour containing the leucocin C produced during one hour (Table 8, III). Actually, no evident inhibition was observed after one hour incubation of the *Listeria* cells in the spent growth supernatant into which the cells of the leucocin C expressing *E. coli* had prior incubation with the *Listeria* cells secreted leucocin C. Presuming that the leucocin C producing *E. coli* strains, with or without *Listeria*-binding capacity, secreted approximately the same amount of leucocin C during the one-hour leucocin C producing period, the cell-mediated killing was probably even more efficient than observed compared to the cell-free killing since the bacteriocin concentration in the spent growth media at time point zero corresponded to the concentration of time point one hour with leucocin C producer cells. In other words, in the beginning of the incubation with *Listeria* cells, the *Listeria* encountered either no leucocin C, which gradually increased in concentration during the one-hour incubation with the *Listeria*-binding leucocin C producing cells, or directly at the beginning of the one-hour incubation period encountered the leucocin C concentration of one-hour production. The quantification of the leucocin C concentration was not possible due to the low concentration (no killing observed from spent growth supernatant and no available leucocin C antiserum). However, when the leucocin C production capacities of the *E. coli* strains ECO777 and ECO778 were compared after longer periods of incubation no difference in leucocin C production could be observed based on MIC values against *Listeria* (results not shown, III).

Table 8. Inhibition of *L. monocytogenes* WSLC 1019 by *E. coli* cells and growth supernatants.

Incubation time	*Listeria*	Cells of ECO779 and *Listeria*	Supernatant of ECO779 and *Listeria*	Cells of ECO777 and *Listeria*	Supernatant of ECO777 and *Listeria*	Cells of ECO778 and *Listeria*	Supernatant of ECO778 and *Listeria*
0 h	$3.5\times10^{7}\pm1.5\times10^{6}$	$3.5\times10^{7}\pm1.5\times10^{6}$	$4.2\times10^{7}\pm2.7\times10^{6}$	$3.5\times10^{7}\pm1.5\times10^{6}$	$2.3\times10^{7}\pm5.8\times10^{6}$	$3.5\times10^{7}\pm1.5\times10^{6}$	$5.6\times10^{7}\pm1.6\times10^{7}$
1 h	$6.3\times10^{7}\pm1.2\times10^{6}$	$4.5\times10^{7}\pm5.0\times10^{6}$	$5.7\times10^{7}\pm6.4\times10^{6}$	$2.0\times10^{5}\pm1.7\times10^{5}$	$2.5\times10^{7}\pm4.7\times10^{6}$	$1.0\times10^{5}\pm0.0$	$4.0\times10^{7}\pm5.0\times10^{6}$

Overnight cultured *L. monocytogenes* WSLC 1019 was 1 % inoculated into BHI medium and mixed with 5 µl *E. coli* cells (OD_{600} = 1) for 1 h incubation at 30 °C. *E. coli* supernatants were cell-free spent BHI medium in which the *E. coli* cells had been growing in for 1 h. Survival of *Listeria* is presented as cfu/ml.

Incubation of *Listeria* with ECO769 (= JT1/*fliC*+*papA*) cells decreased approximately 40 % of the *Listeria* cells, whereas the cell-free spent growth medium containing the corresponding amount of pediocin could only inhibit cell growth but did not decrease the number of viable *Listeria* cells after three hours (Fig. 21, IV). The combination effect of *Listeria*-binding and bacteriocin-producing may enhance the antilisterial effect of the bacteriocin by preventing its dilution in the environment and adsorption onto particles before taking effect to the target cells.

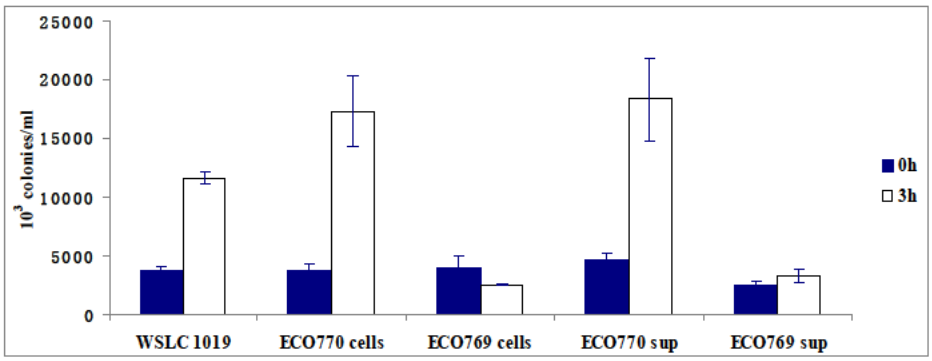

Figure 21. *L. monocytogenes* WSLC 1019 killing test. Survival of *Listeria* after incubation (3 h) with *E. coli* strains or their cell-free spent growth media (3 h).

Conclusions and Future Prospects

The activities of class IIa bacteriocins against food-borne pathogens especially *L. monocytogenes* have gained interest of both academia and industry in developing natural food biopreservatives. In this study, pediocin structural gene *pedA* was cloned into vector pET32a(+) and pET20b(+), respectively, for hybrid expression in *E. coli*. In the former construct, thioredoxin-PedA fusion protein was obtained as inclusion bodies after IPTG induction because of high production level. Renaturation was achieved using GSH/GSSH system. Recombinant pediocin production was 512 AU from 1 ml culture medium of recombinant *E. coli* strain. In the latter construct, fusion protein was localized both intracellularly and periplasmically. Production was 384 AU from 1 ml culture medium of recombinant *E. coli* strain. Both constructs produced more pediocin than natural producing strain *P. acidilactici* PA003. Secretion of pediocin and leucocin C in *E. coli* was realized by insertion of P45-SS_{usp45}-bacteriocin structural gene (*papA*/*lecC*) cluster into pBluescript/$fliC\Delta_{H7}$ vector and transformation of *E. coli* JT1. Pediocin-secreting *Lc. lactis* was constructed by insertion of *pedA* gene into food-grade vector pLEB688 together with SS_{usp45} under the control of P45 promoter. Thus, biologically active bacteriocins were heterologously expressed and the output may even be improved by optimizing the protocol for production or purification.

