Bacteriocins: Identification, structural elucidation, and mechanisms of resistance

by

Cherry Ibarra Romero

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Department of Chemistry
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Abstract

Bacteria employ various antimicrobial compounds to establish themselves within bacterial communities. Bacteriocins, which are ribosomally synthesized antimicrobial compounds, play a crucial role in this dynamic. This thesis focuses on strategies for purifying and identifying diverse bacteriocins and delves into the examination of resistance developed in *Listeria monocytogenes* strains against two distinct types of bacteriocins.

A strain called *Enterococcus canintestini* 49 (EC49), isolated from dog feces, displayed noteworthy antimicrobial activity against *Clostridium perfringens*, vancomycin-resistant enterococci, and *Listeria monocytogenes*. The research on EC49 centered on its bacteriocin production, revealing the identification of two bacteriocins through protein purification and analysis utilizing bacteriocin mining databases. Furthermore, the absence of genes related to virulence factors suggests the potential use of EC49 as a probiotic agent.

Antimicrobial resistance against leucocin A, a type IIa bacteriocin produced by *Leuconostoc gelidum* and against carnocyclin A, a circular bacteriocin produced by *Carnobacterium maltaromaticum* UAL 307, was identified in *L. monocytogenes* strains. The study aimed to determine if genetic changes in the mannose phosphotransferase system (Man-PTS), a docking molecule for type IIa bacteriocins, was responsible for the resistance.

Preface

The content of Chapter 2 was published as Acedo JZ et al. Genome Announc., **2017,** 5;5(40):e01131-17. I performed all the experiments with the assistance of the other authors. Dr. Jeella Acedo wrote the manuscript.

The work done on Chapters 3 and 4 remains unpublished. I carried out approximately 80% of the work described in Chapter 4, namely the strain *E. coli* BL21(DE3) pET-SUMO-LeuA was cloned and provided by Dr. Marco van Belkum. The genome assembly of *Listeria monocytogenes* strains was done with the guidance of Dr. Albert Remus Rosana. All the work done on Chapter 4.2 was done by me.

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ABC	**ATP-binding cassette**
AMR	Antimicrobial resistance
CclA	Carnocyclin A
CFU	Colony forming units
Dha	2,3-didehydroalanine
Dhb	2.3-didehydrobutyrine
DNA	deoxyribonucleic acid
DPC	Dodecylphosphocholine
EC49	*Enterococcus canintestini* 49
FAO	Food and Agriculture Organization
FSA	Food Standards Agency
GDP	Gross domestic product
GRAS	Generally recognized as safe
HP	Hairpin
HPLC	High performance liquid chromatography
InlA	Internalin A
InlB	Internalin B
IPTG	isopropylthio-β-galactoside
LAB	Lactic acid bacteria
Leu A	leucocin A
LtnA1	lacticin A1
LtnA2	lacticin A2

MALDI-TOF MS	Matrix-assisted laser desorption ionization mass spectrometry
Man-PTS	mannose phosphotransferase
MRSA	methicillin-resistant *Staphylococcus aureus*
MurNAc-GlcNAc	N-acetylmuramyl-N-acetylglucosamine
NCBI	National Centre for Biotechnology Information
NMR	Nuclear magnetic resonance
PTMs	posttranslational modifications
PTS	phosphotransferase systems
SDS-PAGE	sulfate polyacrylamide electrophoresis
SPPS	Solid-phase peptide synthesis
SUMO	small ubiquitin-like modifier
TFA	trifluoracetic acid
TFE	(2,2,2-trifluoroethanol)
TM	Transmembrane
UV	Ultraviolet
VRE	Vancomycin-resistant *Enterococcus*
WHO	World Health Organization

CHAPTER 1

1. Introduction

1.1 Lactic acid bacteria

Lactic acid bacteria (LAB) represent an ancient group characterized as non-sporing Gram-positive bacteria, typically existing in the form of rods or cocci, with the ability to ferment carbohydrates into lactic acid. Over time, LAB have adapted to diverse life conditions, spanning anaerobic and aerobic environments, evolving various molecular systems for respiration or fermentative pathways[1]. Moreover, LAB have refined their metabolic processes by establishing symbiotic relationships with diverse hosts to acquire essential nutrients such as vitamins, proteins, and notably, sugars. Adapting to a range of sugars like xylose, cellobiose, ribose, arabinose, glucose, and fructose has empowered LAB to thrive within microbial communities. This diverse group encompasses over 60 genera, with the most commonly recognized including lactobacilli, lactococci, enterococci, streptococci, leuconostoc, and pediococci[12].

LAB, extensively employed in the food industry, play a pivotal role in fermenting diverse products to enhance their quality, prolong shelf-life, and ensure food safety [3]. This bacterial group possesses the capability to decompose indigestible macromolecules in food, such as polysaccharides, while generating an array of advantageous compounds during their metabolic processes, including short-chain fatty acids, amines, bacteriocins, vitamins, and exopolysaccharides[4]. Additionally, LAB exhibit lipolytic and proteolytic activity, enabling the conversion of free amino acids into various metabolites, such as alcohols, aldehydes, acids, and ester compounds, which significantly contribute to the aroma and texture of the fermented products[4].

LAB are also broadly used to produce lactic acid, which has a wide range of industrial applications in food and pharmaceutical industry[5].

A wide array of LAB species have been recognized as integral components of the human gut microbiota. Here, they serve a vital function in regulating various physiological processes in the

host by producing a range of metabolites that influence the molecular communication between bacteria and the host[6]. This type of symbiotic connection is not confined solely to humans. Similar interactions, ranging from mutualism to commensalism, or even parasitism, are also observed in plants, insects, and diverse mammals. These interactions are currently subjects of study with a diverse range of applications, notably in the fields of agriculture and medicine[7].

Amongst LAB, the genera lactococci and lactobacilli are generally recognized as safe (GRAS), from which usage has been exploited in the food industry[8]. Antimicrobial compounds such as bacteriocins produced by this large group of bacteria have gained significant interest in recent years[9].

1.2 Bacteriocins

Since the discovery of penicillin in 1928 by Alexander Fleming[10], it is known that microorganisms can produce antimicrobial substances as a competitive advantage in bacterial communities[7,11]. LAB produce numerous antimicrobial compounds including bacteriocins, organic acids, ethanol, diacetyl, carbon dioxide and hydrogen peroxide[12]. In recent years, the study of bacteriocins from LAB has gained more interest due to their use in food preservation, food safety and human and veterinary medicine[13].

Bacteriocins are ribosomally synthesized bacterial peptides, usually smaller than 10 kDa, which are active in low concentrations against closely related species[14]. Some studies suggest that the activity spectrum of bacteriocins may cross domains, as they can be active against yeast, fungi and eukaryotic cells[11]. Bacteria that produce bacteriocins protect themselves from being killed by their own compounds through the use of immunity mechanisms, including immunity proteins, efflux pumps or a combination of both[13,15].

Most bacteriocins are charged molecules, and as a result of their charge distribution, they can easily interact with components on the microbial cell membrane to perform their antimicrobial activity[16]. The list of known and characterized bacteriocins is quite broad and continue to expand.

Their classification has been challenging since it should consider the origin, complexity of production, structure, physical properties and mechanism of action[13,16].

1.3 Gram-positive bacteriocin classification

Both Gram-positive and Gram-negative bacteria produce bacteriocins, and each group has its own classification system although they may share similarities. Furthermore, the food-grade nature of many LAB species has inspired the search for new bacteriocins produced by this group in the last 30 years, leading to the discovery of many new compounds[17]. The classification scheme has had to evolve as the list has grown. The first classification attempt involved eight groups primarily based on physical properties such as heat resistance, protease sensitivity and cross-reactivity[11]. Later on, this scheme was upgraded by Klaenhammer, who proposed four classes of bacteriocins based on their biochemical properties and genetics(Table 1.1)[18]

Table 1.1 Klaenhammer bacteriocin classification[18].

Bacteriocin class		**Features**	
Class I: **Lantibiotics**		· Small membrane-active peptides (<5 kDa) · Post-translationally modified; they usually contain non-canonical amino acids (lanthionine or β-methyl lanthionine)	
Class II	*IIa*	· Small heat-stable peptides · Lack post-translational modifications (they do not contain lanthionine) · Gly-Gly processing site in the bacteriocin's precursor peptide · Presence of amphiphilic helices	· *Listeria*-active peptides · Consensus N-terminal sequence: -Y-G-N-G-V-X-C
	IIb		· Two-peptide poration complexes
	IIc		· Thiol-activated peptides (require reduced cysteine residues for activity)
Class III		· Large heat-labile proteins with enzymatic activity	
Class IV		· Complex proteins, usually contain one or more non-amino acid chemical moieties (lipid or carbohydrates)	

Recent classification systems have reached a consensus on retaining Class I and Class II with minor adjustments. Class I has been broadened to incorporate all peptides undergoing post-translational modifications, while Class II now comprises four subclasses featuring non-post-translationally modified bacteriocins, including peptides with disulphide bridge formation. On the other hand, Class III has undergone reclassification as large peptides, including bacteriolysins, enzymatically active bacteriocins, large non-lytic bacteriocins, and tailocins, also known as phage tail-like bacteriocins[16]. Class IV has been omitted from the classification schemes, as only a few bacteriocins could be categorized under this class, and they are now incorporated into Class III **(**Table 1.2**)** [17].

Table 1.2 Current bacteriocin classification[16,17].

Class	Category	Features
Class I **Post-translationally modified peptides**	Lantibiotics	(Methyl)lanthionine residues
	Lipolanthines	N-terminal fatty acid, avionin moiety (aminovinylcysteine-labionin-hybrid)
	Linear azol(in)e-containing bacteriocins	Thiazole and (methyl)oxazole rings, linear backbone
	Thiopeptides	6-membered nitrogen heterocycle, azol(in)e rings, dehydro residues
	Bottromycins	Macrocyclic amidine, decarboxylated C-terminal thiazole, β-methylated residues
	Sactibiotics	Cysteine sulphur to α-carbon bridges
	Lasso peptides	N-terminal amine to γ-acid residue cyclization, C-terminal tail threaded through a ring
	Glycocins	Contain glycosylated residue(s)
	Head-to-tail cyclized bacteriocins	N- to C-terminal cyclization
Class II **Unmodified peptides**	**IIa**: Pediocin like bacteriocins	YGNG-motif containing bacteriocins
	IIb: Two-peptide bacteriocins	Act as one unit for synergistic activity
	IIc: Leaderless bacteriocins	Produced with no leader sequence
	IId: Non-pediocin-like bacteriocins, single peptides	Not YGNG-like, linear peptides
Class III **Large peptides**	Bacteriolysins	Large polypeptides, bacteriolytic
	Non-lytic large bacteriocins	Large polypeptides, not bacteriolytic
	Tailocins	Multi-protein complex, bacteriophage tail-like structure

1.4 Production and general use of bacteriocins

Bacteriocins from both Gram-positive and Gram-negative bacteria can demonstrate bactericidal, bacteriostatic, or a combination of both effects through diverse mechanisms within bacterial species closely associated with the producing strain. While these molecules possess an antibiotic effect, they cannot be classified as antibiotics since they differ in certain aspects: bacteriocins are peptides ribosomally produced through the primary phase of bacterial growth, bacteriocin's antimicrobial activity is narrow and usually, they are easily inactivated by digestive enzymes such as trypsin and pepsin[20]. However, the proteinaceous nature of bacteriocins and their unique mechanism of action prevent the development of quick resistance amongst pathogenic strains, which is an ideal property for developing new antibiotics[14].

Multiple Gram-positive and Gram-negative bacteriocins have been discovered and broadly studied in recent years. Still, despite these efforts, few bacteriocins have been approved by regulatory agencies for medical or veterinary applications since oral administration studies concerning safety in humans and animals have been insufficient. Additionally, the cost of bacteriocin production is high due to the low yields in production, which discourages researchers and companies from investing and pursuing this research[21,22].

Multiple alternatives for bacteriocin production have been proposed to solve this problem, including the design of cheaper media and more straightforward purification steps. Producing bacteriocins at a large scale is costly since the fermentation process requires nutritionally rich media and large bioreactors to satisfy the growth conditions that a particular bacteriocin needs to be produced, such as pH, temperature, and oxygen concentration. This procedure necessitates thorough purification steps, involving multiple techniques such as dialysis, centrifugation, lyophilization, and various chromatographic methods like high-performance liquid chromatography (HPLC), cation-exchange chromatography, or hydrophobic interaction chromatography. While these methods are effective at a laboratory scale, they are not ideally suited for industrial-scale production.[23,24].

Various media derived from by-products of the food and dairy sectors, like cheese whey or soybean residues, have undergone testing for bacteriocin production. (Table 1.3), followed by simpler purification steps such as liquid-liquid extractions. Nevertheless, the yield of bacteriocin

production remains low. The development of bacteriocin over-producer strains by genetic engineering, which are easy to grow in combination with the designed media and improved purification methods, could provide a possible solution to this problem[25].

Table 1.3 Examples of by-products used for LAB fermentations to produce bacteriocins.

Fermentation Residue	Bacteriocin producer strain	Reference
Chestnut burr	*Lactobacillus plantarum* CECT 211	Costa-Trigo et al. (2021)[26]
Corn stover	*Lactococcus lactis s*ubsp. *lactis* ATCC 11454	Cheng et al. (2018)[27]
Spent coffee ground	*Lactobacillus plantarum* BCRC 10069 *Lactobacillus paracasei* LCW23	Ponrasu et. al (2020)[23]
Cheese whey	*Lactobacillus sakei* DSMZ 6333	Musatti et al. (2020)[28]
Cheese whey supplemented with soybean	*Lactobacillus plantarum* ST16Pa	Silva Sabo et al. (2017)[29]

Chemical synthesis could be another solution to the bacteriocin production problem. For example, many peptides with either commercial use, like aspartame or pharmaceutical compounds like oxytocin, are commonly produced by solid-phase peptide synthesis (SPPS). Bruce Merrifield developed this chemistry in 1963 and was later awarded a Nobel prize for it[30,31,32].

Merrifield's chemistry is based on running multiple cycles of the same reaction over insoluble polymeric support. Nα-protected amino acids are attached to the N-terminal amine of the nascent peptide on the solid support by using coupling reagents which activate the incoming amino acid's carboxylic acid. An amino group deprotection follows this coupling reaction. Purification steps between cycles are unnecessary, and the excess soluble reagents are removed by filtration and washing. Once the elongation of the peptide has been completed, the peptide can be easily released from the resin and later purified (Figure 1.1).

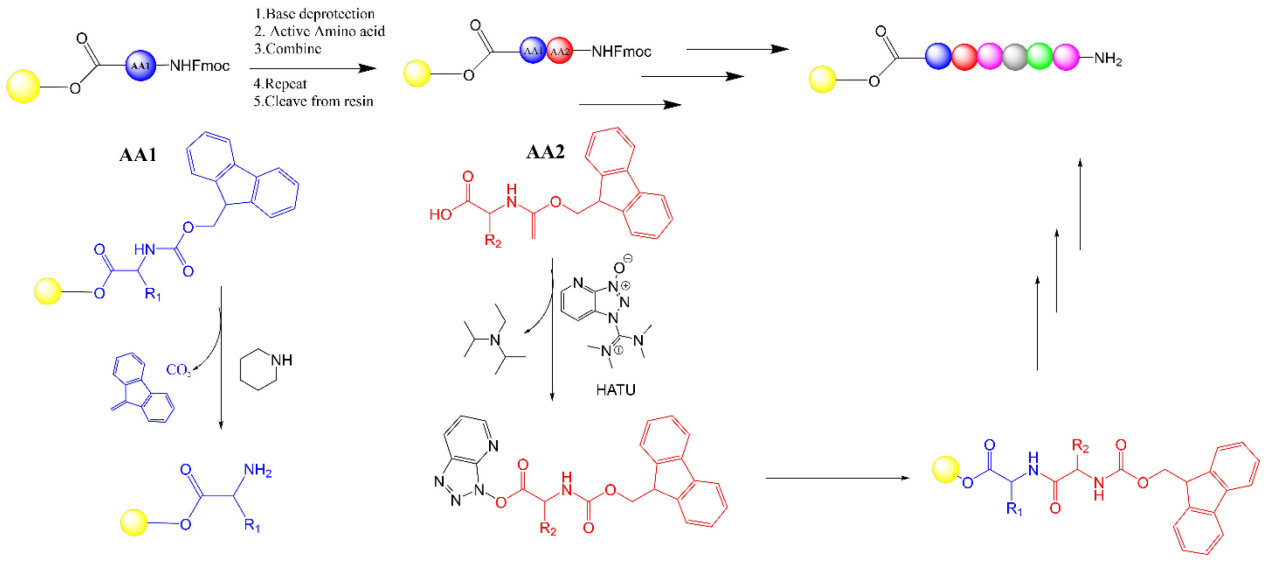


Figure 1.1 Fmoc solid-phase peptide synthesis (Adapted figure from Bédard et al., 2018).

The chemistry involved in solid-phase peptide synthesis (SPPS) presents an alternative approach that may offer a more effective solution compared to biotechnological methods. Peptides from 5-50 amino acids in length can be synthesized with the possibility of introducing unnatural amino acids. However, one of the most significant problems in the chemical synthesis of bacteriocins is to mimic challenging chemical structures such as lasso loops, large macrocycles, long hydrophobic segments, lanthionines or glycosylated side chains of these peptides, which are key structural elements since antimicrobial activity relies on them[33,30].

1.5 The general application of bacteriocins

To date, bacteriocins are mainly used for food preservation rather than being developed as antibiotics since there are still several limitations to overcome as it was previously mentioned. Food spoiling is a major threat to food quality since can cause several pathologies in consumers due to the presence of important pathogens in food such as *Clostridium botulinum*, *Listeria monocytogenes*, *Escherichia coli, Enterococcus*, *Streptococcus*, *Staphylococcus*, *Bacillus*, which can cause inflammatory diseases, respiratory infections, or intestinal disorders[34].

Within the food industry, bacteriocins are applied either independently or as part of hurdle technology, a method that amalgamates various preservation techniques to manage the proliferation of pathogenic bacteria[14].

The industrial application of bacteriocins has been extensively evaluated during the last 60 years since they offer a safe way to preserve food as an alternative to chemical additives, some of which may be toxic for humans. Only two bacteriocins have been successfully commercialized as food preservatives even though the number of known and well-characterized bacteriocins is broad[35]. One of these two approved bacteriocins is nisin, a class I bacteriocin produced by some Gram-positive bacteria species in the *Lactococcus* and *Streptococcus* genus. This bacteriocin exhibits antimicrobial effects against foodborne bacteria, including *Bacillus cereus*, *Clostridium botulinum*, *Listeria monocytogenes*, and *Staphylococcus aureus*. It is commercially available under various names such as Nisaplin™ (Danisco), Novasin™ (Gillco Products Inc.), Chrisin® (Chr. Hansen), NisinA® and NisinZ® (Handary)[36,35].

The other commercialized bacteriocin is Pediocin PA-1, a class IIa bacteriocin which is produced by species in the *Pediococcus* genus. It presents antimicrobial activity against food spoilage bacteria such as *Clostridium perfringens* and the foodborne pathogen *Listeria monocytogenes,* and it has been sold under the names of ALTA 2431™ (Quest International) and Microgard™ (Danisco). Currently, only two more bacteriocins have gained enough technological interest to be evaluated for their industrial application: enterocin AS-48 and lacticin 3147[37,35,38].

The selection criteria for bacteriocins to be used as bio preservatives are quite rigorous. The producer strain must be either GRAS or Qualified Presumption of Safety (QRS), and the bacteriocin should have a broad antimicrobial activity spectrum, and its biological activity should not be related to health risks in animals or humans[39]. The bacteriocin should demonstrate stability within the food it's applied to and contribute positively to the product's overall quality. It is vital to assess the comprehensive efficacy of bacteriocins in various food systems under differing environmental conditions, given that studies indicate variability in their antimicrobial activity across diverse food matrices[37].

The direct usage of strains from the genera *Lactococcus*, *Streptococcus*, *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Enterococcus*, *Carnobacterium* and *Propionibacterium* that produce bacteriocins has also been evaluated and commercialized [40].Various research studies have indicated

that solely applying bacteriocins can lead to a decrease in their concentration due to enzymatic degradation. Consequently, this could compromise the antimicrobial effectiveness of the compound[41].

Bacteriocins have also been used in creative ways in food production, like in food packaging where the bacteriocin is included in a coating film which helps to improve the compound's stability and antimicrobial activity. They are also employed to regulate the fermentation process of various products by inhibiting the growth of microorganisms that may introduce undesired flavors or textures to the finished product [35].

1.6 Conventional methods to produce, identify and purify bacteriocins

The conventional method to identify new bacteriocins includes three main steps: purification, screening, and characterization. Once the bacterial fermentation is completed, multiple sequential rounds of purification will be carried out, these multiple rounds may involve one or more techniques such as hydrophobic interaction chromatography, reversed-phase HPLC, cation exchange chromatography, gel chromatography, liquid-liquid extractions and other techniques[24].

The screening assays involve antimicrobial evaluations, and also, it may include the usage of genomic tools such as databases, which make bacteriocin identification easier[42].

Bacteriocin characterization involves tests to evaluate physicochemical, biochemical, and structural properties. It requires techniques such as genome sequencing to study the genetics of the bacteriocin biosynthetic machinery or the usage of NMR spectroscopy or crystallography to study their structural features. In the last ten years, novel Gram-positive bacteriocins such as laterosporulin produced by the *Brevibacillus* sp. GI-9 strain, plantaricin ZJ008 produced by *Lactobacillus plantarum* ZJ00 and enterocin DD14 produced by *Enterococcus faecalis* 14 have been identified and characterized by this conventional method[42].

1.7 Bacteriocin-producer probiotics

The utilization of lactic acid bacteria in fermenting various products and the associated advantages linked to their consumption have been recognized for an extensive duration. Elie Metchnikoff, working at the Pasteur Institute in 1908, described the healthy benefits of frequent yogurt consumption[43]. He was first to propose that the LAB on yogurt were responsible for conferring a healthy and long life to people in Bulgaria. Many studies have been done to develop functional probiotics ever since[44].

The World Health Organization defines a probiotic as "*a live microorganism that when administered in adequate amounts confers a health benefit on the host*." Many species of the lactic acid bacteria family, such as *Bifidobacterium* sp. and *Lactobacillus* sp., have been approved for use as probiotics[44]. According to the Food and Agriculture Organization (FAO), to approve a particular strain as a probiotic, it must pass first *in vitro* tests such as resistance to gastric acidity and bile acids, adherence to mucus and human epithelial cells, as well as adherence to multiple cell lines. The tested strain must also present antimicrobial activity against potential pathogenic bacteria, the ability to reduce pathogen adhesion to surfaces. If the strain is aimed to be used as a vaginal probiotic, it must present resistance to spermicides[45].

Bacteria which are aimed to be used as probiotics must also comply with several safety considerations. Broad screening is carried out to evaluate these aspects, such as antibiotic resistance patterns, assessment of metabolic activities (like D-lactate production or bile salt deconjugation), assessment of side effects during human studies, epidemiological surveillance of adverse incidents in consumers, and an extensive evaluation of hemolytic effects and the production of toxins[45]. Furthermore, one of the most desirable characteristics of candidates to be used as probiotics is the ability to compete in populated niches, such as the gut microbiota. This skill can be accomplished by the bacterium's ability to produce antimicrobial compounds, such as bacteriocins[46].

The use of bacteriocins as direct bio-preservatives in food can be highly effective since a specific concentration is directly applied to the product to achieve an antimicrobial effect. Nevertheless, the overall function and production of bacteriocins in bacterial communities are not yet well understood. In contrast, some studies have shown that strains that can produce bacteriocins are more successful in thriving in colonization experiments; other studies suggest that the

production of bacteriocin is energetically costly to the producer strain, which reduces the probability of colonization in such competitive environments[47].

The efficacy of bacteriocin producer strains as probiotics has been tested in veterinary medicine in animal models. *Enterococcus faecium* J96 was used in newly hatched broiler chicks. After four days of being born, the chicks were infected with the poultry pathogen *Salmonella pullorum*. The results showed that the strain is effective in preventing infections against this pathogen but not as a treatment following infection[48].

The usage of *E. faecium* EK13, which produces enterocin A, was tested in gnotobiotic Japanese quails, and studies have shown that the administration of this strain as a probiotic reduces the establishment of *Salmonella dusseldorf*. *S. dusseldorf* does not cause any disease in birds, but it does cause intestinal disease in humans upon consumption of meat derived from these birds[49]. *E. faecium* EK13 has also been evaluated in piglets, and the results showed that the strain effectively reduces the colonization of pathogenic *Staphylococcus* spp[50].

Probiotics that can produce bacteriocins may also be an excellent alternative to antibiotics, not only in poultry, but also in aquaculture. Aquatic cultures are heavily populated ecosystems where numerous species of microorganisms coexist. The usage of varying antibiotics has been employed in aquaculture to control the growth of pathogenic bacteria. However, the continuous use of antibiotics has promoted the development of antimicrobial resistance in this environment, and the metabolites produced upon antibiotic consumption by microorganisms can cause toxicity in humans[44].

1.8 *Enterococcus* sp. as probiotics, food additives and supplements

Enterococci are part of the LAB family, over 26 species have been classified in this genus and are commonly found as symbionts on the human and animal gastrointestinal tract[51]. They can also be found inhabiting soil, water, and fermented products. Over time, many *Enterococcus* species have been isolated and used in fermentation cultures to produce various products along with other bacterial genera such as *Lactococcus*, *Pediococcus* and *Lactobacillus,* which are used to provide specific organoleptic properties like taste, texture, and flavor[52].

The consumption of enterococci as probiotics may be useful as supplemental treatment in humans after antibiotic medication, viral infections, chemotherapy and food-borne diseases. Some advantages that have been observed in hosts include growth decrease of pathogenic bacteria, stimulation of the immune system, reinforcement of the mucus barrier and better cholesterol assimilation[53].

The genus *Enterococcus* includes several species, but just *E. faecalis*, *E. faecium*, *E. lactis* and *E. hirae* have been approved worldwide as probiotics in pharmaceutical preparations or as animal feed additives[51]. Examples to be mentioned: *E. faecium* SF68®, which was initially used in Switzerland, but is now approved in many countries as a dietary supplement for humans and animals, *E. faecalis* Symbioflor®1 which is used in Germany for the treatment of symptoms related to irritable bowel syndrome or respiratory illness like chronic sinusitis or bronchitis and *E. faecium* Medilac-Vita which is used in China for the treatment of infantile enteritis[54].

While enterococci are often praised for their potential as probiotics, they have concurrently been recognized as opportunistic pathogens with resistance to multiple drugs. They have the potential to induce serious urinary infections, endocarditis, and bacteremia in individuals with compromised or suppressed immune systems. Consequently, in locations such as Taiwan, the utilization of *E. faecium* and *E. faecalis* as dietary supplements has been prohibited since 2018 as a precautionary measure to curb the extensive emergence of multidrug-resistant enterococci. These bacteria are increasingly exhibiting resistance, notably to vancomycin—a critical antibiotic reserved for treating severe infections caused by Gram-positive bacteria[53,55].

The usage of molecular epidemiology has helped scientists to distinguish *Enterococcus* that are commensals, from those that can cause nosocomial infections. This is achieved by running multiple analyses like molecular fingerprinting, multi-locus sequence typing, phenotypic studies, and whole genome screening. For instance, *E. faecium* has been subtyped into three main clades: clade A1 strains that are not commonly found in healthy patients and are mainly associated with causing nosocomial infections, clade A2 strains that are generally found on animals, and clade B strains that are associated with healthy individuals and seldomly cause infections[51].

Multiple studies suggest that enterococci strains that can cause nosocomial infections are genotypically different from commensal strains. Nevertheless, no *Enterococcus* species is recommended for human consumption either by the European or the American Safety Authorities

(QPS or GRAS status). However, local organizations like the Advisory Committee on Novel Foods and Processes (ACNFP), who advises the Food Standards Agency (FSA) in England, Wales and Northern Ireland permit the use of certain enterococcal strains as food additives, supplements or probiotics[51].

Bacterial strains which are aimed to be used as probiotics need to be investigated in diverse ways. The screening for genes involved in the production of virulence factors is one of the most important studies. Virulence factors are molecules that enhance the capacity of a microorganism to cause illness. Some of the most critical virulence determinant genes to identify in enterococci are listed on Table 1.4[52,55]. The transmission of antimicrobial resistance is another crucial factor to be tested amongst probiotic candidates. *Enterococcus* sp. may have intrinsic antibiotic resistance in their chromosomes to some cephalosporins, sulphonamides, lincosamides, β-lactams, and aminoglycosides[52].

Identifying plasmids or transposons, DNA segments that can be translocated as whole between chromosomal, phage and plasmid DNA, that can be involved in transmitting non-intrinsic AMR against antibiotics like chloramphenicol, erythromycin, fluoroquinolones, tetracyclines, and glycopeptides like vancomycin, is crucial. Studies have shown that the continuous exposure of enterococci to antibiotics has increased the incidence of AMR. In addition, this group of bacteria is well-known for presenting genome plasticity, which is the ability to integrate and use mobile genetic elements such as plasmids, transposons, prophage, and insertions sequences into their genome, which has been evolutionary beneficial for them to succeed in bacterial niches[52,56].

Table 1.4 Genes involved in the production of virulence factors in *Enterococcus* sp.

Virulence gene	Protein	Protein function
agg, *asa1*	Surface aggregation protein	Aggregation in conjugation and infection processes
cyl	Cytolysin	Lyse cells by pore formation in bacterial membranes
gelE	Gelatinase	Zinc-dependent-endopeptidase which hydrolase gelatin, collagen, β-insulin, hemoglobin, and casein
esp	Enterococcal surface protein	Biofilm formation, cell-cell adhesion factors
ace, acm	Collagen adhesion proteins	Adhesion to collagen I and IV. Collagens I and IV are the main components of the endothelial and epithelial cell network, both are found on the epithelial basement membrane, which serves as a barrier between tissue compartments in eukaryotic cells
efa_{fm}, efa_{fc}	Adhesion-like endocarditis antigens	Adhesion to cells

1.9 Enterococcal bacteriocins

Whereas enterococci have indeed become pathogenic to humans due to their evolutionary adaptation to antibiotics, other *Enterococcus* species are still studied for their usage as probiotics, and strains such as *E. faecium* M74 and *E. faecium* SF-68 have complied with most of the functional and safety requirements to be used as probiotics.[57] Bacteriocin production is nowadays considered an important selection criterion amongst bacteria to be used as probiotics (**Table 1.5**). Studies suggest that probiotic functionality is enhanced when the probiotic bacteria can produce

Table 1.5 Examples of classes of enterocins isolated from enterococci[57]

Class I:			
Strain	Bacteriocin	Type	Source of isolation
E. faecalis	Cytolysin CylL and Cyl S	Two-peptide lantibiotic	Clinical isolates
	Enterocin Wα and Wβ		Thai fermented fish
	AS-48	Circular bacteriocin	Bird uropyal glands
Class IIa bacteriocins:			
E. faecium	Enterocin A	sec pathway ABC transportation	Spanish dry fermented sausage
	Enterocin P		
	Bacteriocin GM-1		Feces infants
E. avium	Avicin A		Feces from babies
E. mundtii	Mundticin KS, enterocin CRL35, mundticin QU2		Grass silage, artesanal cheese
E. durans	Durancin GL		Hispanic-style cheese sample
Class IIb			
E.faecalis	Enterocin C	Two-peptide bacteriocins	Human colostrum
	Enterocin 1071 A and B	Two-peptide bacteriocin	Feces from mini-pigs
E.faecium	Enterocin A a and V	Two peptide bacteriocin	Sugar apples
Class II			
E.faecium	Enterocin Q	Leaderless bacteriocin	Dry fermented sausage
Class III			
E. faecalis	Enterolysin A	Heat label enterlolysin	Fish milk

these antimicrobial compounds since bacteriocins can provide dominance to the producer strain in an already colonized ecosystem or they can help to inhibit the growth of competing strains, or they can also serve as signalling peptides which can be sensed by other bacteria or by the host immunological cells[52].

Enterococci typically have the ability to produce multiple enterocins, which are the bacteriocins synthesized by these bacteria. These antimicrobial peptides are predominantly small (20 – 60 amino acids), hydrophobic, heat-stable, and cationic, and they are typically resistant to proteases and stable under different pH values[57].

The study of enterococcal strains, which can produce multiple types of enterocins, is gaining more interest for its application in the food industry as starters, protective cultures or probiotics since their enterocins can inhibit the growth of spoilage bacteria or food-borne pathogens such as *Listeria monocytogenes*, *Bacillus cereus*, *Staphylococcus* spp. and *Clostridium* spp. They also present antagonistic activity against Gram-negative pathogens like *E. coli*, *P. aeruginosa*, *V. cholerae*, yeast as *Candida albicans*, fungi like *Aspergillus niger* and viruses like herpes simplex type 1 and type 2[58,59,60].

Like many other bacteriocins, the cytoplasmic cell membrane is the main target of enterocins. These compounds disrupt the cellular pH gradient by forming pores in the membrane, causing the leakage of essential molecules, which will ultimately result in cellular lysis of the sensitive strain[61].

The bacteriocins produced by enterococci are diverse and most of the strains have been isolated from food, waste, and human and animal feces. *E. faecium* and *E. faecalis* are the most common, but a few more have also been identified as producer strains like *E. muntii*, *E. avium*, *E. hirae* and *E. durans*. Table 1.5 provides some examples of the classification and source from identified enterocins[62].

1.9.1 Antimicrobial resistance and the usage of bacteriocins to face the problem

Antimicrobial resistance (AMR) is the natural evolutionary response developed by bacteria to withstand the extensive usage of antibiotics. Since antibiotics were discovered, the usage of antibiotics has not been limited to treating bacterial infections. Antibiotics have been widely used in veterinary medicine as growth promoters in livestock and aquaculture, which have also contributed enormously to the global AMR problem[63].

When a bacterial community is exposed to any antibiotic, a non-competitive environment is promoted where those bacteria which are non-resistant are easily killed, and those with resistance easily thrive. Bacteria have developed multiple strategies to become resistant to antibiotics. AMR mechanisms can be classified into four main categories: i) inactivation or modification of the antimicrobial molecule, ii) modification of the bacterial target, iii) reduction of antibiotic penetration/accumulation, and iv) bacterial biofilm formation (Figure 1.2)[64].

The emergence of multidrug-resistant bacteria is a serious global concern since available antibiotic treatments are becoming ineffective against bacterial infections, which are becoming more severe. Patients having minimal surgeries who get infected by antimicrobial-resistant organisms can have a higher risk of death or become more prone to develop chronic conditions such as renal failure or cardiovascular diseases[65]. AMR is not only a significant threat to human health but is also threatening the world economy. Calculating the total AMR global economic impact is complicated since there are many things to consider, but close estimations can be calculated by including the most critical factors[64].

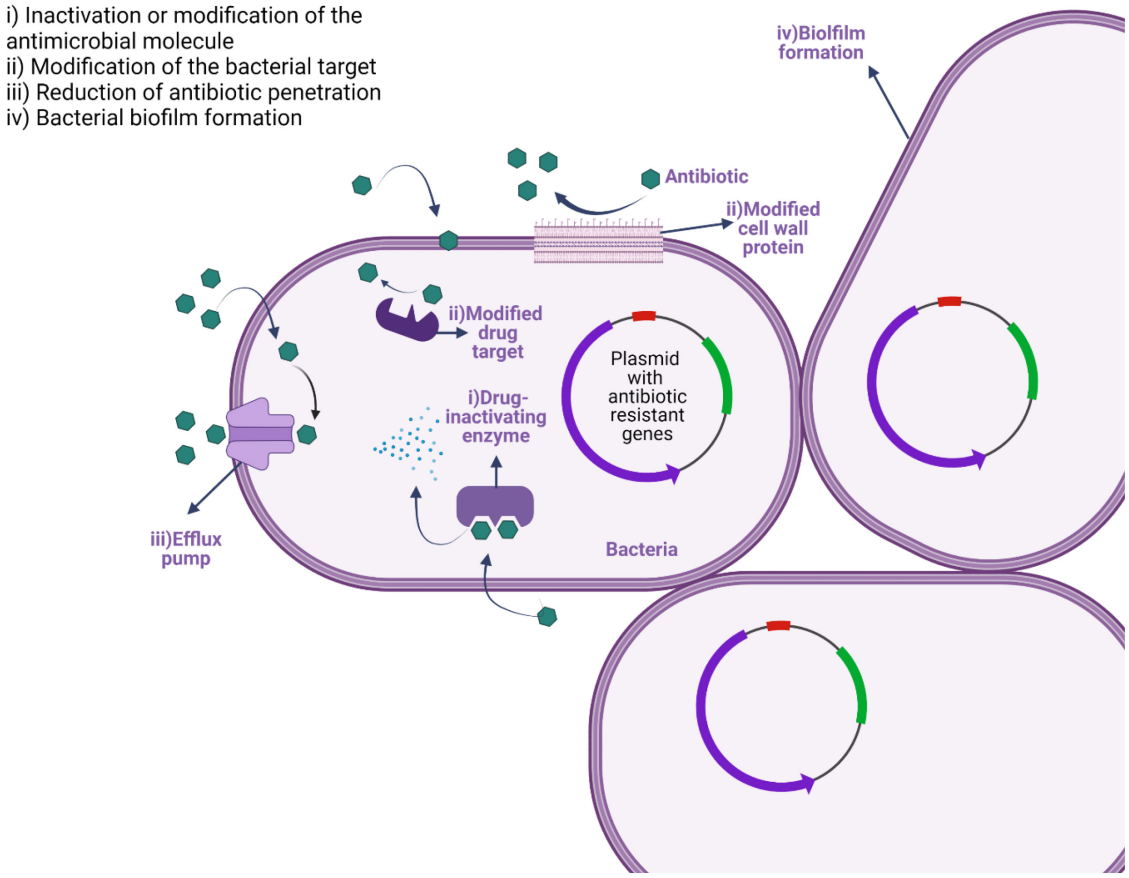


Figure 1.2 Four main categories of antimicrobial resistance (created with BioRender.com)[64] .

For example, if there is a 1% increase in resistance against a specific antibiotic, it is necessary to consider which pathogen has developed the resistance, the prevalence of the pathogen, the type of infection that it causes, the disease burden of that specific infection, the transmissibility of the pathogen (including the implicated genes of the microorganism and how contagious is the infection), and lastly, the available treatments[66].

A study made by Klein et al. in 2015 on the global increase of antibiotic consumption suggests that by 2030, worldwide antibiotic consumption will rise another 15%, mainly by population growth and rapid urbanization, which will bring a higher occurrence of infectious diseases[67].

The antimicrobial resistance problem can be tackled by governments in multiple ways. Countries can ban the usage of antibiotics in livestock and aquaculture, create more robust policies

surrounding the prescription of antibiotics, and more investment can be made in the development of new antibiotics and faster ways to detect AMR microorganisms in ongoing infections[63].

In February 2017, the World Health Organization published a list of "priority status" pathogens for which an urgent new antibiotic development is needed which includes *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species. New antibiotics should present novel chemistry at an accessible cost of production, and the effectiveness of these new antibiotics should be comparable to the existing ones. Still, the killing mechanism should be distinct, so that these new antibiotics remain valuable in the future[64].