Different surface display systems were explored for presenting *Listeria* phage endolysin CBDs and therefore could have the ability to bind to *Listeria* cells. The anchor protein YadA, OmpA and flagella variable domain of FliC were tested successively. CBD-YadA fusion was toxic to *E. coli*, while OmpA-CBD fusion was not present well on the surface. Only chimeric FliC::CBDP35 was located on the surface of the cells. Interestingly, both the chimeric flagella containing CBDP35 and wt flagella of the *E. coli* JT1 strain were capable of binding to *Listeria* cells. A lactococcal anchor protein, PrtP 344 aa, which resulted in the best surface expression of cellulose-binding domain was adopted for displaying of CBD on the surface of *Lc. lactis* cells. Both CBD500 and CBD006 were displayed on the lactococcal cell surface. Thus, *Listeria*-binding strains were obtained.

The aim of this study was to test the hypothesis that bacteriocin secreting cells kill target cells more efficiently if they can also bind to the target cells. We showed that this holds true for *Listeria* and *E. coli* secreting bacteriocin and having *Listeria*-binding flagella. The killing efficiency was increased when the flagella was able to bind to the *Listeria* cells and secrete bacteriocin at the same time. Interaction between the bacteriocin producing strain and sensitive cells may enhance killing effect. A similar strategy could be used to construct a set of probiotic strains that would specifically bind to different pathogens and efficiently kill them by bacteriocins thereby protecting the host from intestinal infections. However, it would be difficult to get permission for such strains due to the present legislation at least in Europe (No. 1829/2003). In addition, the public acceptance of genetically engineered organisms for consumption is very low in Europe (Lynch and Vogel 2001).

Acknowledgements

This work was carried out at the Department of Food and Environmental Sciences, Faculty of Agriculture and Forestry, University of Helsinki. Financial support was received from China Scholarship Council and University of Helsinki. The National Natural Science Foundation of China, Tekes, the National Technology Agency and Academy of Finland are acknowledged.

I am deeply grateful to my supervisor Per Saris for allowing me to join this group and guiding me throughout this project. I would like to thank you for your kindness, patience and advice during these years. Without your support, it is impossible for me to finish my thesis in this timetable. You have always been encouraging even though I was depressed in dilemma. I learned not only how to do research in academia but also to be optimistic and strong in life.

I wish to express my warmest thanks to the reviewers Professor Matti Karp and Docent Arto Pulliainen for critical revision and constructive comments.

I express my gratitude to all the collaborators in this work. I wish to thank Professor Zhijiang Zhou and Ye Han from Tianjin University for guiding me at the beginning of my studies. I thank the co-authors, Timo Takala, Xing Wan, Justus Reunanen, Kari Kylä-Nikkilä, and Ossian Saris for their professional cooperation and contribution to the study. I am grateful to all the lab members, current and former, including Ruiqing, Sanna, Navdeep, and Anne for helpful discussions and relaxing moments. Special thanks to Yurui for sharing experimental expertise and living skills. Also thanks to all the colleagues in the department for creating an inspiring working atmosphere.

I wish to acknowledge Professor Marko Dolinar and Mingqiang Qiao for sharing experiences in scientific research and also in life.

I want to thank Seija Oikarinen for arrangement of my PhD study and answering questions patiently.

All my friends, in- and outside Finland are appreciated for their support in my graduating. I am grateful to Jun Qiao for caring, understanding and being there. Your comfortable words always calm me down when I was frustrated with “endless bugs” during thesis writing period. Thanks go to Yi Tang and Samuli Nikula for taking care of me when I needed. Having hotpot at Xiaoyu’s home with Yurui, Fang Wang, Li Ma and other friends was the best way of consoling my homesickness. I also wish to thank Paula for showing me an amazing Lapland and driving out of the way at several nights for the aurora, Suzhen for being a good partner of visiting all kinds of museums in Helsinki, Vytas for giving useful comments on scientific English writing, Lin Ning for looking after me during my illness, Liwei for providing new perspectives in conversations. Also many other lovely people are thanked for friendship in Finland.

Finally, I’d like to thank my parents for your unconditional love to me. Your understanding, believing and unshaken support are the source of my courage.

In Helsinki, July 2014

References

Ackermann, N., Tiller, M., Anding, G., Roggenkamp, A. and Heesemann, J. (2008) Contribution of trimeric autotransporter C-terminal domains of oligomeric coiled-coil adhesin (Oca) family members YadA, UspA1, EibA, and Hia to translocation of the YadA passenger domain and virulence of *Yersinia enterocolitica*. J. Bacterial. 190: 5031-5043.

Aizawa, S. I. (1996) Flagellar assembly in *Salmonella typhimurium*. Mol. Microbiol. 19: 1-5.

Alegría, Á., Delgado, S., Flórez, A. B. and Mayo, B. (2013) Identification, typing, and functional characterization of *Leuconostoc* spp. strains from traditional, starter-free cheeses. Dairy Sci. & Technol. 93: 657-673.

Arachchi, G. J. G., Cridge, A. G., Dias-Wanigasekera, B. M., Cruz, C. D., McIntyre, L., Liu, R., Flint, S. H. and Mutukumira, A. N. (2013) Effectiveness of phages in the decontamination of *Listeria monocytogenes* adhered to clean stainless steel, stainless steel coated with fish protein, and as a biofilm. J. Ind. Microbiol. Biotechnol. 40: 1105-1116.

Austin, C. (2003) Novel approach to obtain biologically active recombinant heterodimeric proteins in *Escherichia coli*. J. Chromatogr. B. 786: 93-107.

Bassi, A. S., Ding, D. N., Gloor, G. B. and Margaritis, A. (2000) Expression of single chain antibodies (ScFvs) for c-myc oncoprotein in recombinant *Escherichia coli* membranes by using the ice-nucleation protein of *Pseudomonas syringae*. Biotechnol. Prog. 16: 557-563.