Antibiotic innovation is challenging since developing drugs is highly expensive and risky, and the chances of being successful are low. Furthermore, new antibiotics are often restricted to prevent the early development of antimicrobial resistance, so several pharmaceutical companies have decided not to invest in this matter. Therefore, government intervention is crucial to develop sustainable solutions for developing new antibiotics and creating strategies for prevention, control, surveillance and monitoring of AMR[66].

Despite the drawbacks that the usage of bacteriocin can present for their clinical applications, the rising problem of antimicrobial resistance has pushed researchers efforts to develop creative strategies to use bacteriocins in the medicinal or veterinary field by using either synthetic chemistry or bioengineering to produce analogues that are on compliance with the FDA regulations[14].

Bacteriocins possess great qualities to be used as antibiotics such as their potency and low toxicity. In vitro studies have shown promising results in experiments with lantibiotics and thiopeptides. Amongst the successful examples, Duramycin (Moli1901) was developed by a collaboration between Lantibio and AOP Orphan Pharmaceuticals and has completed Phase II clinical trials in the treatment of cystic fibrosis[68]. The company Oragenics has developed a synthetic analogue of the lantibiotic mutacin 1140, MU1140-S, in pre-clinical trials[69]. Oragenics also has bioengineered OG253, a lantibiotic which is under investigation for the treatment of *Clostridium difficile* infections[70]. Novacta Byosistems Ltd developed a mersacidin analog that is under evaluation to be used as an antibiotic against pathogens such as methicillin-resistant

Staphylococcus aureus (MRSA), vancomycin-resistant *Enterococcus* (VRE), *Clostridium difficile* and *Streptococcus pyogenes*[14,71].

Various nisin-derived products have gained approval for application in veterinary medicine. For instance, products like Wipe Out® or Mast Out®, based on nisin, are utilized by Immucell Corporation as an intramammary solution to treat mastitis in lactating dairy cows. Another example is Teatseal®, marketed by Cross Vetpharm Group Ltd[14,35].

An important constraint in utilizing bacteriocins as pharmaceutical products lies in their vulnerability to proteases. Over the past two decades, various formulations for bacteriocins have been extensively advocated to overcome this challenge. Encapsulation of bacteriocins has been explored using diverse systems, including liposomes, film coatings, nanofibers, and nanoparticles made from materials like gold, silver, polysaccharides (such as phytoglycogen or chitosan). The primary aim of these systems is to enhance the delivery of the compounds to specific targets by ameliorating their pharmacokinetics, thereby increasing their bioavailability and extending their half-life[20,21,72].

1.10 Resistance to bacteriocins

Similar to antibiotics, prolonged exposure of bacteria to bacteriocins can lead to the development of bacteriocin-resistant strains. To assess the clinical implications of bacteriocins, studies have investigated the emergence of resistance to widely used bacteriocins such as nisin, lacticin 3147, and type IIa bacteriocins in vitro. These evaluations have been conducted across various significant Gram-positive bacterial species, including *Enterococcus faecalis*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Clostridium difficile*, *Lactococcus lactis*, and *Streptococcus pneumoniae*[73].

These studies have found that the developed resistance has similar mechanisms to the ones shown in antibiotics [74]. Interestingly, the genes involved in these mechanisms are not commonly found amongst bacteria which may suggest bacteriocin resistance is not easily developed or transferred as antibiotic resistance. Bacteriocin resistant populations grow and then are selected in the presence of the bacteriocin, whereas in antibiotic resistance spontaneous mutations and natural selection can occur just at the moment bacteria face antibiotics[73,75].

One of the mechanisms of action of bacteriocins relies in the direct interaction of the bacteriocin with charged components in the cytoplasmic membrane of the target bacteria. This interaction causes the formation of pores which will lead to the leakage of intracellular metabolites to cause cell death. An alternative mechanism of action of these compounds relies on the prevention of the cell wall synthesis by the binding of the peptide with the precursor molecule, lipid II. Therefore, the acquired resistance of strains involves changes in the cell wall by producing membrane stiffness, reduction of bacteriocin receptors or by the presence of modified lipoteichoic acids[76].

Bacteriocin resistance can be classified as innate or acquired. Distinct genetic loci have been identified to provide either innate or acquired resistance[77].

1.10.1 Innate resistance

1.10.1.1 Immune mimicricy

Immune mimicricy is an example of innate resistance where strains that do not produce the bacteriocin can encode homologues immunity systems to the producer strain to protect themselves from the bacteriocin. These genes have been designated as "orphan immunity genes"[75].

1.10.1.2 Resistance due to bacteriocin degradation

In *Bacillus cereus* and *Paenibacillus polymixa* strains, it has been identified the production of nisinase, an enzyme which degrades nisin[75]. This enzyme can break the C-terminal lanthionine ring. Nisin degradation can also be performed by other proteases like NSR protease which was found in non-nising-producing *Lactococcus lactis* strains, this protease removes the nisin C-terminal tail[78,41].

1.10.1.3 Resistance associated with growth conditions

Through experiments conducted on various *L. monocytogenes* strains, it has been noted that the tolerance to specific bacteriocins, such as Pediocin PA-1 or nisin, varies notably in osmotically stressed and cold-stressed cultures. This observed variation is thought to be attributed to each strain's capability to adapt to diverse environmental changes, resulting in alterations in cell

morphology and components of the cell membrane. Consequently, these adaptations are presumed to affect interactions with bacteriocins, although the precise mechanisms of this interaction alteration remain not entirely understood[79,80].

1.10.1.4 Resistance due to changes in the bacterial cell wall composition

Gram-positive bacterial cell wall has teichoic acids and lipoteichoic acids as main constituents. Teichoic acids form a polymer which contain a highly negatively charged backbone provided by deprotonized phosphate groups[81]. In some Gram-positive bacteria, this negative charge can change by the introduction of basic amino groups, the introduction of this groups is mediated by proteins produced by the *dlt* operon. Mutations in this operon have shown an increased negative surface charge which ultimately promote the interaction with bacteriocin causing more sensitive strains, in the other hand, *Clostridium difficile* and *Bacillus cereus* resistant strains, have shown an increased expression of the *dlt* genes when bacteria are grown in the presence of bacteriocins[82,83].

1.10.2 Acquired resistance

Mechanisms involved in acquired resistance are diverse. The developed mechanisms depend on the microorganism, the bacteriocin, the strain and the environmental conditions. These mechanisms include mutations in regulatory elements, alterations in cell wall or cytoplasmic membrane synthesis[77].

1.11 *L. monocytogenes* and its resistance to bacteriocins

1.11.1 The clinical importance of *L. monocytogenes.*

Food-borne diseases are defined as illnesses caused by the consumption of food contaminated with chemicals, toxins, and harmful microorganisms such as bacteria, parasites, fungi and viruses. This contamination is mainly produced by unhygienic practices in the food production process. They can cause either mild symptoms such as nausea, vomiting and diarrhea or severe sicknesses such as cancer, kidney or liver failure or neuronal disorders[84].

Food-borne diseases are a growing problem which significantly impact the economy of many countries, either by having repercussions on the money spent on health care systems, productivity of the individuals who get sick or by jeopardizing international trades. During the last decade, more than 30 agents have been identified as causative of these illnesses, not only in humans but also in livestock. The microorganisms that have been identified in most of the outbreaks around the globe are *Salmonella*, *Listeria* and *E. coli,* for which reason, research on the prevention and control of these microorganisms has gained importance and studies to prevent the spread and development of antimicrobial resistance amongst them is crucial[85].

L. monocytogenes is a Gram-positive bacterium which can cause listeriosis, an infectious food-borne disease that in healthy individuals can cause a mild infection, but in elders or immunocompromised people can develop septicemia, endocarditis, meningitis, or infections in other parts of the body like bones and joints. In a pregnant woman, listeriosis can be severe for the expected baby, and cause an abortion[86].

This widely distributed bacterium is commonly found in various food types such as dairy products, fermented meats, and vegetables. It is recognized for its propensity to create biofilms and its resilience in harsh conditions like low temperatures, low pH, and high salt concentrations. Among the 17 identified species of *Listeria*, only two, *L. monocytogenes* and *L. ivanovii*, have proven pathogenic for humans and ruminants. The molecular factors enabling *Listeria* to thrive in diverse microenvironments are multifaceted and numerous, involving the production of various substances including internalins and multiple proteases. Specifically, *L. monocytogenes* serotypes 1/2a, 1/2b, and 4b are primarily associated with human outbreaks[87].

1.11.2 The infection process of *L. monocytogenes*

Listeriosis, the infection caused by *L. monocytogenes,* initiates upon ingesting contaminated food with an estimated 10 – 100 million colony forming units (CFU) in healthy hosts, and only 0.1 to 10 million CFU in immunocompromised individuals[88]. Upon ingestion, *L. monocytogenes* find the intestinal epithelium. It crosses the epithelial barrier and then the lamina propria to find the lymphatic system and the bloodstream, where it will be spread to the liver and spleen, which are *L. monocytogenes'* target organs. *L. monocytogenes* can cross the blood-brain barrier in

immunocompromised people, and it can cross the fetoplacental barrier in pregnant women, where it can cause premature delivery or spontaneous abortion (Figure 1.3)[86,87].

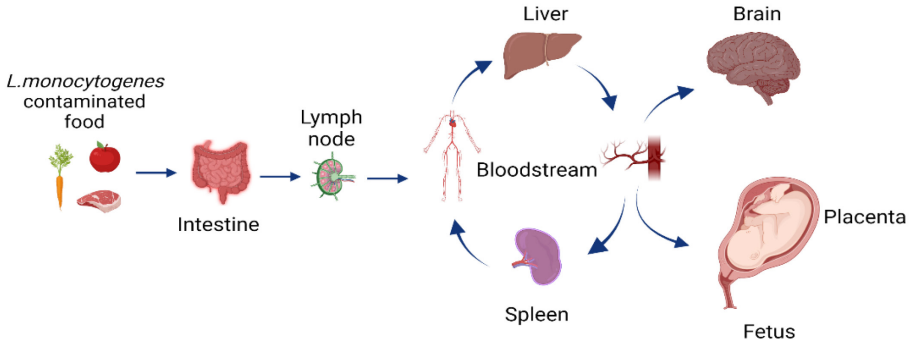


Figure 1.3 *L. monocytogenes* infection (adapted from L. Radoshevich et al., 2018; created with BioRender.com).

L. monocytogenes is a facultative intracellular bacterium which uses endothelial cells as its primary target. To enable bacterial cell binding, *L. monocytogenes* produces two proteins of around 80 kDa each on its surface, known as internalin A and B (InlA, InlB). Once *L. monocytogenes* has been internalized by receptor-mediated endocytosis, the bacteria escape the vacuole by secreting listeriolysin O, phospholipase A and B, and then start to replicate in the cytoplasm[86].

Listeriolysin O (LLO) is a potent toxin which is optimally expressed at 37ºC[89]. LLO not only promotes vacuole lysis but also promotes several changes in cell organelles like the endoplasmic reticulum, where it induces changes in the cell to make it more susceptible to infection, or it can also cause changes on histone post-translational modifications which are of great importance to control chromatin packing and hence the access to specific genes necessary for cell functionality get disrupted[90]. Furthermore, *L. monocytogenes* can also rearrange the host cell's actin filaments into an actin tail thanks to an actin filament crosslinking protein, ActA, which endows motility to this bacterium inside of the cell and allows the bacteria to infect adjacent cells (Figure 1.4)[90,91,92].

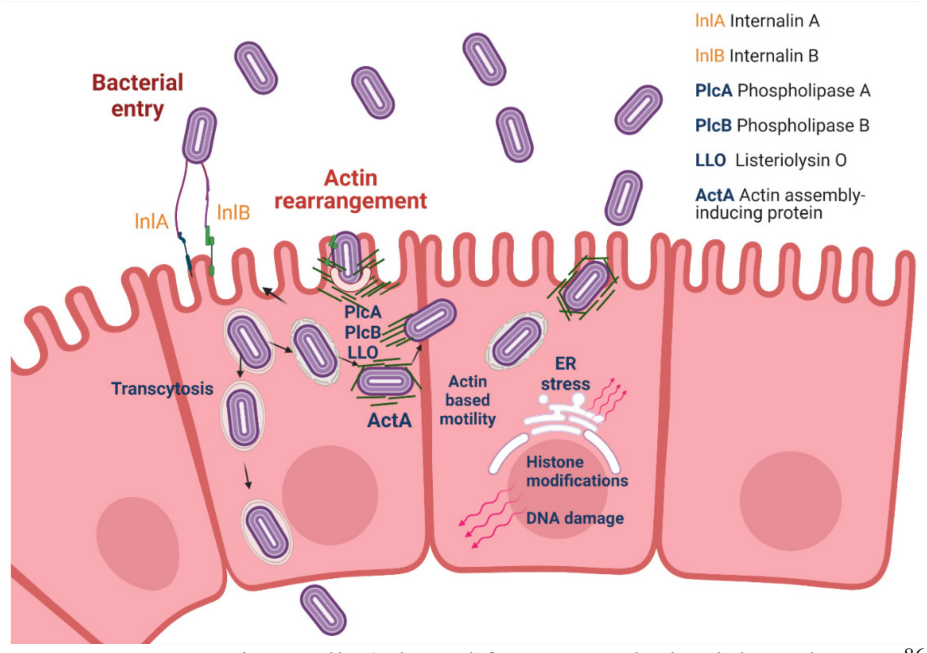


Figure 1.4 *L. monocytogenes* entry into cells (adapted from L. Radoshevich et al., 2018[86]; created with BioRender.com).

1.11.3 Antilisterial bacteriocins

Recent reports point out that bacteriocins which belong to Class I or II may have antilisterial properties, but mainly Class IIa bacteriocins have been broadly studied for this purpose. Circular bacteriocins, which are peptides linked by their N-to-C termini, exhibit a broad-spectrum antimicrobial activity. These peptides have also been studied to be applied as bio preservatives and for their usage against *Listeria sp*[93].

1.12 Class IIa bacteriocins

Class IIa bacteriocins are also known as YGNG-motif containing bacteriocins, and they additionally possess great antimicrobial activity against multiple foodborne pathogens apart from *L. monocytogenes*[94].

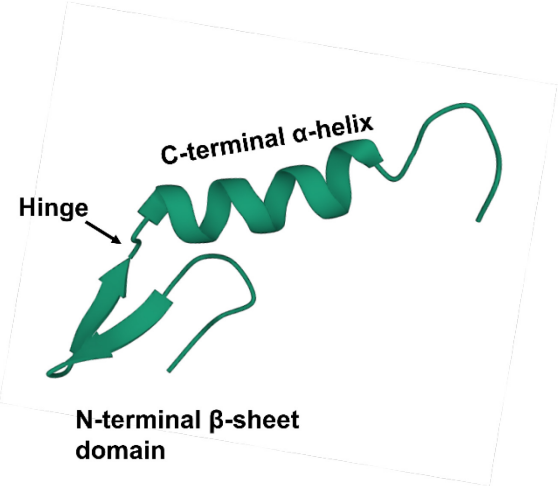


Figure 1.5 Leucocin A. Domain structure of type IIa bacteriocins. (Adapted from L. Johnsen et al., 2005[95]).

Type IIa bacteriocins are usually 35 – 48 amino acids long, they are stable under heat, and they are cationic. They share a common feature, an N-terminal sequence (KYYGNGL/VXCXKXX) which has been found to be unstructured in water but in the presence of membrane mimicking environments such as detergents, DPC (dodecylphosphocholine) micelles, or in the presence of TFE (2,2,2-trifluoroethanol), they shape into an antiparallel β-sheet where one disulphide bond helps to stabilize the structure (Figure 1.5). This feature has been identified to play an important role in antimicrobial activity [96]. Studies have shown that the absence of the disulphide bond can drastically diminish the antimicrobial activity in this type of bacteriocins[97].

The N-terminal β-sheet is followed by a C-terminal domain, which is less conserved in its sequence. This domain has been identified as the domain associated in recognition of target strains[96]. Type IIa bacteriocins may have a hairpin structure stabilized with a second disulphide bond between a cysteine residue in the middle of the helix and a cysteine located in the terminal part but most of the type IIa bacteriocins have just one disulfide bridge. In the middle of the two domains, there is a flexible hinge which is believed to provide flexibility to the structure so it can enable the C-terminal domain to dip into the hydrophobic part of the bacterial membrane [98]. Type

IIa bacteriocins can lack the two cysteine residues in the C-terminal region. Instead, they can contain two tryptophan residues which accommodate into the bacterial membrane to exert their mechanism of action[95].

In 2010, Haugen et al. conducted an investigation to comprehend the role of each domain in type IIa bacteriocins. They constructed diverse chimeras via site-directed mutagenesis, amalgamating the principal domains of various type IIa bacteriocins. These hybrid constructs were subsequently tested against a diverse panel of bacteria., and the results showed that by exchanging the C-terminal part, there was a difference in the targeted strains, suggesting this domain dictates the antimicrobial profile. It is involved in receptor recognition, whereas the N-terminal region is necessary to exert the mechanism of action[96].

Although most of the IIa bacteriocins share these common features, it has been found that the structure may change even when they contain similar amino acid sequences. Carnobacteriocin B2 has similarities to the sequence of other IIa bacteriocins like leucocin A and it contains a disulphide bridge between the two conserved cysteines, Cys9 and Cys14 but the N-terminus part is highly disordered in comparison to the well defined β-sheet of the other type IIa bacteriocins, which suggests that the interaction with the membrane occurs in the central amphipathic helix (Figure 1.6)[97,98].

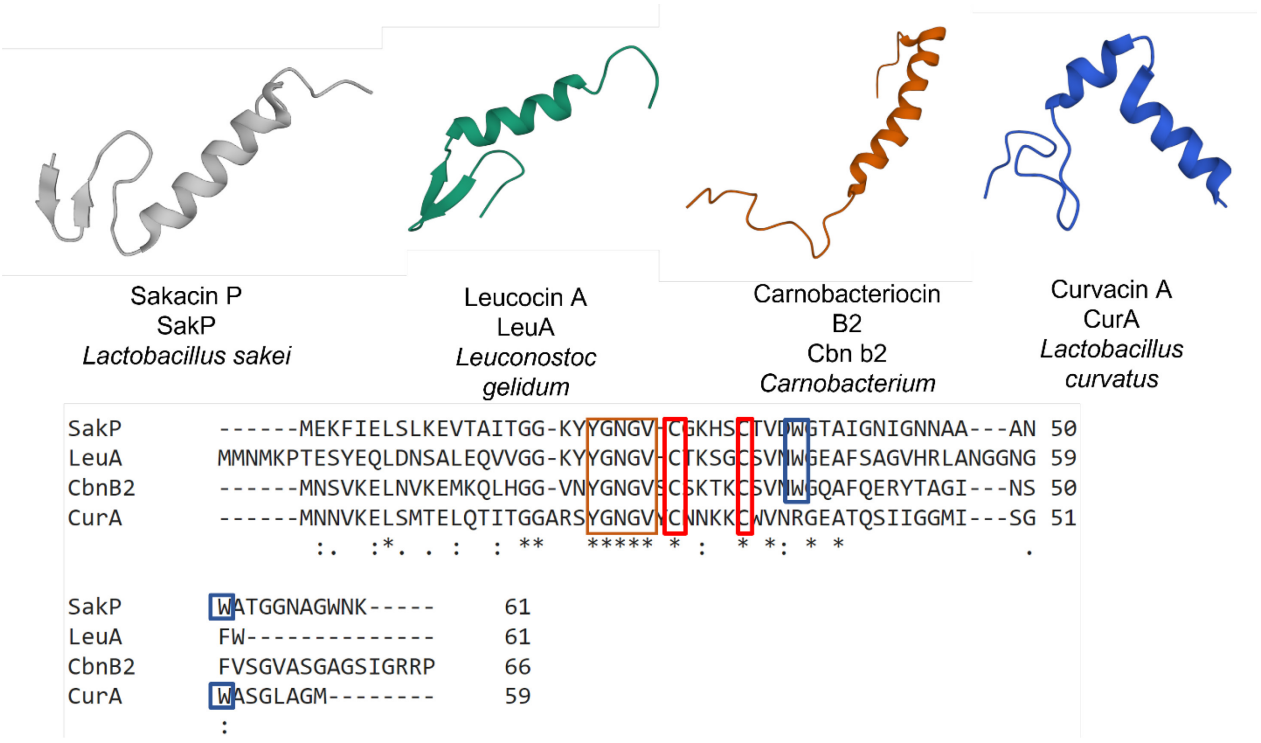


Figure 1.6 Different types of IIa bacteriocins and their 3D structure. Orange box: Common N-terminal sequence amongst type IIa bacteriocins. Red boxes: Cysteines which usually form disulphide bridges. Blue boxes: tryptophans that play an essential role in the stabilization and mechanism of action of some type IIa bacteriocins.

1.12.1 Genetics and immunity of type IIa bacteriocins

Type IIa bacteriocins require at least four genes for their production: i) The structural bacteriocin gene, ii) a gene which encodes an immunity protein which helps to protect the producer bacteria from its bacteriocin, iii) an ABC transporter (ATP-binding cassette) gene, which is necessary to export the bacteriocin and, iv) a gene that encodes a protein whose function is still unknown[99]. These genes are primarily located on plasmids, but it has been found that genes from some IIa bacteriocins can also be located on the bacterial chromosome, like sakacin P or carnobacterium B2 and BM2. These operons can be in different loci, and the organization of the four genes may vary[94].

These bacteriocins are firstly produced as prebacteriocins with an N-terminal leader sequence which gets removed by proteolytic cleavage during their secretion to yield a mature bacteriocin. Some studies suggest that without the signal peptide, the bacteriocin would not be

recognized by the ABC transporter[100]. This sequence also protects the bacteria from being killed by its bacteriocin by keeping the bacteriocin inactive in the cytosol[99].

The bacteriocin and immunity genes are usually located in the same operon, and they are expressed simultaneously. Immunity proteins contain around 88 – 115 amino acids, and it has been found that they have a high degree of specificity according to the bacteriocin that they protect against, but it can also be possible to find cross immunity amongst type IIa bacteriocins[95].

1.12.2 General mechanism of action of type IIa bacteriocins

The reported antimicrobial spectrum of IIa bacteriocins is distinct for every member of this family. Still, most studies have found that these bacteriocins have a general bactericidal activity against Gram-positive bacteria such as *Bacillus* spp., *Staphylococcus* spp., *Brochotrix* spp., but mainly against *L. monocytogenes*[101].

Multiple mechanisms of action have been proposed for type IIa bacteriocins. Like other LAB bacteriocins, one of the most accepted mechanisms relies on electrostatic interactions where it is known that bacteriocins can form poration complexes that produce ionic imbalance and leakage of vital molecules, which has been demonstrated with PA-1, mesentericin Y105 and Bavaricin MN[94]. Type IIa bacteriocins seem to cause a partial dissipation of the proton motive force by disrupting the transmembrane potential and pH gradient, which then causes intracellular depletion of ATP, followed by other biochemical changes that will eventually cause cellular lysis[102].

Studies have shown that the damage caused by IIa bacteriocins depends on bacteriocin concentration and lipid composition of the target membrane, and that the pH also plays an essential role in these bacteriocin's mechanism of action[99,98]. Some experiments on type IIa bacteriocins suggest pH 7.5 to 6.0 can improve membrane binding and permeabilization[101].

Since type IIa bacteriocins are amphiphilic, these bacteriocins can form pores by following the "barrel-stave" model, where the initial step occurs with an electrostatic bonding between the positively charged residues of the bacteriocin located in the N-terminal β-sheet and the anionic phospholipid groups of the bacterial membrane[99]. To form the pore, a structural rearrangement of the α-helixes of the bacteriocin and the lipid acyl chain occurs on the bacterial membrane (Figure

1.7). Membrane destabilization by the accumulation of bacteriocin molecules along the bacterial membrane has also been suggested, but with less evidence available[94].

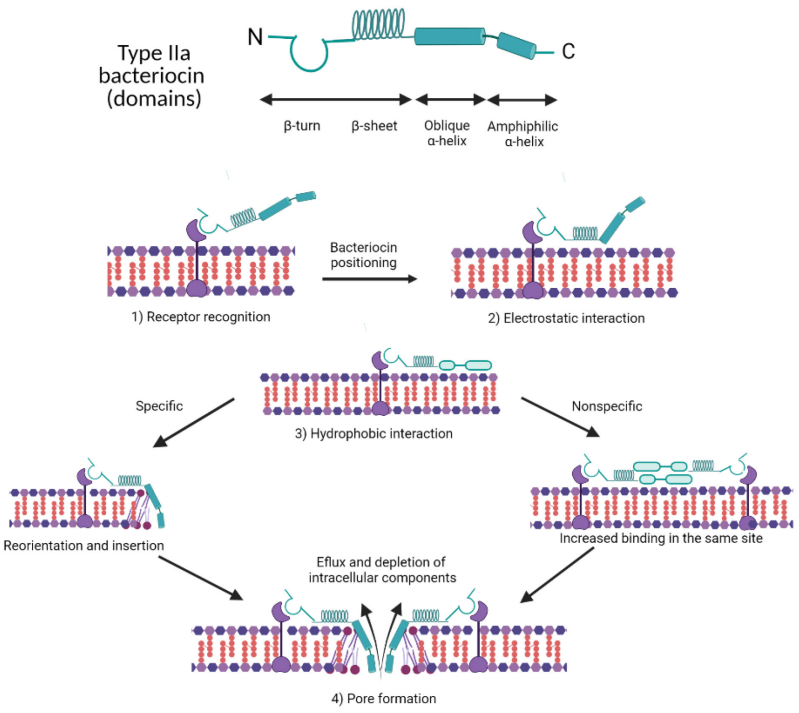


Figure 1.7 Main domains and representation of the proposed mechanisms of action of type IIa bacteriocins (Adapted from S. Ennhanar et al., 2000[94]; created with BioRender.com).

The idea of a mechanism of action dependent on a specific receptor has been studied over the past few years. In an experiment by Nissen-Meyer, mutated fragments of pediocin PA-1 were obtained and tested along with non-modified bacteriocin Pediocin PA-1. One of the mutants, a 15-mer, was able to inhibit the activity of the native bacteriocin, suggesting that the fragment was able to function as a competitive inhibitor of type IIa bacteriocins.[96,102]

Meanwhile, in another study, Diep *et al*. found that the immunity protein of lactococcin A, a type IId bacteriocin[103,104] , binds to subunits C and D of the mannose phosphotransferase system (Man-PTS), a cell membrane protein, to prevent the producer strain *L. lactis* from being killed by its bacteriocin. Thus, their work suggested that subunits C and D of this receptor might be the target of type IIa bacteriocins[105]. Phosphotransferase systems can only be found in bacteria but not in

eukaryotes. Therefore, understanding their role in the mechanism of action of bacteriocins is crucial to developing new strategies to target this attractive system.

1.12.3 The mannose phosphotransferase system

Bacteria have adapted to utilize various forms of carbohydrates as their energy sources. The Man-PTS, a transmembrane protein, serves as the primary transporter for mannose uptake in certain bacteria; however, it also demonstrates the ability to recognize and absorb glucose. The Man-PTS has been widely suggested as the potential receptor for type IIa bacteriocins. Multiple studies, involving resistant strains of *L. lactis* and *L. monocytogenes*, have observed a decreased expression of Man-PTS genes, further supporting this hypothesis[80,106].

Man-PTS belongs to a big family of sugar transporters known as the phosphoenolpyruvate: carbohydrate phosphotransferase systems (PTS), which are essential for bacterial survival and adaptation. PTS are found in only about 60% of bacterial species, few archaea but not in eukaryotic cells[107].

PTS consist of two cytoplasmic proteins: EI and HPr, and a transmembrane enzyme known as EII. The EII complex is important for sugar recognition, and it consists of three subunits: IIA, IIB and IIC. Man-PTS is unique in this family since it is the only PTS which possesses an IID subunit. EI and HPr transfer phosphoryl groups from phosphoenolpyruvate to the IIA and IIB units, which then pass the phosphoryl group to the sugar translocated by the IIC/IID subunits. Studies have shown that subunits IIC and IID have evolved in parallel and when these subunits are expressed separately, the units are unstable and nonfunctional[108].

Interestingly, amongst all the PTS families, the mannose phosphotransferase system seems to be the only PTS involved in the mechanism of action of bacteriocins type IIa, IId, and IIe. Many aspects of the man-PTS are unique: as it has been mentioned, man-PTS is the only one with an IID subunit and also is the only member where there is the presence of a phosphorylated histidine residue instead of a cysteine in the IIB subunit and besides its function of sugar transportation, studies have showed that the man-PTS plays a role in the infection of *E.coli* by the bacteriophage λ[109].

Multiple studies have attempted to understand which part of the receptor is involved in the mechanism of type IIa bacteriocins since they are important prospects to be used as antibiotics or food preservatives[96,97]. An important breakthrough in this field involved aligning numerous amino acid sequences of Man-PTS from various bacteria. These alignments revealed high conservation among the sequences, with minor variances observed in specific regions of the subunit IIC and IID sequences. These slight differences might play a crucial role in the recognition process by type IIa bacteriocins.[110],[111].

In a heterologous expression experiment[111], the sequences of subunits IIC and IID from *L. monocytogenes* were expressed in a resistant *L. lactis* strain. The mutated strain showed sensitivity against a panel of type IIa bacteriocins. Furthermore, this experiment involved creating various strains where subunits IIC and IID were expressed together and separately. The findings revealed that when only one subunit is expressed, it does not confer sensitivity to the bacteriocins. This suggests that both subunits are crucial for the mechanism of action of bacteriocins, while the remaining components of the Man-PTS appear to be less significant in this process.[105].

In the identical investigation, scientists cloned and expressed the genes *mptC* and *ptnC*. These genes code for the mannose-specific IIC component in *L. monocytogenes* and *L. lactis*, respectively, leading to the generation of various chimeras. One of these chimeras consisted of the N-terminal segment of MptC and the matching sequence of PtnC along with the *L. lactis* IID component. Conversely, the other chimera featured the N-terminal region of PtnC and the complementary segment of MptD[111].

The findings indicated that only the chimeras incorporating the N-terminal section from MptC displayed sensitivity to class IIa bacteriocins. Additionally, the results revealed that the interaction between the bacteriocins and the receptor is contingent on the sequence; the bacteriocins specifically interact with a defined region of 40 amino acids within subunit C. Notably, sequence alignment highlighted that the most conserved areas on subunit C corresponded to residues 85–99 in *mptC* and 87–103 in *ptnC*, predicting the formation of an extracellular loop. These predicted extracellular loops shared similarities with other transporters, where such sequences have been recognized as critical for sugar recognition and transport[111].

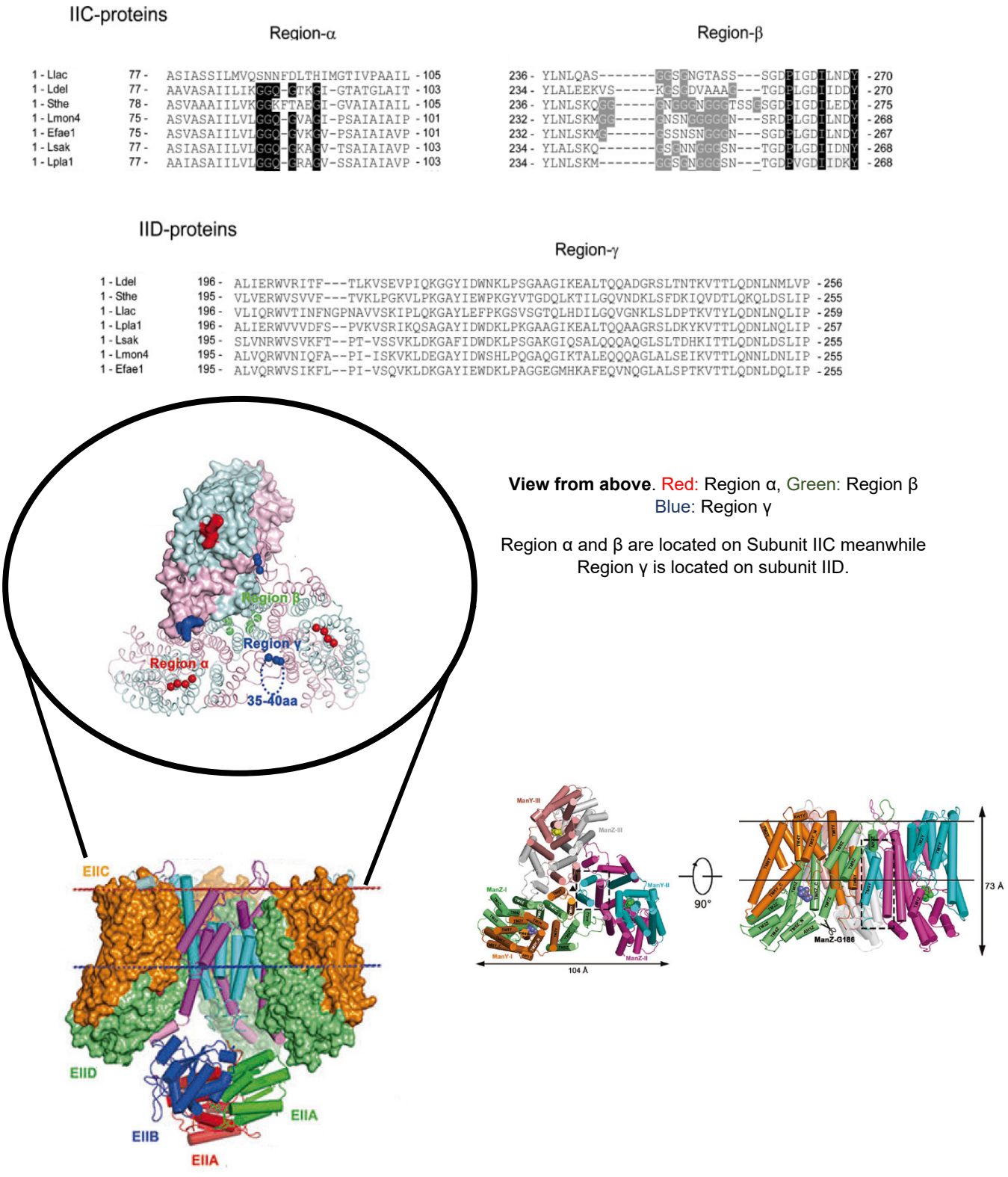
In a separate investigation [112], a multiple sequence alignment was conducted to determine whether Man-PTS from various bacteria could serve as a receptor for class IIa bacteriocins. The study also involved heterologous expression experiments of these sequences in a *L. lactis* resistant strain. The findings revealed that the cloned sequences exhibited differing degrees of sensitivity ranging from high sensitivity to low sensitivity or no sensitivity at all in the mutants. The study suggested the presence of three key regions (α, β, and γ) within the Man-PTS, where the interaction between bacteriocin and the receptor takes place. Variances in the amino acid sequences within these regions were found to influence bacteriocin sensitivity or resistance, with two of these regions situated in subunit C and one in subunit D [113].

Region α was identified in the N-terminal half of the IIC subunit, and it contains the conserved sequence GGQGXXG. Region β is located in the C-terminal end of the IIC protein, and presents the conserved sequence DP[I/LV]GD[I/L] [D/E/N]XY. Region γ can be found in the IID subunit, and is a 35 – 40 amino acid sequence which was not found in all the sensitive bacteria, but when it is present, bacteria showed higher sensitivity to type IIa bacteriocins [114,109].

In a study made by Liu X. et al.[115], a cryo-EM structure of *E.coli* man-PTS at a resolution of 3.52 Å was reported. In this report, they describe how the ManY and ManZ components (which correspond to subunits IIC and IID respectively) are organized along the cellular membrane.

The cryo-EM structure showed that the ManYZ complex is composed of three protomers that are related to each other. Each unit is composed of two subunits: ManY and ManZ which share a similar folding. This whole structure is parallel to the membrane.

According to the reported by Liu X. et al., "ManY contains nine transmembrane helices (TM1-9Y) and one horizontal periplasmatic amphipathic α-helix (AH1Y), with N- and C-termini on periplasmic and cytoplasmic sides. On the other hand, ManZ also contains nine transmembrane helices (TM1-9Z) and two horizontal amphipathic α-helices (AH1Z and AH2Z), with N-and C-termini on cytoplasmic and periplasmic sides". The oligomerization of ManYZ occurs by the interaction of the C-terminal of TM8Y and TM9Y of ManY through hydrophobic residues. In this study, the α, β, and γ are also shown in the cryo-EM structure (Figure 1.8)[109].



View from above. Red: Region α, Green: Region β Blue: Region γ

Region α and β are located on Subunit IIC meanwhile Region γ is located on subunit IID.

Figure 1.8 Region α, region β and region γ in the MPT receptor (Figure adapted from Kjos M.[110], and Liu X.[115]).

As previously mentioned, bacteriocins resistance mechanisms can be grouped into two: acquired resistance or innate resistance. Acquired resistance is believed to be obtained after continuous exposure to a particular antimicrobial agent by generating a spontaneous mutation in genes associated with the bacteriocin's mechanism of action, like host-cell receptors, cell wall synthesis or transcriptional regulation components of metabolism. In contrast, innate resistance is the intrinsic ability of a bacterial species to resist the activity of a specific antibiotic or antimicrobial agent due to its inherent structure[116,117].

Listeria monocytogenes can detect and adjust to various environmental conditions through the utilization of gene regulators [90]. The expression of virulence factors gets repressed by high concentrations of fermentable carbohydrates, which can only be found outside the host[86]. Bacteria sense environmental conditions through receptors of two-components, which rely on phosphorylation for signal transduction. These systems are usually found embedded in the bacterial membrane with a cytoplasmic component which responds to extracellular changes by mediating changes in gene expression. For example, resD-ResE is a two-component regulation system that is necessary to activate the expression of the transporters involved in the intake of glucose and β-glucosides such as arbutin, salicilon or cellobiose[118].

In a study of a *L. monocytogenes* resistant strain to leucocin A, a type IIa bacteriocin, it was observed that the expression of a mannose transporter, the IIAB Man-PTS operon was decreased[119]. In a similar work, resistant *L. lactis* strains to the same bacteriocin, were mutated to heterologously express the mannose transporter operon of sensitive strains of *L. monocytogenes*. The mutated strains ended up becoming sensitive to LeuA[120]. The correlation of ResD-ResE expression and the expression of the Man-PTS operon have been conducted, with a focus on studying how these expression changes are linked to developed resistance against bacteriocins[73,121].

The mechanism of action of many bacteriocins relies on interactions with the bacterial membrane. Therefore, bacterial changes in the membrane by mutations through acquired resistance can decrease or block these interactions by changing the membrane fluidity or modifying the overall charge of their membrane components[122,22].