Beasley, S. S., Takala, T. M., Reunanen, J., Apajalahti, J. and Saris, P. E. J. (2004) Characterization and electrotransformation of *Lactobacillus crispatus* isolated from Chicken crop and intestine. Poult. Sci. 83: 45-48.

Benkerroum, N., Ghouati, Y., Ghalfi, H., Elmejdoub, T., Roblain, D., Jacques, P. and Thonart, P. (2002) Biocontrol of *Listeria monocytogenes* in a model cultured milk (lben) by *in situ* bacteriocin production from *Lactococcus lactis* ssp. *lactis*. Int. J. Dairy Technol. 55: 145-151.

Bernhardt, T. G., Wang, I. N., Struck, D. K. and Young, R. (2002) Breaking free: "protein antibiotics" and phage lysis. Res. Microbiol. 153: 493-501.

Beshkova, D. and Frengova, G. (2012) Bacteriocins from lactic acid bacteria: Microorganisms of potential biotechnological importance for the dairy industry. Eng. Life Sci. 12: 419-432.

Borrero, J., Jiménez, J. J., Gútiez, L., Herranz, C., Cintas, L. M. and Hernández, P.E. (2011) Use of the usp45 lactococcal secretion signal sequence to drive the secretion and functional expression of enterococcal bacteriocins in *Lactococcus lactis*. Appl. Microbial. Biotechnol. 89: 131-143.

Borysowski, J., Weber-Dabrowska, B. and Górski, A. (2006) Bacteriophage endolysins as a novel class of antibacterial agents. Exp. Biol. Med. 231: 366-377.

Bren, L. (2007) Bacteria-eating virus approved as food additive. FDA Consum. 41: 20-22.

Budde, B. B., Hornbaek, T., Jacobsen, T., Barkholt, V. and Koch, A. G. (2003) *Leuconostoc carnosum* 4010 has the potential for use as a protective culture for vacuum-packed meats: culture isolation, bacteriocin identification, and meat application experiments. Int. J. Food Microbiol. 83: 171-184.

Buist, G., Kok, J., Leenhouts, K. J., Dabrowska, M., Venema, G. and Haandrikman, A. J. (1995) Molecular cloning and nucleotide sequence of the gene encoding the major peptidoglycan hydrolase of *Lactococcus lactis*, a muramidase needed for cell separation. J. Bacteriol. 177: 1554-1563.

Carlton, R. M., Noordman, W. H., Biswas, B., de Meester, E. D. and Loessner, M. J. (2005) Bacteriophage P100 for control of *Listeria monocytogenes* in foods: genome sequence, bioinformatic analyses, oral toxicity study, and application. Regul. Toxicol. Pharmacol. 43: 301-312.

Centers for Disease Control and Prevention. (2013) *Listeria* (Listeriosis): general information available at: http://www.cdc.gov/listeria/risk.html/ Accessed 2 January 2013.

Chikindas, M., Emond, E., Haandrikman, A. J., Kok, J., Leenhouts, K., Pandian, S., Venema, G. and Venema, K. (2010) Heterologous processing and export of the bacteriocins pediocin PA-1 and lactococcin A in *Lactococcus lactis*: a study with leader exchange. Probiotics & Antimicro. Prot. 2: 66-76.

Cornelis, P. (2000) Expressing genes in different *Escherichia coli* compartments. Curr. Opin. Biotechnol. 11: 450-454.

Cossart, P. and Jonquières, R. (2000) Sortase, a universal target for therapeutic agents against Gram-positive bacteria? PNAS 97: 5013-5015.

Cotter, P. D., Hill, C. and Ross, R. P. (2005) Bacteriocins: developing innate immunity for food. Nat. Rev. Microbiol. 3: 777-788.

Cui, Y., Zhang, C., Wang, Y., Shi, J., Zhang, L., Ding, Z., Qu, X. and Cui, H. (2012) Class IIa bacteriocins: diversity and new development. Int. J. Mol. Sci. 13: 16668-16707.

da Silva, E. P. and De Martinis, E. C. P. (2013) Current knowledge and perspectives on biofilm formation: the case of *Listeria monocytogenes*. Appl. Microbiol. Biotechnol. 97: 957-968.

Dabour, N., Zihler, A., Kheadr, E., Lacroix, C. and Fliss, I. (2009) *In vivo* study on the effectiveness of pediocin PA-1 and *Pediococcus acidilactici* UL5 at inhibiting *Listeria monocytogenes*. Int. J. Food Microbiol. 133: 225-233.

De Vuyst, L. and Leroy, F. (2007) Bacteriocins from lactic acid bacteria: production, purification, and food application. J. Mol. Microbiol. Biotechnol. 13: 194-199.

Devi, S. M. and Halami, P. M. (2013) Conjugal transfer of bacteriocin plasmids from different genera of lactic acid bacteria into *Enterococcus faecalis* JH2-2. Ann. Microbiol. 63: 1611-1617.

Diep, D. B., Skaugen, M., Salehian, Z., Holo, H. and Nes, I. F. (2007) Common mechanisms of target cell recognition and immunity for class II bacteriocins. PNAS 104: 2384-2389.

Drider, D., Fimland, G., Héchard, Y., McMullen, L. M. and Prévost, H. (2006) The continuing story of class IIa bacteriocins. Microbiol. Mol. Biol. Rev. 70: 564-582.

EEC. (1983) EEC Commission Directive 83/463/EEC.

Eom, J. E. and Moon, G. S. (2010) Heterologous production of pediocin PA-1 in *Lactobacillus reuteri*. J. Microbiol. Biotechnol. 20: 1215-1218.

Eugster, M. R., Haug, M. C., Huwiler, S. G. and Loessner, M. J. (2011) The cell wall binding domain of *Listeria* bacteriophage endolysin PlyP35 recognizes terminal GlcNAc residues in cell wall teichoic acid. Mol. Microbiol. 81: 1419-1432.