In a study done by Balay et al. in 2018[123], the impact of utilizing various carbohydrates (glucose, sucrose, fructose, mannose, and cellobiose) on the growth of strains of *L. monocytogenes* in the presence of carnocyclin A and leucocin A was examined. The *L. monocytogenes* strains were

cultivated to a cell density of approximately 10^4 CFU in basic media containing each carbohydrate. These strains were initially exposed to bacteriocins at a concentration of 3.3 mM, which has been identified as the minimum inhibitory concentration for carnocyclin A and leucocin A in broth. Upon a second exposure to bacteriocins, two of the five *L. monocytogenes* strains, FSL R2-499 and FSL C1-056, developed resistance. This resistance was evident in the presence of cellobiose and sucrose, characterized by a brief lag phase in the bacterial growth curve compared to other strains and cultures without bacteriocin. Intriguingly, it was observed that these strains took longer to develop resistance against carnocyclin A in comparison to leucocin A. These findings indicate that the presence of distinct carbohydrates does indeed impact the capacity to generate bacteriocin resistance in *L. monocytogenes.* However, the mechanism behind this process remains unknown[123].

In unpublished work from our research group, Dr. Kaur demonstrated that mutations in a singular amino acid in the sequence of the IID protein of the Man-PTS could be implicated in conferring resistance to *L. monocytogenes* against leucocin A. Five *L. monocytogenes* strains were tested for sensitivity against leucocin A and one out of five strains presented resistance (*L. monocytogenes* 507) (Table 1.6). In the same way, six *L. lactis* strains were tested against the same bacteriocin. *L. lactis* it is known to be naturally resistant to leucocin A[120], nevertheless, in this experiment, one out of the six strains was found to be sensitive (*L. lactis* ATCC 14365).

When the Man-PTS IID DNA sequence was obtained from all the tested strains, it was found that *L. monocytogenes* 507 presented a mutation in just one amino acid in comparison to the other *L. monocytogenes* strains (Figure 1.9).

Table 1.6 Results from Dr. Kaur spot-on-lawn assays where *L. monocytogenes* and *L. lactis* strains were tested for their sensitivity against leucocin A.

	Strain	Resistance (R)/ No Resistance (NR) against leucocin A
L. monocytogenes	EGDe	NR
	LI052	NR
	LI0507	R
	LI0510	NR
L. lactis	IL403	R
	MG1363	R
	DSM4645	R
	ATCC 11454	R
	ATCC 19435	R
	ATCC 14365	NR

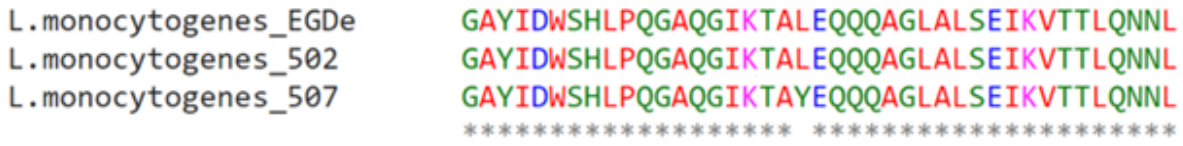


Figure 1.9 Amino acid sequence of the MPT-IID extracellular γ-region of *L. monocytogenes* 502 and 507 compared to the genomic strain (EGDe).

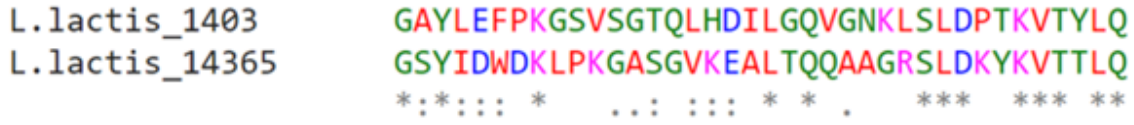


Figure 1.10 Amino acid sequence of the MPT-IID extracellular region of *L. lactis* 1403 and 14365.

Interestingly, *L. lactis* ATCC 14365 share similarities to the sequences of *L. monocytogenes*, suggesting that the changes in this sequence might be implicated in the conferred sensitivity to leucocin A and pediocin PA-1 (Figure 1.10, Figure 1.11).

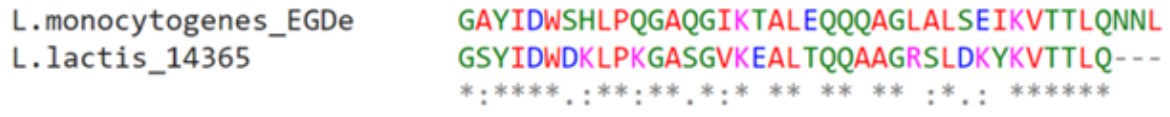


Figure 1.11 Amino acid sequence of the MPT-IID extracellular γ-region of *L. monocytogenes* EGDe compared to amino acid sequence of the MPT-IID extracellular γ-region of *L. lactis* 14365.

As discussed in the cryogenic electron microscopy structure of *E. coli* Man-PTS published by Liu et al. (2019), subunit IIC and IID do not share a similar amino acid sequence but fold similarly. They are related to each other by two-fold pseudosymmetry axis passing through the center of both subunits and running along the midplane of the membrane. The pseudosymmetric subunits are composed of two re-entrant loops (hairpins HP1 and HP2) and five transmembrane helices (TM1–5). Interestingly, the 28 amino acid sequence which is believed to be involved in the conferred resistance to bacteriocins in the unpublished experiments done by Kaur, falls into one of the re-entrant loops, the gamma-loop region of MPT systems in the IID subunit (Figure 1.12)[108,115]

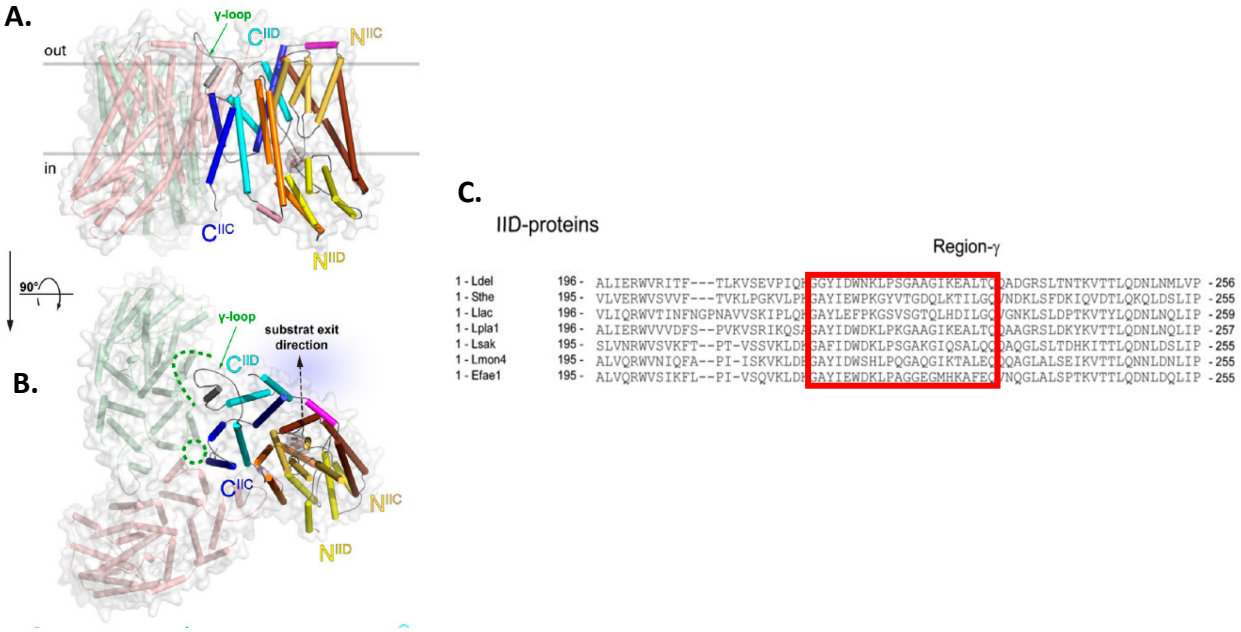


Figure 1.12 Trimer of three IIC/IIDMan protomers. **A.** Parallel to the membrane. **B.** The periplasmatic side in bacteria. IIC subunit (blue, brown, dark yellow), IID subunit (cyan, ochre, yellow). The helix bundle is composed of TM4, TM5 of IIC (blue) and TM3 of IID (cyan), and the gamma loop (green) provides the contact interface between the promoters of the trimer. **C.** Sequence of the region-γ located on the IID subunit from different bacteria. The red square shows the mutated region found in unpublished experiments done by Kaur.

1.12.3.1 Leucocin A and the Man-PTS

Leucocin A is a class IIa bacteriocin produced by *Leuconostoc gelidum*, a LAB that was isolated from vacuum packaged meat. This bacteriocin is 37 amino acids long with a calculated molecular weight of 3930.3. It is stable at low pH and is heat resistant. It can be inactivated by proteolysis, and it is usually isolated by acid precipitation and several sequential chromatographic techniques. Leucocin A has been found to be active against Gram-positive bacteria such as *Enterococcus*, *Carnobacterium*, *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Clostridium*[124,125].

Leucocin A, in the presence of membrane-like environments, folds as most of the type IIa bacteriocins: the N-terminus is shaped as a three-strand antiparallel beta-sheet from residues 2 – 16, while the C-terminus forms an alpha helix which contains one disulphide bond between Cys9 and Cys14 [126] (Figure 1.13).

Type IIa bacteriocins share multiple structural similarities, but the difference in their biological activity suggests that a critical molecular interaction must occur between a membrane receptor and the bacteriocin, which still is not well understood.

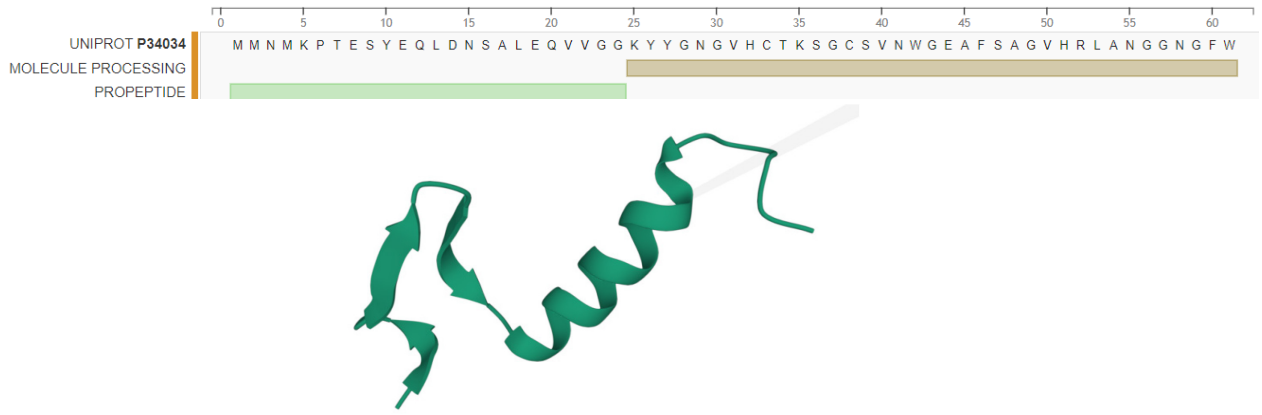


Figure 1.13 Leucocin A structure in DPC micelles (P34034).

1.13 Circular bacteriocins

Circular bacteriocins are antimicrobial peptides produced by Gram-positive bacteria, forming cyclic structures with an N-to-C terminal covalent linkage. These peptides are classified into Class I bacteriocins (modified peptides), and they are active against a broad range of bacteria which includes *L. monocytogenes* and *C. perfringens*. Circular bacteriocins are initially produced as linear structures that contain a leader sequence that gets cleaved during maturation. The mature cyclic structure is usually 58 – 70 amino acids long, and the molecular weights are around 5.6 – 7.2 kDa[127,128].

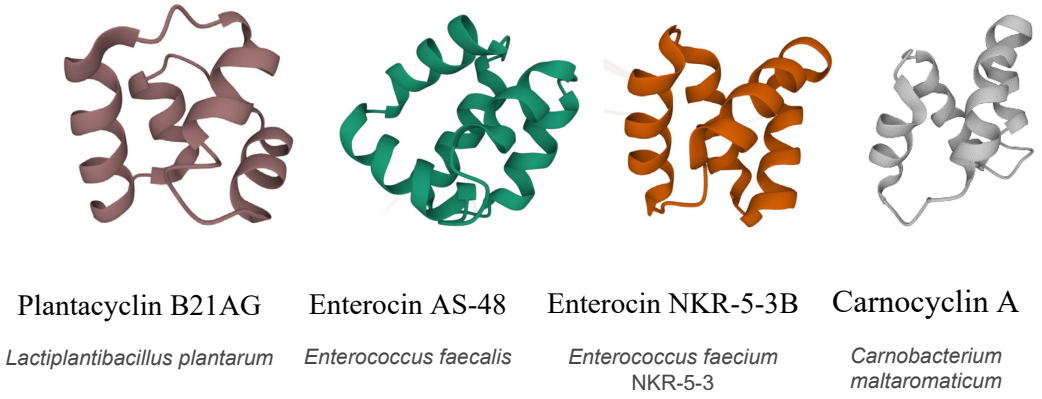


Figure 1.14 3D structure of circular bacteriocins (PDB: 6WI6, 1E68, 2MP8, 2KJF).

These peptides are stable under a wide pH range. In addition, they are heat and proteolytically stable. Until now, 20 circular bacteriocins have been characterized and isolated from sources including fermented foods, mammalian feces and wound exudates. They have been classified into two main families according to sequence similarity and biochemical characteristics[129]. Family I circular bacteriocins are cationic and have a high isoelectric point (pI ~10) and include enterocin NKR-5-3B, amylocyclicin, amylocyclicin CMW1, enterocin AS-48, bacteriocin 21(identical to AS-48), carnocyclin A, circularin A, thermocin 458, garvicin ML, lactocyclicin Q, leucocyclicin Q, pumilarin, uberolysin and cerecyclin[130]. Family II members are more hydrophobic with a lower isoelectric point (pI ~5) and include gassericin A/reutericin 6, butyrivibriocin AR10, acidocin B, paracyclicin, plantaricyclin A and plantacyclin B21AG[127,130].

NMR spectroscopy has been a helpful tool to elucidate the complete structure of circular bacteriocins. These compounds contain four to five alpha helices with a hydrophobic core (Figure 1.14). Studies on Enterocin AS-48 suggest that the circular shape of these peptides provides significant stability against physical stress, but it is not essential for the antimicrobial activity since the linear peptide has still shown antimicrobial activity. However, this activity seems to be considerably less than the one shown by the circular peptide[131,132].

1.13.1 Carnocyclin A

Carnocyclin A (CclA) was isolated from *Carnobacterium maltaromaticum* UAL3017 through activity-guided purification. Carnocyclin A is a cationic peptide of 60 amino acids and has a molecular weight of 5862 Da. It possesses a high isoelectric point (~10) due to the presence of many hydrophobic residues, and it is stable under different temperatures (-80 °C – 100 °C), pH (2 – 12) and it is resistant to proteases. Carnocyclin A is active against *L. monocytogenes*, *S. aureus*, *E. faecium*, *E. faecalis* and other Gram-positive bacteria[93]. CclA forms anion-selective pores by interacting electrostatically with the bacterial lipid bilayer. NMR experiments on the structure of CclA showed that the peptide is shaped as a globular structure with four helices with a hydrophobic core where the amide bond linking the N- and C- termini of the peptide has been located in helix α4 (Figure 1.15)[133,134].

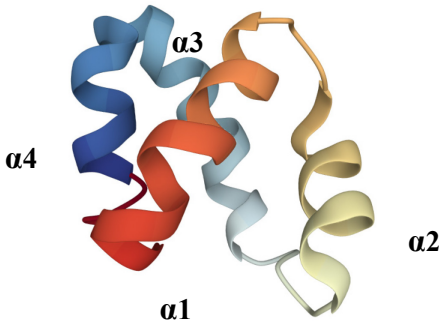


Figure 1.15 Carnocyclin A 3D NMR structure (PDB:2KJF).

1.13.2 Genetics and Biosynthesis

The genes for circular bacteriocin biosynthesis are located either in the bacterial chromosome or on plasmids. In addition, most of the encoded proteins are predicted to be hydrophobic, so the biosynthesis of these peptides might occur at the membrane[127,128].

The number of genes required for circular bacteriocin production and immunity is between five to ten. One of these genes encodes a leader sequence of 2 to 48 amino acids long, along with the core peptide. Another gene encodes a cationic and hydrophobic protein which provides immunity to the host organism[127]. The other genes include the information to produce an ABC transporter which consists of an ATPase and putative proteins whose function remains unknown. However, some studies have shown they are necessary for bacteriocin immunity or biosynthesis[105].

The production of circular bacteriocins requires three main steps: cleavage of the leader sequence, circularisation, and peptide export. To date, it still is unclear how the circularisation process occurs. The N- and C- terminal are believed to play a significant role, since it has been found that most of the circular bacteriocin ends contain aromatic and hydrophobic residues. As the circulation point is located in the internal part of the alpha helix, it has been hypothesized that a predominantly hydrophobic and sterically hindered region is needed for this process[128,131].

1.13.3 Mechanism of action of circular bacteriocins

Similar to some other types of bacteriocins, the general mechanism of action of circular bacteriocins relies on pore formation by electrostatic interactions with the membrane. The position of every amino acid in the sequence of these peptides plays a vital role in providing stability to the structure, but their position is also crucial for the mechanism of pore formation[135]. The position of buried aromatic residues appears to be conserved amongst circular bacteriocins, along with cationic residues like lysine and histidine in a helix α3 and α4, which appear in polycationic clusters[128,130].

From crystallography experiments on enterocin AS-48, it is known that this bacteriocin can form dimers. This dimeric structure is pH dependent. At a pH lower than three, the peptide is monomeric, and the protonation of glutamic acid residues play an important role in this conformational change[132]. Two mechanisms have been proposed for pore formation with circular

bacteriocins: dimeric and monomeric. In both mechanisms, the initial step starts with the attraction of the peptide to the anionic cell membrane. In the dimeric mechanism, following the binding to the bacterial membrane, a conformational change occurs on the peptide, where the enclosed structure with the hydrophilic residues in the inside, opens due to the interaction with the lipidic environment of the bacterial membrane[132]. In this mechanism, aromatic residues such as tryptophan or tyrosine promote peptide anchoring to the membrane to initiate pore formation. Mechanism 2 occurs similarly but just with one peptide (Figure 1.16)[131].

Currently, there are not many studies on the mechanism of action of circular bacteriocins belonging to Family II; it has been only suggested that gassericin A can cause cell disruption resulting in potassium ions efflux[136].

Further research has indicated variations in the pore-forming properties of these peptides. For instance, enterocin AS-48 has been observed to generate non-selective pores in liposomes, leading to the release of low molecular weight compounds. In contrast, carnocyclin A forms pores in lipid membranes that are anion selective and voltage dependent.[132,133]. Circular bacteriocins can also be active against Gram-negative bacteria only when the integrity of the outer membrane has been perturbed with EDTA[131].

It has also been proposed the mechanism of action of circular bacteriocins could be mediated by interaction with a receptor, this idea is still unclear but there is evidence which supports it[128].

Experiments in a membrane-like environments have shown that these peptides can be active without the presence of a receptor. However, the concentration of bacteriocin needed for these experiments was significantly higher than the concentration used in *in vivo* experiments, which suggests there is an alternative mechanism that may require the presence of a receptor[131,128].

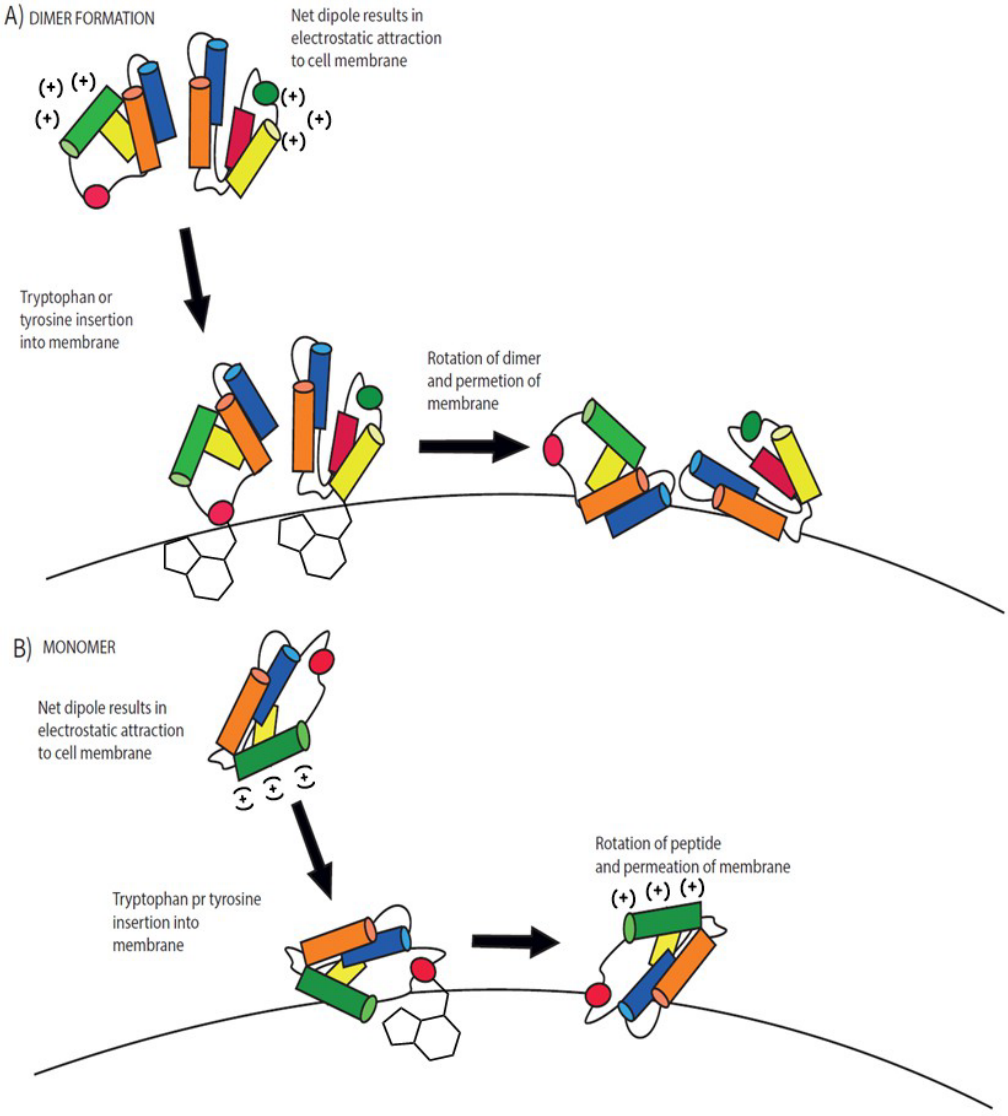


Figure 1.16 Circular bacteriocins' mechanism of action (Figure adapted from Towle *et al*., 2017)[131].

1.14 Lantibiotics

Lantibiotics are ribosomally synthesized antimicrobial peptides classified as type I bacteriocins, which are produced by many Gram-positive bacteria. These peptides contain lanthionine rings which are formed as posttranslational modifications (PTMs)[137].

The first common PTM in these peptides is the formation of 2,3-didehydroalanine (Dha) or 2,3-didehydrobutyrine (Dhb), which occurs by the dehydration of serine or threonine, respectively. This reaction is usually followed by the lanthionine ring formation, which occurs by a thioether linkage formed through a Michael-type addition of a cysteine side chain with either Dha or Dhb. This reaction yields a meso-lanthionine (from Dha) or (3-methyl)-lanthionine (from Dhb) (Figure 1.17)[138].

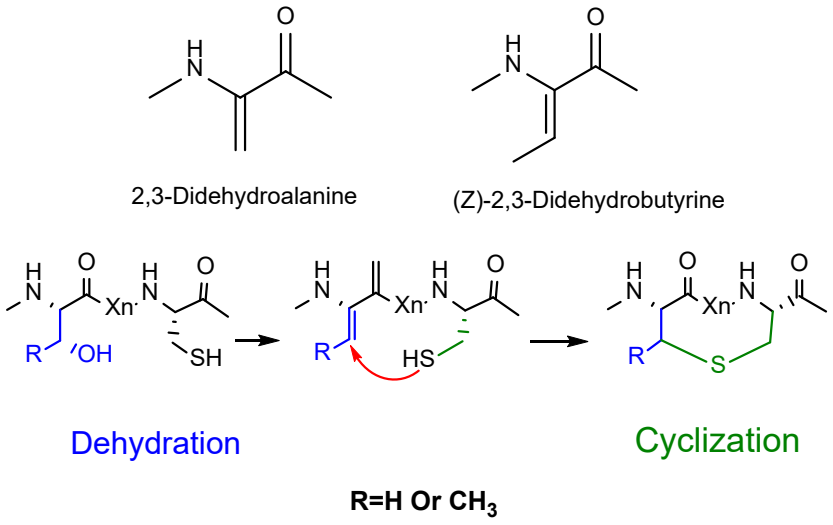


Figure 1.17 Lanthionine ring formation.

The lanthionine rings are present in all lantibiotics, which are classified based on their biosynthetic pathway. Class I lantibiotics are modified by two enzymes, LanB (dehydratase) and LanC (cyclase). Class II, III and IV require only one enzyme with dehydratase and cyclase activity: Lan M in class II, LanKC in class III and LanL in class IV(Figure 1.18)[138, 139]. These compounds are initially produced as a precursor peptide termed LanA, composed of an N-terminal leader peptide and a C-terminal core peptide. All the PTMs occur in the core peptide. In contrast, the

leader peptide promotes the PTMs in the core peptide and keeps it inactive before its translocation to the extracellular space by its ABC transporter[140].

These compounds' general mechanism of action can be grouped into two groups: i) mersacidin-like, where the bacteriocin inhibits cell wall biosynthesis by scavenging the peptidoglycan precursor, lipid II, or ii) nisin-like mechanism, where the peptide forms a pore that lyses the cell[141].

Lantibiotics can also be grouped according to their functional and structural features. Type A lantibiotics are long peptides with a similar arrangement. This group is also subdivided into two: Type-A(I), which contain peptides with several charged residues which either provide a negative charge overall or a slightly positive charge; meanwhile, type-A(II) are highly negatively charged. Type-B lantibiotics are peptides shorter than type-A and have a globular structure. All the lantibiotics exert their mechanism of action through interactions with lipid II, although there are important differences in how they interact with lipid II[142].

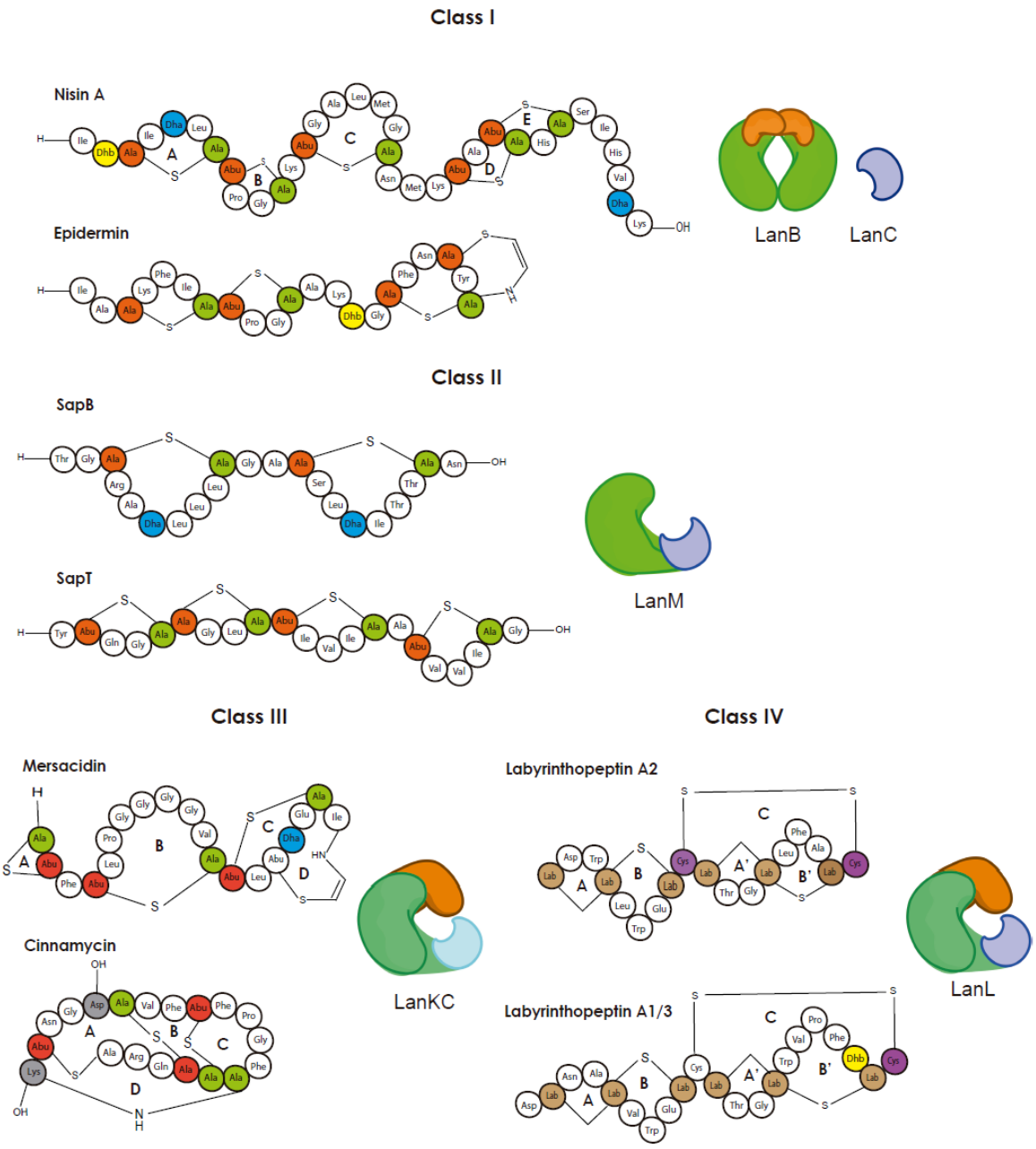


Figure 1.18 Representative examples of the four main classes of lantibiotics. This classification depends on the modifying enzyme. In class I the first PTM is mediated by LanB, which has a glutamylation/elimination domain (green-orange), whereas in class II the reaction is catalyzed by LanM with a kinase domain (green). Class III and IV are modified by the lyase/kinase-domain in LanKC and LanL (dark green/dark orange). The second PTM, cyclization, is a done by a zinc dependent enzyme, LanC in Class I and the LanC-like domain in Class II and IV (light blue). Class III peptides are modified by the LanC-like domain in LanKC which is independent from a zinc-ion (cyan).

1.15 The role of lipid II in the mechanism of action of lantibiotics

1.15.1.1 Type A bacteriocins

The shape of bacteria is maintained and adapted due to the presence of the peptidoglycan layer also known as “the sacculus”. The peptidoglycan is a conserved structure amongst bacteria which serves as an envelope to protect the bacterial cell and maintain its shape. Various bacteria exhibit diverse types of peptidoglycans. However, the majority of these peptidoglycans are created using identical building blocks, which are biosynthesized in the cytoplasm. Subsequently, these blocks are transported and connected to a polyprenyl phosphate lipid referred to as lipid II[143].

The sacculus always prevents bacterial lysis and it is composed of glycan chains linked by short peptides. In Gram-negative bacteria, the peptidoglycan layer is single whereas in Gram-positive bacteria is multilayered[144]. The growth of this layer requires the synthesis of new peptidoglycan which is incorporated into the existing layer. This process occurs in three main steps: first, the activated precursors, UDP-N-acetylglucosamine and UDP-N-acetylmuramyl pentapeptide are synthesized inside the cell. Second, in the existing peptidoglycan layer found in the inner membrane, the nucleotide precursors are added through its undecaprenyl phosphate moiety to form the lipid-anchored disaccharide-pentapeptide monomer subunit, lipid II. Lastly, lipid II is polymerized releasing undecaprenyl pyrophosphate resulting in a glycan chain which is inserted into the sacculus[145]. This mechanism is regulated and guided by enzymes and elements of the bacterial skeleton, and it varies amongst species (Figure 1.19)[144,145].

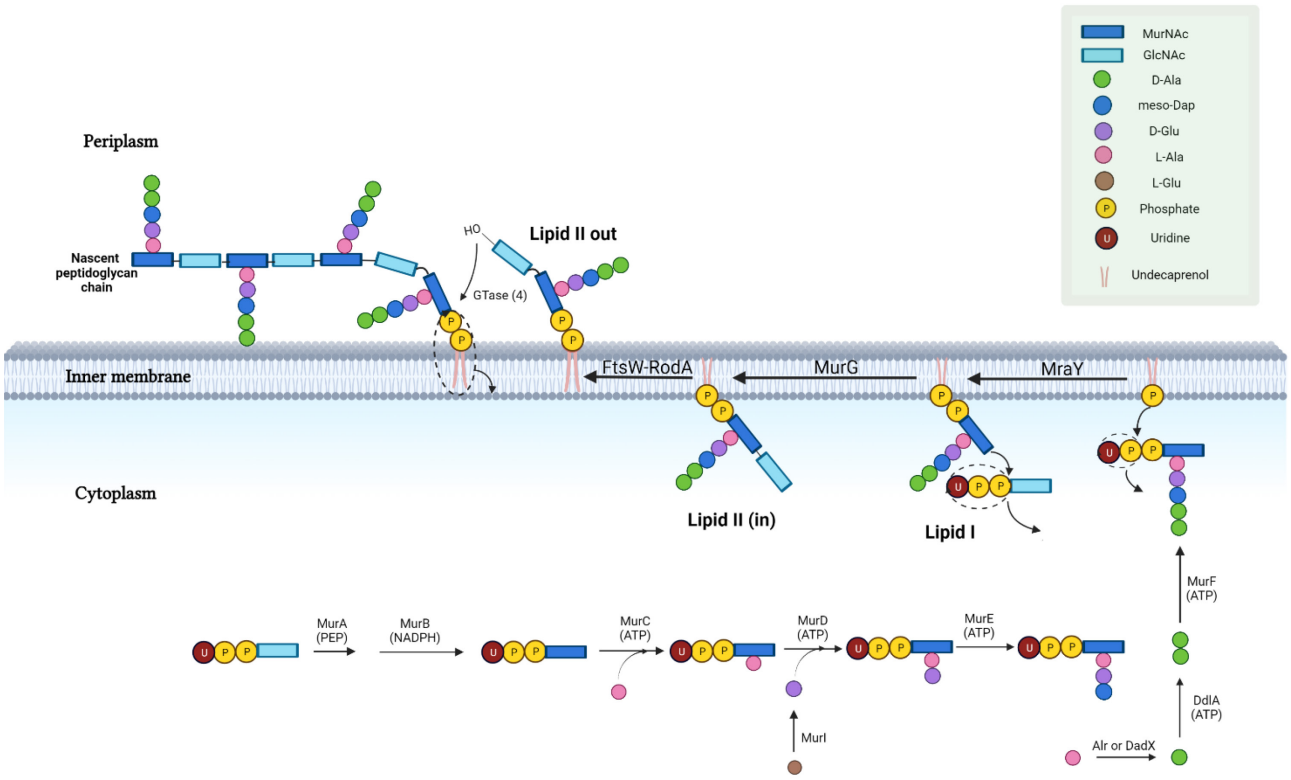


Figure 1.19 General mechanism of peptidoglycan biosynthesis in bacteria (adapted figure from Typas A. et al.[145] in BioRender)

Lipid II is the essential building block molecule of peptidoglycan biosynthesis. Each lipid II monomer contains the disaccharide unit N-acetylmuramyl-N-acetylglucosamine (MurNAc-GlcNAc) and a pentapeptide, as well as a bactoprenol carrier lipid (C_{55}-P), which is linked to the disaccharide unit via a pyrophosphate bridge (Figure 1.20)[146].

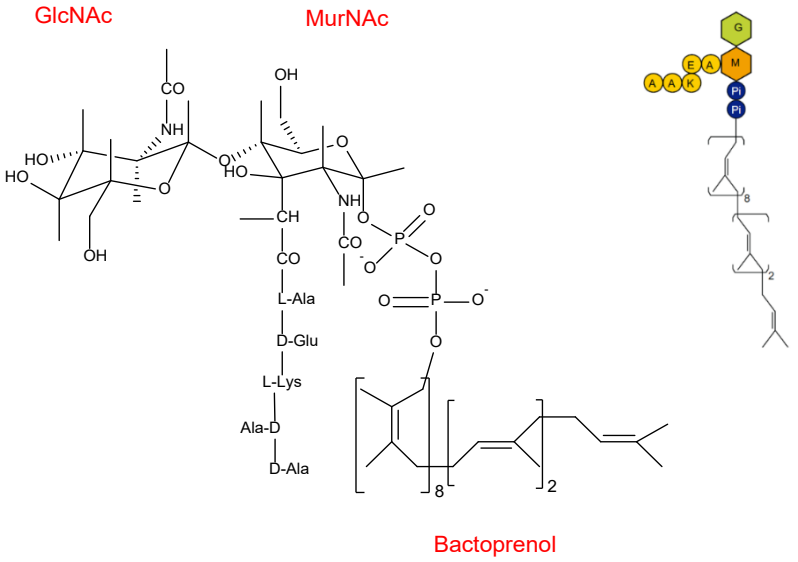


Figure 1.20 Chemical structure of lipid II in Gram-positive bacteria. The amino acid sequence in lipid II varies amongst species (adapted figure from Breukink et. al.[147]).

Nisin-like bacteriocins are elongated flexible peptides with a positive net charge. It is known that nisin forms pore complexes through a multi-step process which includes binding and insertion into the bacterial membrane, but how exactly the pores are formed is still not well understood[141].

The bactericidal activity of nisin was first described in a study done by Breukink et al., in 1999[148]. The effect of nisin was compared to maganin, a peptide of animal origin that is also known to cause dissipation of the proton-motive force. Both peptides were tested in a viability assay which correlated the ability of the peptides to permeabilize essential ions such as potassium and cause cell death in *Micrococcus flavus* a Gram-positive bacteria. The results showed that nisin was active in nanomolar concentrations whereas magainin required two orders higher to cause the same effect in *M. flavus* cells nevertheless, when the same assay was carried out in vesicles composed of an *M. flavus* lipid isolate from a chloroform/methanol extraction, the membrane permeabilizing activity of nisin considerably dropped, suggesting the presence of one or more specific molecules was needed to allow nisin's activity[148].

In the same study, the effect of nisin and maganin was tested on *M. Flavus* cells in parallel to vancomycin whose mechanism of action relies on blocking the cell wall biosynthesis. It was found that the presence of vancomycin inhibited the membrane leaking effect of nisin but did not affect the activity of magainin, thus these results suggested that lipid II is used as a docking molecule by nisin to permeabilize the membrane[148].

After this publication, the same author proposed nisin's mechanism of action, which starts with the binding of nisin to the bacterial membrane through an electrostatic interaction with the negatively charged phospholipid headgroups in lipid II and the C-terminal part of the peptide, where most of the positive charge is found. This step is followed by peptide insertion, which is believed to occur through the C & D nisin rings (containing multiple hydrophobic residues), which allows the peptide to twist into the membrane. Multiple monomers of nisin are needed to form a pore in the membrane[148].