Eugster, M. R. and Loessner, M. J. (2012) Wall teichoic acid restrict access of bacteriophage endolysin Ply118, Ply511, and PlyP40 cell wall binding domains to the *Listeria monocytogenes* peptidoglycan. J. Bacteriol. 194: 6498-6506.

FDA. (1988) Nisin preparation: affirmation of GRAS status as a direct human food ingredient. Federal Register 53: 247-11.

Ferreira, A., Sue, D., O'Byrne, C. P. and Boor, K. J. (2003) Role of *Listeria monocytogenes* sigmaB in survival of lethal acidic conditions and in the acquired acid tolerance response. Appl. Environ. Microbiol. 69: 2692-2698.

Fimland, G., Johnsen, L., Axelsson, L., Brurberg, M. B., Nes, I. F., Eijsink, V. G. H. and Nissen-Meyer, J. (2000) A C-terminal disulfide bridge in pediocin-like

bacteriocins renders bacteriocin activity less temperature dependent and is a major determinant of the antimicrobial spectrum. J. Bacteriol. 182: 2643-2648.

Fimland, G., Johnsen, L., Dalhus, B. and Nissen-Meyer, J. (2005) Pediocin-like antimicrobial peptides (class IIa bacteriocins) and their immunity proteins: biosythesis, structure, and mode of action. J. Peptide Sci. 11: 688-696.

Fimland, G., Sletten, K. and Nissen-Meyer, J. (2002) The complete amino acid sequence of the pediocin-like antimicrobial peptide leucocin C. Biochem. Biophys. Res. Commun. 295: 826-827.

Fischetti, V. A., Pancholi, V. and Schneewind, O. (1990) Conservation of a hexapeptide sequence in the anchor region of surface proteins from Gram-positive cocci. Mol. Microbiol. 4: 1603-1605.

Gálvez, A., Abriouel, H., López, R. L. and Omar, N. B. (2007) Bacteriocin-based strategies for food biopreservation. Int. J. Food Microbiol. 120: 51-70.

García, P., Rodríguez, L., Rodríguez, A. and Martínez, B. (2010) Food biopreservation: promising strategies using bacteriocins, bacteriophages and endolysins. Trends Food Sci. Technol. 21: 373-382.

Garsa, A. K., Kumariya, R., Sood, S. K., Kumar, A. and Kapila, S. (2014) Bacteriocin production and different strategies for their recovery and purification. Probiotics & Antimicro. Prot. 6: 47-58.

Gasson, M. J. (1983) Plasmid complements of *Streptococcus lactis* NCDO712 and other lactic streptococci after protoplast-induced curing. J. Bacteriol. 154: 1-9.

Georgiou, G., Stahopoulos, C., Daugherty, P. S., Nayak, A. R., Iverson, B. L. and Curtiss III, R. (1997) Display of heterologous proteins on the surface of microorganisms: From the screening of combinatorial libraries to live recombinant vaccines. Nat. Biotechnol. 15: 29-34.

Gibson, T. J. (1984) Studies on the Epstein-Barr virus genome. PhD thesis, University of Cambridge, UK.

Gratia, A. (1925) Sur un remarquable exemple d'antagonism entre deux souches de colibacille. C.R. Soc. Biol. 93: 1040-1041.

Griffiths, A. J. F., Gelbart, W. M., Miller, J. H. and Lewontin, R. C. (1999) Modern genetic analysis. Freeman, New York, USA.

Hagens, S., de Wouters, T., Vollenweider, P. and Loessner, M. J. (2011) Reporter bacteriophage A511::celB transduces a hyperthermostable glycosidase from *Pyrococcus furiosus* for rapid and simple detection of viable *Listeria* cells. Bacteriophage 1: 143-151.

Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. 166: 557-580.

Hartmann, H. A., Wilke, T. and Erdmann, R. (2011) Efficacy of bacteriocin-containing cell-free culture supernatants from lactic acid bacteria to control *Listeria monocytogenes* in food. Int. J. Food Microbiol. 146: 192-199.

Hastings, J. W., Sailer, M., Johnson, K., Roy, K. L., Vederas, J. C. and Stiles, M. E. (1991) Characterization of leucocin A-UAL 187 and cloning of the bacteriocin gene from *Leuconostoc gelidum*. J. Bacteriol. 173: 7491-7500.

Haugen, H. S., Fimland, G. and Nissen-Meyer, J. (2011) Mutational analysis of residues in the helical region of the class IIa bacteriocin pediocin PA-1. Appl. Environ. Microbiol. 77: 1966-1972.

Haugen, H. S., Fimland, G., Nissen-Meyer, J. and Kristiansen, P. E. (2005) Three-dimensional structure in lipid micelles of the pediocin-like antimicrobial peptide curvacin A. Biochemistry 44: 16149-16157.

Hildén, L. and Johansson, G. (2004) Recent developments on cellulases and carbohydrate-binding modules with cellulose affinity. Biotechnol. Lett. 26: 1683-1693.

Holo, H. and Nes, I. F. (1989) High-frequency transformation, by electroporation, of *Lactococcus lactis* subsp. *cremoris* grown with glycine in osmotically stabilized media. Appl. Environ. Microbiol. 55: 3119-3123.

Huang, C. J., Lin, H. and Yang, X. (2012) Industrial production of recombinant therapeutics in *Escherichia coli* and its recent advancements. J. Ind. Microbiol. Biotechnol. 39: 383-399.

ISO 10560. (1999) Milk and milk products-detection of *Listeria monocytogenes*. Geneva: International Organization for Standardization.

ISO 11290-1. (1996) Microbiology of food and animal feeding stuffs-horizontal method for the detection and enumeration of *Listeria monocytogenes*-Part 1: detection method. Geneva: International Organization for Standardization.

Isoda, R., Simanski, S. P., Pathangey, L., Stone, A. E. and Brown, T. A. (2007) Expression of a *Porphyromonas gingivalis* hemagglutinin on the surface of a *Salmonella* vaccine vector. Vaccine 25: 117-126.