A study done in 2001, showed that lipid II enhances nisin's mechanism of action by forming more stable pores[149]. In an NMR study done by Hsu *et al*. in 2003, it was also found that in comparison to the glycopeptide vancomycin, nisin binds to lipid II in a unique way since it forms a well-defined network of five intermolecular hydrogen bonds between the first ten N-terminal amino acids (rings A and B) and the pyrophosphate moiety of lipid II. Studies done on nisin-like bacteriocins such as subtilin, epidermin, gallidermin and mutacin 1140 have shown that since these peptides share similar structure, the mechanism of action is the same[150].

Experiments of the activity of nisin analogues in bacteria and in the presence of lipid II vesicles has been also tested to understand better the mechanism of action and efficacy of these compounds[151,152]. The tested variants included versions where rings A and B have been modified through mutations or fragments where only rings C, D and E are present. The results showed that these fragments bind tightly to lipid II in the vesicles, and *in vivo* experiments it was observed that these fragments could kill bacteria efficiently. The effect was also studied under fluorescence microscopy, and it was found that the fragments bind tightly to lipid II and cluster the molecule, thus relocating it from its functional location and blocking cell wall synthesis[153]. Nisin's dual mechanism of action of pore formation and inhibition of peptidoglycan synthesis make this bacteriocin a potent antimicrobial agent that is still broadly used[14].

1.15.1.2 Two-component lantibiotics

Two-component lantibiotics are composed of two peptides which are biosynthesized by the same gene cluster. Multiple studies have shown that these peptides can act either synergistically or individually to perform their antimicrobial effect on a broad range of Gram-positive bacteria. It has been observed however, for two-component lantibiotics such as haloduracin or lacticin 3147, that when both peptides interact together at equimolar concentrations (1:1 stoichiometry), the antimicrobial impact differs when these compounds are tested independently. They can either inhibit bacterial growth or eliminate bacteria entirely, showcasing either a bacteriostatic or bactericidal effect[141].

1.15.1.3 Lacticin 3147

Lacticin 3147 is a lantibiotic with two components: lacticin A1 a type B peptide and lacticin A2 a type A peptide. This peptide was first isolated from *L. lactis* subsp. *lactis* DPC3147, a strain found in Irish kefir grains. This bacteriocin has a broad-spectrum activity against Gram-positive bacteria which includes important pathogens such as *L. monocytogenes*, methicillin resistant *S. aureus* (MRSA), vancomycin resistant *enterococci* (VRE), penicillin-resistant *Pneumococcus* (PRP) and *B. subtilis*[154].

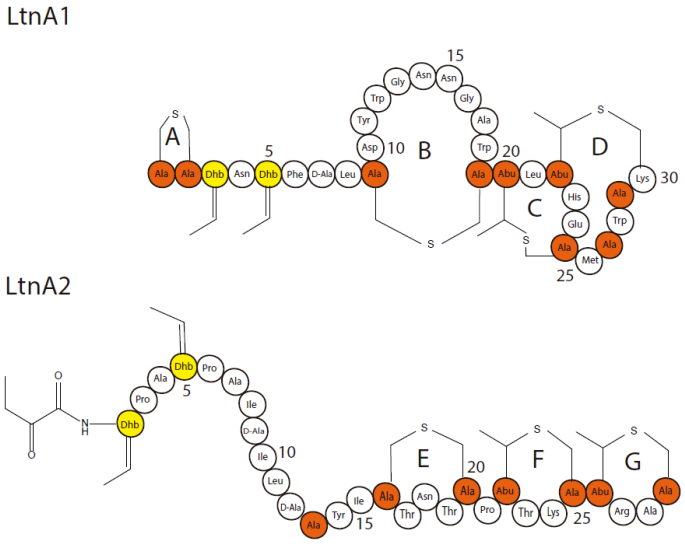


Figure 1.21 Two components of lacticin 3147, LtnA1 and LtnA2.

Lacticin A1 (Ltn A1) has a molecular weight of 3306.69 Da, and the active peptide is formed of 30 amino acids, whereas Lacticin A2 (Ltn A2) is formed of 29 amino acids with a molecular weight of 2847.40 Da (Figure 1.21)[154].

In 2017, a study done on the mechanism of action of lacticin 3147 by members of our research group was published where through NMR and isothermal titration calorimetry experiments, it was found that LtnA1 has a bacteriostatic effect in Gram-positive bacteria[142]. LtnA1 binds to lipid II in a 2:1 ratio forming a pyrophosphate cage through its C-terminal residues, thus inhibiting peptidoglycan biosynthesis (Figure 1.19). This binding stabilizes the conformation of LtnA1, which helps to promote the binding of LtnA2. The formed LtnA: LtnA2:LipidII complex adopts a transmembrane conformation, leading to a pore formation in a 2:2:1 ratio[142]. Lacticin 3147 forms pores that cause the dissipation of the membrane potential by leaking potassium and phosphate. The cell tries to recover these losses through the hydrolysis of ATP, but this event will eventually cause cell death[155].

On the other hand, LtnA2 has shown an alternative mechanism when tested separately. At micromolar concentrations, LtnA2 can kill bacteria through membrane lysis, but this effect must be further studied to understand how this mechanism works. When LtnA2 was tested on artificial membranes, no membrane lysis was observed with or without the presence of lipid II. In contrast, when LtnA2 was tested *in vivo* in whole-cell experiments, the bactericidal effect suggested Ltn2 requires an embedded biomolecule| to induce membrane lysis[142].

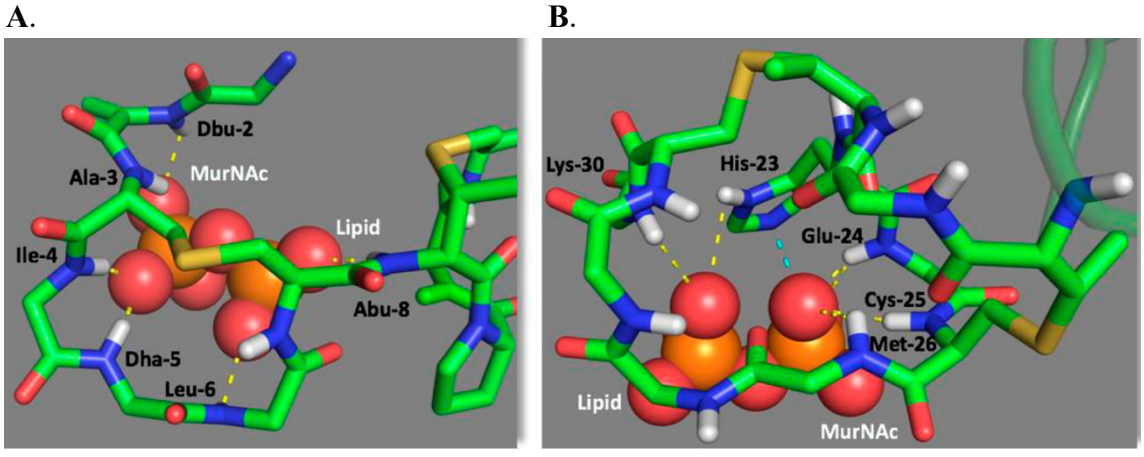


Figure 1.22 A. Nisin-lipid II pyrophosphate cage, **B.** LtnA1-lipid II cage (Modified figure from Bakhtiary et al, 2017).

1.16 Hypothesis

If bacteriocins are systematically investigated for their applications as antibiotics and in food technology, then their diverse antimicrobial properties will demonstrate significant potential for clinical use and food preservation. Additionally, an in-depth examination of bacteriocins' role in the evolutionary process will provide valuable insights into the adaptation strategies of bacteria, offering a nuanced understanding of the interplay between bacteriocin production, microbial competition, and potential implications for both medical and food-related contexts. Experimentation described in this thesis therefore aimed to test the following hypotheses:

1) Inter-family gene transfer transfers bacterocin genes and horizontal gene transfer results in strains with useful properties for probiotic applications.

2) Listeria monocytogenes develops bacteriocin resistance by mutations in phosphortransferase systems (PTS).

3) Isotope labeling of bacteriocins allow elucidation of the interaction of bacteriocins with the mannose-PTS, or their interactions with Lipid II

To test these hypotheses, I aimed to meet the following objectives:

1) To analyse the genome of Enterococcus canintestini to determine whether the genome encodes for novel bacteriocins, or for bacteriocins that were acquired by horizontal gene transfer from other organisms.

2) To analyse the genome of Listeria monocytogenes before and after development of stable resistance to carnocyclin A to determine whether resistance relates to mutations in PTS systems, or to unrelated mutations.

3) Labeling of leucocin A through SUMO fusion technology

4) Labeling of leucocin A through the design of a labelling media

Chapter 2

2. Bacteriocin mining in *Enterococcus canintestini* 49 for bacteriocin discovery

Bacteriocin mining, a flourishing field in microbiology and biotechnology, focuses on the systematic exploration and discovery of these natural peptides from various microbial sources. This process involves the isolation, characterization, and evaluation of bacteriocins for their potential applications in food preservation, probiotics, and clinical therapies. The quest for novel bacteriocins involves leveraging genomic, metagenomic, and bioinformatic tools to identify, isolate, and engineer these antimicrobial peptides. The interdisciplinary nature of bacteriocin mining combines molecular biology, genomics, bioinformatics, and protein chemistry, driving the search for new bacteriocins and expanding our understanding of their diverse functionalities. This pursuit aims not only to discover new antimicrobial agents but also to comprehend the complex interactions and mechanisms underlying these peptides, potentially revolutionizing the landscape of antimicrobial strategies[157].

2.1 Objectives

The main objective of this project was to purify, identify and characterize the bacteriocins produced by *Enterococcus canintestini* EC49. This strain was provided by CanBiocin, a biotec company working on producing and studying animal probiotics. The provided strain was isolated from dog feces and showed antimicrobial activity against *L. monocytogenes, C. perfringens* vancomycin-resistant enterococci in unpublished studies done by CanBiocin. This project's goals also included identifying potential new enterocins by using bacteriocin mining databases, for which reason a DNA extraction and genome assembly was carried out.

2.1.1 Results and discussion

To confirm that *E. canintestini* 49 was able to produce antimicrobial compounds, an initial spot-on-lawn assay was performed. EC49 was grown overnight in 5 ml of APT broth. The indicator strains *E. faecalis* 710C and *Lactococcus lactis* subsp. *cremoris* HP as positive controls, were grown under the same conditions as that of EC49.

The EC49 inoculated broth was centrifuged to separate the cell pellet, and 100 µl of the supernatant was removed and concentrated *in vacuo*. The concentrate was used to perform the spot-on-lawn assay. In a spot-on-lawn assay, a positive result is considered when a halo (zone of inhibition) is formed. Both supernatant and concentrated samples showed antimicrobial activity (Figure 2.1). Since the results for EC49 were positive, a large-scale fermentation was performed.

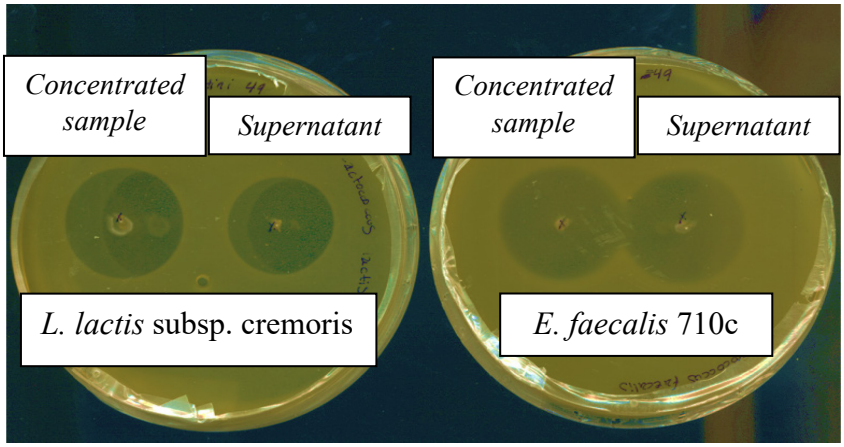


Figure 2.1 Spot-on-lawn assay results. *E. canintestini* 49 supernatant sample and *E. canintestini* 49 supernatant concentrated sample against *L. lactis* subsp. *cremoris* (left) and *E. faecalis* 710c (right).

2.1.2 Purification

The produced bacteriocins were purified from a 1L overnight culture. First, the bacterial culture was centrifuged to pellet the cells. The Supernatant was loaded onto an Amberlite resin which was consequently washed with increasing concentrations of isopropanol in increments as described in experimental procedures. From each wash, 200 µl were collected for a spot-on-lawn assay. Each sample was concentrated in vacuo. The results are shown in Figure 2.2.

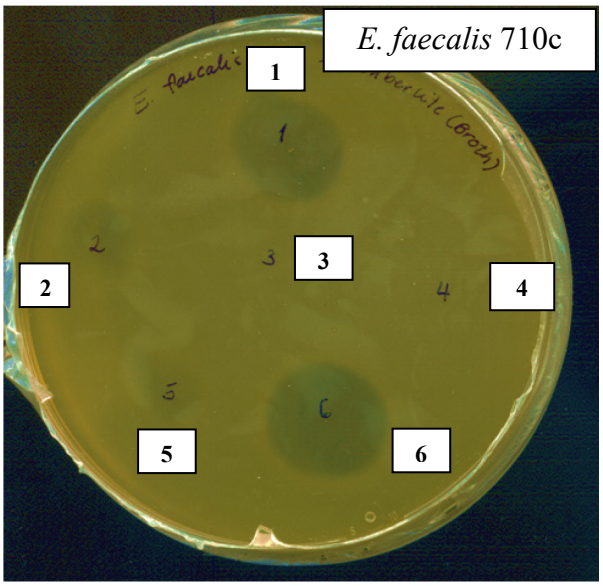


Figure 2.2 Spot-on-lawn assay for amberlite purification fractions. Indicator strain: *E.faecalis 710c* 1) Clarified supernatant, 2) Flow through, 3) H_2O wash, 4) 20% 2-Propanol, 5) 40% 2-Propanol, 6) 80% 2-Propanol + 0.1% TFA.

Since the results showed that the 80% 2-Propanol + 0.1% TFA fraction was active against the indicator strain, it was collected and concentrated using a rotavap for further purification steps. A Phenomenex Strata C8 reverse phase cartridge was used for the next step. After loading the sample into the cartridge, it was washed with 50 ml of solvents of increasing polarity added successively The full method is described in experimental procedures. From each fraction, 200 µl were collected and concentrated for a spot-on-lawn assay in order to look for antimicrobial activity. The obtained results are shown in Figure 2.3.

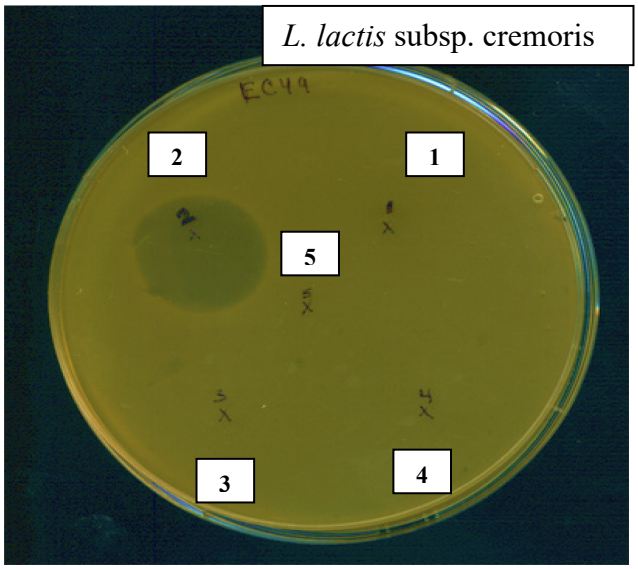


Figure 2.3 Spot-on-lawn assay for the Strata C8 purification. Indicator strain: *Lactococcus lactis* subsp. cremoris 1) Flow through, 2) 80% 2-Propanol + 0.1% TFA, 3) 40%2-Propanol, 4) 30% Ethanol, 5) 20% 2-Propanol.

The obtained results showed that the fraction with 80% 2-Propanol + 0.1% TFA contained the active compounds. This fraction was collected for high performance liquid chromatography (HPLC) purification, from which 100 µl were taken and concentrated *in vacuo* to analyze with matrix-assisted laser desorption ionization mass spectrometry (MALDI-TOF MS), where a variety of m/z peaks were observed, suggesting the presence of several compounds. For HPLC purification, an analytical Phenomenex column (Jupiter type 5u C18 300 Å; 160 x 4.60 mm) was used with the following solvents: Solvent A: water with 0.1% TFA, Solvent B: acetonitrile with 0.1% TFA (full method described in Chapter 6). The chromatogram showed eight main peaks, which were collected and re-evaluated in a spot-on-lawn assay (Figure 2.4).

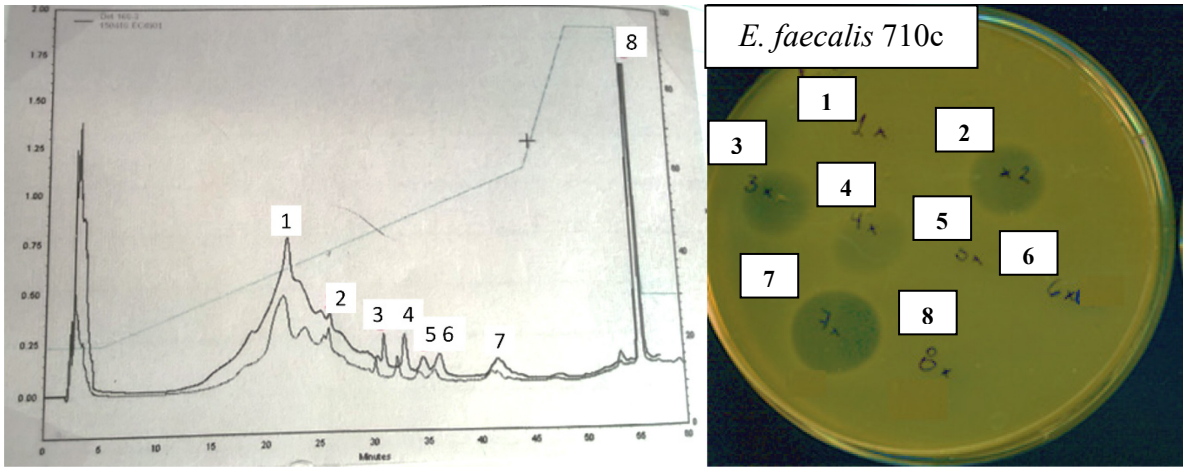


Figure 2.4 HPLC chromatogram and spot-on-lawn assay results obtained from individual HPLC isolated peaks. Indicator strain: *E. faecalis* 710c.

After the fractions from the peaks observed on the HPLC chromatogram were collected, a mass spectrometry analysis was run for each (MALDI-TOF). The fractions were concentrated and lyophilized for MS/MS analysis.

To investigate the nature of the compounds, a protease K digestion was performed on fractions 3,4,5,6,7 and 8. Ten microliters of every fraction were spotted along with protease K (Figure 2.5). From the results in the proteolytic assay, it can be observed that the antimicrobial activity was entirely or partially lost, which suggested the active isolated compounds were proteinaceous.

The MS/MS analysis revealed the elucidation of two distinct amino acid sequences from two of the eight samples (Sample No. 3, 4, and 7). Findings indicated that samples 3 and 4 contained identical compounds, while compound 7 was identified as a different peptide. The obtained query sequences were used for a search in the protein database BLAST, where a match was found, the sequences corresponded to enterocin NKR-5-3D and NKR-5-3Z, peptides from *E. faecium NKR-5-3*.

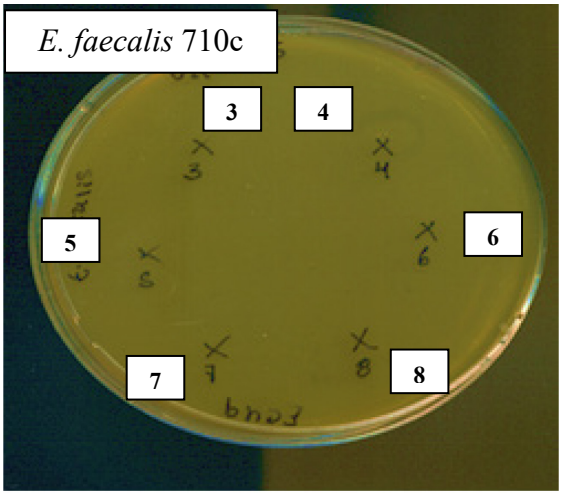


Figure 2.5 Results from the proteolysis assay for the active fractions from HPLC purification. Indicator strain: *E. faecalis* 710c. 10µl of proteinase-K (10 mg/ml) were spotted besides 10 µl of each fraction to corroborate the proteinaceous nature of the compounds. The obtained results show that there is no antimicrobial activity in the presence of the protease due to peptide degradation.

2.2 Genome analysis

The chromosomal DNA of EC49 was extracted by using a DNeasy blood & tissue kit and later submitted to the Molecular Biology Facility of the University of Alberta for genome sequencing and assembly. The obtained draft genome contains 24 contigs and 2,734,830 bases with 36.2% GC content, and it was deposited on the NCBI database with the accession number: LHUG00000000[156].

The EC49 genome assembly was analyzed with the help of Dr. Jeella Acedo on the antimicrobial peptide databases BAGEL3 and antiSMASH, which revealed the presence of a gene cluster with 96.7% nucleotide sequence homology with the bacteriocin gene cluster found in *E. faecium* NKR-5-3 which contains the genes involved in the biosynthesis of enterocins NKR-5-3A, -C, -D-and Z. Enterocin NKR-5-3A and -Z constitute a two-peptide bacteriocin (Class IIb) whereas NKR-5-3-C is a class IIa bacteriocin. Enterocin NKR-5-3-D possesses antimicrobial activity, but it also functions as an inductive peptide[158].

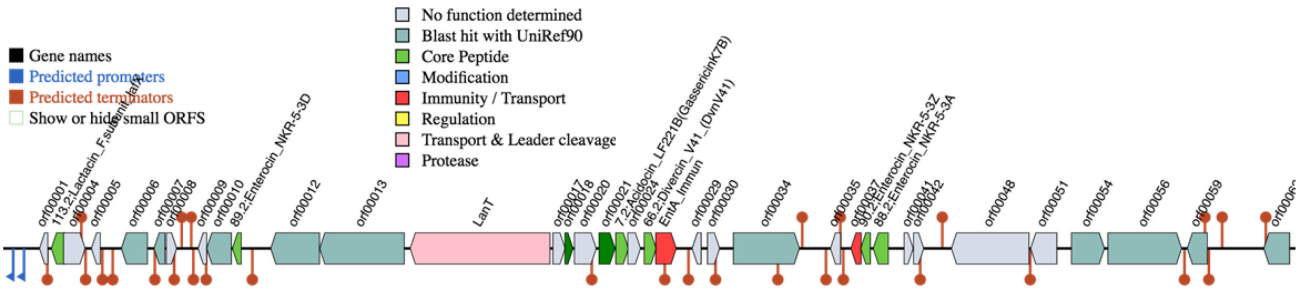


Figure 2.6 BAGEL3 bacteriocin prediction based on the EC49 draft genome. Colored green arrows show the genes which code for the core peptides. As the labels in the top of the arrows indicate, from left to right: lacticin F, which was not isolated, enterocin NKR-5-3D isolated, gassericin K7B not isolated, divercin V41 not isolated, enterocin NKR-5-3Z isolated, enterocin NKR-5-3A not isolated.

E. canintestini 49 encodes enterocins NKR-5-3A, -Z, and -D and a homolog of enterocin NKR-5-3C that differs by one amino acid. BAGEL3 predicted three additional putative bacteriocins: lacticin F, gassericinK7B and divercin V41 (Figure 2.6). The production of enterocin NKR-5-3D and -Z were detected by tandem mass spectrometry. In the genome sequence, genes that encode virulence factors were not found, which suggests the strain can be tested for its use as a probiotic.

2.2.1 Conclusions and overview

Most new and novel bacteriocins are isolated from bacteria that coexist in well-established microbial communities. For bacteriocin production, it is necessary to have a good understanding of the natural production process so that when fermentation is carried out, it is assured that the bacteriocin producer strain is incubated under the right conditions and the suitable media to produce a good yield of these compounds.

The comprehensive research methodology employed in this study, involving genomic DNA isolation, sequencing, assembly, annotation, and bioinformatic analyses, proved crucial in understanding the genetic composition of *E. canintestini* 49.

Enterococcus canintestini 49 was incubated using a standard media and standard conditions for enterococcal species, and although the genome analysis showed EC49 possesses the complete machinery to produce enterocins NKR-5-3A, -Z, and -D only enterocins NKR-5-3-Z and

-D were identified, which could be the result of the purification method or the incubation conditions, or perhaps there are missing genetic components which go beyond our current knowledge about bacteriocin production.

Upon conducting a BLAST search on the nucleotide sequences associated with the enterocins present in *E. canintestini* 49, the results indicated matches primarily with *Enterococcus faecium* (Table 2.1-2.3)). These matches exhibited low E values and a substantial percentage of query cover, indicative of considerable alignment. These findings strongly suggest a probable shared evolutionary origin between the sequences, highlighting a higher likelihood of a common genetic heritage.

Table 2.1 Nucleotide BLAST of Enterocin NKR-5-3D from *E. canintestini* 49

Description	Scientific Name	Query Cover	E value	Percent identity
Enterococcus faecium DNA, enterocins NKR-5-3 gene cluster, strain: NKR-5-3	*Enterococcus faecium*	100%	1.00E-53	96.3
Enterococcus faecium ent53D gene for enterocin NKR-5-3D prepeptide, complete cds	*Enterococcus faecium*	100%	1.00E-53	96.3
Latilactobacillus curvatus strain TMW 1.1928 chromosome, complete genome	*Latilactobacillus curvatus*	100%	6.00E-52	95.56
Latilactobacillus curvatus strain TMW 1.624 chromosome, complete genome	*Latilactobacillus curvatus*	100%	6.00E-52	95.56
Lactobacillus sakei IP-TX (stxP), StxR (stxR), StxK (stxK), StxT (stxT), SakT alpha (sakT alpha), SakT beta (sakT beta), SakTIM (sakTIM), SakX (sakX), SakXIM (sakXIM), brochocin immunity protein BcrI-like protein, hypothetical protein, and unknown protein genes, complete cds; and hypothetical protein	*Latilactobacillus sakei*	100%	6.00E-52	95.56

Table 2.2 Nucleotide BLAST of Enterocin NKR-5-3Z from *E. canintestini* 49

Description	Scientific Name	Query Cover	E value	Percent identity
Enterococcus faecium DNA, enterocins NKR-5-3 gene cluster, strain: NKR-5-3	*Enterococcus faecium*	100%	8.00E-87	100
Enterococcus faecium ent53A, ent53Z genes for enterocin NKR-5-3A prepeptide, enterocin NKR-5-3Z prepeptide, complete cds	*Enterococcus faecium*	100%	8.00E-87	100

Table 2.3 Nucleotide BLAST of Enterocin NKR-5-3A from *E. canintestini* 49

Description	Scientific Name	Query Cover	E value	Percent identity
Enterococcus faecium DNA, enterocins NKR-5-3 gene cluster, strain: NKR-5-3	*Enterococcus faecium*	100%	8.00E-87	100
Enterococcus faecium ent53A, ent53Z genes for enterocin NKR-5-3A prepeptide, enterocin NKR-5-3Z prepeptide, complete cds	*Enterococcus faecium*	100%	8.00E-87	100

The observed genetic similarities between the enterocins of *E. canintestini* 49 and *Enterococcus faecium*, inferred from the BLAST results, may indicate the possibility of lateral gene transfer events (Table 2.1, Table 2.2, Table 2.3).

Lateral gene transfer, a fundamental mechanism in bacterial evolution, involves the transfer of genetic material between different species or lineages. Such events could involve the horizontal exchange of genetic elements—such as plasmids, transposons, or mobile genetic elements—housing these enterocin sequences between these bacterial species.

In the search for new antibiotic peptides, comprehending lateral gene transfer is crucial as it enables the acquisition and exchange of genetic material related to antimicrobial agents between diverse bacterial species.

However, while the BLAST results strongly suggest genetic similarities, the direct inference of lateral gene transfer as the sole mechanism requires further investigation. Detailed genomic analyses, including phylogenetic studies, analysis of genomic contexts, and examination of mobility elements associated with these genes, would be essential to robustly establish lateral gene transfer as the specific mechanism behind the observed genetic affinities.

Understanding the potential occurrences and impacts of lateral gene transfer in these bacteria is crucial for comprehending their evolutionary adaptations, ecological interactions, and the diversity of genetic elements contributing to their functionalities. This knowledge could shed light on how bacterial species acquire beneficial traits and adapt to environmental challenges, thus shaping the dynamics and evolution of microbial communities.

The research findings on *E. canintestini* 49 also underscore its potential as a probiotic strain due to its capacity to inhibit various pathogenic bacteria without the presence of virulence-associated genes. This discovery holds significant promise, particularly amid concerns regarding the virulence factors prevalent in enterococcal strains, which require thorough scrutiny before their application in biotechnological contexts.

CHAPTER 3

3. Understanding *Listeria monocytogene*'s defense strategies: Carnocyclin A resistance

3.1 Objectives

The objective of this project was to study the correlation between the Man-PTS and the developed resistance against carnocyclin A in the *L. monocytogenes* strains, FSL R2-499 and FSL C1-056, which were obtained from Dr. Balay and Dr. McMullen. We hoped that this would help us understand if the mechanism of action of circular bacteriocins relies on the presence of this receptor.

3.2 Results and discussion

Glycerol stocks from each strain, *L. monocytogenes* FSL-C1056 and *L. monocytogenes* FSL-R2-499, were obtained from Dr. Balay along with one glycerol stock of *L. monocytogenes* FSL-J1-177, a strain which did not develop any resistance during previous studies[159]. One of the glycerol stocks of each *L. monocytogenes* FSL-C1056 and *L. monocytogenes* FSL-R2-499 provided by Dr. Balay contained the strain before it was exposed to carnocyclin A (CclA), and the other glycerol stock had the strain collected after it was exposed to CclA and developed resistance. Dr. Balay performed DNA extractions on all the strains involved in the study for DNA sequencing. The DNA extraction was done initially only on the strains before exposure to CclA.

Once the strains were obtained, production of carnocyclin A was needed to test these strains' resistance against this circular bacteriocin. The method developed by Dr. Leah Martin-Visscher was used to isolate carnocyclin A from APT media[93]. The *L. monocytogenes* strains were then challenged in spot-on-lawn assays with five concentrations of carnocyclin A: 250 μM, 62 μM, 16 μM, 8 μM, and 2 μM in media prepared with different carbon sources (cellobiose, mannose and dextrose). The obtained results are summarized on Table 3.1.

In this assay, 10 µL of each solution with the corresponding bacteriocin concentration were spotted on a plate inoculated with a lawn of the corresponding *L. monocytogenes* strain. These plates were later incubated at 37 ℃ for 48 h. After incubation, the growth in the plate was evaluated.

Full growth in the plate indicated resistance against the tested compound, whereas the presence of a halo where the bacteriocin was spotted indicated no resistance.

The *L. monocytogenes* strains designated as FSL-R2-499, FSL-C1056, and FSL-J1-177 from the glycerol stocks exhibited sensitivity to all the tested concentrations. The halo formed was a faint halo with defined borders, suggesting the bacteriocin acts as a bactericidal agent.

Table 3.1 Results of spot-on-lawn assays. Resistance (-), No resistance (+).

	Cellobiose media					**Mannose media**					**APT media**				
	Carnocyclin A concentration (µM)					**Carnocyclin A concentration (µM)**					**Carnocyclin A concentration (µM)**				
L. monocytogenes strain	250	62	16	8	2	250	62	16	8	2	250	62	16	8	2
FSL-R2-499	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FSL-R2-499 CclAR	-	-	-	-	-	+	+	+	+	+	+	+	+	-	-
FSL-C1056	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FSL-C1056 CclAR	-	-	-	-	-	+	+	+	-	-	+	-	-	-	-
FSL-J1-177	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

On the contrary, strains FSL-R2-499 CclAR and FSL-C1056 CclAR exhibited resistance with intriguing variations. FSL-C1056 CclAR demonstrated resistance in all tested media. In the dextrose (APT) and cellobiose media, resistance was observed up to a maximum concentration of 62 µM. However, in the mannose media, resistance was limited to a concentration of only 8 µM. In contrast, strain FSL-R2-499 CclAR displayed no resistance in the mannose media, complete resistance with cellobiose, and resistance up to 8 µM with dextrose. These differing resistance profiles suggest that sugar intake receptors may be involved in the mechanism of action of the circular bacteriocin carnocyclin A.

L. monocytogenes is recognized for its capability to adapt to various surroundings through signal-sensing responses, which initiate gene regulation to ensure survival. Carbohydrate availability is thought to be a key factor in influencing the expression of various virulent traits. *L.*

monocytogenes possesses many transport systems for carbohydrates and sugar derivatives which can trigger virulence factors. *L. monocytogenes* FSL-C1056 and *L. monocytogenes* FSL-R2-499 are clinical strains isolated from outbreaks which may suggest that the more virulent a strain is, the bigger the adaptability will be[90,160].

Most carbohydrates used by *L. monocytogenes* are transported by PTSs, as has been previously mentioned. The differences in the resistance shown by the two *L. monocytogenes* strains suggested that the mannose receptor may play a role in the mechanism of action of carnocyclin A, as shown for type IIa bacteriocins in studies done by Kjos *et al*.[113,111] If circular bacteriocins use the Man-PTS as a docking molecule similar to type IIa bacteriocins, then it is possible that mutations in this receptor could confer resistance in strains FSL-C1056 and FSL-R2-499.

To find mutations in the α, β or γ regions in subunits C and D of the Man-PTS of the resistant *L. monocytogenes* strains, the genome annotations from the non-resistant strains, obtained from Dr. Balay were examined, and the amino acid sequences of these subunits were extracted. We first compared these protein sequences to the sequences of the wild type of strain *L. monocytogenes* EGD-e, which has been identified as a non-resistant strain against carnocyclin A.

The alignment and comparison of the three obtained amino acid sequences were carried out in the multiple sequence alignment program, CLUSTAL OMEGA. In the alignment, only one amino acid was different in the sequence of subunit IIC of *L. monocytogenes* FSL-C1-056 when compared to the sequence of *L. monocytogenes* EGD-e (valine instead of isoleucin). This mutation was found outside the reported regions α, β, or γ, as shown in Figure 3.1. Despite the similarity in both these amino acid residues, this mutation could lead to conformational changes in the protein in further generations, which could also explain the slight resistance (up to 8 uM) in the mannose media, which was not observed in strain FSL-R2-499. Further structural studies like NMR experiments should be done to test this idea.

Figure 3.1 Sequence alignment of the MPT/IIC and IID sequences from strains *L. monocytogenes* EGD-e, *L. monocytogenes* FSL-R2-499 and *L. monocytogenes* FSL-C1-056 ran in CLUSTAL OMEGA. In the red squares, regions α, β and γ, reported by Kjos et al[111,113].

Mutations in microorganisms can be either permanent or temporary. They can be spontaneously induced by environmental changes like the presence of chemicals, radiation, simple changes in carbon sources, or viruses. Since the genome assemblies of the *L. monocytogenes* strains were carried out before the strains were exposed to carnocyclin A, the mutations causing resistance to this bacteriocin might have occurred after the strains were exposed to carnocyclin A. It was also possible that resistance was not permanent. To evaluate this idea, an experiment was carried out where the resistant strains of *L. monocytogenes* FSL-R2-499 CclAR, *L. monocytogenes* FSL-C1-056 CclAR and *L. monocytogenes* EGD-e were grown for sixty generations on APT media in the

absence of carnocyclin A, and then a spot-on-lawn assay was carried out to evaluate if the resistance against carnocyclin A was still present. In the first experiment, the same concentrations of bacteriocin were used (250 μM, 62 μM, 16 μM, 8 μM, 2 μM). Interestingly, the obtained results were the same as in the first experiment, the resistant strains remained resistant, and the control strain, *L. monocytogenes* EGD-e, showed no resistance as expected (Table 3.1).