Jacob, F. and Fuerst, C. R. (1958) The mechanism of lysis by phage studied with defective lysogenic bacteria. J. Gen. Microbiol. 18: 518-526.

Johnsen, L., Fimland, G., Eijsink, V. and Nissen-Meyer, J. (2000) Engineering increased stability in the antimicrobial peptide pediocin PA-1. Appl. Environ. Microbiol. 66: 4798-4802.

Johnsen, L., Fimland, G. and Nissen-Meyer, J. (2005) The C-terminal domain of pediocin-like antimicrobial peptides (class IIa bacteriocins) is involved in specific recognition of the C-terminal part of cognate immunity proteins and in determining the antimicrobial spectrum. J. Biol. Chem. 280: 9243-9250.

Junttila, J. and Brander, M. (1989) *Listeria monocytogenes* septicaemia associated with consumption of salted mushrooms. Scand. J. Infect. Dis. 21: 339-342.

Kheadr, E., Zihler, A., Dabour, N., Lacroix, C., Le Blay, G. and Fliss, I. (2010) Study of the physicochemical and biological stability of pediocin PA-1 in the upper gastrointestinal tract conditions using a dynamic *in vitro* model. J. Appl. Microbiol. 109: 54-64.

Kim, E-J and Yoo, S-K (1998) Cell surface display of CD8 ecto domain on *Escherichia coli* using ice nucleation protein. Biotechnol. Tech. 12: 197-201.

Kjos, M., Borrero, J., Opsata, M., Birri, D. J., Holo, H., Cintas, L. M., Snipen, L., Hernández, P. E., Nes, I .F. and Diep, D. B. (2011a) Target recognition, resistance, immunity and genome mining of class II bacteriocins from Gram-positive bacteria. Microbiology 157: 3256-3267.

Kjos, M., Nes, I. F. and Diep, D. B. (2009) Class II one-peptide bacteriocins target a phylogenetically defined subgroup of mannose phosphotransferase systems on sensitive cells. Microbiology 155: 2949-2961.

Kjos, M., Nes, I. F. and Diep, D. B. (2011b) Mechanisms of resistance to bacteriocins targeting the mannose phosphotransferase system. Appl. Environ. Microbiol. 77: 3335-3342.

Klumpp, J. and Loessner, M. J. (2013) *Listeria* phages: Genomes, evolution, and application. Bacteriophage 3.

Kos, B., Šušković, J., Vuković, S., Šimpraga, M., Frece, J. and Matošić, S. (2003) Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. J. Appl. Microbiol. 94: 981-987.

Kovačević, M., Burazin, J., Pavlović, H., Kopjar, M. and Piliižota, V. (2013) Prevalence and level of *Listeria monocytogenes* and other *Listeria* sp. in ready-to-eat minimally processed and refrigerated vegetables. World J. Microbiol. Biotechnol. 29: 707-712.

Kuenne, C., Billion, A., Mraheil, M. A., Strittmatter, A., Daniel, R., Goesmann, A., Barbuddhe, S., Hain, T. and Chakraborty, T. (2013) Reassessment of the *Listeria*

monocytogenes pan-genome reveals dynamic integration hotspots and mobile genetic elements as major components of the accessory genome. BMC Genomics 14: 47.

Kuipers, O. P., de Ruyter, P. G. G. A., Kleerebezem, M. and de Vos, W. M. (1998) Quorum sensing-controlled gene expression in lactic acid bacteria. J. Biotechnol. 64: 15-21.

Kumar, B., Balgir, P. P., Kaur, B. and Garg, N. (2011) Cloning and expression of bacteriocins of Pediococcus spp.: A review. Arch. Clin. Microbiol. 2.

Kuwajima, G. (1988) Flagellin domain that affects H antigenicity of *Esherichia coli* K-12. J. Bacteriol. 170: 485-488.

Kylä-Nikkilä, K., Alakuijala, U. and Saris, P. E. J. (2010) Immobilization of *Lactococcus lactis* to cellulosic material by cellulose-binding domain of *Cellvibrio japonicus*. J. Appl. Microbiol. 109: 1274-1283.

Le Blay, G., Hammami, R., Lacroix, C. and Fliss, I. (2012) Stability and inhibitory activity of pediocin PA-1 against *Listeria* sp. in simulated physiological conditions of the human terminal ileum. Probiotics & Antimicro. Prot. 4: 250-258.

Lee, J. H., Kim, J. H., Hwang, S. W., Lee, W. J., Yoon, H. K., Lee, H. S. and Hong, S. S. (2000) High-level expression of antimicrobial peptide mediated by a fusion partner reinforcing formation of inclusion bodies. Biochem. Biophys. Res. Commun. 277: 575-580.

Lee, S. Y., Choi, J. H. and Xu, Z. (2003) Microbial cell-surface display. Trends Biotechnol. 21: 45-52.

Leenhouts, K., Buist, G. and Kok, J. (1999) Anchoring of proteins to lactic acid bacteria. Antonie Van Leeuwenhoek 76: 367-376.

Leo, J. C., Elovaara, H., Brodsky, B., Skurnik, M. and Goldman, A. (2008) The Yersinia adhesion YadA binds to a collagenous triple-helical conformation but without sequence specificity. Protein Eng. Des. Sel. 21: 475-484.

Li, R., Takala, T. M., Qiao, M., Xu, H. and Saris, P. E. J. (2011) Nisin-selectable food-grade secretion vector for *Lactococcus lactis*. Biotechnol. Lett. 33: 797-803.

Loessner, M. J. (2005) Bacteriophage endolysins-current state of research and applications. Curr. Opin. Microbiol. 8: 480-487.

Loessner, M. J., Kramer, K., Ebel, F. and Scherer, S. (2002) C-terminal domains of *Listeria monocytogenes* bacteriophage murein hydrolases determine specific recognition and high-affinity binding to bacterial cell wall carbohydrates. Mol. Microbiol. 44: 335-349.