After growing the strains for 60 generations and doing the spot-on-lawn assay, new DNA extractions were carried out on both strains for genome sequencing. The genome assembly and protein annotation were carried out once the genome sequence was obtained from the Molecular Biology Services Unit provided by the Department of Biological Sciences at the University of Alberta. The genome assemblies were done using MiGa and MeDuSa and the genome annotation was done using RAST. The genome assemblies were deposited on the NCBI database (accession numbers: JAALEI000000000). The protein sequences from subunits IIC and IID from the Man-PTSs were obtained from the annotations, which were aligned with the sequences from the strains before they were exposed to the bacteriocin, however there were no changes in the sequences as observed in Table 3.1 (Figure 3.2,Figure 3.3).

a) *L. monocytogenes* FSL-C1-056

```
Mannose-specific_IIC_component_[Listeria_monocytogenes_FSL-C1-056-WT]        MSVISIILVVLIAFLAGIEGILDEFQFHQPLIACTLIGLVTGNLTACIILGGTLQMIALG    60
Mannose-specific_IIC_component_[Listeria_sp.monocytogenes_FSL-C1-056-60g]    MSVISIILVVLIAFLAGIEGILDEFQFHQPLIACTLIGLVTGNLTACIILGGTLQMIALG    60
                                                                            ************************************************************

Mannose-specific_IIC_component_[Listeria_monocytogenes_FSL-C1-056-WT]        WANIGAAVAPDAALASVASAIILVLGGQGVAGIPSAIAIAIPLAVAGLFLTMIVRTLAVP    120
Mannose-specific_IIC_component_[Listeria_sp.monocytogenes_FSL-C1-056-60g]    WANIGAAVAPDAALASVASAIILVLGGQGVAGIPSAIAIAIPLAVAGLFLTMIVRTLAVP    120
                                                                            ************************************************************

Mannose-specific_IIC_component_[Listeria_monocytogenes_FSL-C1-056-WT]        IVHLMDRAAEKGNIRSVEWLHISAICMQGIRIAIPAAALLFIPADSVQSFLEAMPAWLTD    180
Mannose-specific_IIC_component_[Listeria_sp.monocytogenes_FSL-C1-056-60g]    IVHLMDRAAEKGNIRSVEWLHISAICMQGIRIAIPAAALLFIPADSVQSFLEAMPAWLTD    180
                                                                            ************************************************************

Mannose-specific_IIC_component_[Listeria_monocytogenes_FSL-C1-056-WT]        GMAIGGGMVVAVGYALVINMMATKEVWPFFVIGFVVAAISQLTLIAIGALGVALALIYLN    240
Mannose-specific_IIC_component_[Listeria_sp.monocytogenes_FSL-C1-056-60g]    GMAIGGGMVVAVGYALVINMMATKEVWPFFVIGFVVAAISQLTLIAIGALGVALALIYLN    240
                                                                            ************************************************************

Mannose-specific_IIC_component_[Listeria_monocytogenes_FSL-C1-056-WT]        LSKMGGGNSNGGGGGNSRDPLGDILNDY     268
Mannose-specific_IIC_component_[Listeria_sp.monocytogenes_FSL-C1-056-60g]    LSKMGGGNSNGGGGGNSRDPLGDILNDY     268
                                                                            ****************************
```

***b) L. monocytogenes* FSL-R2-499**

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Mannose-specific_IIC_component_[Listeria_monocytogenes_FSL-R2-499-WT]        MSVISIILVVLIAFLAGIEGILDEFQFHQPLIACTLIGLVTGNLTACIILGGTLQMIALG    60
Mannose-specific_IIC_component_[Listeria_sp.monocytogenes_FSL-R2-499-60g]    MSVISIILVVLIAFLAGIEGILDEFQFHQPLIACTLIGLVTGNLTACIILGGTLQMIALG    60
                                                                            ************************************************************

Mannose-specific_IIC_component_[Listeria_monocytogenes_FSL-R2-499-WT]        WANIGAAVAPDAALASVASAIILVLGGQGVAGIPSAIAIAIPLAVAGLFLTMIVRTLAVP    120
Mannose-specific_IIC_component_[Listeria_sp.monocytogenes_FSL-R2-499-60g]    WANIGAAVAPDAALASVASAIILVLGGQGVAGIPSAIAIAIPLAVAGLFLTMIVRTLAVP    120
                                                                            ************************************************************

Mannose-specific_IIC_component_[Listeria_monocytogenes_FSL-R2-499-WT]        IVHLMDRAAEKGNIRSVEWLHISAICMQGVRIAIPAAALLFIPADSVQSFLEAMPAWLTD    180
Mannose-specific_IIC_component_[Listeria_sp.monocytogenes_FSL-R2-499-60g]    IVHLMDRAAEKGNIRSVEWLHISAICMQGVRIAIPAAALLFIPADSVQSFLEAMPAWLTD    180
                                                                            ************************************************************

Mannose-specific_IIC_component_[Listeria_monocytogenes_FSL-R2-499-WT]        GMAIGGGMVVAVGYALVINMMATKEVWPFFVIGFVVAAISQLTLIAIGALGVALALIYLN    240
Mannose-specific_IIC_component_[Listeria_sp.monocytogenes_FSL-R2-499-60g]    GMAIGGGMVVAVGYALVINMMATKEVWPFFVIGFVVAAISQLTLIAIGALGVALALIYLN    240
                                                                            ************************************************************

Mannose-specific_IIC_component_[Listeria_monocytogenes_FSL-R2-499-WT]        LSKMGGGNSNGGGGGNSRDPLGDILNDY     268
Mannose-specific_IIC_component_[Listeria_sp.monocytogenes_FSL-R2-499-60g]    LSKMGGGNSNGGGGGNSRDPLGDILNDY     268
                                                                            ****************************
```

Figure 3.2 Sequence alignment of the Man-PTS subunit IIC from strains *L. monocytogenes* FSL-R2-499 and *L. monocytogenes* FSL-C1-056 before they were exposed to CclA (top) and after (bottom) growing the strain for 60 generations.

a) *L. monocytogenes* FSL-02-499

```
Mannose-specific_IID_component_[Listeria_monocytogenes_FSL-R2-499-WT]        MAEKIELSKRDRLRVAWRSTFIQGSWNYERMQNGGWAFSMIPAIKKLYKTKEDRSQALKR     60
Mannose-specific_IID_component_[Listeria_sp.monocytogenes_FSL-R2-499-60g]    MAEKIELSKRDRLRVAWRSTFIQGSWNYERMQNGGWAFSMIPAIKKLYKTKEDRSQALKR     60
                                                                            ************************************************************

Mannose-specific_IID_component_[Listeria_monocytogenes_FSL-R2-499-WT]        HLEFFNTHPYIASPILGVTLALEEERANGAEVDDVAIQGVKVGMMGPLAGVGDPVFWFTI     120
Mannose-specific_IID_component_[Listeria_sp.monocytogenes_FSL-R2-499-60g]    HLEFFNTHPYIASPILGVTLALEEERANGAEVDDVAIQGVKVGMMGPLAGVGDPVFWFTI     120
                                                                            ************************************************************

Mannose-specific_IID_component_[Listeria_monocytogenes_FSL-R2-499-WT]        RPMLGALGASLALSGNILGPILFFVAWNVIRWGFMWYTQEFGYKAGSKITDDLSGGLLQD     180
Mannose-specific_IID_component_[Listeria_sp.monocytogenes_FSL-R2-499-60g]    RPMLGALGASLALSGNILGPILFFVAWNVIRWGFMWYTQEFGYKAGSKITDDLSGGLLQD     180
                                                                            ************************************************************

Mannose-specific_IID_component_[Listeria_monocytogenes_FSL-R2-499-WT]        ITKGASILGMFVLAALVQRWVNIQFAPIISKVKLDEGAYIDWSHLPQGAQGIKTALEQQQ     240
Mannose-specific_IID_component_[Listeria_sp.monocytogenes_FSL-R2-499-60g]    ITKGASILGMFVLAALVQRWVNIQFAPIISKVKLDEGAYIDWSHLPQGAQGIKTALEQQQ     240
                                                                            ************************************************************

Mannose-specific_IID_component_[Listeria_monocytogenes_FSL-R2-499-WT]        AGLALSEIKVTTLQNNLDNLIPGLAAVALTFLCMWLLKKKISPIIIILGLFVVGIVGHLI     300
Mannose-specific_IID_component_[Listeria_sp.monocytogenes_FSL-R2-499-60g]    AGLALSEIKVTTLQNNLDNLIPGLAAVALTFLCMWLLKKKISPIIIILGLFVVGIVGHLI     300
                                                                            ************************************************************

Mannose-specific_IID_component_[Listeria_monocytogenes_FSL-R2-499-WT]        GLL      303
Mannose-specific_IID_component_[Listeria_sp.monocytogenes_FSL-R2-499-60g]    GLL      303
                                                                            ***
```

b) *L. monocytogenes* FSL-C1-056 CclAR

```
CLUSTAL O(1.2.4) multiple sequence alignment

Mannose_specific_IID_component_Listeria_monocytogenes_FSL_C1_056_WT      MAEKIELSKRDRLRVAWRSTFIQGSWNYERMQNGGWAFSMIPAIKKLYKTKEDRSQALKR
Mannose_specific_IID_component_Listeria_monocytogenes_FSL_C1_056_60g     MAEKIELSKRDRLRVAWRSTFIQGSWNYERMQNGGWAFSMIPAIKKLYKTKEDRSQALKR
                                                                         ************************************************************

Mannose_specific_IID_component_Listeria_monocytogenes_FSL_C1_056_WT      HLEFFNTHPYIASPILGVTLALEEERANGAEVDDVAIQGVKVGMMGPLAGVGDPVFWFTI
Mannose_specific_IID_component_Listeria_monocytogenes_FSL_C1_056_60g     HLEFFNTHPYIASPILGVTLALEEERANGAEVDDVAIQGVKVGMMGPLAGVGDPVFWFTI
                                                                         ************************************************************

Mannose_specific_IID_component_Listeria_monocytogenes_FSL_C1_056_WT      RPMLGALGASLALSGNILGPILFFVAWNVIRWGFMWYTQEFGYKAGSKITDDLSGGLLQD
Mannose_specific_IID_component_Listeria_monocytogenes_FSL_C1_056_60g     RPMLGALGASLALSGNILGPILFFVAWNVIRWGFMWYTQEFGYKAGSKITDDLSGGLLQD
                                                                         ************************************************************

Mannose_specific_IID_component_Listeria_monocytogenes_FSL_C1_056_WT      ITKGASILGMFVLAALVQRWVNIQFAPIISKVKLDEGAYIDWSHLPQGAQGIKTALEQQQ
Mannose_specific_IID_component_Listeria_monocytogenes_FSL_C1_056_60g     ITKGASILGMFVLAALVQRWVNIQFAPIISKVKLDEGAYIDWSHLPQGAQGIKTALEQQQ
                                                                         ************************************************************

Mannose_specific_IID_component_Listeria_monocytogenes_FSL_C1_056_WT      AGLALSEIKVTTLQNNLDNLIPGLAAVALTFLCMWLLKKKISPIIIILGLFVVGIVGHLI
Mannose_specific_IID_component_Listeria_monocytogenes_FSL_C1_056_60g     AGLALSEIKVTTLQNNLDNLIPGLAAVALTFLCMWLLKKKISPIIIILGLFVVGIVGHLI
                                                                         ************************************************************

Mannose_specific_IID_component_Listeria_monocytogenes_FSL_C1_056_WT      GLL
Mannose_specific_IID_component_Listeria_monocytogenes_FSL_C1_056_60g     GLL
                                                                         ***
```

Figure 3.3 Sequence alignment of the Man-PTS subunit IID from strains *L. monocytogenes* FSL-R2-499 and *L. monocytogenes* FSL-C1-056 before it was exposed to CclA and after growing the strain for 60 generations.

Studies done by Stoll in 2010, suggest that the transport of cellobiose and glucose are mediated by different PTS transporters. Currently, seven families of PTS have been identified in *L. monocytogenes*: PTS glucose-glucoside (Glc), PTS mannose-fructose-sorbose (Man), PTS lactose-N, N'-diacetylchitobiose-β-glucoside (Lac), PTS fructose-mannitol (Fru), PTS galactitol (Gat), PTS L-ascorbate and PTS glucitol. Cellobiose seems to be transported by one of the members of the PTS Lac family, PTS^{Lac-4}. Meanwhile, glucose and mannose use all 4 PTSMan permeases.

As the findings indicated a notable rise in observed resistance in the presence of cellobiose, an investigation into mutations within these receptors was conducted. It's crucial to note that the genome assembly annotation identified these components as "lactose/cellobiose PTS system components," yet no mutations were detected in any of these genes, as depicted in the subsequent figures (Figure 3.4, Figure 3.5, Figure 3.6, Figure 3.7, Figure 3.8, Figure 3.9).

A.

```
fig|1639.7679.peg.2432PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-WT]          MTISERQRSLLEKLNDSQKTVTAKALSEMLGVSSKTVRNDIMQINQSFSSTIIASKAGKG      60
fig|6666666.523413.peg.256PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-60g]    MTISERQRSLLEKLNDSQKTVTAKALSEMLGVSSKTVRNDIMQINQSFSSTIIASKAGKG      60
                                                                                                   ************************************************************

fig|1639.7679.peg.2432PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-WT]          YFLMPNEQLSQMNLTKNNENLHFELLRHIIEQDYTNFYDLADQFFISESTLARIIKELNI      120
fig|6666666.523413.peg.256PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-60g]    YFLMPNEQLSQMNLTKNNENLHFELLRHIIEQDYTNFYDLADQFFISESTLARIIKELNI      120
                                                                                                   ************************************************************

fig|1639.7679.peg.2432PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-WT]          VIAEKDESLCIIRKNNELLTEGGEEEKRRIFNLFLNQEIENHQLSLDKYADYFDYCNLKQ      180
fig|6666666.523413.peg.256PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-60g]    VIAEKDESLCIIRKNNELLTEGGEEEKRRIFNLFLNQEIENHQLSLDKYADYFDYCNLKQ      180
                                                                                                   ************************************************************

fig|1639.7679.peg.2432PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-WT]          LSELIIAYHKKHEFFMNDFSTISFILHIAVLIERISMGSYIERTALLEQDKTSLEMAAHL      240
fig|6666666.523413.peg.256PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-60g]    LSELIIAYHKKHEFFMNDFSTISFILHIAVLIERISMGSYIERTALLEQDKTSLEMAAHL      240
                                                                                                   ************************************************************

fig|1639.7679.peg.2432PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-WT]          AETLENELQINIPTQELSYIARLYSGKLTTTSTIDAQVFGSVVTRLLEAVDQNFHIDFSA      300
fig|6666666.523413.peg.256PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-60g]    AETLENELQINIPTQELSYIARLYSGKLTTTSTIDAQVFGSVVTRLLEAVDQNFHIDFSA      300
                                                                                                   ************************************************************

fig|1639.7679.peg.2432PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-WT]          DEKIATYLVAHISALYKRANHKQYLTNPLTEELKNKFPFIYNVSVYASAFIQKELAITFP      360
fig|6666666.523413.peg.256PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-60g]    DEKIATYLVAHISALYKRANHKQYLTNPLTEELKNKFPFIYNVSVYASAFIQKELAITFP      360
                                                                                                   ************************************************************

fig|1639.7679.peg.2432PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-WT]          DDEIAYIALHFLSASETINHGKKRKILLVTPYGAGSQRLIHNQLKKIPDFSIDLLVSQSI      420
fig|6666666.523413.peg.256PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-60g]    DDEIAYIALHFLSASETINHGKKRKILLVTPYGAGSQRLIHNQLKKIPDFSIDLLVSQSI      420
                                                                                                   ************************************************************

fig|1639.7679.peg.2432PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-WT]          FDIKQFTLDKEIHLILTAEPLNLTTDIPVYHYDLLLAESDLQKIKHILETKQKTESISRK      480
fig|6666666.523413.peg.256PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-60g]    FDIKQFTLDKEIHLILTAEPLNLTTDIPVYHYDLLLAESDLQKIKHILETKQKTESISRK      480
                                                                                                   ************************************************************

fig|1639.7679.peg.2432PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-WT]          FFKKELFFPKQNFKSKEETITFLCEQLTAFDYCDPDYVAKVFEREQLSSTCYGNYYAIPH      540
fig|6666666.523413.peg.256PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-60g]    FFKKELFFPKQNFKSKEETITFLCEQLTAFDYCDPDYVAKVFEREQLSSTCYGNYYAIPH      540
                                                                                                   ************************************************************

fig|1639.7679.peg.2432PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-WT]          AIQRSAKKNAVAVCSLDKPIDWGGNRVKLVLLLTMKEERDNSFEELFGQLVTILNERSFV      600
fig|6666666.523413.peg.256PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-60g]    AIQRSAKKNAVAVCSLDKPIDWGGNRVKLVLLLTMKEERDNSFEELFGQLVTILNERSFV      600
                                                                                                   ************************************************************

fig|1639.7679.peg.2432PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-WT]          KKLAKQEDFQQFIELCEQKTLDS    623
fig|6666666.523413.peg.256PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-60g]    KKLAKQEDFQQFIELCEQKTLDS    623
                                                                                                   ***********************
```

B.

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fig|1639.7679.peg.2388PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenesFSL-R2-499-WT]          MDNKNLEAIMGLIMHGGNAKSNAMEAIQAAKNGAFELAEEKISEAEQSIVEAHHSQTGLL        60
fig|6666666.523413.peg.300PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenesFSL-R2-499-60g]      MDNKNLEAIMGLIMHGGNAKSNAMEAIQAAKNGAFELAEEKISEAEQSIVEAHHSQTGLL        60
                                                                                                    ************************************************************

fig|1639.7679.peg.2388PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenesFSL-R2-499-WT]          TEEAKGNHMEVTLLTVHSQDHLMTAMTFTDLAKELIDVYRKLLK        104
fig|6666666.523413.peg.300PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenesFSL-R2-499-60g]      TEEAKGNHMEVTLLTVHSQDHLMTAMTFTDLAKELIDVYRKLLK        104
                                                                                                    ****************************************
```

C.

```
fig|1639.7679.peg.2929PTScellobiose-specificIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-WT]          MDLEQTIMSLIVFGGNAKSDAMLAIDSAKKGDFAQADEQIAQAEQALLEAHHSQTKLIQG    60
fig|6666666.523413.peg.1891;PTScellobiose-specificIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-60g]    MDLEQTIMSLIVFGGNAKSDAMLAIDSAKKGDFAQADEQIAQAEQALLEAHHSQTKLIQG    60
                                                                                                              ************************************************************

fig|1639.7679.peg.2929PTScellobiose-specificIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-WT]          EARGEKTEVSLLLVHAQDHLMNAITFKDLAKEIVDLYKNK        100
fig|6666666.523413.peg.1891;PTScellobiose-specificIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-60g]    EARGEKTEVSLLLVHAQDHLMNAITFKDLAKEIVDLYKNK        100
                                                                                                              ****************************************
```

D.

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fig|1639.7679.peg.2842PTS_cellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-WT]          MNEMETVIFGMISQVGSARSSYLEGLRAAREGNFEEAEAKLKEGGETLANGHHEHHKLIQ      60
fig|6666666.523413.peg.1607PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-60g]      MNEMETVIFGMISQVGSARSSYLEGLRAAREGNFEEAEAKLKEGGETLANGHHEHHKLIQ      60
                                                                                                      ************************************************************

fig|1639.7679.peg.2842PTS_cellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-WT]          KEASGEKVEIQLLLIHAEDLLITTETLREVVTEFVHVYKKIN        102
fig|6666666.523413.peg.1607PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-60g]      KEASGEKVEIQLLLIHAEDLLITTETLREVVTEFVHVYKKIN        102
                                                                                                      ****************************************
```

E.

```
fig|1639.7679.peg.1767_PTS_cellobiose_IIA_component(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-WT]          MSEQDYVEETDSLNELSMNILIHAGNARNDLVKGLNHLEELEFNEAEEFIASAKREIVIA 60
fig|6666666.523413.peg.2088_PTS_cellobiose_IIA_component(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-60g]    MSEQDYVEETDSLNELSMNILIHAGNARNDLVKGLNHLEELEFNEAEEFIASAKREIVIA 60
                                                                                                                ************************************************************

fig|1639.7679.peg.1767_PTS_cellobiose_IIA_component(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-WT]          HSLQTDTLQLEASGNQIRYSTLFCHAQDTLMTAKSEILIGEHMLRLFKKMTELTKK     116
fig|6666666.523413.peg.2088_PTS_cellobiose_IIA_component(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-60g]    HSLQTDTLQLEASGNQIRYSTLFCHAQDTLMTAKSEILIGEHMLRLFKKMTELTKK     116
                                                                                                                ************************************************************
```

F.

```
fig|1639.7679.peg.968_PTSIIAcomponent[Listeria_monocytogenes_FSL-R2-499-WT]          MELEQTIMQLIVHGGNAKSDAMLAIEAAKKGDFDVADEQIKNAEAALLEAHHSQTSLIQG    60
fig|6666666.523413.peg.163_PTSIIAcomponent[Listeria_monocytogenes_FSL-R2-499-60g]   MELEQTIMQLIVHGGNAKSDAMLAIEAAKKGDFDVADEQIKNAEAALLEAHHSQTSLIQG    60
                                                                                   ************************************************************

fig|1639.7679.peg.968_PTSIIAcomponent[Listeria_monocytogenes_FSL-R2-499-WT]          EARGEKAEVSLLLVHAQDHLMNAITFKDLAKEIVDLYRSK 100
fig|6666666.523413.peg.163_PTSIIAcomponent[Listeria_monocytogenes_FSL-R2-499-60g]   EARGEKAEVSLLLVHAQDHLMNAITFKDLAKEIVDLYRSK 100
                                                                                   ****************************************
```

Figure 3.4 A-F Sequence alignment of the Lactose/cellobiose-PTS subunits IIA from strains *L. monocytogenes* FSL-R2-499 before it was exposed to CclA and after growing the strain for 60 generations.

A.

```
fig|1639.7679.peg.2389PTScellobioseIIB(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-WT]          MSKKTIMLICSAGMSTSLLVTKMQKAAAEKSLELDIFAVAASDADNQLASKTIDVVLLGP      60
fig|6666666.523413.peg.299PTScellobioseIIB(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-60g]   MSKKTIMLICSAGMSTSLLVTKMQKAAAEKSLELDIFAVAASDADNQLASKTIDVVLLGP      60
                                                                                                  ************************************************************

fig|1639.7679.peg.2389PTScellobioseIIB(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-WT]          QVRFMEKQMEEKLAPKNIPSAVINMTDYGTMNGQNVLNLALNLIKGA    107
fig|6666666.523413.peg.299PTScellobioseIIB(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-60g]   QVRFMEKQMEEKLAPKNIPSAVINMTDYGTMNGQNVLNLALNLIKGA    107
                                                                                                  *********************************************
```

B.

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fig|1639.7679.peg.967_PTS_system_IIB_component_(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-WT]          MNNIMLVCSAGMSTSLLVKKMTEAIEKQQVDATVIAVAEADFDKYKGNVDVVLLAPQVRF     60
fig|6666666.523413.peg.164_PTS_system_IIB_component_(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-60g]   MNNIMLVCSAGMSTSLLVKKMTEAIEKQQVDATVIAVAEADFDKYKGNVDVVLLAPQVRF     60
                                                                                                            ************************************************************

fig|1639.7679.peg.967_PTS_system_IIB_component_(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-WT]          LEKNLKRVLDPLGIPVAIINGIDYGTMDGEKVLNDALAMIEK      102
fig|6666666.523413.peg.164_PTS_system_IIB_component_(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-60g]   LEKNLKRVLDPLGIPVAIINGIDYGTMDGEKVLNDALAMIEK      102
                                                                                                            ****************************************
```

C.

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fig|1639.7679.peg.1769PTS_cellobiose-specificIIB_component(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-WT]          MKNILLICGSGASSGFMAAAIRKAAKKRGEQVTVKAASESQIDERINEIDYLLIGPHLAY  60
fig|6666666.523413.peg.2086PTS_cellobiose-specificIIB_component(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-60g]   MKNILLICGSGASSGFMAAAIRKAAKKRGEQVTVKAASESQIDERINEIDYLLIGPHLAY  60
                                                                                                                       ************************************************************

fig|1639.7679.peg.1769PTS_cellobiose-specificIIB_component(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-WT]          MLDDLKQKVADKNVLVSIIPQATYGTLNGEKALDLILTMEG     101
fig|6666666.523413.peg.2086PTS_cellobiose-specificIIB_component(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-60g]   MLDDLKQKVADKNVLVSIIPQATYGTLNGEKALDLILTMEG     101
                                                                                                                       *****************************************
```

D.

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fig|1639.7679.peg.2327PTS_cellobioseIIB(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-WT]        MKNILLVCNAGMSTSFLVEKMKAAGAEQGVEANIWAVSDAELHENWEKADVILLGPQVGY  60
fig|6666666.523413.peg.88PTScellobioseIIB(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-60g]    MKNILLVCNAGMSTSFLVEKMKAAGAEQGVEANIWAVSDAELHENWEKADVILLGPQVGY  60
                                                                                                  ************************************************************

fig|1639.7679.peg.2327PTS_cellobioseIIB(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-WT]        LKGNTEKVVGGKIPVEVINMLDYGRVNGAAVLERAIELIG  100
fig|6666666.523413.peg.88PTScellobioseIIB(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-60g]    LKGNTEKVVGGKIPVEVINMLDYGRVNGAAVLERAIELIG  100
                                                                                                  ****************************************
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E.

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fig|1639.7679.peg.2826PTScellobioseIIB(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-WT]        MKNIMLMCNAGMSTSVLVRKMERVVEERNLELTIWAISETDFEKNWRKADAILLGPQVNY  60
fig|6666666.523413.peg.1591PTScellobioseIIB(EC.2.7.1.205)[Listeria_monocytogenesFSL-R2-499-60g]   MKNIMLMCNAGMSTSVLVRKMERVVEERNLELTIWAISETDFEKNWRKADAILLGPQVNY  60
                                                                                                  ************************************************************

fig|1639.7679.peg.2826PTScellobioseIIB(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-WT]        MKDKVIDTVGDNVSVAVIDIVDYGRMNGEKVLDLAISLL  99
fig|6666666.523413.peg.1591PTScellobioseIIB(EC.2.7.1.205)[Listeria_monocytogenesFSL-R2-499-60g]   MKDKVIDTVGDNVSVAVIDIVDYGRMNGEKVLDLAISLL  99
                                                                                                  ****************************************
```

F.

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fig|1639.7679.peg.2845PTScellobioseIIB(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-WT]        MKKILLVCAAGMSTSLLVTKMKAHATSIGEEIEIEALPVSEASNVVDKMDIVMLGPQVRY  60
fig|6666666.523413.peg.1610PTScellobioseIIB(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-60g]  MKKILLVCAAGMSTSLLVTKMKAHATSIGEEIEIEALPVSEASNVVDKMDIVMLGPQVRY  60
                                                                                                  ************************************************************

fig|1639.7679.peg.2845PTScellobioseIIB(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-WT]        QKPQVDELVQGRIPVVVIDMKDYGMLNGKAVLEKAFAEIG  100
fig|6666666.523413.peg.1610PTScellobioseIIB(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-60g]  QKPQVDELVQGRIPVVVIDMKDYGMLNGKAVLEKAFAEIG  100
                                                                                                  ****************************************
```

G.

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fig|1639.7679.peg.2931PTScellobioseIIB(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-WT]        MKTIMLVCSAGMSTSLLVTKMEKAAAEKGLEAKIFAVAEAEAANHLDEIDVLLLGPQVRF  60
fig|6666666.523413.peg.1889                                                                       MKTIMLVCSAGMSTSLLVTKMEKAAAEKGLEAKIFAVAEAEAANHLDEIDVLLLGPQVRF  60
                                                                                                  ************************************************************

fig|1639.7679.peg.2931PTScellobioseIIB(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-WT]        LEGNMKKKLEPKGIPLAVINSVDYGMMKGDKVLEQALELMK  101
fig|6666666.523413.peg.1889                                                                       LEGNMKKKLEPKGIPLAVINSVDYGMMKGDKVLEQALELMK  101
                                                                                                  ****************************************
```

H.

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fig|1639.7679.peg.2965PTSlactose/cellobioseIIB[Listeria_monocytogenes_FSL-R2-499-WT]              MKILAVCGLGQGTSLILRMNVETVLRDMGVDADVEHIDVSAARSMNVDIIVTSQELAETL  60
fig|6666666.523413.peg.1855PTSlactose/cellobioseIIB[Listeria_monocytogenes_FSL-R2-499-60g]        MKILAVCGLGQGTSLILRMNVETVLRDMGVDADVEHIDVSAARSMNVDIIVTSQELAETL  60
                                                                                                  ************************************************************

fig|1639.7679.peg.2965PTSlactose/cellobioseIIB[Listeria_monocytogenes_FSL-R2-499-WT]              GTDTSAKVVIVNNYFDNAEIKNALSAAINS  90
fig|6666666.523413.peg.1855PTSlactose/cellobioseIIB[Listeria_monocytogenes_FSL-R2-499-60g]        GTDTSAKVVIVNNYFDNAEIKNALSAAINS  90
                                                                                                  ******************************
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I.

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fig|1639.7679.peg.2430PTScellobioseIIB(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-WT]          MKNIMLVCNAGMSTGMLAKKIEAASGNTLNVTAYSESEYTDYLDGVDLVLIGPQIRFLMP      60
fig|6666666.523413.peg.258PTScellobioseIIB(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-60g]    MKNIMLVCNAGMSTGMLAKKIEAASGNTLNVTAYSESEYTDYLDGVDLVLIGPQIRFLMP      60
                                                                                                  ************************************************************

fig|1639.7679.peg.2430PTScellobioseIIB(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-WT]          QIKQAVSVPVHAISPVKYGIMDGKGVYEDIQKLIGG      96
fig|6666666.523413.peg.258PTScellobioseIIB(EC.2.7.1.205)[Listeria_monocytogenes_FSL-R2-499-60g]    QIKQAVSVPVHAISPVKYGIMDGKGVYEDIQKLIGG      96
                                                                                                  ********************************
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Figure 3.5 A-I Sequence alignment of the Lactose/cellobiose-PTS subunits IIB from strains *L. monocytogenes* FSL-R2-499 before it was exposed to CclA and after growing the strain for 60 generations.

A.

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fig|1639.7679.peg.1768PTS_system_cellobioseIICcomponent[Listeria_monocytogenes_FSL-R2-499-WT]          MNNKVMDFMTNKFAPKVNKVVKNPWVSAIQDAIMSALPLVFVGSLVTIVSLLKNLFPGMP   60
fig|6666666.523413.peg.2087PTS-system_cellobioseIICcomponent[Listeria_monocytogenes_FSL-R2-499-60g]  MNNKVMDFMTNKFAPKVNKVVKNPWVSAIQDAIMSALPLVFVGSLVTIVSLLKNLFPGMP   60
                                                                                                     ************************************************************

fig|1639.7679.peg.1768PTS_system_cellobioseIICcomponent[Listeria_monocytogenes_FSL-R2-499-WT]          DFSMISNFSFGMFGLVVAFLIPYYLMEKKGNSSQKLISGATGLVLFLMLLFPTISADGDA   120
fig|6666666.523413.peg.2087PTS-system_cellobioseIICcomponent[Listeria_monocytogenes_FSL-R2-499-60g]  DFSMISNFSFGMFGLVVAFLIPYYLMEKKGNSSQKLISGATGLVLFLMLLFPTISADGDA   120
                                                                                                     ************************************************************

fig|1639.7679.peg.1768PTS_system_cellobioseIICcomponent[Listeria_monocytogenes_FSL-R2-499-WT]          VFILSRFGATGMFLSITTGLFVGCVMNFAAKRSFFSEDTPIPDFVVGWFNSLLPITFILI   180
fig|6666666.523413.peg.2087PTS-system_cellobioseIICcomponent[Listeria_monocytogenes_FSL-R2-499-60g]  VFILSRFGATGMFLSITTGLFVGCVMNFAAKRSFFSEDTPIPDFVVGWFNSLLPITFILI   180
                                                                                                     ************************************************************

fig|1639.7679.peg.1768PTS_system_cellobioseIICcomponent[Listeria_monocytogenes_FSL-R2-499-WT]          VGWLITVQFNIDFFEVIVAVFSPLASIVQSYPGFVLSVFIPAFLYTFGISGWVMMPAIYP   240
fig|6666666.523413.peg.2087PTS-system_cellobioseIICcomponent[Listeria_monocytogenes_FSL-R2-499-60g]  VGWLITVQFNIDFFEVIVAVFSPLASIVQSYPGFVLSVFIPAFLYTFGISGWVMMPAIYP   240
                                                                                                     ************************************************************

fig|1639.7679.peg.1768PTS_system_cellobioseIICcomponent[Listeria_monocytogenes_FSL-R2-499-WT]          VYMAGLAENSQAVANGASASNIATQETVYALISIGGVGTTLSLSIMMLILSKSLQLKAIG   300
fig|6666666.523413.peg.2087PTS-system_cellobioseIICcomponent[Listeria_monocytogenes_FSL-R2-499-60g]  VYMAGLAENSQAVANGASASNIATQETVYALISIGGVGTTLSLSIMMLILSKSLQLKAIG   300
                                                                                                     ************************************************************

fig|1639.7679.peg.1768PTS_system_cellobioseIICcomponent[Listeria_monocytogenes_FSL-R2-499-WT]          KAVIVPSIFNINEPLFFGAPIAFNPYLMIPTWINAFLVPSIAYFVMSMNLVSIPAQSFLL   360
fig|6666666.523413.peg.2087PTS-system_cellobioseIICcomponent[Listeria_monocytogenes_FSL-R2-499-60g]  KAVIVPSIFNINEPLFFGAPIAFNPYLMIPTWINAFLVPSIAYFVMSMNLVSIPAQSFLL   360
                                                                                                     ************************************************************

fig|1639.7679.peg.1768PTS_system_cellobioseIICcomponent[Listeria_monocytogenes_FSL-R2-499-WT]          WYMPYPVTSYLATQDFRGVIACLAIIVITWLVYLPFFKAYDNSLLKQEKLDAVETEKEMV   420
fig|6666666.523413.peg.2087PTS-system_cellobioseIICcomponent[Listeria_monocytogenes_FSL-R2-499-60g]  WYMPYPVTSYLATQDFRGVIACLAIIVITWLVYLPFFKAYDNSLLKQEKLDAVETEKEMV   420
                                                                                                     ************************************************************

fig|1639.7679.peg.1768PTS_system_cellobioseIICcomponent[Listeria_monocytogenes_FSL-R2-499-WT]          TN   422
fig|6666666.523413.peg.2087PTS-system_cellobioseIICcomponent[Listeria_monocytogenes_FSL-R2-499-60g]  TN   422
                                                                                                     **
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B.

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fig|1639.7679.peg.2328PTScellobiose-specificIIC[Listeria_monocytogenesFSL-R2-499-WT]     MAETKKKSIVNGFINVAQRLGGQIHLRSLRDAFASIMPFMILAGFVTLINYVILEPTGFM  60
fig|6666666.523413.peg.89PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]        MAETKKKSIVNGFINVAQRLGGQIHLRSLRDAFASIMPFMILAGFVTLINYVILEPTGFM  60
                                                                                        ************************************************************

fig|1639.7679.peg.2328PTScellobiose-specificIIC[Listeria_monocytogenesFSL-R2-499-WT]     GKIVNPDTLRTWQEIGISIGNGTLSVITLLVTVAISYHLCLNRGYKNVIAPILVALSSFI  120
fig|6666666.523413.peg.89PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]        GKIVNPDTLRTWQEIGISIGNGTLSVITLLVTVAISYHLCLNRGYKNVIAPILVALSSFI  120
                                                                                        ************************************************************

fig|1639.7679.peg.2328PTScellobiose-specificIIC[Listeria_monocytogenesFSL-R2-499-WT]     VVTPIAMTFLPEGASKSIEVPNVIPVSYTGASGMFVGIIVGLIATDLFIKLSKNKRMQIN  180
fig|6666666.523413.peg.89PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]        VVTPIAMTFLPEGASKSIEVPNVIPVSYTGASGMFVGIIVGLIATDLFIKLSKNKRMQIN  180
                                                                                        ************************************************************

fig|1639.7679.peg.2328PTScellobiose-specificIIC[Listeria_monocytogenesFSL-R2-499-WT]     LTGNIPPAVIKSFNVLIPIMITVIIFSVMSFAVNQIFSMDFNTLVTTIITKPLSYVTTSL  240
fig|6666666.523413.peg.89PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]        LTGNIPPAVIKSFNVLIPIMITVIIFSVMSFAVNQIFSMDFNTLVTTIITKPLSYVTTSL  240
                                                                                        ************************************************************

fig|1639.7679.peg.2328PTScellobiose-specificIIC[Listeria_monocytogenesFSL-R2-499-WT]     PGFLLITSIANLFFGLGIHQAVISGPLLDPFLLQNMQENMVAYANHQEIPHIINMAFKDT  300
fig|6666666.523413.peg.89PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]        PGFLLITSIANLFFGLGIHQAVISGPLLDPFLLQNMQENMVAYANHQEIPHIINMAFKDT  300
                                                                                        ************************************************************

fig|1639.7679.peg.2328PTScellobiose-specificIIC[Listeria_monocytogenesFSL-R2-499-WT]     FAVMGGSGNTIGLLIAIFIFGKRKDYKDISKLSAAPSLFNISEPIIFGLPIVFNPLLIIP  360
fig|6666666.523413.peg.89PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]        FAVMGGSGNTIGLLIAIFIFGKRKDYKDISKLSAAPSLFNISEPIIFGLPIVFNPLLIIP  360
                                                                                        ************************************************************

fig|1639.7679.peg.2328PTScellobiose-specificIIC[Listeria_monocytogenesFSL-R2-499-WT]     FVLAPIFSLTTAYYATAAGWINHVVVQTPWTTPPIISGFLATGGDWRASVLQVIIIVVTV  420
fig|6666666.523413.peg.89PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]        FVLAPIFSLTTAYYATAAGWINHVVVQTPWTTPPIISGFLATGGDWRASVLQVIIIVVTV  420
                                                                                        ************************************************************

fig|1639.7679.peg.2328PTScellobiose-specificIIC[Listeria_monocytogenesFSL-R2-499-WT]     FIYLPFLRMDEKVAFATAQKSDAK      444
fig|6666666.523413.peg.89PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]        FIYLPFLRMDEKVAFATAQKSDAK      444
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C.

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fig|1639.7679.peg.2390PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          MNKSNVFIDKLLVFANKFSSQRHIAAIRDGFVTLIPMTIIASFWVLVNNLILSPTNGLLK     60
fig|6666666.523413.peg.298PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]     MNKSNVFIDKLLVFANKFSSQRHIAAIRDGFVTLIPMTIIASFWVLVNNLILSPTNGLLK     60
                                                                                      ************************************************************

fig|1639.7679.peg.2390PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          NYSAAVKWADLGNQIYNGTLGIMALMIAVTIGYKLAVSYGDEGLIGAVMGLVSYMIVLPA     120
fig|6666666.523413.peg.298PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]     NYSAAVKWADLGNQIYNGTLGIMALMIAVTIGYKLAVSYGDEGLIGAVMGLVSYMIVLPA     120
                                                                                      ************************************************************

fig|1639.7679.peg.2390PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          QIIITDVEGKTFTAAAALTQTQTSATGMFLAIIATLLSVTWLSKFSKIERLKIKMPESVP     180
fig|6666666.523413.peg.298PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]     QIIITDVEGKTFTAAAALTQTQTSATGMFLAIIATLLSVTWLSKFSKIERLKIKMPESVP     180
                                                                                      ************************************************************

fig|1639.7679.peg.2390PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          PSIAKSFNVLIPIFFVTLILGLIEVLIVWVFNTNIPALILTFFQAPLISSFQSIGGILLY     240
fig|6666666.523413.peg.298PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]     PSIAKSFNVLIPIFFVTLILGLIEVLIVWVFNTNIPALILTFFQAPLISSFQSIGGILLY     240
                                                                                      ************************************************************

fig|1639.7679.peg.2390PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          VCFSNLLWAFGLHGTFILGSIGEPIMLTAIQENMEALKNGLALPNIVTKPFLDAFGWMGG     300
fig|6666666.523413.peg.298PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]     VCFSNLLWAFGLHGTFILGSIGEPIMLTAIQENMEALKNGLALPNIVTKPFLDAFGWMGG     300
                                                                                      ************************************************************

fig|1639.7679.peg.2390PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          GGMIICLVIAIMIASRREDYRSITKVGIIPSLFNVSEPLMFGLPVVFNPLLAVPLIIVPA     360
fig|6666666.523413.peg.298PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]     GGMIICLVIAIMIASRREDYRSITKVGIIPSLFNVSEPLMFGLPVVFNPLLAVPLIIVPA     360
                                                                                      ************************************************************

fig|1639.7679.peg.2390PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          VTVTIAYYATALGWIAKTSVLIPWTTPPVISGYLATNGDWRAAVLQIFLIAIGVLIYLPF     420
fig|6666666.523413.peg.298PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]     VTVTIAYYATALGWIAKTSVLIPWTTPPVISGYLATNGDWRAAVLQIFLIAIGVLIYLPF     420
                                                                                      ************************************************************

fig|1639.7679.peg.2390PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          VKASNRTVIKENAK   434
fig|6666666.523413.peg.298PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]     VKASNRTVIKENAK   434
                                                                                      **************
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D.