Loessner, M. J., Rudolf, M. and Scherer, S. (1997) Evaluation of luciferase reporter bacteriophage A511:luxAB for detection of *Listeria monocytogenes* in contaminated foods. Appl. Environ. Microbiol. 63: 2961-2965.

Loessner, M. J., Schneider, A. and Scherer, S. (1996) Modified *Listeria* bacteriophage lysin genes (ply) allow efficient overexpression and one-step purification of biochemically active fusion proteins. Appl. Environ. Microbiol. 62: 3057-3060.

Lohans, C. T. and Vederas, J. C. (2012) Development of class IIa bacteriocins as therapeutic agents. Int. J. Microbiol. doi:10.1155/2012/386410.

Lynch, D. and Vogel, D. (2001) The regulation of GMOs in Europe and the United States: A case-study of contemporary European regulatory politics. Council on Foreign Relations.

Mahony, J., McAuliffe, O., Ross, R. P. and van Sinderen, D. (2011) Bacteriophages as biocontrol agents of food pathogens. Curr. Opin. Biotechnol. 22: 157-163.

Maischberger, T., Mierau, I., Peterbauer, C. K., Hugenholtz, J. and Haltrich, D. (2010) High-level expression of *Lactobacillus* β-galactosidases in *Lactococcus lactis* using the food-grade, nisin-controlled expression system NICE. J. Agric. Food. Chem. 58: 2279-2287.

Majander, K., Anton, L., Antikainen, J., Lång, H., Brummer, M., Korhonen, T. K. and Westerlund-Wikström, B. (2005a) Extracellular secretion of polypeptides using a modified *Escherichia coli* flagellar secretion apparatus. Nat. Biotechnol. 23: 475-481.

Majander, K., Korhonen, T. K. and Westerlund-Wikström, B. (2005b) Simultaneous display of multiple foreign peptides in the FliD capping and FliC filament proteins of the *Escherichia coli* flagellum. Appl. Environ. Microbiol. 71: 4263-4268.

Makhloufi, K. M., Carré-Mlouka, A., Peduzzi, J., Lombard, C., van Reenen, C. A., Dicks, L. M. T. and Rebuffat, S. (2013) Characterization of Leucocin B-KM432Bz from *Leuconostoc pseudomesenteroides* isolated from boza, and comparison of its efficiency to pediocin PA-1. PLoS ONE 8: e70484.

Makrides, S. C. (1996) Strategies for achieving high-level expression of genes in *Escherichia coli*. Microbiol. Rev. 60: 512-538.

Martín, M., Gutiérrez, J., Criado, R., Herranz, C., Cintas, L. M. and Hernández, P. E. (2007) Chimeras of mature pediocin PA-1 fused to the signal peptide of enterocin P permits the cloning, production, and expression of pediocin PA-1 in *Lactococcus lactis*. J. Food Prot. 12: 2792-2798.

Marugg, J. D., Gonzalez, C. F., Kunka, B. S., Ledeboer, A. M., Pucci, M. J., Toonen, M. Y., Walker, S. A., Zoetmulder, L. C. and Vandenbergh, P. A. (1992) Cloning, expression, and nucleotide sequence of genes involved in production of pediocin PA-1, and bacteriocin from *Pediococcus acidilactici* PAC1.0. Appl. Environ. Microbiol. 58: 2360-2367.

Mattick, A. T. R., Hirsch, A. and Berridge, N. J. (1947) Further observations on an inhibitory substance (nisin) from lactic streptococci. The Lancet 250: 5-8.

Mazmanian, S. K., Liu, G., Ton-That, H. and Schneewind, O. (1999) *Staphylococcus aureus* sortase, an enzyme that anchors surface proteins to the cell wall. Science 285: 760-763.

Miller, K. W., Schamber, R., Chen, Y. and Ray, B. (1998) Production of active chimeric pediocin AcH in *Escherichia coli* in the absence of processing and secretion genes from the *Pediococcus pap* operon. Appl. Environ. Microbiol. 64: 1587.

Moon, G. S., Pyun, Y. R. and Kim, W. J. (2005a) Characterization of the pediocin operon of *Pediococcus acidilactici* K10 and expression of his-tagged recombinant pediocin PA-1 in *Escherichia coli*. J. Microbiol. Biotechnol. 15: 403-411.

Moon, G. S., Pyun, Y. R., Park, M. S., Ji, G. E. and Kim, W. J. (2005b) Secretion of recombinant pediocin PA-1 by *Bifidobacterium longum*, using the signal sequence for bifidobacterial α-amylase. Appl. Environ. Microbiol. 71: 5630-5632.

Motlagh, A., Bukhtiyarova, M. and Ray, B. (1994) Complete nucleotide sequence of pSMB 74, a plasmid encoding the production of pediocin AcH in *Pediococcus acidilactici*. Lett. Appl. Microbiol. 18: 305-312.

Nagarajan, S. (2012) New tools for exploring "old friends-microbial lipases". Appl. Biochem. Biotechnol. 168: 1163-1196.

Narsaiah, K., Jha, S. N., Wilson, R. A., Mandge, H. M., Manikantan, M. R., Malik, R. K. and Vij, S. (2013) Pediocin-loaded nanoliposomes and hybrid alginate-nanoliposome delivery systems for slow release of pediocin. BioNanoSci. 3: 37-42.

Navarre, W. W. and Schneewind, O. (1994) Proteolytic cleavage and cell wall anchoring at the LPXTG motif of surface proteins in Gram-positive bacteria. Mol. Microbiol. 14: 115-121.

Nieto-Lozano, J. C., Reguera-Useros, J. I., Peláez-Martínez, M. D. C., Sacristán-Pérez-Minayo, G., Gutiérrez-Fernández, Á. J. and la Torre, A. H. D. (2010) The effect of the pediocin PA-1 produced by *Pediococcus acidilactici* against *Listeria monocytogenes* and *Clostridium perfringens* in Spanish dry-fermented sausages and frankfurters. Food Control 21: 679-685.