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fig|1639.7679.peg.2431PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          MRFNTISEKMDQYISPLANKLSQQRHLKATRDAFMSMLPITLFGSIPIILKAAPVTDDTK     60
fig|6666666.523413.peg.257PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]    MRFNTISEKMDQYISPLANKLSQQRHLKATRDAFMSMLPITLFGSIPIILKAAPVTDDTK     60
                                                                                     ************************************************************

fig|1639.7679.peg.2431PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          NGFLLGWANFAEKYDLILNWISGITLGAMSLYICVGITYYLCKHYHEDFLRPLMFSIAGF     120
fig|6666666.523413.peg.257PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]    NGFLLGWANFAEKYDLILNWISGITLGAMSLYICVGITYYLCKHYHEDFLRPLMFSIAGF     120
                                                                                     ************************************************************

fig|1639.7679.peg.2431PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          FMLVLNPIKMGWDGKEVDISFLDGRGILLSIFVAIVTVEGYHWMRKKNVGRITMPDSVPA     180
fig|6666666.523413.peg.257PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]    FMLVLNPIKMGWDGKEVDISFLDGRGILLSIFVAIVTVEGYHWMRKKNVGRITMPDSVPA     180
                                                                                     ************************************************************

fig|1639.7679.peg.2431PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          SLSETFAALVPGIILMTFFSIIFIIFNLLDTTAIQFLYEKMAPSFKAADSLPFTILSIFL     240
fig|6666666.523413.peg.257PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]    SLSETFAALVPGIILMTFFSIIFIIFNLLDTTAIQFLYEKMAPSFKAADSLPFTILSIFL     240
                                                                                     ************************************************************

fig|1639.7679.peg.2431PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          VHLFWFFGVHDAALAGILGPIRDGNLSINAAEKMAGQDLTNIFTTPFWTYFVIIGGSGSV     300
fig|6666666.523413.peg.257PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]    VHLFWFFGVHDAALAGILGPIRDGNLSINAAEKMAGQDLTNIFTTPFWTYFVIIGGSGSV     300
                                                                                     ************************************************************

fig|1639.7679.peg.2431PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          LALAFLMMRSKSKQLSTVGKVGFLPSIFGISEPLIFGTPLMLNPIFFFPFIFTSVFNGVI     360
fig|6666666.523413.peg.257PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]    LALAFLMMRSKSKQLSTVGKVGFLPSIFGISEPLIFGTPLMLNPIFFFPFIFTSVFNGVI     360
                                                                                     ************************************************************

fig|1639.7679.peg.2431PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          TYLCMYFGIVGKTFAVFSWQMPAPIGAFLSTMDWRAIVLVFILIFLNGLMYYPFLKVYEK     420
fig|6666666.523413.peg.257PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]    TYLCMYFGIVGKTFAVFSWQMPAPIGAFLSTMDWRAIVLVFILIFLNGLMYYPFLKVYEK     420
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fig|1639.7679.peg.2431PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          NLVALEKEAEEENIAAN       437
fig|6666666.523413.peg.257PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]    NLVALEKEAEEENIAAN       437
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E.

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fig|1639.7679.peg.2710PTScellobioseIIC[Listeria_monocytogenesFSL-R2-499-WT]          MSSERSLSSRFIEGLSIFAQKISSQKHIMAIRDGFAAMIPITIIAAFFLLVNNVLLQPEN   60
fig|6666666.523413.peg.1476PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]  MSSERSLSSRFIEGLSIFAQKISSQKHIMAIRDGFAAMIPITIIAAFFLLVNNVLLQPEN   60
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fig|1639.7679.peg.2710PTScellobioseIIC[Listeria_monocytogenesFSL-R2-499-WT]          GLLKFIPNVENYLGVGIQVYNATLGIMAILAAFLIGNFLAKSYGMEGRTEGVIAVAAYVV   120
fig|6666666.523413.peg.1476PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]  GLLKFIPNVENYLGVGIQVYNATLGIMAILAAFLIGNFLAKSYGMEGRTEGVIAVAAYVV   120
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fig|1639.7679.peg.2710PTScellobioseIIC[Listeria_monocytogenesFSL-R2-499-WT]          LIPASSHLMSVDGKAFEAGGVLTQEMTSSTGMFLAIIAALVSITMLAKFSKSKKLKISMP   180
fig|6666666.523413.peg.1476PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]  LIPASSHLMSVDGKAFEAGGVLTQEMTSSTGMFLAIIAALVSITMLAKFSKSKKLKISMP   180
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fig|1639.7679.peg.2710PTScellobioseIIC[Listeria_monocytogenesFSL-R2-499-WT]          ESVPPAIAKSFNILIPSFLVLSILAIIEVLVVSFVSMSIPEIIVKVLQIPLVGGFQTLPG   240
fig|6666666.523413.peg.1476PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]  ESVPPAIAKSFNILIPSFLVLSILAIIEVLVVSFVSMSIPEIIVKVLQIPLVGGFQTLPG   240
                                                                                    ************************************************************

fig|1639.7679.peg.2710PTScellobioseIIC[Listeria_monocytogenesFSL-R2-499-WT]          ILLYVFLAGFLWVFGIHGAFVLGAISGPVLLTSLQQNIDAVNAGTALPNIVTQPFLDAFV   300
fig|6666666.523413.peg.1476PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]  ILLYVFLAGFLWVFGIHGAFVLGAISGPVLLTSLQQNIDAVNAGTALPNIVTQPFLDAFV   300
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fig|1639.7679.peg.2710PTScellobioseIIC[Listeria_monocytogenesFSL-R2-499-WT]          YMGGGGTIICLVIAIFIASKRPDHRMVTKFGLIPSIFNVSEPLMFGLPVVFNPIYGIPLV   360
fig|6666666.523413.peg.1476PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]  YMGGGGTIICLVIAIFIASKRPDHRMVTKFGLIPSIFNVSEPLMFGLPVVFNPIYGIPLV   360
                                                                                    ************************************************************

fig|1639.7679.peg.2710PTScellobioseIIC[Listeria_monocytogenesFSL-R2-499-WT]          IAPLASTAMAYFATSWGWISQTYILIPWVTPPVLSGYLATGGDIRASILQIAIIIVGTLI   420
fig|6666666.523413.peg.1476PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]  IAPLASTAMAYFATSWGWISQTYILIPWVTPPVLSGYLATGGDIRASILQIAIIIVGTLI   420
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fig|1639.7679.peg.2710PTScellobioseIIC[Listeria_monocytogenesFSL-R2-499-WT]          YLPFVLVANRAYVLEQKAAGKVEAVTNGEV  450
fig|6666666.523413.peg.1476PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]  YLPFVLVANRAYVLEQKAAGKVEAVTNGEV  450
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F.

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fig|1639.7679.peg.2431PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          MRFNTISEKMDQYISPLANKLSQQRHLKATRDAFMSMLPITLFGSIPIILKAAPVTDDTK     60
fig|6666666.523413.peg.257PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]     MRFNTISEKMDQYISPLANKLSQQRHLKATRDAFMSMLPITLFGSIPIILKAAPVTDDTK     60
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fig|1639.7679.peg.2431PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          NGFLLGWANFAEKYDLILNWISGITLGAMSLYICVGITYYLCKHYHEDFLRPLMFSIAGF     120
fig|6666666.523413.peg.257PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]     NGFLLGWANFAEKYDLILNWISGITLGAMSLYICVGITYYLCKHYHEDFLRPLMFSIAGF     120
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fig|1639.7679.peg.2431PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          FMLVLNPIKMGWDGKEVDISFLDGRGILLSIFVAIVTVEGYHWMRKKNVGRITMPDSVPA     180
fig|6666666.523413.peg.257PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]     FMLVLNPIKMGWDGKEVDISFLDGRGILLSIFVAIVTVEGYHWMRKKNVGRITMPDSVPA     180
                                                                                     ************************************************************

fig|1639.7679.peg.2431PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          SLSETFAALVPGIILMTFFSIIFIIFNLLDTTAIQFLYEKMAPSFKAADSLPFTILSIFL     240
fig|6666666.523413.peg.257PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]     SLSETFAALVPGIILMTFFSIIFIIFNLLDTTAIQFLYEKMAPSFKAADSLPFTILSIFL     240
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fig|1639.7679.peg.2431PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          VHLFWFFGVHDAALAGILGPIRDGNLSINAAEKMAGQDLTNIFTTPFWTYFVIIGGSGSV     300
fig|6666666.523413.peg.257PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]     VHLFWFFGVHDAALAGILGPIRDGNLSINAAEKMAGQDLTNIFTTPFWTYFVIIGGSGSV     300
                                                                                     ************************************************************

fig|1639.7679.peg.2431PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          LALAFLMMRSKSKQLSTVGKVGFLPSIFGISEPLIFGTPLMLNPIFFFPFIFTSVFNGVI     360
fig|6666666.523413.peg.257PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]     LALAFLMMRSKSKQLSTVGKVGFLPSIFGISEPLIFGTPLMLNPIFFFPFIFTSVFNGVI     360
                                                                                     ************************************************************

fig|1639.7679.peg.2431PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          TYLCMYFGIVGKTFAVFSWQMPAPIGAFLSTMDWRAIVLVFILIFLNGLMYYPFLKVYEK     420
fig|6666666.523413.peg.257PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]     TYLCMYFGIVGKTFAVFSWQMPAPIGAFLSTMDWRAIVLVFILIFLNGLMYYPFLKVYEK     420
                                                                                     ************************************************************

fig|1639.7679.peg.2431PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          NLVALEKEAEEENIAAN       437
fig|6666666.523413.peg.257PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]     NLVALEKEAEEENIAAN       437
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G.

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fig|1639.7679.peg.2844PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          MSMMSKFEHGMERVLVPVANKLNSQRHIAAIRDAFILVFPLIMAGSIITLINFAVLSPDG   60
fig|6666666.523413.peg.1609PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]    MSMMSKFEHGMERVLVPVANKLNSQRHIAAIRDAFILVFPLIMAGSIITLINFAVLSPDG   60
                                                                                     ************************************************************

fig|1639.7679.peg.2844PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          FIAKILFLGKIFPNLADAQAVFSPVMQGSTNIMAILIVFLVARNLAIFFKQDDLLCGLTS   120
fig|6666666.523413.peg.1609PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]    FIAKILFLGKIFPNLADAQAVFSPVMQGSTNIMAILIVFLVARNLAIFFKQDDLLCGLTS   120
                                                                                     ************************************************************

fig|1639.7679.peg.2844PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          IGAFFIVYTPYTVVDNASYMTIKFLGAQGLFVAIIVAIITGEVFSRLARSPRLMIKMPDQ   180
fig|6666666.523413.peg.1609PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]    IGAFFIVYTPYTVVDNASYMTIKFLGAQGLFVAIIVAIITGEVFSRLARSPRLMIKMPDQ   180
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fig|1639.7679.peg.2844PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          VPPAVARSFKVLIPVIIITILFSVINYLITLVAPEGLNDLVYTVIQAPLKDMGTNVFSVI   240
fig|6666666.523413.peg.1609PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]    VPPAVARSFKVLIPVIIITILFSVINYLITLVAPEGLNDLVYTVIQAPLKDMGTNVFSVI   240
                                                                                     ************************************************************

fig|1639.7679.peg.2844PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          IIGLVSNLLWVLGIHGPNTVAAIRDTIFTEPNLDNLSYVAQHGSAWGAPYPATWAGLNDG   300
fig|6666666.523413.peg.1609PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]    IIGLVSNLLWVLGIHGPNTVAAIRDTIFTEPNLDNLSYVAQHGSAWGAPYPATWAGLNDG   300
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fig|1639.7679.peg.2844PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          FANYGGSGMTLGLLIAIFIASRRADYRDIAKLSLAPGIFNINEPVIFGLPIVLNPIMVIP   360
fig|6666666.523413.peg.1609PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]    FANYGGSGMTLGLLIAIFIASRRADYRDIAKLSLAPGIFNINEPVIFGLPIVLNPIMVIP   360
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fig|1639.7679.peg.2844PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          FIITPAINTLIGYFFISTKLIPPVAYQVPWTTPGPLIPFLGTGGNWLALLVGLLCLAVAT   420
fig|6666666.523413.peg.1609PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]    FIITPAINTLIGYFFISTKLIPPVAYQVPWTTPGPLIPFLGTGGNWLALLVGLLCLAVAT   420
                                                                                     ************************************************************

fig|1639.7679.peg.2844PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          VIYLPFVLVSNKIAASDAAMDKNATASTEQ  450
fig|6666666.523413.peg.1609PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]    VIYLPFVLVSNKIAASDAAMDKNATASTEQ  450
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H.

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fig|1639.7679.peg.2902PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          MNGLTAFLEKYFVPVAAKIGSQKHLVALRDAFISTMPITMAGSIAVLLNAFFRDFPTDWG    60
fig|6666666.523413.peg.1924PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]    MNGLTAFLEKYFVPVAAKIGSQKHLVALRDAFISTMPITMAGSIAVLLNAFFRDFPTDWG    60
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fig|1639.7679.peg.2902PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          WTGFVEAMQPLIDINGYVYNGTLAIVSIIFAFSLGYNLSKAYEVDRLAGGLVSLAAFVMN    120
fig|6666666.523413.peg.1924PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]    WTGFVEAMQPLIDINGYVYNGTLAIVSIIFAFSLGYNLSKAYEVDRLAGGLVSLAAFVMN    120
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fig|1639.7679.peg.2902PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          LTVTVSLDAVKAAIAASNANFDVATLPKEFAGIYGFFSLSQVNGTGLFTAMIFGFISTII    180
fig|6666666.523413.peg.1924PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]    LTVTVSLDAVKAAIAASNANFDVATLPKEFAGIYGFFSLSQVNGTGLFTAMIFGFISTII    180
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fig|1639.7679.peg.2902PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          YAKLMRRNIIIKMPDSVPPAVSKAFAAIIPALVALYVVGIIDWAFFKITNMDVITWISKT    240
fig|6666666.523413.peg.1924PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]    YAKLMRRNIIIKMPDSVPPAVSKAFAAIIPALVALYVVGIIDWAFFKITNMDVITWISKT    240
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fig|1639.7679.peg.2902PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          IQEPLLSLSQGYGAVLLVTFLVQLLWFFGIHGPNVLAPVLESLWGTAQLQNISAAQEGAK    300
fig|6666666.523413.peg.1924PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]    IQEPLLSLSQGYGAVLLVTFLVQLLWFFGIHGPNVLAPVLESLWGTAQLQNISAAQEGAK    300
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fig|1639.7679.peg.2902PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          LPFEWVRGSFDAYVWMGGSGGTLVLIIALLMFSKRADARTVAKLSLAPGIFNINEPIMFG    360
fig|6666666.523413.peg.1924PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]    LPFEWVRGSFDAYVWMGGSGGTLVLIIALLMFSKRADARTVAKLSLAPGIFNINEPIMFG    360
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fig|1639.7679.peg.2902PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          LPIVLNTIYLIPFLIAPMVMVTIAYFATTLGIVGPVKIAVVWVMPPLLNSFLATGGDWMA    420
fig|6666666.523413.peg.1924PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]    LPIVLNTIYLIPFLIAPMVMVTIAYFATTLGIVGPVKIAVVWVMPPLLNSFLATGGDWMA    420
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fig|1639.7679.peg.2902PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          PVISLINMVVAFLIWVPFVITANRVGVPEEEMKA      454
fig|6666666.523413.peg.1924PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]    PVISLINMVVAFLIWVPFVITANRVGVPEEEMKA      454
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I.

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fig|1639.7679.peg.2930PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          MNGFIAFMEKYFIPYAAKIGGQRHLVAIRDGFITTMPLMILGSFAVLINNFPIPAYQKFM   60
fig|6666666.523413.peg.1890PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]    MNGFIAFMEKYFIPYAAKIGGQRHLVAIRDGFITTMPLMILGSFAVLINNFPIPAYQKFM   60
                                                                                     ************************************************************

fig|1639.7679.peg.2930PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          NNLFGEGTWQAFGGNVWNGTFAILALLIAFTVAYNLAKSYDKDPLSSAVVSVATFFTIGA   120
fig|6666666.523413.peg.1890PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]    NNLFGEGTWQAFGGNVWNGTFAILALLIAFTVAYNLAKSYDKDPLSSAVVSVATFFTIGA   120
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fig|1639.7679.peg.2930PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          IAPGADGIANTGGLGSTGLFLALIIAILSTEIFTRLSGSPKLIINMPDGVPPAVSRSFAA   180
fig|6666666.523413.peg.1890PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]    IAPGADGIANTGGLGSTGLFLALIIAILSTEIFTRLSGSPKLIINMPDGVPPAVSRSFAA   180
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fig|1639.7679.peg.2930PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          LFPAMITVSIFGLITAFFQAAGVTNLVISFYELVQEPFMGLANSLPAALLLAFVSAFLWF   240
fig|6666666.523413.peg.1890PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]    LFPAMITVSIFGLITAFFQAAGVTNLVISFYELVQEPFMGLANSLPAALLLAFVSAFLWF   240
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fig|1639.7679.peg.2930PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          FGLHGANIIDPFMQTINIPAIEANVKALEAGKELPYIVNKPFFDSFVNLGGTGATIGLII   300
fig|6666666.523413.peg.1890PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]    FGLHGANIIDPFMQTINIPAIEANVKALEAGKELPYIVNKPFFDSFVNLGGTGATIGLII   300
                                                                                     ************************************************************

fig|1639.7679.peg.2930PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          AIFIVARKHKAYMTVSKLSAAPGIFNINEPMMFGLPIVLNPIMFIPYILAPLVLVTVAYF   360
fig|6666666.523413.peg.1890PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]    AIFIVARKHKAYMTVSKLSAAPGIFNINEPMMFGLPIVLNPIMFIPYILAPLVLVTVAYF   360
                                                                                     ************************************************************

fig|1639.7679.peg.2930PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          ATAIGWVPACTIVTPWTTPPIIGGALATQSIAGGVLAAVNLGLSILIFLPFAKIAQIQEL   420
fig|6666666.523413.peg.1890PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]    ATAIGWVPACTIVTPWTTPPIIGGALATQSIAGGVLAAVNLGLSILIFLPFAKIAQIQEL   420
                                                                                     ************************************************************

fig|1639.7679.peg.2930PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-WT]          RREKEALAAE      430
fig|6666666.523413.peg.1890PTScellobioseIIC[Listeria_monocytogenes_FSL-R2-499-60g]    RREKEALAAE      430
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Figure 3.6 A-I Sequence alignment of the Lactose/cellobiose-PTS subunits IIC from strains *L. monocytogenes* FSL-R2-499 before it was exposed to CclA and after growing the strain for 60 generations.

A.

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fig|1639.7678.peg.949PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-WT]          MTISERQRSLLEKLNDSQKTVTAKALSEMLGVSSKTVRNDIMQINQSFSSTIIASKAGKG      60
fig|6666666.523397.peg.2279PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-60g]  MTISERQRSLLEKLNDSQKTVTAKALSEMLGVSSKTVRNDIMQINQSFSSTIIASKAGKG      60
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fig|1639.7678.peg.949PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-WT]          YFLMPNEQLSQMNLTKNNENLHFELLRHIIEQDHTNFYDLADQFFISESTLARIIKELNI      120
fig|6666666.523397.peg.2279PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-60g]  YFLMPNEQLSQMNLTKNNENLHFELLRHIIEQDHTNFYDLADQFFISESTLARIIKELNI      120
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fig|1639.7678.peg.949PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-WT]          VIAEKDESLCIIRKNNELLTEGGEEEKRRIFNLFLNQEIENHQLSLDKYADYFDYCNLKQ      180
fig|6666666.523397.peg.2279PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-60g]  VIAEKDESLCIIRKNNELLTEGGEEEKRRIFNLFLNQEIENHQLSLDKYADYFDYCNLKQ      180
                                                                                                 ************************************************************

fig|1639.7678.peg.949PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-WT]          LSELIIAYHKKHEFFMNDFSTISFILHIAVLIERISMGSYIERTALLEQDKTSLEMAAHL      240
fig|6666666.523397.peg.2279PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-60g]  LSELIIAYHKKHEFFMNDFSTISFILHIAVLIERISMGSYIERTALLEQDKTSLEMAAHL      240
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fig|1639.7678.peg.949PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-WT]          AETLENELQINIPTQELSYIARLYSGKLTTTSTIDAQVFGSVVTRLLEAVDQNFHIDFSA      300
fig|6666666.523397.peg.2279PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-60g]  AETLENELQINIPTQELSYIARLYSGKLTTTSTIDAQVFGSVVTRLLEAVDQNFHIDFSA      300
                                                                                                 ************************************************************

fig|1639.7678.peg.949PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-WT]          DEKIATYLVAHISALYKRANHKQYLTNPLTEELKNKFPFIYNVSVYASAFIQKELAITFP      360
fig|6666666.523397.peg.2279PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-60g]  DEKIATYLVAHISALYKRANHKQYLTNPLTEELKNKFPFIYNVSVYASAFIQKELAITFP      360
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fig|1639.7678.peg.949PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-WT]          DDEIAYIALHFLSASETINHGKKRKILLVSPYGAGSQRLVHNQLKKIPDFSIDLLVSQSI      420
fig|6666666.523397.peg.2279PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-60g]  DDEIAYIALHFLSASETINHGKKRKILLVSPYGAGSQRLVHNQLKKIPDFSIDLLVSQSI      420
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fig|1639.7678.peg.949PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-WT]          FDIKQFILDKEIHLIITAEPLNLTTDIPVYHYDLLLTEADLQKIKHILETKPKADSISRK      480
fig|6666666.523397.peg.2279PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-60g]  FDIKQFILDKEIHLIITAEPLNLTTDIPVYHYDLLLTEADLQKIKHILETKPKADSISRK      480
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fig|1639.7678.peg.949PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-WT]          FFKKELFFPKQNFKSKEETITFLCEQLTAFDYCDPDYVAKVFEREQLSSTCYGNYYAIPH      540
fig|6666666.523397.peg.2279PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-60g]  FFKKELFFPKQNFKSKEETITFLCEQLTAFDYCDPDYVAKVFEREQLSSTCYGNYYAIPH      540
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fig|1639.7678.peg.949PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-WT]          AIQRSAKKNAVAVCSLDKPIDWGGNRVKLVLLLTMKEERDNSFEELFGQLVTILNERSFV      600
fig|6666666.523397.peg.2279PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-60g]  AIQRSAKKNAVAVCSLDKPIDWGGNRVKLVLLLTMKEERDNSFEELFGQLVTILNERSFV      600
                                                                                                 ************************************************************

fig|1639.7678.peg.949PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-WT]          KKLAKQEDFQQFIELCEQKTLDS   623
fig|6666666.523397.peg.2279PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-60g]  KKLAKQEDFQQFIELCEQKTLDS   623
                                                                                                 ***********************
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B.

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fig|1639.7678.peg.2766PTSIIAcellobiose(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-WT]          MDLEQTIMSLIVFGGNAKSDAMLAIDSAKKGDFAQADEQIAQAEQALLEAHHSQTKLIQG      60
fig|6666666.523397.peg.2398PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenesFSL-C1-056-60g]   MDLEQTIMSLIVFGGNAKSDAMLAIDSAKKGDFAQADEQIAQAEQALLEAHHSQTKLIQG      60
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fig|1639.7678.peg.2766PTSIIAcellobiose(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-WT]          EARGEKTEVSLLLVHAQDHLMNAITFKDLAKEIVDLYKNK   100
fig|6666666.523397.peg.2398PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenesFSL-C1-056-60g]   EARGEKTEVSLLLVHAQDHLMNAITFKDLAKEIVDLYKNK   100
                                                                                                 ***************************************
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C.

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fig|1639.7678.peg.1596PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-WT]          MSEQDYVEETDSLNELSMNILIHAGNARNDLVKGLNHLEELEFNEAEEFIASAKREIVIA      60
fig|6666666.523397.peg.1181PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenesFSL-C1-056-60g]   MSEQDYVEETDSLNELSMNILIHAGNARNDLVKGLNHLEELEFNEAEEFIASAKREIVIA      60
                                                                                                 ************************************************************

fig|1639.7678.peg.1596PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-WT]          HSLQTDTLQLEASGNQIRYSTLFCHAQDTLMTAKSEILIGEHMLRLFKKVTELTKK   116
fig|6666666.523397.peg.1181PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenesFSL-C1-056-60g]   HSLQTDTLQLEASGNQIRYSTLFCHAQDTLMTAKSEILIGEHMLRLFKKVTELTKK   116
                                                                                                 ************************************************************
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D.

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fig|1639.7678.peg.2682PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-WT]          MNEMETVIFGMISQVGSARSSYLEGLRAAREGNFEEAEAKLKEGGETLANGHHEHHKLIQ      60
fig|6666666.523397.peg.530PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-60g]   MNEMETVIFGMISQVGSARSSYLEGLRAAREGNFEEAEAKLKEGGETLANGHHEHHKLIQ      60
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fig|1639.7678.peg.2682PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-WT]          KEASGEKVEIQLLLIHAEDLLITTETLREVVTEFVHVYKKIN 102
fig|6666666.523397.peg.530PTScellobioseIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-60g]   KEASGEKVEIQLLLIHAEDLLITTETLREVVTEFVHVYKKIN 102
                                                                                                 ******************************************
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E.

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fig|1639.7678.peg.833PTSIIAcellobiose(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-WT]       MELEQTIMQLIVHGGNAKSDAMLAIEAAKKGDFDVADEQIKNAEAALLEAHHSQTSLIQG      60
fig|6666666.523397.peg.2534PTSIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-60g]           MELEQTIMQLIVHGGNAKSDAMLAIEAAKKGDFDVADEQIKNAEAALLEAHHSQTSLIQG      60
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fig|1639.7678.peg.833PTSIIAcellobiose(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-WT]       EARGEKAEVSLLLVHAQDHLMNAITFKDLAKEIVDLYRSK 100
fig|6666666.523397.peg.2534PTSIIA(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-60g]           EARGEKAEVSLLLVHAQDHLMNAITFKDLAKEIVDLYRSK 100
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Figure 3.7 A-E Sequence alignment of the Lactose/cellobiose-PTS subunits IIA from strains *L. monocytogenes* FSL-C1-056 before it was exposed to CclA and after growing the strain for 60 generations.

A.

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fig|1639.7678.peg.951PTScellobioseIIB(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-WT]          MKNIMLVCNAGMSTGMLAKKIEAASGNTLNVTAYSESEYTDYLDGVDLVLIGPQIRFLMP     60
fig|6666666.523397.peg.2277PTScellobioseIIB(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-60g]    MKNIMLVCNAGMSTGMLAKKIEAASGNTLNVTAYSESEYTDYLDGVDLVLIGPQIRFLMP     60
                                                                                                   ************************************************************

fig|1639.7678.peg.951PTScellobioseIIB(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-WT]          QIKQAVSVPVHAISPVKYGIMDGKGVYEDIQKLIGG      96
fig|6666666.523397.peg.2277PTScellobioseIIB(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-60g]    QIKQAVSVPVHAISPVKYGIMDGKGVYEDIQKLIGG      96
                                                                                                   ********************************
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B.

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fig|1639.7678.peg.832PTScellobioseIIB(EC.2.7.1.205)[FSL-C1-056-WT]          MNNIMLVCSAGMSTSLLVKKMTEAIEKQQVDATVIAVAEADFDKYKGNVDVVLLAPQVRF     60
fig|6666666.523397.peg.2535PTScellobioseIIB(EC.2.7.1.205)[FSL-C1-056-60g]   MNNIMLVCSAGMSTSLLVKKMTEAIEKQQVDATVIAVAEADFDKYKGNVDVVLLAPQVRF     60
                                                                            ************************************************************

fig|1639.7678.peg.832PTScellobioseIIB(EC.2.7.1.205)[FSL-C1-056-WT]          LEKNLKRVLDPLGIPVAIINGIDYGTMDGEKVLNDALAMIEK       102
fig|6666666.523397.peg.2535PTScellobioseIIB(EC.2.7.1.205)[FSL-C1-056-60g]   LEKNLKRVLDPLGIPVAIINGIDYGTMDGEKVLNDALAMIEK       102
                                                                            ***************************************
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C.

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fig|1639.7678.peg.2801PTScellobioseIIB[Listeria_monocytogenes_FSL-C1-056-WT]          MKILAVCGLGQGTSLILRMNVETVLRDMGVDADVEHIDVSAARSMNVDIIVTSQELAETL     60
fig|6666666.523397.peg.2433PTScellobioseIIB[Listeria_monocytogenes_FSL-C1-056-60g]    MKILAVCGLGQGTSLILRMNVETVLRDMGVDADVEHIDVSAARSMNVDIIVTSQELAETL     60
                                                                                      ************************************************************

fig|1639.7678.peg.2801PTScellobioseIIB[Listeria_monocytogenes_FSL-C1-056-WT]          GTDTSAKVVIVNNYFDNAEIKNALSAAINS  90
fig|6666666.523397.peg.2433PTScellobioseIIB[Listeria_monocytogenes_FSL-C1-056-60g]    GTDTSAKVVIVNNYFDNAEIKNALSAAINS  90
                                                                                      ******************************
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D.

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fig|1639.7678.peg.1022PTScellobioseIIB(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-WT]          MKNILLVCNAGMSTSFLVEKMKAAGAEQGVEANIWAVSDAELHENWEKADVILLGPQVGY     60
fig|6666666.523397.peg.608PTScellobioseIIB(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-60g]     MKNILLVCNAGMSTSFLVEKMKAAGAEQGVEANIWAVSDAELHENWEKADVILLGPQVGY     60
                                                                                                   ************************************************************

fig|1639.7678.peg.1022PTScellobioseIIB(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-WT]          LKGNTEKVVGGKIPVEVINMLDYGRVNGAAVLERAIELIG   100
fig|6666666.523397.peg.608PTScellobioseIIB(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-60g]     LKGNTEKVVGGKIPVEVINMLDYGRVNGAAVLERAIELIG   100
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E.

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fig|1639.7678.peg.1594PTScellobioseIIB(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-WT]          MKNILLICGSGASSGFMAAAIRKAAKKRGEQVTVKAASESQIDERINEIDYLLIGPHLAY      60
fig|6666666.523397.peg.1179PTScellobioseIIB(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-60g]    MKNILLICGSGASSGFMAAAIRKAAKKRGEQVTVKAASESQIDERINEIDYLLIGPHLAY      60
                                                                                                    ************************************************************

fig|1639.7678.peg.1594PTScellobioseIIB(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-WT]          MLDDLKQKVADKNVLVSIIPQATYGTLNGEKALDLILTMEG 101
fig|6666666.523397.peg.1179PTScellobioseIIB(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-60g]    MLDDLKQKVADKNVLVSIIPQATYGTLNGEKALDLILTMEG 101
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F.

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fig|1639.7678.peg.2768PTScellobioseIIB(EC.2.7.1.205)[Listeria_monocytogenesFSL-C1-056-WT]          MKTIMLVCSAGMSTSLLVTKMEKAAAEQGLEAKIFAVAEAEAANHLDEIDVLLLGPQVRF      60
fig|6666666.523397.peg.2400PTScellobioseIIB(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-60g]    MKTIMLVCSAGMSTSLLVTKMEKAAAEQGLEAKIFAVAEAEAANHLDEIDVLLLGPQVRF      60
                                                                                                    ************************************************************

fig|1639.7678.peg.2768PTScellobioseIIB(EC.2.7.1.205)[Listeria_monocytogenesFSL-C1-056-WT]          LEGNMKKKLEPKGIPLAVINSVDYGMMKGDKVLEQALELMK 101
fig|6666666.523397.peg.2400PTScellobioseIIB(EC.2.7.1.205)[Listeria_monocytogenes_FSL-C1-056-60g]    LEGNMKKKLEPKGIPLAVINSVDYGMMKGDKVLEQALELMK 101
                                                                                                    *****************************************
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G.

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fig|1639.7678.peg.2666PTScellobioseIIB(EC.2.7.1.205)[Listeria_monocytogenesFSL-C1-056-WT]          MKNIMLMCNAGMSTSVLVRKMERVVEERNLELTIWAISETDFEKNWRKADAILLGPQVNY      60
fig|6666666.523397.peg.546PTScellobioseIIB(EC.2.7.1.205)[Listeria_monocytogenesFSL-C1-056-60g]     MKNIMLMCNAGMSTSVLVRKMERVVEERNLELTIWAISETDFEKNWRKADAILLGPQVNY      60
                                                                                                    ************************************************************

fig|1639.7678.peg.2666PTScellobioseIIB(EC.2.7.1.205)[Listeria_monocytogenesFSL-C1-056-WT]          MKDKVIDTVGDNVSVAVIDIVDYGRMNGEKVLDLAISLL     99
fig|6666666.523397.peg.546PTScellobioseIIB(EC.2.7.1.205)[Listeria_monocytogenesFSL-C1-056-60g]     MKDKVIDTVGDNVSVAVIDIVDYGRMNGEKVLDLAISLL     99
                                                                                                    ***************************************
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Figure 3.8 A-G Sequence alignment of the Lactose/cellobiose-PTS subunits IIB from strains *L. monocytogenes* FSL-C1-056 before it was exposed to CclA and after growing the strain for 60 generations.

A.

```
fig|1639.7678.peg.950PTSIIC[Listeria_monocytogenes_FSL-C1-056-WT]                    MRFNTISEKMDQYISPLANKLSQQRHLKATRDAFMSMLPITLFGSIPIILKAAPVTDDTQ  60
fig|6666666.523397.peg.2278PTScellobiose-specific_IIC[Listeria_monocytogenes_FSL-C1-056-60g]  MRFNTISEKMDQYISPLANKLSQQRHLKATRDAFMSMLPITLFGSIPIILKAAPVTDDTQ  60
                                                                                     ************************************************************

fig|1639.7678.peg.950PTSIIC[Listeria_monocytogenes_FSL-C1-056-WT]                    NGFLLGWANFAEKYDLILNWISGITLGAMSLYICVGITYYLCKHYHEDFLRPLMFSIAGF  120
fig|6666666.523397.peg.2278PTScellobiose-specific_IIC[Listeria_monocytogenes_FSL-C1-056-60g]  NGFLLGWANFAEKYDLILNWISGITLGAMSLYICVGITYYLCKHYHEDFLRPLMFSIAGF  120
                                                                                     ************************************************************

fig|1639.7678.peg.950PTSIIC[Listeria_monocytogenes_FSL-C1-056-WT]                    FMLVLNPIKMGWDGKEVDISFLDGRGILLSIFVAIVTVEGYHWMRKKNVGRITMPDSVPA  180
fig|6666666.523397.peg.2278PTScellobiose-specific_IIC[Listeria_monocytogenes_FSL-C1-056-60g]  FMLVLNPIKMGWDGKEVDISFLDGRGILLSIFVAIVTVEGYHWMRKKNVGRITMPDSVPA  180
                                                                                     ************************************************************

fig|1639.7678.peg.950PTSIIC[Listeria_monocytogenes_FSL-C1-056-WT]                    SLSETFAALVPGIILMTFFSIIFIIFNLLDTTAIQFLYEKMAPSFKAADSLPFTILSIFL  240
fig|6666666.523397.peg.2278PTScellobiose-specific_IIC[Listeria_monocytogenes_FSL-C1-056-60g]  SLSETFAALVPGIILMTFFSIIFIIFNLLDTTAIQFLYEKMAPSFKAADSLPFTILSIFL  240
                                                                                     ************************************************************

fig|1639.7678.peg.950PTSIIC[Listeria_monocytogenes_FSL-C1-056-WT]                    VHLFWFFGVHDAALAGILGPIRDGNLSINAAEKMAGQDLTNIFTTPFWTYFVIIGGSGSV  300
fig|6666666.523397.peg.2278PTScellobiose-specific_IIC[Listeria_monocytogenes_FSL-C1-056-60g]  VHLFWFFGVHDAALAGILGPIRDGNLSINAAEKMAGQDLTNIFTTPFWTYFVIIGGSGSV  300
                                                                                     ************************************************************

fig|1639.7678.peg.950PTSIIC[Listeria_monocytogenes_FSL-C1-056-WT]                    LALAFLMMRSKSKQLSTVGKVGFLPSIFGISEPLIFGTPLMLNPIFFFPFIFTSVFNGVI  360
fig|6666666.523397.peg.2278PTScellobiose-specific_IIC[Listeria_monocytogenes_FSL-C1-056-60g]  LALAFLMMRSKSKQLSTVGKVGFLPSIFGISEPLIFGTPLMLNPIFFFPFIFTSVFNGVI  360
                                                                                     ************************************************************

fig|1639.7678.peg.950PTSIIC[Listeria_monocytogenes_FSL-C1-056-WT]                    TYLCMYFGIVGKTFAVFSWQMPAPIGAFLSTMDWRAIVLVFILIFLNGLMYYPFLKVYEK  420
fig|6666666.523397.peg.2278PTScellobiose-specific_IIC[Listeria_monocytogenes_FSL-C1-056-60g]  TYLCMYFGIVGKTFAVFSWQMPAPIGAFLSTMDWRAIVLVFILIFLNGLMYYPFLKVYEK  420
                                                                                     ************************************************************

fig|1639.7678.peg.950PTSIIC[Listeria_monocytogenes_FSL-C1-056-WT]                    NLVALEKEAEEENIAAN     437
fig|6666666.523397.peg.2278PTScellobiose-specific_IIC[Listeria_monocytogenes_FSL-C1-056-60g]  NLVALEKEAEEENIAAN     437
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B.

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fig|1639.7678.peg.1021PTSIIC[Listeria_monocytogenes_FSL-C1-056-WT]                MAETKKKSIVNGFINVAQRLGGQIHLRSLRDAFASIMPFMILAGFVTLINYVILEPTGFM     60
fig|6666666.523397.peg.607PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-60g]  MAETKKKSIVNGFINVAQRLGGQIHLRSLRDAFASIMPFMILAGFVTLINYVILEPTGFM     60
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fig|1639.7678.peg.1021PTSIIC[Listeria_monocytogenes_FSL-C1-056-WT]                GKIVNPDTLRTWQEIGISIGNGTLSVITLLVTVAISYHLCLNRGYKNVIAPILVALSSFI     120
fig|6666666.523397.peg.607PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-60g]  GKIVNPDTLRTWQEIGISIGNGTLSVITLLVTVAISYHLCLNRGYKNVIAPILVALSSFI     120
                                                                                 ************************************************************

fig|1639.7678.peg.1021PTSIIC[Listeria_monocytogenes_FSL-C1-056-WT]                VVTPIAMTFLPEGASKSIEVPNVIPVSYTGASGMFVGIIVGLIATDLFIKLSKNKRMQIN     180
fig|6666666.523397.peg.607PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-60g]  VVTPIAMTFLPEGASKSIEVPNVIPVSYTGASGMFVGIIVGLIATDLFIKLSKNKRMQIN     180
                                                                                 ************************************************************

fig|1639.7678.peg.1021PTSIIC[Listeria_monocytogenes_FSL-C1-056-WT]                LTGNIPPAVIKSFNVLIPIMITVIIFSVMSFAVNQIFSMDFNTLVTTIITKPLSYVTTSL     240
fig|6666666.523397.peg.607PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-60g]  LTGNIPPAVIKSFNVLIPIMITVIIFSVMSFAVNQIFSMDFNTLVTTIITKPLSYVTTSL     240
                                                                                 ************************************************************

fig|1639.7678.peg.1021PTSIIC[Listeria_monocytogenes_FSL-C1-056-WT]                PGFLLITSIANLFFGLGIHQAVISGPLLDPFLLQNMQENMVAYANHQEIPHIINMAFKDT     300
fig|6666666.523397.peg.607PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-60g]  PGFLLITSIANLFFGLGIHQAVISGPLLDPFLLQNMQENMVAYANHQEIPHIINMAFKDT     300
                                                                                 ************************************************************

fig|1639.7678.peg.1021PTSIIC[Listeria_monocytogenes_FSL-C1-056-WT]                FAVMGGSGNTIGLLIAIFIFGKRKDYKDISKLSAAPSLFNISEPIIFGLPIVFNPLLIIP     360
fig|6666666.523397.peg.607PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-60g]  FAVMGGSGNTIGLLIAIFIFGKRKDYKDISKLSAAPSLFNISEPIIFGLPIVFNPLLIIP     360
                                                                                 ************************************************************

fig|1639.7678.peg.1021PTSIIC[Listeria_monocytogenes_FSL-C1-056-WT]                FVLAPIFSLTTAYYATAAGWINHVVVQTPWTTPPIISGFLATGGDWRASVLQVIIIVVTV     420
fig|6666666.523397.peg.607PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-60g]  FVLAPIFSLTTAYYATAAGWINHVVVQTPWTTPPIISGFLATGGDWRASVLQVIIIVVTV     420
                                                                                 ************************************************************

fig|1639.7678.peg.1021PTSIIC[Listeria_monocytogenes_FSL-C1-056-WT]                FIYLPFLRMDEKVAFATAQKSDAK 444
fig|6666666.523397.peg.607PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-60g]  FIYLPFLRMDEKVAFATAQKSDAK 444
                                                                                 ***********************
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C.

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fig|1639.7678.peg.1595PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-WT]          MNNKVMDFMTNKFAPKVNKVVKNPWVSAIQDAIMSALPLVFVGSLVTIVSLLKNLFPGMP   60
fig|6666666.523397.peg.1180PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-60g]    MNNKVMDFMTNKFAPKVNKVVKNPWVSAIQDAIMSALPLVFVGSLVTIVSLLKNLFPGMP   60
                                                                                     ************************************************************

fig|1639.7678.peg.1595PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-WT]          DFSMISNFSFGMFGLVVAFLIPYYLMEKKGNSSQKLISGATGLVLFLMLLFPTISADGDA   120
fig|6666666.523397.peg.1180PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-60g]    DFSMISNFSFGMFGLVVAFLIPYYLMEKKGNSSQKLISGATGLVLFLMLLFPTISADGDA   120
                                                                                     ************************************************************

fig|1639.7678.peg.1595PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-WT]          VFILSRFGATGMFLSITTGLFVGCVMNFAAKRSFFSEDTPIPDFVVGWFNSLLPITFILI   180
fig|6666666.523397.peg.1180PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-60g]    VFILSRFGATGMFLSITTGLFVGCVMNFAAKRSFFSEDTPIPDFVVGWFNSLLPITFILI   180
                                                                                     ************************************************************

fig|1639.7678.peg.1595PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-WT]          VGWLITVQFNIDFFEVIVAVFSPLASIVQSYPGFVLSVFIPAFLYTFGISGWVMMPAIYP   240
fig|6666666.523397.peg.1180PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-60g]    VGWLITVQFNIDFFEVIVAVFSPLASIVQSYPGFVLSVFIPAFLYTFGISGWVMMPAIYP   240
                                                                                     ************************************************************

fig|1639.7678.peg.1595PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-WT]          VYMAGLAENSQAVANGASASNIATQETVYALISIGGVGTTLSLSIMMLILSKSLQLKAIG   300
fig|6666666.523397.peg.1180PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-60g]    VYMAGLAENSQAVANGASASNIATQETVYALISIGGVGTTLSLSIMMLILSKSLQLKAIG   300
                                                                                     ************************************************************

fig|1639.7678.peg.1595PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-WT]          KAVIVPSIFNINEPLFFGAPIAFNPYLMIPTWINAFLVPSIAYFVMSMNLVSIPAQSFLL   360
fig|6666666.523397.peg.1180PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-60g]    KAVIVPSIFNINEPLFFGAPIAFNPYLMIPTWINAFLVPSIAYFVMSMNLVSIPAQSFLL   360
                                                                                     ************************************************************

fig|1639.7678.peg.1595PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-WT]          WYMPYPVTSYLATQDFRGVIACLAIIVITWLVYLPFFKAYDNSLLKQEKLDAVETEKEMV   420
fig|6666666.523397.peg.1180PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-60g]    WYMPYPVTSYLATQDFRGVIACLAIIVITWLVYLPFFKAYDNSLLKQEKLDAVETEKEMV   420
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fig|1639.7678.peg.1595PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-WT]          TN      422
fig|6666666.523397.peg.1180PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-60g]    TN      422
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D.

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fig|1639.7678.peg.2558PTScellobioseIIC[Listeria_monocytogenesFSL-C1-056-WT]          MSSERSLSSRFIEGLSIFAQKISSQKHIMAIRDGFAAMIPITIIAAFFLLVNNVLLQPEN      60
fig|6666666.523397.peg.484PTScellobioseIIC[Listeria_monocytogenesFSL-C1-056-60g]    MSSERSLSSRFIEGLSIFAQKISSQKHIMAIRDGFAAMIPITIIAAFFLLVNNVLLQPEN      60
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fig|1639.7678.peg.2558PTScellobioseIIC[Listeria_monocytogenesFSL-C1-056-WT]          GLLKFIPNVENYLGVGIQVYNATLGIMAILAAFLIGNFLAKSYGMEGRTEGVIAVAAYVV      120
fig|6666666.523397.peg.484PTScellobioseIIC[Listeria_monocytogenesFSL-C1-056-60g]    GLLKFIPNVENYLGVGIQVYNATLGIMAILAAFLIGNFLAKSYGMEGRTEGVIAVAAYVV      120
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fig|1639.7678.peg.2558PTScellobioseIIC[Listeria_monocytogenesFSL-C1-056-WT]          LIPASSHLMSVDGKAFEAGGVLTQEMTSSTGMFLAIIAALVSITMLAKFSKSKKLKISMP      180
fig|6666666.523397.peg.484PTScellobioseIIC[Listeria_monocytogenesFSL-C1-056-60g]    LIPASSHLMSVDGKAFEAGGVLTQEMTSSTGMFLAIIAALVSITMLAKFSKSKKLKISMP      180
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fig|1639.7678.peg.2558PTScellobioseIIC[Listeria_monocytogenesFSL-C1-056-WT]          ESVPPAIAKSFNILIPSFLVLSILAIIEVLVVSFVSMSIPEIIVKVLQIPLVGGFQTLPG      240
fig|6666666.523397.peg.484PTScellobioseIIC[Listeria_monocytogenesFSL-C1-056-60g]    ESVPPAIAKSFNILIPSFLVLSILAIIEVLVVSFVSMSIPEIIVKVLQIPLVGGFQTLPG      240
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fig|1639.7678.peg.2558PTScellobioseIIC[Listeria_monocytogenesFSL-C1-056-WT]          ILLYVFLAGFLWVFGIHGAFVLGAISGPVLLTSLQQNIDAVNAGTALPNIVTQPFLDAFV      300
fig|6666666.523397.peg.484PTScellobioseIIC[Listeria_monocytogenesFSL-C1-056-60g]    ILLYVFLAGFLWVFGIHGAFVLGAISGPVLLTSLQQNIDAVNAGTALPNIVTQPFLDAFV      300
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fig|1639.7678.peg.2558PTScellobioseIIC[Listeria_monocytogenesFSL-C1-056-WT]          YMGGGGTIICLVIAIFIASKRPDHRMVTKFGLIPSIFNVSEPLMFGLPVVFNPIYGIPLV      360
fig|6666666.523397.peg.484PTScellobioseIIC[Listeria_monocytogenesFSL-C1-056-60g]    YMGGGGTIICLVIAIFIASKRPDHRMVTKFGLIPSIFNVSEPLMFGLPVVFNPIYGIPLV      360
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fig|1639.7678.peg.2558PTScellobioseIIC[Listeria_monocytogenesFSL-C1-056-WT]          IAPLASTAMAYFATSWGWISQTYILIPWVTPPVLSGYLATGGDIRASILQIAIIIVGTLI      420
fig|6666666.523397.peg.484PTScellobioseIIC[Listeria_monocytogenesFSL-C1-056-60g]    IAPLASTAMAYFATSWGWISQTYILIPWVTPPVLSGYLATGGDIRASILQIAIIIVGTLI      420
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fig|1639.7678.peg.2558PTScellobioseIIC[Listeria_monocytogenesFSL-C1-056-WT]          YLPFVLVANRAYVLEQKAAGKVEAVTNGEV    450
fig|6666666.523397.peg.484PTScellobioseIIC[Listeria_monocytogenesFSL-C1-056-60g]    YLPFVLVANRAYVLEQKAAGKVEAVTNGEV    450
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E.

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fig|1639.7678.peg.2684PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-WT]          MSMMSKFEHGMERVLVPVANKLNSQRHIAAIRDAFILVFPLIMAGSIITLINFAVLSPDG     60
fig|6666666.523397.peg.528PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-60g]    MSMMSKFEHGMERVLVPVANKLNSQRHIAAIRDAFILVFPLIMAGSIITLINFAVLSPDG     60
                                                                                     ************************************************************

fig|1639.7678.peg.2684PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-WT]          FIAKILFLGKIFPNLADAQAVFSPVMQGSTNIMAILIVFLVARNLAIFFKQDDLLCGLTS     120
fig|6666666.523397.peg.528PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-60g]    FIAKILFLGKIFPNLADAQAVFSPVMQGSTNIMAILIVFLVARNLAIFFKQDDLLCGLTS     120
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fig|1639.7678.peg.2684PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-WT]          IGAFFIVYTPYTVVDNASYMTIKFLGAQGLFVAIIVAIITGEVFSRLARSPRLMIKMPDQ     180
fig|6666666.523397.peg.528PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-60g]    IGAFFIVYTPYTVVDNASYMTIKFLGAQGLFVAIIVAIITGEVFSRLARSPRLMIKMPDQ     180
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fig|1639.7678.peg.2684PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-WT]          VPPAVARSFKVLIPVIIITILFSVINYLITLVAPEGLNDLVYTVIQAPLKDMGTNVFSVI     240
fig|6666666.523397.peg.528PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-60g]    VPPAVARSFKVLIPVIIITILFSVINYLITLVAPEGLNDLVYTVIQAPLKDMGTNVFSVI     240
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fig|1639.7678.peg.2684PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-WT]          IIGLVSNLLWVLGIHGPNTVAAIRDTIFTEPNLDNLSYVAQHGSAWGAPYPATWAGLNDG     300
fig|6666666.523397.peg.528PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-60g]    IIGLVSNLLWVLGIHGPNTVAAIRDTIFTEPNLDNLSYVAQHGSAWGAPYPATWAGLNDG     300
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fig|1639.7678.peg.2684PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-WT]          FANYGGSGMTLGLLIAIFIASRRADYRDIAKLSLAPGIFNINEPVIFGLPIVLNPIMVIP     360
fig|6666666.523397.peg.528PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-60g]    FANYGGSGMTLGLLIAIFIASRRADYRDIAKLSLAPGIFNINEPVIFGLPIVLNPIMVIP     360
                                                                                     ************************************************************

fig|1639.7678.peg.2684PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-WT]          FIITPAINTLIGYFFISTKLIPPVAYQVPWTTPGPLIPFLGTGGNWLALLVGLLCLAVAT     420
fig|6666666.523397.peg.528PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-60g]    FIITPAINTLIGYFFISTKLIPPVAYQVPWTTPGPLIPFLGTGGNWLALLVGLLCLAVAT     420
                                                                                     ************************************************************

fig|1639.7678.peg.2684PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-WT]          VIYLPFVLVSNKIAASDAAMDKNATASTEQ   450
fig|6666666.523397.peg.528PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-60g]    VIYLPFVLVSNKIAASDAAMDKNATASTEQ   450
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F.

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fig|1639.7678.peg.2741PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-WT]          MNGLTAFLEKYFVPVAAKIGSQKHLVALRDAFISTMPITMAGSIAVLLNAFFRDFPTDWG   60
fig|6666666.523397.peg.2373PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-60g]    MNGLTAFLEKYFVPVAAKIGSQKHLVALRDAFISTMPITMAGSIAVLLNAFFRDFPTDWG   60
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fig|1639.7678.peg.2741PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-WT]          WTGFVEAMQPLIGINGYVYNGTLAIVSIIFAFSLGYNLSKAYEVDRLAGGLVSLAAFVMN   120
fig|6666666.523397.peg.2373PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-60g]    WTGFVEAMQPLIGINGYVYNGTLAIVSIIFAFSLGYNLSKAYEVDRLAGGLVSLAAFVMN   120
                                                                                      ************************************************************

fig|1639.7678.peg.2741PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-WT]          LTVTVSLDAVKAAIAASNANFDVATLPKEFAGIYGFFSLSQVNGTGLFTAMIFGFISTII   180
fig|6666666.523397.peg.2373PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-60g]    LTVTVSLDAVKAAIAASNANFDVATLPKEFAGIYGFFSLSQVNGTGLFTAMIFGFISTII   180
                                                                                      ************************************************************

fig|1639.7678.peg.2741PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-WT]          YAKLMRRNIIIKMPDSVPPAVSKAFAAIIPALVALYVVGIIDWAFFKITNMDVITWISKT   240
fig|6666666.523397.peg.2373PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-60g]    YAKLMRRNIIIKMPDSVPPAVSKAFAAIIPALVALYVVGIIDWAFFKITNMDVITWISKT   240
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fig|1639.7678.peg.2741PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-WT]          IQEPLLSLSQGYGAVLLVTFLVQLLWFFGIHGPNVLAPVLESLWGTAQLQNISAAQEGAK   300
fig|6666666.523397.peg.2373PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-60g]    IQEPLLSLSQGYGAVLLVTFLVQLLWFFGIHGPNVLAPVLESLWGTAQLQNISAAQEGAK   300
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fig|1639.7678.peg.2741PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-WT]          LPFEWVRGSFDAYVWMGGSGGTLVLIIALLMFSKRADARTVAKLSLAPGIFNINEPIMFG   360
fig|6666666.523397.peg.2373PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-60g]    LPFEWVRGSFDAYVWMGGSGGTLVLIIALLMFSKRADARTVAKLSLAPGIFNINEPIMFG   360
                                                                                      ************************************************************

fig|1639.7678.peg.2741PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-WT]          LPIVLNTIYLIPFLIAPMVMVTIAYFATTLGIVGPVKIAVVWVMPPLLNSFLATGGDWMA   420
fig|6666666.523397.peg.2373PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-60g]    LPIVLNTIYLIPFLIAPMVMVTIAYFATTLGIVGPVKIAVVWVMPPLLNSFLATGGDWMA   420
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fig|1639.7678.peg.2741PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-WT]          PVISLINMVVAFLIWVPFVITANRVGVPEEEMKA      454
fig|6666666.523397.peg.2373PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-60g]    PVISLINMVVAFLIWVPFVITANRVGVPEEEMKA      454
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G.

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fig|1639.7678.peg.2767PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-WT]          MNGFIAFMEKYFIPYAAKIGGQRHLVAIRDGFITTMPLMILGSFAVLINNFPIPAYQKFM    60
fig|6666666.523397.peg.2399PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-60g]    MNGFIAFMEKYFIPYAAKIGGQRHLVAIRDGFITTMPLMILGSFAVLINNFPIPAYQKFM    60
                                                                                      ************************************************************

fig|1639.7678.peg.2767PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-WT]          NNLFGEGTWQAFGGNVWNGTFAILALLIAFTVAYNLAKSYDKDPLSSAVVSVATFFTIGA    120
fig|6666666.523397.peg.2399PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-60g]    NNLFGEGTWQAFGGNVWNGTFAILALLIAFTVAYNLAKSYDKDPLSSAVVSVATFFTIGA    120
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fig|1639.7678.peg.2767PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-WT]          IAPGADGIANTGGLGSTGLFLALIIAILSTEIFTRLSGSPKLIINMPDGVPPAVSRSFAA    180
fig|6666666.523397.peg.2399PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-60g]    IAPGADGIANTGGLGSTGLFLALIIAILSTEIFTRLSGSPKLIINMPDGVPPAVSRSFAA    180
                                                                                      ************************************************************

fig|1639.7678.peg.2767PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-WT]          LFPAMITVSIFGLITAFFQAAGVTNLVISFYELVQEPFMGLANSLPAALLLAFVSAFLWF    240
fig|6666666.523397.peg.2399PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-60g]    LFPAMITVSIFGLITAFFQAAGVTNLVISFYELVQEPFMGLANSLPAALLLAFVSAFLWF    240
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fig|1639.7678.peg.2767PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-WT]          FGLHGANIIDPFMQTINIPAIEANVKALEAGKELPYIVNKPFFDSFVNLGGTGATIGLII    300
fig|6666666.523397.peg.2399PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-60g]    FGLHGANIIDPFMQTINIPAIEANVKALEAGKELPYIVNKPFFDSFVNLGGTGATIGLII    300
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fig|1639.7678.peg.2767PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-WT]          AIFIVARKHKAYMTVSKLSAAPGIFNINEPMMFGLPIVLNPIMFIPYILAPLVLVTVAYF    360
fig|6666666.523397.peg.2399PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-60g]    AIFIVARKHKAYMTVSKLSAAPGIFNINEPMMFGLPIVLNPIMFIPYILAPLVLVTVAYF    360
                                                                                      ************************************************************

fig|1639.7678.peg.2767PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-WT]          ATAIGWVPACTIVTPWTTPPIIGGALATQSIAGGVLAAVNLGLSILIFLPFAKIAQIQEL    420
fig|6666666.523397.peg.2399PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-60g]    ATAIGWVPACTIVTPWTTPPIIGGALATQSIAGGVLAAVNLGLSILIFLPFAKIAQIQEL    420
                                                                                      ************************************************************

fig|1639.7678.peg.2767PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-WT]          RREKEALAAEGVTAE 435
fig|6666666.523397.peg.2399PTScellobioseIIC[Listeria_monocytogenes_FSL-C1-056-60g]    RREKEALAAEGVTAE 435
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Figure 3.9 A-G Sequence alignment of the Lactose/cellobiose-PTS subunits IIC from strains *L. monocytogenes* FSL-C1-056 before it was exposed to CclA and after growing the strain for 60 generations.

3.3 Conclusions and overview

The process of antimicrobial resistance is complex, involving various contributing mechanisms. Given that the mode of action of bacteriocins differs for each type, and the emergence of resistance is often shaped by the characteristics of the targeted bacteria and their environment, the exploration of resistance to bacteriocins can be approached from multiple perspectives.

The research involving *L. monocytogenes* strains FSL-C1056, FSL-R2-499, and the non-resistant FSL-J1-177 provided crucial insights into the resistance development against carnocyclin A and its potential association with sugar intake receptors.

The experimental tests showed varying resistance patterns between the non-resistant and resistant strains when exposed to different concentrations of carnocyclin A. These results suggested that resistance to carnocyclin A might be affected by the type of sugar used as a carbon source. The diverse responses to the bacteriocin, especially with cellobiose, glucose, and mannose, indicated a complex relationship between sugar metabolism and the development of resistance. The differences in resistance observed across carbon sources hinted at a possible connection between specific phosphotransferase systems (PTS), possibly, the mannose transporter.

No significant mutations in the Man-PTS structure were found in resistant strains in this study, just a single amino acid variation in the IIC subunit of FSL-C1056 compared to the non-resistant reference strain was discovered. This variation might lead to slight conformational changes, potentially explaining the resistance in mannose media. Further research and structural studies are imperative to validate this hypothesis.

The stability and permanency of mutations in conferring resistance were also explored, revealing persistent resistance in the strains even after 60 generations. Mutations in cellobiose transporters was also evaluated but no mutations were identified in these transporters.

Studies have shown that *L. monocytogenes* displays a dual lifestyle, exhibiting virulence traits based on available carbon sources, impacting its pathogenicity when growing inside human cells. According to what has been reported in literature, it exists an intriguing connection between the expression of specific PTS systems and the modulation of *prfA*, a critical transcriptional

activator of virulence genes in *L. monocytogenes*, suggesting a vital role of sugar metabolism in regulating virulence factors and resistance pathways. It would also be interesting to investigate changes in the expression of transcription factors such as regulator components in both strains. These studies should be done on the strains before and after they were exposed to the bacteriocin.

Another alternative avenue for investigating the development of carnocyclin resistance in the *L. monocytogenes* strains, involves exploring prophage sequences that have the potential to introduce stop codons into sequences crucial for sugar transporters.

Carnocyclin A might not necessitate a specific docking molecule for its action. It's plausible that resistance is facilitated by alterations in various cell wall components, potentially leading to changes in the overall bacterial cell wall polarity to hinder bacteriocin binding.

The evolutionary selection process operates through various mechanisms to select the fit, eliminate the unfit, and overlook neutral changes. Single Nucleotide Polymorphisms (SNPs) are variations in a single nucleotide (A, T, C, or G) at a specific position in the DNA sequence. These variations can occur naturally within a population and contribute to genetic diversity. The importance of SNPs in evolution lies in their role as a source of genetic variation that can be subject to natural selection. Single changes in the DNA sequence that occur at a specific position within the genome and can affect various phenotypic traits, including virulence, antibiotic resistance, and adaptation to multiple environments.

It is important to highlight that controlled environment, characterized by significant selective pressure, often result in parallel evolution among replicates subjected to identical conditions. Parallel evolution refers to the independent development of similar traits, characteristics, or genetic changes in separate but related lineages or populations.

Research conducted by Orsi et al. in 2008[161] revealed a significant similarity in the genome backbone sequences of two *L. monocytogenes* isolates from 1988, associated with sporadic listeriosis, and two isolates from 2000, linked to substantial outbreaks. These findings provide valuable insights that could guide the investigation of *L. monocytogenes* strains FSL-C1056 and FSL-R2-499. Another noteworthy example, observed in a study by Bergthorsson et al. in 1999[162], demonstrated that after 20,000 generations of cultivation under uniform conditions, *Escherichia*

coli populations derived from a common parent strain exhibited substitutions in identical candidate genes.

According to studies done by Harrand S, in 2020[163] in the evolution of *L. monocytogenes* in a food processing plant, it was reported that even with a low single nucleotide mutation rate (1.15×10^{-7} changes per nucleotide per year in a genome of 2.88 Mb), *Listeria monocytogenes* was able to evolve and adapt to the usage of different sanitizers. It would be interesting to run a WGS data analysis of *L. monocytogenes* FSL-R2-499 and *L. monocytogenes* FSL-C1-056 to study the presence of SNPs after the *L. monocytogenes* strains were exposed to carnocyclin A, to understand better the developed resistance.

CHAPTER 4

4. Exploring labeling approaches to elucidate the mechanism of action of bacteriocins

4.1 Isotopic labeling of peptides

Isotopic labeling of peptides plays an important role in enhancing the accuracy and depth of analysis through Nuclear Magnetic Resonance (NMR) techniques. Incorporating isotopes, such as ^{13}C, ^{15}N, or ^{2}H, into peptides offers a substantial advantage allowing for more precise and detailed structural elucidation. With isotopic labeling, distinct atoms within the peptide structure can be selectively observed and traced, providing a clear and unambiguous signal in NMR spectra. This labeling strategy assists in resolving complex spectral overlaps and enables the assignment of resonances to specific amino acids, which is particularly beneficial in larger or more intricate peptide structures. Moreover, isotopic labeling enhances the sensitivity of NMR experiments, facilitating the study of dynamics, interactions, and conformational changes within peptides. Consequently, the precise structural information obtained from isotopically labeled peptides via NMR techniques is indispensable in understanding the intricate mechanisms of biological processes, drug development, and protein-protein interactions, making it an indispensable tool in biochemical and pharmaceutical research[164,165].

Isotopically labeling peptides, while an invaluable technique for advanced structural and functional studies can present several challenges. One primary difficulty is the cost and complexity associated with the labeling process. Incorporating stable isotopes into peptides often requires specialized equipment, reagents, and expertise, which can be expensive and time-consuming. Additionally, the synthesis of isotopically labeled peptides may pose challenges in terms of yield and efficiency, especially for longer or more complex sequences. Ensuring high labeling efficiency and uniform incorporation of isotopes across the peptide sequence is crucial, as non-uniform labeling can complicate spectral interpretation and data analysis. Furthermore, the use of isotopically labeled amino acids or reagents might introduce impurities or side products that could affect the purity of the final labeled peptide. These challenges in the isotopic labeling process highlight the need for careful optimization, specialized techniques, and rigorous quality control to

ensure the production of accurately labeled peptides for precise and reliable analyses in biochemical and biophysical studies[165,166].

The amount of labeled peptide required for an NMR (Nuclear Magnetic Resonance) experiment can vary based on several factors, including the specific experiment, the sensitivity of the NMR instrument, the complexity of the peptide, and the desired resolution of the spectra[165].

For standard solution NMR experiments, the required amount of labeled peptide typically ranges from a few nanomoles to several hundred micromoles. The minimum amount of peptide necessary is often determined by the sensitivity of the NMR instrument, as well as the desired signal-to-noise ratio for accurate data collection and analysis[164,165].

Isotopically labeled peptides generally require higher amounts compared to unlabeled peptides due to the costs and effort involved in the labeling process. For example, for peptides labeled with ^{13}C or ^{15}N, higher quantities may be necessary to compensate for the natural abundance of isotopes in the sample[164,165].

In general, the minimum amount needed can depend on the specific NMR technique being used, the sensitivity of the spectrometer, the complexity of the sample, and the desired resolution[164,165].

4.2 Leucocin A isotopic labeling through SUMO fusion technology

4.2.1 Objectives

The main goal of this project was to produce ^{15}N and ^{13}C labelled leucocin A (LeuA) through small ubiquitin-like modifier (SUMO) fusion technology in order to run 2D-NMR experiments on this type IIa bacteriocin. The NMR experiment focus was to study the structure of the peptide with oxygenated analogues of dodecylphosphocholine (DPC) micelles, prepared by Dr. Isaac Antwi. Furthermore, isotopically labelled LeuA in DPC micelles was also to be studied to observe interactions between peptide sequences that emulate the Man-PTS IID extracellular loop regions of *L. monocytogenes* 502, *L. monocytogenes* 507 and *L. monocytogenes* EGDe to have a better understanding of the mechanism of action of leucocin A.

4.2.2 Results and discussion

SUMO proteins can be attached to other proteins *via* an isoamide covalent bond as posttranslational modifications which occur naturally in eukaryotic cells. SUMO tags play an important role in the signalling of numerous cellular processes such as apoptosis, protein activation, nuclear-cytosolic transport, response to stress and others. The isoamide bond formed between a target protein and the SUMO protein can also be easily removed by SUMO proteases in cells[167].

SUMO tags have served as a dependable biotechnological tool for the efficient expression and purification of various proteins in both eukaryotic and prokaryotic hosts. In this process, a plasmid containing a gene that encodes the SUMO tag with an N-terminal His tag is fused to a gene encoding a peptide of interest and a stop codon at the 3' end of the open reading frame. When this plasmid is transformed into an appropriate host organism, expression of a SUMO fusion protein is initiated by the the T7 promoter that can be induced by addition of isopropylthio-β-galactoside (IPTG). SUMO fusion proteins are favorable expression constructs because the peptide of interest can be produced with no extra residues attached, as the SUMO protease (which will eventually cleave the SUMO tag from the peptide) specifically recognizes the 3D structure of the SUMO tag, and cleaves after a conserved Gly-Gly motif[168].

The strain *E. coli* BL21(DE3) containing pET-SUMO-LeuA was constructed and provided by Dr. Marco van Belkum. The strain was initially inoculated onto an agar plate of LB media with 50μg/mL of kanamycin. Protein expression and purification were done according to the methods described on experimental procedures (heterologus expression of proteins) followed by a Ni-NTA affinity chromatography which offered the binding of the six-histidine tag of the expressed protein to the resin.

After the performance of Ni-NTA affinity chromatography, the resin was loaded into a column which was washed with different concentrations of imidazole as described on experimental procedures. The imidazole washes were done to replace the nickel-protein interactions by nickel-imidazole interactions to release the expressed protein. After the imidazole washes, a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to corroborate the presence of the expressed protein (Figure 4.1).

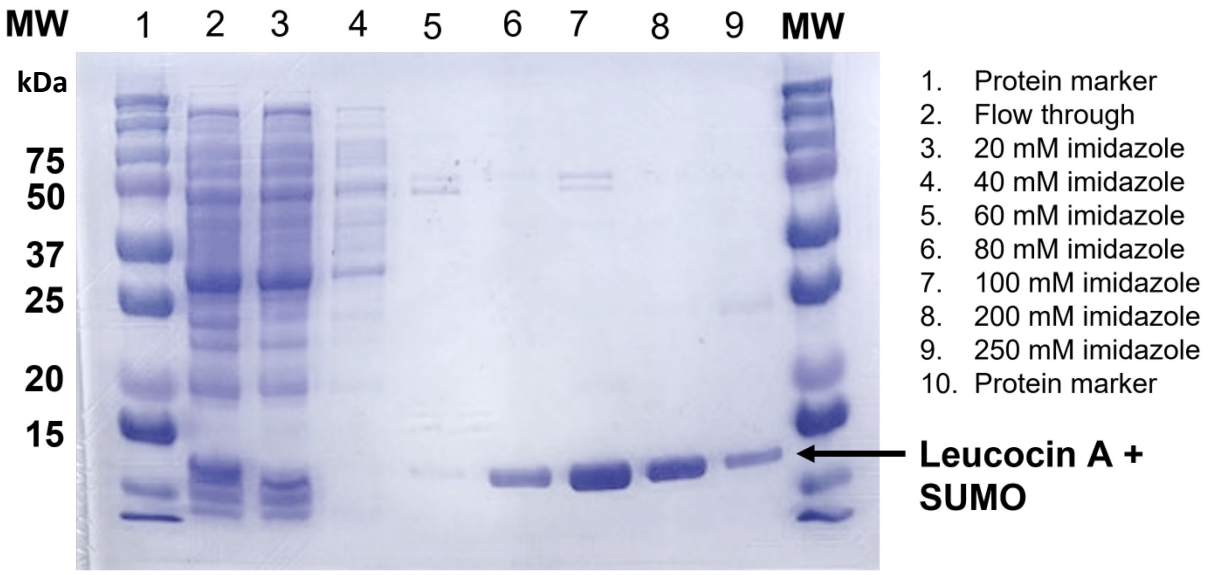

Figure 4.1 SDS-PAGE gel of Ni-NTA affinity chromatography fractions from the purification of proteins from pET-SUMO-LeuA expression.

As observed in Figure 4.1, the protein eluted in fractions with imidazole concentrations of 80 mM to 250 mM. These fractions were combined to run a dialysis using a SPECTRA/POR® membrane of 6000-8000 MW according to what it is described on experimental procedures.

After the dialysis, the bag content was isolated and concentrated to an approximate volume of 4 mL using an Amicon tube (3000 MW, 5000 g, 50 min). The SUMO-protease digestion was performed using the concentrated solution as described on Chapter 5.

To evaluate whether the cleavage of the peptide was successful, MALDI-TOF MS, a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and a spot-on-lawn assay were also carried out, as described in experimental procedures (Figure 4.2).

The MALDI-TOF spectra showed a peak with the expected molecular weight of 3938 Da which is in accordance with the molecular weight reported for Leucocin A and the biological assay showed that the cleaved peptide is biologically active[169]. The spot-on-lawn assay was done after the cleavage, which may explain why a high concentration was necessary to show antimicrobial activity, since the peptide still needed to be purified.

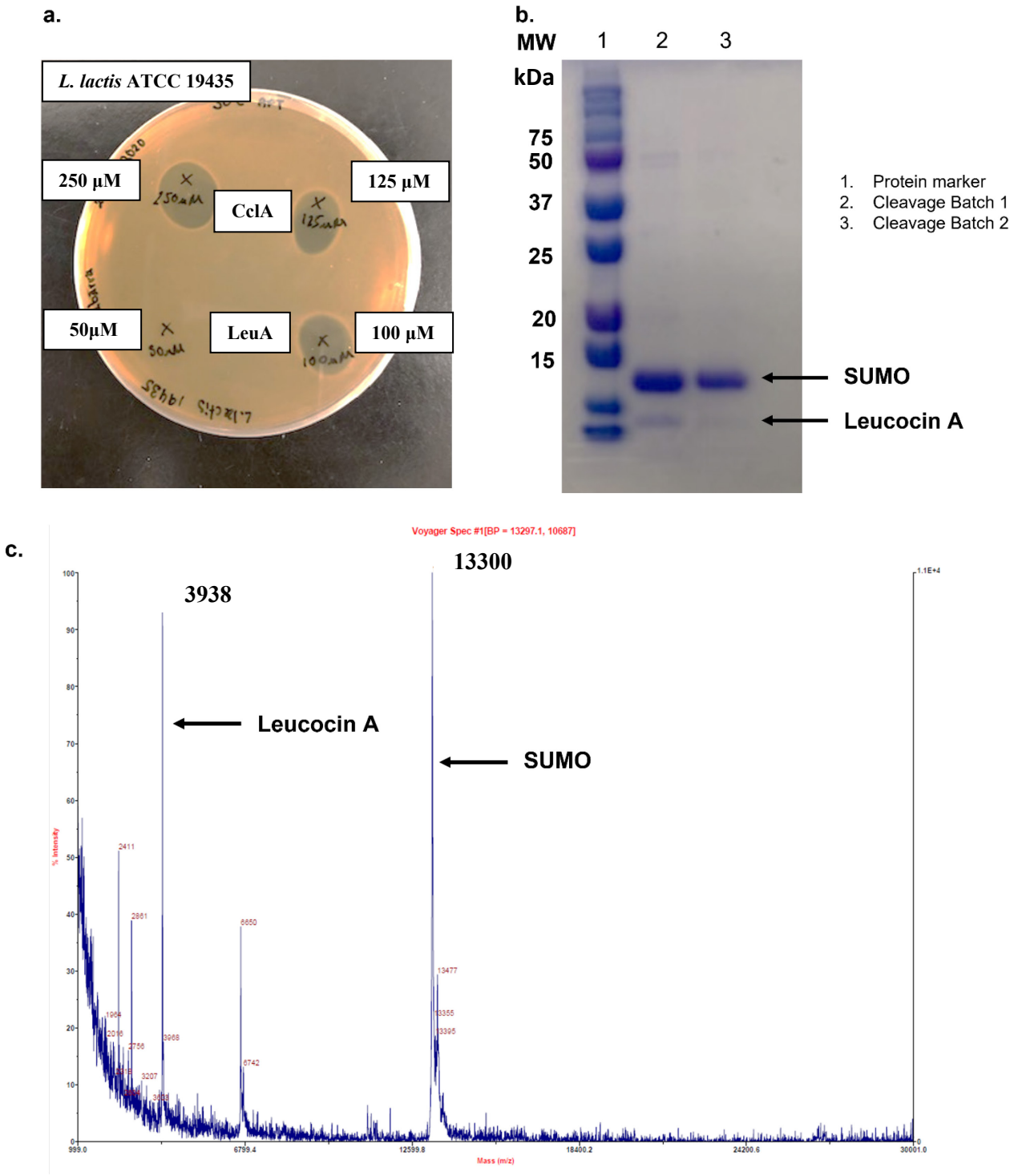

Figure 4.2 a. Spot-on-lawn assay which shows antimicrobial activity of the expressed peptide with two different concentrations (50 µM and 100 µM) after the cleavage. Indicator strain: *L. lactis* ATCC 19435. CclA was used as positive control. **b.** The SDS-PAGE after the cleavage showed a successful procedure as seen in the two separated bands below the 15 kDa protein marker **c.** MALDI-TOF spectra showed the two expected peaks, one for leucocin A, 3938 Da and one for the SUMO protein, 13300 Da.

The obtained peptide was biologically active, as it is shown in Figure 4.2a. Along with MALDI-TOF and SDS-PAGE data, we could conclude that the peptide was expressed.