Oliveira, J., Castilho, F., Cunha, A. and Pereira, M. J. (2012) Bacteriophage therapy as a bacterial control stratigy in aquaculture. Aquacult. Int. 20: 879-910.

Orsi, R. H., Bakker, H. C. D. and Wiedmann, M. (2011) *Listeria monocytogenes* lineages: genomics, evolution, ecology, and phenotypic characteristics. Int. J. Med. Microbiol. 301: 79-96.

Pamer, E. G. (2004) Immune responses to *Listeria monocytogenes*. Nat. Rev. Immunol. 4: 812-823.

Papagianni, M. and Anastasiadou, S. (2009) Pediocins: the bacteriocins of Pediococci. Sources, production, properties and application. Microb. Cell Fact. 8: 3.

Petrović, D. M., Leenhouts, K., van Roosmalen, M. L., KleinJan, F. and Broos, J. (2012) Monitoring lysin motif-ligand interactions via tryptophan analog fluorescence spectroscopy. Anal. Biochem. 428: 111-118.

Poquet, I., Saint, V., Seznec, E., Simoes, N., Bolotin, A. and Gruss, A. (2000) HtrA is the unique surface housekeeping protease in *Lactococcus lactis* and is required for natural protein processing. Mol. Microbiol. 35: 1042-1051.

Portnoy, D. A., Chakraborty, T., Goebel, W. and Cossart, P. (1992) Molecular determinants of *Listeria monocytogenes* pathogenesis. Infect. Immun. 60: 1263-1267.

Ray, B. and Miller, K. W. (2000) Pediocin. pp. 525-566. *In*: Naidu, A.S. (eds.). Natural food antimicrobial systems. Boca Raton, FL: CRC Press.

Ray, S. K., Kim, W. J., Johnson, M. C. and Ray, B. (1989) Conjugal transfer of a plasmid encoding bacteriocin production and immunity in *Pediococcus acidilactici* H. J. Appl. Bacteriol. 66: 393-399.

Renye Jr., J. A. and Somkuti, G. A. (2010) Nisin-induced expression of pediocin in dairy lactic acid bacteria. J. Appl. Microbiol. 108: 2142-2151.

Roces, C., Rodríguez, A. and Martínez, B. (2012) Cell wall-active bacteriocins and their applications beyond antibiotic activity. Probiotic & Antimicro. Prot. 4: 259-272.

Rodríguez, J. M., Martinez, M. I., Horn, N. and Dodd, H. M. (2003) Heterologous production of bacteriocins by lactic acid bacteria. Int. J. Food Microbiol. 80: 101-116.

Rodríguez, J. M., Martinez, M. I. and Kok, J. (2002) Pediocin PA-1, a wide-spectrum bacteriocin from lactic acid bacteria. Crit. Rev. Food Sci. Nutr. 42: 91-121.

Rogers, L. A. and Whittier, E. O. (1928) Limiting factors in lactic fermentation. J. Bacteriol. 16: 211-229.

Rosef, O., Klaeboe, H., Paulauskas, A. and Ambrasienė, D. (2012) Diversity of *Listeria monocytogenes* isolated from humans, food, and environmental sources in Norway. Vet. Med. Zoot. 59: 71-79.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA.

Samuelson, P., Gunneriusson, E., Nygren, P. Å. and Ståhl, S. (2002) Display of proteins on bacteria. J. Biotechnol. 96: 129-154.

Santiago-Silva, P., Soares, N. F. F., Nóbrega, J. E., Júnior, M. A. W., Barbosa, K. B. F., Volp, A. C. P., Zerdas, E. R. M. A. and Würlitzer, N. J. (2009) Antimicrobial efficiency of film incorporated with pediocin (ALTA® 2351) on preservation of sliced ham. Food Control 20: 85-89.

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M. A., Roy, S. L., Jones, J. L. and Griffin, P. M. (2011) Foodborne illness acquired in the United States-major pathogens. Emerg. Infect. Dis. 17: 7-15.

Schmelcher, M., Shabarova, T., Eugster, M. R., Eichenseher, F., Tchang, V. S., Banz, M. and Loessner, M. J. (2010) Rapid multiplex detection and differentiation of *Listeria* cells by use of fluorescent phage endolysin cell wall binding domains. Appl. Environ. Microbiol. 76: 5745-5756.

Schmelcher, M., Waldherr, F. and Loessner, M. J. (2012) *Listeria* bacteriophage peptidoglycan hydrolases feature high thermoresistance and reveal increased activity after divalent metal cation substitution. Appl. Microbiol. Biotechnol. 93: 633-643.

Shimazu, M., Nguyen, A., Mulchandani, A. and Chen, W. (2003) Cell surface display of organophosphorus hydrolase in *Pseudomonasputida* using an ice-nucleation protein anchor. Biotechnol. Prog. 19: 1612-1614.

Smartt, A. E. and Ripp, S. (2011) Bacteriophage reporter technology for sensing and detecting microbial targets. Anal. Bioanal. Chem. 400: 991-1007.

Smartt, A. E., Xu, T., Jegier, P., Carswell, J. J., Blount, S. A., Sayler, G. S. and Ripp, S. (2012) Pathogen detection using engineered bacteriophages. Anal. Bioanal. Chem. 402: 3127-3146.

Somkuti, G. A. and Steinberg, D. H. (2010) Pediocin production in milk by *Pediococcus acidilactici* in co-culture with *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. J. Ind. Microbiol. Biotechnol. 37: 65-69.

Sood, S. K., Simha, B. V., Kumariya, R., Garsa, A. K., Mehla, J., Meena, S. and Lather, P. (2013) Highly specific culture-independent detection of YGNGV motif-containing pediocin-producing strains. Probiotics & Antimicro. Prot. 5: 37-42.

Stathopoulos, C., Georgiou, G. and Earhart, C. F. (1996) Characterization of *Escherichia coli* expression an Lpp'OmpA (46-159)-PhoA fusion protein localized in the outer membrane. Appl. Microbiol. Biotechnol. 45: 112-119.

Ståhl, S. and Uhlén, M. (1997) Bacterial surface display: trends and progress. Trends Biotechnol. 15: 185-192.

Steidler, L., Viaene, J., Fiers, W. and Remaut, E. (1998) Functional display of a heterologous protein on the surface of *Lactococcus lactis* by means of the cell wall anchor of *Staphylococcus aureus* protein A. Appl. Environ. Microbiol. 64: 342-345.

Stiles, M. E. (1996) Biopreservation by lactic acid bacteria. Antonie Van Leeuwenhoek 70: 331-345.

Strauss, A. and Götz, F. (1996) *In vivo* immobilization of enzymatically active polypeptides on the cell surface of *Staphylococcus carnosus*. Mol. Microbiol. 21: 491-500.

Tafakori, V., Ahmadian, G. and Amoozegar, M. A. (2012) Surface display of bacterial metallothioneins and a chitin binding domain on *Escherichia coli* increase cadmium adsorption and cell immobilization. Appl. Biochem. Biotechnol. 167: 462-473.

Takala, T., Saris, P. and Tynkkynen, S. (2003) Food-grade host/vector expression system for *Lactobacillus casei* base on complementation of plasmid-associated phospho-β-galactosidase gene *lacG*. Appl. Microbiol. Biotechnol. 60: 564-570.

Tian, Z., Teng, D., Yang, Y., Luo, J., Feng, X., Fan, Y., Zhang, F. and Wang, J. (2007) Multimerization and fusion expression of bovine lactoferricin derivative LfcinB15-W4,10 in *Escherichia coli*. Appl. Microbiol. Biotechnol. 75: 117-124.

Tominaga, T. and Hatakeyama, Y. (2006) Determination of essential and variable residues in pediocin PA-1 by NNK scanning. Appl. Environ. Microbiol. 72: 1141-1147.

Trias, R., Badosa, E., Montesinos, E. and Bañeras, L. (2008) Bioprotective *Leuconostoc* strains against *Listeria monocytogenes* in fresh fruits and vegetables. Int. J. Food Microbiol. 127: 91-98.

van Bloois, E., Winter, R. T., Kolmar, H. and Fraaije, M. W. (2011) Decoration microbes: surface display of proteins on *Escherichia coli*. Trends Biotechnol. 29: 79-86.

van Heijenoort, J. (1998) Assembly of the monomer unit of bacterial peptidoglycan. Cell Mol. Life Sci. 54: 300-304.

Venema, K., Kok, J., Marugg, J. D., Toonen, M. Y., Ledeboer, A. M., Venema, G. and Chikindas, M. L. (1995) Functional analysis of the pediocin operon of

Pediococcus acidilactici PAC1.0: PedB is the immunity protein and PedD is the percursor processing enzyme. Mol. Microbiol. 17: 515-522.

Vollmer, W., Blanot, D. and De Pedro, M. A. (2008) Peptidoglycan structure and architecture. FEMS Microbiol. Rev. 32: 149-167.

Wan, X. (2012) Genetic characterisation and heterologous expression of leucocin C, a class IIa bacteriocin from *Leuconostoc carnosum* 4010. Master thesis. University of Helsinki, Finland.

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. Appl. Microbiol. Biotechnol. 97: 3509-3518.

Wang, J. Y. and Chao, Y. P. (2006) Immobilization of cells with surface-displayed chitin-binding domain. Appl. Environ. Microbiol. 72: 927-931.

Wang, P., Yang, H., Hu, Y., Yuan, F., Zhao, G., Zhao, Y. and Chen, Y. (2012) Characterization of *Listeria monocytogenes* isolates in import food products of China from 8 provinces between 2005 and 2007. J. Food Sci. 77: 212-216.

Watson, R. M., Woody, R. M., Lewis, R. V., Bohle, D. S., Andreotti, A. H., Ray, B. and Miller, K. W. (2001) Conformational changes in pediocin AcH upon vesicle binding and approximation of the membrane-bound structure in detergent micelles. Biochemistry 40: 14037-14046.

Westerlund-Wikström, B., Tanskanen, J., Virkola, R., Hacker, J., Lindberg, M., Skurnik, M. and Korhonen, T. K. (1997) Functional expression of adhesive peptides as fusions to *Escherichia coli* flagellin. Prot. Eng. 10: 1319-1326.

WHO Expert Committee on Food Additives. (1969) Specifications for the identity and purity of food additives and their toxicological evaluation: some antibiotics. Twelfth Report. WHO Technical Report Series No. 430.

Winkelströter, L. K. and De Martinis, E. C. P. (2013) Effect of bacteriocins and conditions that mimic food and digestive tract on biofilm formation, in vitro invasion of eukaryotic cells and internalin gene expression by *Listeria monocytogenes*. Probiotics & Antimicro. Prot. 5: 153-164.

Wollmann, P., Zeth, K., Lupas, A. N. and Linke, D. (2006) Purification of the YadA membrane anchor for secondary structure analysis and crystallization. Int. J. Biol. Macromol. 39: 3-9.

Yang, Z., Liu, Q., Wang, Q. and Zhang, Y. (2008) Novel bacterial surface display systems based on outer membrane anchoring elements from the marine bacterium *Vibrio anguillarum*. Appl. Environ. Microbiol. 74: 4359-4365.

Yim, S. S., An, S. J., Han, M-J, Choi, J. W. and Jeong, K. J. (2013) Isolation of a potential anchoring motif based on proteome analysis of *Escherichia coli* and its use for cell surface display. Appl. Biochem. Biotechnol. 170: 787-804.

Young, R., Wang, I-N and Roof, W. D. (2000) Phages will out: strategies of host cell lysis. Trends Microbiol. 8: 120-128.

Young, R. Y. (2002) Bacteriophage holins: deadly diversity. J. Mol. Microbiol. Biotechnol. 4: 21-36.