To label peptides with ^{15}N and ^{13}C isotopes, a bacterial media with containing carbon and nitrogen sources with ^{15}N and ^{13}C isotopes needed to be used for fermentation of the desired strain. Isotopic labelling media is usually expensive; therefore two types of media were tested to label LeuA in a cost-effective way. The first tested labeling media was a modified version of the M9 media, reported by Cai et al. in 1998[170], and the recipe is shown in Table 4.1.

Table 4.1 M9 recipe for 1 L of media.

1. 970 mL basic solution (anhydrous salts, autoclaved) per 970 ml
KH_2PO_4 13.0 g
K_2HPO_4 10.0 g
Na_2HPO_4 9.0 g
K_2SO_4 2.4 g
NH_4Cl 2 g
2. 10 mL trace element solution (syringe filtered) per 100 ml
$FeSO_4$ ($7H_2O$) 0.60 g
$CaCl_2$ ($2H_2O$) 0.60 g
$MnCl_2$ ($4H_2O$) 0.12 g
$CoCl_2$ ($6H_2O$) 0.08 g
$ZnSO_4$($7H_2O$) 0.07 g
$CuCl_2$ ($2H_2O$) 0.03 g
H_3BO_3 2 mg
$(NH_4)_6Mo_7O_{24}$ ($4H_2O$) 0.025 g
EDTA 0.50 g
3. 10 mL 1 M $MgCl_2$ (syringe filtered)
4. 6 mL thiamine (5 mg/mL, syringe filtered)
5. 1 mL ampicillin (100 mg/mL, syringe filtered)
6. D-Glucose 2.5 g + 2.5 g at the moment of induction
7. 10 mL 10% yeast extract (syringe filtered)

The modified M9 media was only used in the induction step since for the initial inoculation, LB media was used as described before. A fermentation on LB media was also carried out in parallel as control. After MALDI-TOF MS was done to verify if the SUMO cleavage was successfully complete, a protein concentration test was carried out in a spectrophotometer at UV 280 nm to determine how much fused protein was approximately produced per litre.

The results showed that 13.9 mg/L was produced in the LB media, whereas 8 mg was produced in the modified M9 media. The dialyzed sample still had to undergo a final HPLC

purification step. For that reason, the yield for this media was considered low since during HPLC purifications a significant amount of sample is usually lost. A yield higher than 15 mg/L is considered good since NMR experiments usually require several mg of peptide.

The second tested media was Spectra 9, a commercially available media produced by Cambridge Isotope Laboratories. This media contains labelled ^{15}N NH_4 salts and ^{13}C labelled glucose. Spectra 9 is supplemented with amino acids, vitamins, peptides, and other essential nutrients, along with 1 g of Celtone Base Powder (another labelled media from the same company). Celtone media has been reported as an efficient media for protein expression, and it is broadly used for labelling purposes; nevertheless, it is costly.

Spectra 9 media is commercially available as a ready-to-use solution. LeuA production was first tested on its non-labeled version, which means it did not contain either ^{13}C or ^{15}N in its formulation. Following the same protocol as when M9 media was tested, growth of the strain *E. coli* BL21(DE3) pet-SUMO-LeuA in 1 L of Spectra 9 was carried out along with 1 L of LB media, which was used as a positive control.

After purification, the protein concentration analysis showed a good yield of production of the fused protein; 17.2 mg of protein were measured in the sample in comparison to 9.0 mg produced in the LB media. After the non labeled media showed promising results, the labeled version was tried as well in a similar manner as the non-labeled version obtaining 14.3 mg/ L.

To remove the SUMO tag from the solution, a final purification step was carried out in both samples, labeled and non-labeled. The HPLC method is described in detail in experimental procedures. By running the first 2 mL of sample in the instrument, three main peaks were observed on the HPLC chromatogram. The fractions with the compounds which produced these peaks were collected and further analyzed with MALDI-TOF MS to determine which corresponded to leucocin A. Results from MALDI-TOF MS showed the sample collected at ~13 min corresponded to leucocin A (3934 Da for the non-labelled peptide, 4136 Da for the labelled peptide). After all the solution with the cleaved peptide was purified through HPLC (~10mL), the collected HPLC sample ~10 mL was concentrated to ~5 mL using a rotary evaporator.

The concentrated sample was again analyzed with MALDI-TOF MS. The results showed still the presence of compounds with a molecular weight of 13290 and 13956 Da respectively which

could be still due to the presence of the SUMO-tag protein, and also we observed the presence of compounds with smaller molecular weight than the SUMO-tag ~7000 Da (Figure 4.3) which indicated the sample was still not pure. The sample was further filtered with an Amicon® Ultra-15 Centrifugal Filter Unit (3kDa), but the peptide could not be recovered after this step.

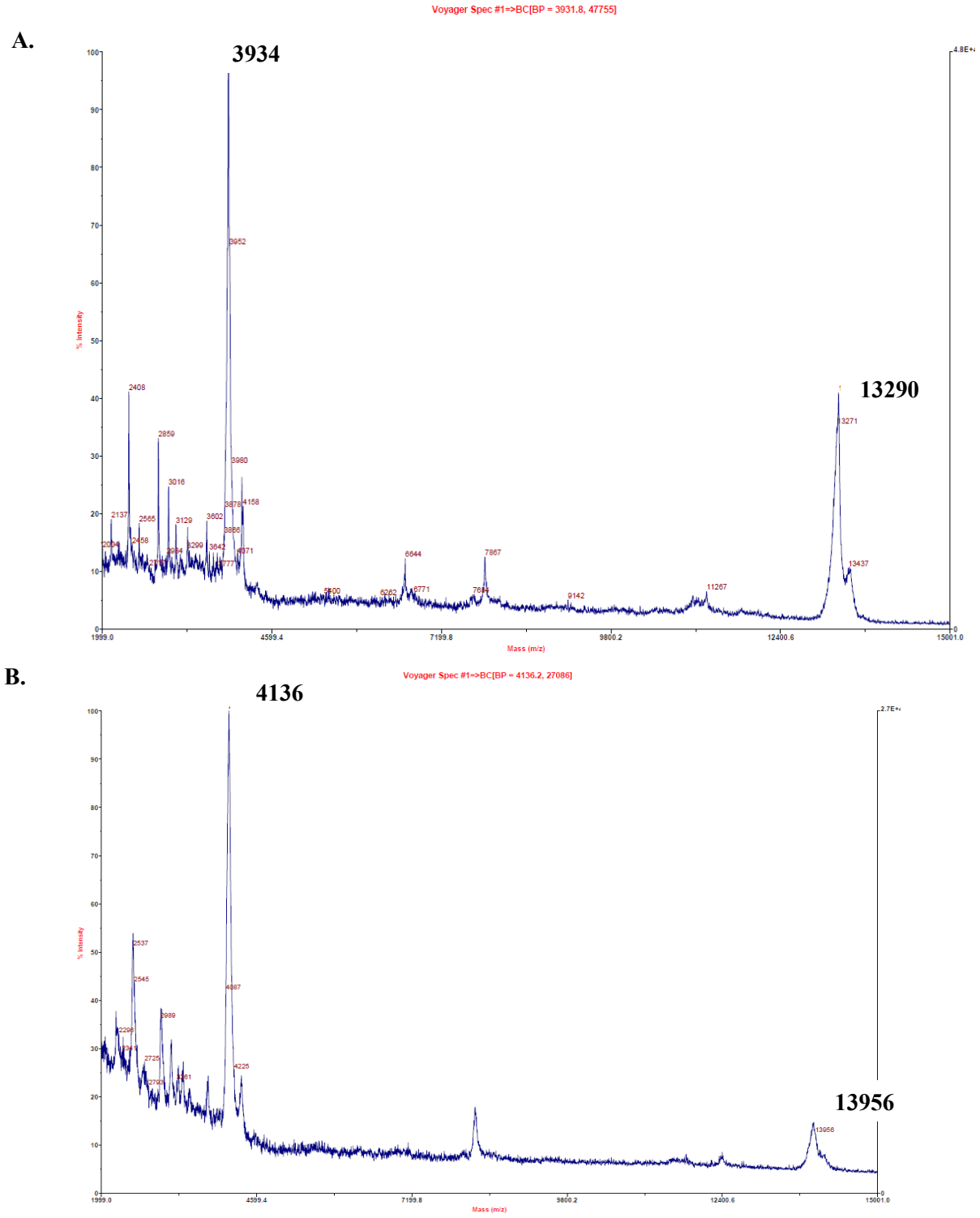

Figure 4.3 MALDI-TOF spectra of the cleaved peptide after HPLC-purification. **A.** Non-labelled Spectra 9 **B.** Labelled Spectra 9. Both spectrums show the presence of SUMO protein.

4.2.3 Conclusions and overview

NMR analysis demands a high degree of purity in the compound being examined. However, the fraction obtained from HPLC contained not just the target compound but also LeuA and other impurities, necessitating a re-purification of the sample. The SUMO tag, with a monoisotopic molecular weight of 13276.6 Da (excluding the N-terminal Met residue), possibly corresponds to compounds with average m/z values of 13290 and 13956 Da. It's plausible that fragments with m/z values lower than that of the SUMO tag but higher than LeuA could have resulted from interactions with the peptide and degradation during the HPLC injection process. Analysis of a sample from LB media, performed in conjunction with those from Spectra 9 labeled and non-labeled media, also showed the presence of the same problematic compounds. This suggests an issue lies with the purification process rather than with the specific media used, as depicted in Figure 4.4 MALDI-TOF spectra of the cleaved peptide after HPLC-purification in the control LB media.

Notably, the MALDI-TOF spectra in Figure 4.4, conducted post SUMO-tag cleavage but prior to HPLC purification, revealed the presence of a compound with a molecular weight of 6650 Da. This suggests a potential problem with the protein expression, prompting the need for a refined purification method to isolate these compounds before the HPLC analysis. Considerations were made towards utilizing methods such as protein exclusion chromatography or an additional dialysis step.

This marked the conclusion of my involvement in the project during my Ph.D., with Tess Lamer taking over for its continuation. This transition indicates persistent challenges in the purification and identification of compounds, stressing the need for further refinements in the purification process to ensure accurate analysis.

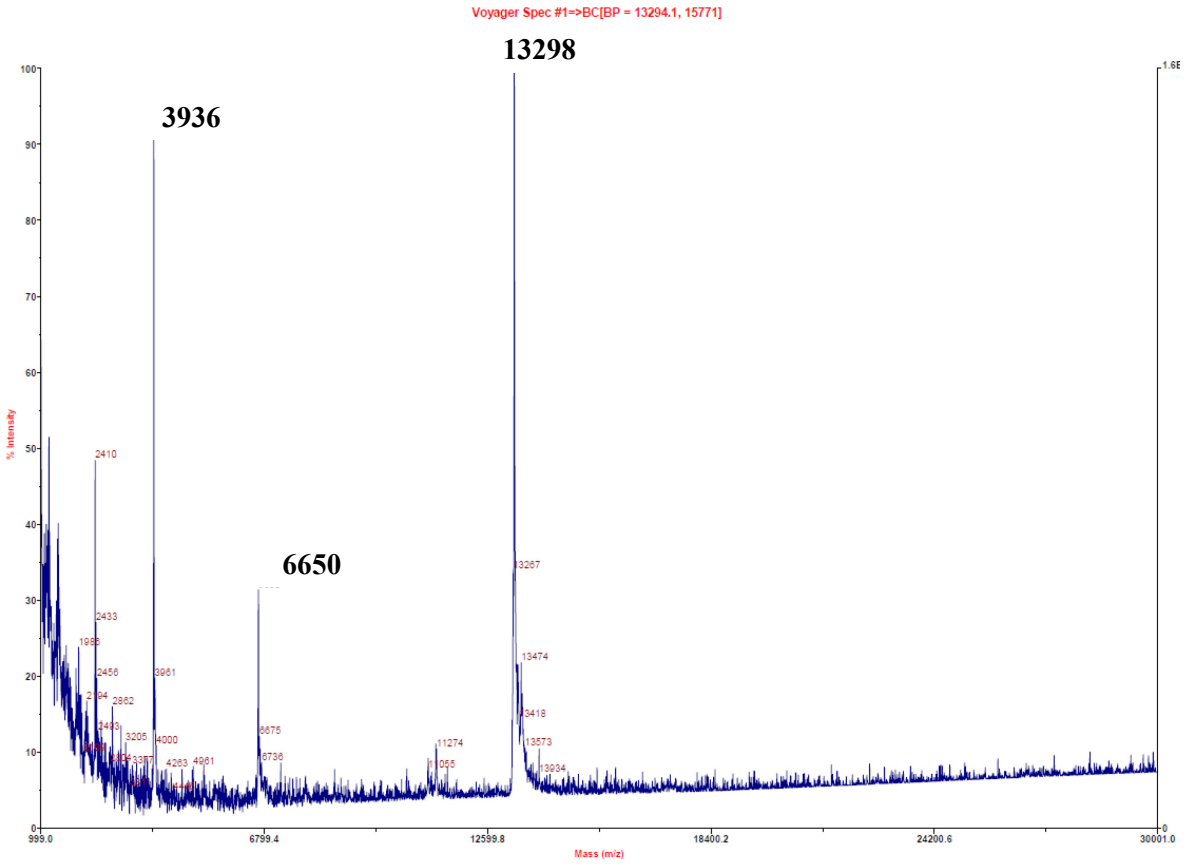

Figure 4.4 MALDI-TOF spectra of the cleaved peptide after HPLC-purification in the control LB media.

4.3 Innovative Labeling Strategies for Lacticin 3147

4.3.1 Objective

The main goal of this project was to produce the two components of lacticin 3147 in ^{15}N and ^{13}C labelled media to run 2D-NMR experiments to study the mechanism of action of LtnA2 and its interaction with the complex LtnA1: Lipid II.

4.3.2 Results and discussion

The production of bacteriocins can rely on several factors, among which media composition stands out as a key influencer. Lactic acid bacteria typically thrive in nutrient-rich environments, where they engage in competitive interactions with other bacteria sharing the same habitat to secure their survival[171].

Other factors like pH, temperature, and exchange of oxygen/CO_2 can also play an important role in inducing bacteriocin production. Many studies have shown that the best yield of bacteriocin production occurs when there is a high biomass production[172].

The first two tested media to produce and label lacticin 3147 were Celtone® and Bioexpress®. These two media are commercial culture formulations from Cambridge Isotope Laboratories, Inc., which has reported that both media are suitable for growing *E. coli* and other bacteria. *E. coli* is Gram-negative bacteria are frequently employed for protein expression and have distinct nutrient demands compared to LAB. However, neither medium appears to contain inhibitory components in their formulation that might impede the growth of LAB.

Cambridge Isotope Laboratories Inc. does not report the exact formulation on any of its commercial media. Bioexpress® is sold as a concentrated solution that needs to be diluted to a final volume of 1000 mL, and it is described as a "fully rich media" which contains a complex mixture of glucose, amino acids, peptides, vitamins, minerals, and cofactors that has been prepared from algal cell hydrolysates.

Celtone® complete media is described in the same way as Bioexpress®, but it is also mentioned that this media contains a similar formulation as LB media, and in comparison, to Bioexpress®, this media is sold as a ready-to-use media in a 1L bottle.

To find out if bacteriocin production was possible using these two media, the unlabeled versions of both were tested along with the reported M17 media used by Martin *et al*. in 2004[154], which has the following formulation:

Per 800 mL:

2.5 g tryptone

5.0 g yeast extract

1.5 g D/L- methionine

50 mg $MnSO_4.4H_2O$

125 mg $MgSO_4$

Before sterilizing this media, the solution was passed through Amberlite XAD-resin to remove hydrophobic components. After sterilization, 100 mL of a filter-sterilized 10% (w/v) D-glucose solution with 19.0 g of β-glycerophosphate was prepared, and then added to the previously sterilized media. This step was done to prevent the caramelization of the media.

Fermentations with 500 mL of the three media types were carried out with a lacticin 3147 overproducer *L. lactis* strain previously used by Dr. Bakhtiary in his studies of Lacticin 3147[146]. Incubation and fermentation were done as described in the general procedures. Once fermentation was finished, the culture media were centrifuged for 20 min at 8,000 rpm, and peptide purification was carried out from either the supernatant or from the cell pellet. The purification methods are described in experimental procedures.

As reported by previous group members who worked with this peptide, the best yield of bacteriocin purification was obtained when the bacteriocin was purified from the cell pellet[154,146]. Both LtnA1 and LtnA2 were produced in all three media formulations, but at too low a concentration to be used for NMR experiments. When the samples were lyophilized after HPLC purification, 1.5 mg of LtnA1 and 0.7 mg of LtnA2 were obtained from the M17 media, which was used as control, whereas less than 0.1 mg of peptide was produced from 500 mL of Bioexpress® and Celtone® media.

It was known that both peptides were produced in small amounts in both media since MALDI-TOF MS analysis of collected HPLC fractions show m/z peaks corresponding to the correct molecular weight of the desired peptides[146]. However, after lyophilization of these HPLC fractions, no considerable amounts of either of the two-components peptide was obtained from any of the tested media.

The produced biomass of each media was collected and weighted; 3.5 g of cells were obtained from 500 mL of the M17 media, whereas 1.2 g were produced from 500 mL of Celtone® and 0.7 g from 500 mL of Bioexpress®. From these results, it can be noticed there may have been a problem with the media formulation for the Celtone® and Bioexpress® media. Knowing that the formulation of both the media was designed for Gram-negative bacteria, three formulations were developed to supplement the Celtone® media. The Bioexpress ® media was not optimized as its supported cell growth was very poor.

According to Cambridge Isotope Laboratories, supplementing Bioexpress® media can improve protein production. Therefore, 500 mL of unlabeled Celtone® media were supplemented with various salts, metals, and cofactors, as mentioned in Table 4.2.

Table 4.2 Supplementation of Bioexpress® media.

Medium P salts	Cofactors	Trace metals
50 mM Na_2HPO_4	10 µg/ml thiamin	10 µM $FeCl_3$
50 mM KH_2PO_4		4 µM $CaCl_2$
5 mM Na_2SO_4		2 µM $MnCl_2$
2 mM $MgSO_4$		2 µM $ZnSO_4$
		0.4 µM $CoCl_2$
		0.4 µM $NiCl_2$
		0.4 µM Na_2MoO_4
		0.4 µM Na_2SO_3
		0.4 mM H_3BO_3

Along with this trial, a 50:50 proportion of APT media:Celtone media was tested. Once more it was found that the components of lacticin 3147 were produced, but at very low amounts.

The collected biomass was 1.8 g for the media supplemented with M9 salts (Table 4.1) and 2.1 g for the 50:50 ATP media:Celtone.

Table 4.3 Amino acid content in Celtone® reported by Cambridge isotopes®.

Free amino acid composition				**Total amino acid composition**			
Ala	11.1%	**Arg**	4.6%	**Ala**	7.6%	**Arg**	5.6%
Asp	17.5%	**Glu**	10.0%	**Asp**	9.6%	**Glu**	10.2%
Gly	11.5%	**His**	1.2%	**Gly**	6.4%	**His**	2.3%
Ile	1.3%	**Leu**	7.7%	**Ile**	3.1%	**Leu**	8.4%
Lys	7.1%	**Met**	1.7%	**Lys**	12.0%	**Met**	1.6
Phe	3.8%	**Pro**	6.2%	**Phe**	8.3%	**Pro**	5.6%
Ser	5.2%	**Thr**	2.8%	**Ser**	4.4%	**Thr**	4.8%
Tyr	3.7%	**Val**	2.1%	**Tyr**	3.8%	**Val**	4.5%
Trp	0.2%	**Cys**	0.2%	**Trp**	0.2%	**Cys**	0.7%

The notable difference between the formulations of the M17 media, which showed a good yield of bacteriocin production, and Celtone® and Bioexpress® media is the nitrogen source. M17 media contains yeast extract, whereas the commercial media seems to have only algal cell hydrolysates, which may not be entirely assimilated by the *L. lactis* strain, thus causing low production of the peptide. According to specifications reported by the supplier, the amino acid content is the reported on Table 4.3 (free amino acid composition is referred to the amount of each unbound amino acid unit not bound to a protein and total amino acids consider both, bound and not bound). Conversely, yeast extracts are more nutritious; they contain a combination of proteins, peptides, amino acids, nucleic acids, B vitamins, minerals, carbohydrates, and other components Table 4.4[162].

Table 4.4 Nutritional content of *S. cerevisiae* yeast extract.

For 100g	Dried yeast	Dried yeast extract	Yeast extract paste
Water g	4	4	37
Calorie kcal	362	334	159
Protein (N x 6.25) g	48	69	27.8
Carbohydrate g	23.7	7-13	11.8
sugar g	8.0		0
starch g	1.5		
Fiber g	13		
Fat g	5.4	<0.5	0.0
SFA (saturated fatty acid) g	0.7		
MUFA (monounsaturated fatty acids) g	2.1		
PUF (polyunsaturated fatty acids) g	2.4		
Vitamins			
B1 (thiamine) mg	40	10-12	9.7
B2 (riboflavine) mg	4	8-12	14.3
B3 (niacin) mg	25	90-110	9.7
B5 (pantothenic acid) mg	9	12-20	
B6 (pyridoxine) mg	2.6	6-8	1.3
B9 (folic acid) µg	2500		
B12 (cobalamine) µg		0.5-1.5	0.5
Minerals			
Calcium mg	130		86
Phosphorus mg	130		106
Magnesium mg	170		180
Potassium mg	2460		2600
Sodium (salt) mg	40		3600
Iron mg	5		3.7
Copper mg	5.3		0.3
Zinc mg	5.6		2.1
Manganese mg	0.4		
Selenium µg	71		18

Partly hydrolyzed protein is the main component of yeast extracts, and the overall content of compounds with nitrogen ranges from 45 – 75%, of which 80% comes from nitrogen in proteins

and 10 – 12% from nucleic acid nitrogen and the other components like glutathione, N-acetylglucosamine, lecithin and others (Table 4.5) [163].

Table 4.5 Amino acid content of *S. cerevisiae* yeast extract.

Amino acid g/100 g	Free amino acid (g)	Total, amino acid (g)
Alanine	3.7	4.4
Arginine	1.8	2.5
Aspartic acid	1.9	4.9
Cysteine	0.3	0.4
Glutamic acid	5.2	8.1
Glycine	1.2	2.4
Histidine	0.9	1.0
Isoleucine	2.2	2.7
Leucine	3.5	3.8
Lysine	2.2	4.0
Methionine	0.7	0.9
Phenylalanine	1.8	2.3
Proline	1.0	2.0
Serine	1.8	2.3
Threonine	1.7	2.1
Tyrosine	1.2	1.4
Tryptophan	0.6	0.6
Valine	2.6	2.9
Total	34.6	48.0

After considering that the formulation of the commercial media may be the problem, the design of a labelled yeast extract that could be used in the formulation of the M17 media along with labelled glucose was investigated. The approach to design and use a ^{15}N, ^{13}C and ^{2}H labelled yeast extract to express a protein in insect cells was done by Opitz *et al*. in 2015. They obtained a ^{15}N and ^{13}C labelling efficiency of up to 90% in their protein of interest[164]. They grew the yeast *Pichia pastoris* on a glucose-and ammonium-based yeast minimal medium, which yielded up to 64

g of cell wet weight, which was then treated with proteases and further purification steps to yield 6.6 g of yeast extract that were used in the final media[164].

Following this approach, an unlabeled yeast extract was produced first from the fermentation of the unlabeled minimal medium based on glucose and yeast nitrogen base (YNB) with *P. pastoris* GS115. The yeast extract was prepared as mentioned in experimental procedures following the Opitz *et al.* protocol[164]. From the first batch, 4.0 g of yeast extract was produced, and then a second fermentation was completed, which yielded 3.5 g of yeast extract, to obtain a total of 7.5 g of yeast extract, which were then used for the preparation of 1 L of M17 media. However, this media produced similar results as Celtone® and Bioexpress® media, and there was not enough peptide produced for NMR experiments.

Lastly, Bioexpress® and Celtone® media were tested again, this time with a proportion of 80:20 of commercial media:M17 media (commercial M17). Once the media was combined, the mixture was passed through an Amberlite-XAD column, sterilized, and then inoculated and incubated. This time, the purification was done from both, the supernatant, and the cell pellet. Interestingly, the results showed an improvement in the purified amount of LtnA1 from the supernatant. 0.8 mg were obtained from the mixture with Celtone® and 1.1 mg from the mixture with Bioexpress®, whereas no considerable amount of peptide was obtained from the cell pellet. Conversely, the production of LtnA2 remained the same as in previous fermentations, and no considerable amount was obtained.

4.3.3 Conclusions and overview

The results obtained from our experiments underscore the significant impact of media formulation on bacteriocin production. In their natural environment, bacteria typically synthesize these compounds in response to the availability of nutrients and in reaction to the presence of other bacterial species. Notably, creating a commercial lab media suitable for Gram-positive bacteria proves to be a costly endeavor due to the high nitrogen and carbon concentrations necessary for efficient bacteriocin production.

Hence, our conclusion emphasizes that an alternative and cost-effective approach to produce and label these compounds involves engineering an *E. coli* strain. In particular, the genetic information points to Lacticin 3147 being encoded by a 12.6kb region of the plasmid pMRC01,

housing ten genes arranged in two distinct clusters. This insight paves the way for our future research endeavors, suggesting the exploration of constructing an *E. coli* strain capable of producing and labeling bacteriocins like Lacticin 3147. This avenue will be a focus for our research group in upcoming studies.

CHAPTER 5

5. General conclusions

The process of antimicrobial resistance is complex, involving various contributing mechanisms. Given that the mode of action of bacteriocins differs for each type, and the emergence of resistance is often shaped by the characteristics of the targeted bacteria and their environment, the exploration of resistance to bacteriocins can be approached from multiple perspectives.

The research involving *L. monocytogenes* strains FSL-C1056, FSL-R2-499, and the non-resistant FSL-J1-177 provided crucial insights into the resistance development against carnocyclin A and its potential association with sugar intake receptors.

The experimental tests showed varying resistance patterns between the non-resistant and resistant strains when exposed to different concentrations of carnocyclin A. These results suggested that resistance to carnocyclin A might be affected by the type of sugar used as a carbon source. The diverse responses to the bacteriocin, especially with cellobiose, glucose, and mannose, indicated a complex relationship between sugar metabolism and the development of resistance. The differences in resistance observed across carbon sources hinted at a possible connection between specific phosphotransferase systems (PTS), possibly, the mannose transporter.

No significant mutations in the Man-PTS structure were found in resistant strains in this study, just a single amino acid variation in the IIC subunit of FSL-C1056 compared to the non-resistant reference strain was discovered. This variation might lead to slight conformational changes, potentially explaining the resistance in mannose media. Further research and structural studies are imperative to validate this hypothesis.

The stability and permanency of mutations in conferring resistance were also explored, revealing persistent resistance in the strains even after 60 generations. Mutations in cellobiose transporters was also evaluated but no mutations were identified in these transporters.

Studies have shown that *L. monocytogenes* displays a dual lifestyle, exhibiting virulence traits based on available carbon sources, impacting its pathogenicity when growing inside human cells. According to what has been reported in literature, it exists an intriguing connection between the expression of specific PTS systems and the modulation of *prfA*, a critical transcriptional activator

of virulence genes in *L. monocytogenes*, suggesting a vital role of sugar metabolism in regulating virulence factors and resistance pathways. It would also be interesting to investigate changes in the expression of transcription factors such as regulator components in both strains. These studies should be done on the strains before and after they were exposed to the bacteriocin.

Another alternative avenue for investigating the development of carnocyclin resistance in the *L. monocytogenes* strains, involves exploring prophage sequences that have the potential to introduce stop codons into sequences crucial for sugar transporters.

Carnocyclin A might not necessitate a specific docking molecule for its action. It's plausible that resistance is facilitated by alterations in various cell wall components, potentially leading to changes in the overall bacterial cell wall polarity to hinder bacteriocin binding.

The evolutionary selection process operates through various mechanisms to select the fit, eliminate the unfit, and overlook neutral changes. Single Nucleotide Polymorphisms (SNPs) are variations in a single nucleotide (A, T, C, or G) at a specific position in the DNA sequence. These variations can occur naturally within a population and contribute to genetic diversity. The importance of SNPs in evolution lies in their role as a source of genetic variation that can be subject to natural selection. Single changes in the DNA sequence that occur at a specific position within the genome and can affect various phenotypic traits, including virulence, antibiotic resistance, and adaptation to multiple environments.

It is important to highlight that controlled environment, characterized by significant selective pressure, often result in parallel evolution among replicates subjected to identical conditions. Parallel evolution refers to the independent development of similar traits, characteristics, or genetic changes in separate but related lineages or populations.

Research conducted by Orsi et al. in 2008[161] revealed a significant similarity in the genome backbone sequences of two *L. monocytogenes* isolates from 1988, associated with sporadic listeriosis, and two isolates from 2000, linked to substantial outbreaks. These findings provide valuable insights that could guide the investigation of *L. monocytogenes* strains FSL-C1056 and FSL-R2-499. Another noteworthy example, observed in a study by Bergthorsson et al. in 1999[162], demonstrated that after 20,000 generations of cultivation under uniform conditions, *Escherichia*

coli populations derived from a common parent strain exhibited substitutions in identical candidate genes.

According to studies done by Harrand S, in 2020[163] in the evolution of *L. monocytogenes* in a food processing plant, it was reported that even with a low single nucleotide mutation rate (1.15 x 10^{-7} changes per nucleotide per year in a genome of 2.88 Mb). A preliminary analysis of the SNPs differentiating wild type and bacteriocin resistant mutants identified many dozen SNPs that accumulated in the few generation of exposure to bacteriocins (Rosana and Vederas, unpublished observations). Further studies are necessary, however, whether *L. monocytogenes* mutates much more rapidly after exposure to bacteriocins, this enabling resistance that relates to multiple genetic loci.

CHAPTER 6

6. Experimental procedures

6.1 General experiments

6.1.1 Media and bacterial strains

6.1.1.1 Media preparation

All culture media were purchased from Benton Dickinson and Company (BD, Franklin Lakes, NJ, USA) and prepared according to manufacturer instructions. To prepare soft agar test tubes, 0.75% (w/v) agar was firstly dissolved in 5 mL of boiling broth (depending on the strain) and then sterilized in the autoclave. Agar plates were prepared with 1.5% (w/v) of agar in the desired broth, and then the agar solution was sterilized in the autoclave. After letting the solution cool down, the desired antibiotic was added (when needed) and the solution was stirred. 25 mL of the solution was poured into Petri dishes under sterile conditions (laminar flow cabinet). The agar plates were collected and stored at 4 – 8 ºC once the agar solidified. A Milli-Q reagent water system (Millipore Co., Milford, MA) was the source of deionized water.

6.1.1.2 Glycerol stocks

Bacterial strains were stored at -80 ºC in a sterile solution of 20% glycerol. Strains were grown on 5 mL of culture media to prepare a glycerol stock. To inoculate a strain either in an agar plate or broth, a sterile microbial loop was used. Thawing the glycerol stocks was avoided.

6.1.1.3 Growth conditions for indicator strains

The growth conditions for the indicator strains that were used for every chapter in this thesis are mentioned in Table 6.1.

Table 6.1 Growth conditions for the used strains.

Indicator strain	Agar Media	Conditions
E. canintestini EC49	APT	37 ºC
E. faecalis 710c	APT	30 ºC
C. maltaromaticum UAL3017	APT	25 ºC
L. monocytogenes sp.	APT	32 ºC
L. lactis sp.	APT	25 ºC
P. pastoris GS115	LB	30 ºC
E. coli BL21(DE3) pET-SUMO-LeuA	LB (50 µg/mL Kanamycin)	37 ºC

6.1.2 Antimicrobial activity assays

6.1.2.1 Spot-on-lawn assay

An agar plate was streaked under sterile conditions (laminar-flow cabinet) with an inoculum obtained from the corresponding glycerol stock. A sterile inoculation loop was used to streak a plate with the four-quadrant streak plate method, which was initially used to separate bacteria and to verify the culture was pure.

The inoculated plate was incubated overnight at the optimal temperature for bacterial growth. After growth, one isolated colony was picked with an inoculation loop, and then used to inoculate 10 mL of the appropriate broth and then incubated. Once the culture grew, 5 mL of melted soft agar (0.75%) were inoculated with 100 µL of the indicator strain culture, mixed and poured into an agar plate (20 mL/plate). Once the soft agar solidified, 10 µL of the sample to be tested for antimicrobial activity, were added on top of the bacterial lawn. Finally, the samples were dried,

incubated overnight at the optimal temperature for the indicator strain, and observed for zones of clearing.

6.1.3 General molecular biology techniques

6.1.3.1 DNA extractions

Chromosomal DNA was obtained from a bacterial culture with an approximate concentration of 2×10^9 cells which was verified through an OD600 assay using an Implen NanoPhotometer P360 (Implen Inc). The DNA extraction was performed according to the DNeasy Blood & Tissue Kit manufacturer instructions (Qiagen). The DNA concentration was measured in a Qubit fluorometer and a Nanodrop One/One®.

6.1.3.2 Agarose gel electrophoresis

0.45 g of Ultra-Pure Agarose (Invitrogen) were dissolved in 30 mL of Tris-borate-EDTA (Sigma-Aldrich) to prepare a 1.5% agarose gel (w/v) and 3 mL of SYBR Safe DNA gel stain (Life Technologies; 10,000X in DMSO) was added to stain the DNA. Sample buffer (6X; Thermo Scientific) was added to DNA samples before loading onto the gel. Gels were run at 90 – 110 V and visualized using a Dark Reader Transilluminator (Clare Chemical Research, Dolores, CO, USA).

6.1.3.3 DNA sequencing

DNA sequencing was done at the University of Alberta Molecular Biology Service Unit using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an ABI 3730 DNA Analyzer (Applied Biosystems).

6.1.4 Heterologous expression of proteins

6.1.4.1 Inoculation

An LB agar plate (50 mg/mL kanamycin) was streaked under sterile conditions (laminar-flow cabinet) with an inoculum obtained from the glycerol stock of *E. coli* BL21(DE3) pET-SUMO-LeuA. A sterile inoculation loop was used to streak plates with the four-quadrant streak plate method, which was initially used to separate bacteria and to verify the culture was pure.

Once the inoculated plate was incubated overnight at the optimal temperature for bacterial growth, one isolated colony was picked with an inoculation loop, and then used to inoculate 50 mL of LB broth with 50 mg/mL of kanamycin and then incubated (O/N, 37 ºC, 225 rpm). Once the culture grew, 1L of tested media (50 mg/mL of kanamycin) was inoculated with 10 mL of previous culture and incubated for an approximate of 3 hours at 37 ºC, 225 rpm until an OD of 0.9 was reached. After this time, the induction step was done by adding IPTG to the flask to a final concentration of 0.5 mM and the media was incubated under the same conditions for another 5 hours.

6.1.4.2 Cell harvesting

The 1 L culture was centrifuged at 4 000 x *g*, 4 ºC, for 10 minutes. The supernatant was discarded, and the cell pellet was used for the peptide purification.

6.1.4.3 Cell lysis

Bacterial cells were lysed using a Branson Sonifier 450 (Branson Ultrasonics Corp., Danbury, CT, USA). A bacterial suspension was prepared with 10 mL of lysis buffer (20 mM Tris-HCl, pH 7.8, 150 mM NaCl, 2 mM DTT) per gram of cells. The bacterial suspension was transferred into a 50 mL conical tube, and the cells were lysed by 7 cycles of 30 s sonication followed by 30 s on ice. Consequently, 150 U of DNase I was added after lysis and the tube was incubated for 10 min. Lastly, the lysate was centrifuged (23,700 × *g*, 30 min, 4 °C), and the supernatant was collected.

6.1.4.4 Tris-glycine SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyze protein samples, and commercial 12% Mini-PROTEAN TGX precast gels (Bio-Rad, Hercules, CA, USA) were used. Protein samples were mixed with 2X Laemmli Sample Buffer (Bio-Rad) and boiled at 100 °C for 5 min. Prepared samples and protein standards (Bio-Rad) were loaded onto the gel, and then the gel was run at 70 V for 15 min to compress the bands, followed by 120 – 180 V until the dye reached the bottom of the gel. Gels were visualized with either coomassie stain (0.1% w/v coomassie R-250, 40% ethanol, 10% acetic acid), followed by destaining (10% ethanol, 7.5% acetic acid), or GelCode Blue stain (Pierce, Rockford, IL, USA) and destained with water.

6.1.5 Purification of peptides and proteins

6.1.5.1 Amberlite XAD-16

To purify bacteriocins from culture media, Amberlite XAD-16 resin (Sigma-Aldrich, St. Louis, MO, USA) was used. First, the resin (40 – 80 g) was soaked in isopropanol (2-Propanol) for 30 min and then loaded into a glass column fitted at the base with Miracloth (EMD Millipore, Billerica, MA, USA). The resin was later washed with 250 mL of 0.1% trifluoroacetic acid (TFA) per 40 g resin at 10 mL/min using a peristaltic pump (Econo Pump, Bio-Rad). The solvent fractions that were used are described later in the subsequent relevant sections. Spot-on-lawn assays were performed on all fractions to identify the bacteriocins.

6.1.5.2 C8 and C18 solid-phase extraction

Bond Elut C8 10 g, 60 mL cartridge (Agilent, Mississauga, ON, Canada) and Strata C18-E (Phenomenex, Torrance, CA, USA) were used. The cartridge was first activated by washing with 50 mL methanol and 100 mL Milli-Q water at 10 mL/min. Then, the samples and solvents used (described later in the subsequent relevant sections) were passed through the cartridge at 5 mL/min using a peristaltic pump (Econo Pump, Bio-Rad).

6.1.5.3 RP-HPLC

Reverse-phase high-performance liquid chromatography (RP-HPLC) was performed on a Gilson system (analytical and preparative) equipped with a model 322 HPLC pump, GX-271 liquid handler, 156 UV/Vis detector and a 10 mL sample loop. HPLC grade solvents were filtered through a Millipore filter under vacuum before use. Details on the columns, solvents, and methods used are described in the subsequent relevant sections.

6.1.5.4 Ni-NTA affinity chromatography

Nickel-nitrilotriacetic acid (Ni-NTA) Superflow resin (Qiagen) was used to purify His-tagged proteins. Typically, 3 mL of slurry (50% suspension in 30% ethanol) was used for every 40 mL of cell-free lysate obtained from the heterologous expression plus 5 mM imidazole. The mixture of resin and sample was left under gently shaking for 1 hour at 4 ºC . Then, a gravity column was set up and samples and buffers were allowed to pass through, and fractions collected. For the purification of leucocin A, solutions of 5 mL of lysis buffer (mentioned in 4.2) with 6 concentrations of imidazole (40 mM, 60 mM, 80 mM, 100 mM, 200 mM, and 250 mM) were passed through the Ni-NTA resin and collected separately.

6.1.5.5 Dialysis

After confirming protein expression by checking the presence of the SUMO-fused protein in the collected Ni-NTA fractions by running a SDS-PAGE gel. The collected fractions from Ni-NTA were all mixed and poured into a dialysis bag (6000-8000 MW, SPECTRA/POR®). The dialysis bag was submerged into 1L of dialysis buffer (20 µM Tris-HCl, 150 µM NaCl, 1 µM DTT, 1 µM EDTA), the dialysis was left under stirring at 4 ºC for 3 hours, the buffer solution was changed for a new one after 1.5 hour. After 3 hours passed, the solution on the dialysis bag was collected into a 50 mL conical tube.

6.1.5.6 SUMO protease digestion

5000 U of the His-tagged SUMO protease (McLab, South San Francisco, CA, USA), together with 20 µL of 10X SUMO protease buffer (500 mM Tris-HCl pH 8.0, 2% IGEPAL® CA-630 (Sigma-Aldrich), 10 mM dithiothreitol and 150 mM NaCl) were added to the protein concentrated solution after it was passed through an Amycon tube (3000 MW).

6.1.6 Protein quantification

6.1.6.1 Spectrophotometric quantification

Protein concentrations were determined by measuring absorbance at 280 nm using an Implen NanoPhotometer P360 (Implen Inc.).

6.1.7 Mass spectrometry

6.1.7.1 MALDI-TOF MS

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) was performed on an AB Sciex Voyager Elite system (Foster City, CA, USA) in positive reflectron mode with delayed extraction, using either 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) or 4-hydroxy-α-cyanocinnamic acid as matrix. A two-layer sample preparation method was employed.

Before data acquisition, samples were acidified, and whenever applicable, samples were cleaned-up either by rinsing with 0.1% TFA or with ZipTip pipette tips (C4 or C18, EMD Millipore) according to the manufacturer's instructions.

6.1.7.2 Bioinformatics

The NCBI-BLAST[166] (National Center for Biotechnology Information-Basic Local Alignment Search Tool) was used for genome mining. BAGEL3[167] was used to find genes encoding bacteriocins. Clustal W and Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo) were used for sequence alignment. To do the genome assembly, the Microbe Genomes Atlas (MiGA)[168], was

initially used to process the genome sequences obtained from the University of Alberta Molecular Biology Service Unit. For scaffolding, the genome, MeDuSa (Multi-Draft-based Scaffolder)[169] was used. The type (Strain) genome server[170] was used to corroborate the taxonomy of the genome assembly. RASTtk[171] was used for genome annotation.

6.2 Experimental procedures for the identification and purification of bacteriocins in *E. canintestini* 49

6.2.1 Bacterial strains and culture conditions

E. canintestini 49 strain provided by CanBiocin was inoculated from a glycerol stock into a 5 mL APT broth. It was incubated at 37 ºC with no shaking overnight. The indicator strains *E. faecalis* 710C and *L. lactis* subsp. *cremoris* HP were inoculated in the same way and grown in the same conditions as that of EC49. APT medium was used for both indicator strains. *L. lactis* subsp. *cremoris* HP and *E. faecalis* 710C were incubated overnight without shaking at 25 ºC and 37 ºC, respectively.

6.2.2 Identification of antimicrobial activity of *E. canintestini* 49

6.2.2.1 Spot-on-lawn assay

An APT agar plate was streaked under sterile conditions (laminar-flow cabinet) with an inoculum obtained from the *E. canintestini* 49 glycerol stock. A sterile inoculation loop was used to streak a plate with the four-quadrant streak plate method, which was initially used to separate bacteria and to verify the culture was pure.

The inoculated plate was incubated overnight at the optimal temperature for bacterial growth. After growth, one isolated colony was picked with an inoculation loop, and then used to inoculate 250 mL of APT sterile broth and then incubated (O/N, 37 ºC, no shaking). After growth, the broth was centrifuged at 4000 x *g*, 4 ºC for 10 min. The supernatant was used for the spot-on-lawn assay. In addition, a 100 µL supernatant was taken and concentrated for 1.5 hours in a SpeedVac concentrator, and this concentrated portion was also used for the spot-on-lawn assay.

In sterile conditions, 6 mL APT soft agar (0.75% agar) were inoculated with 100 uL of the indicator strain. Once the soft agar was inoculated, it was poured evenly into an APT agar plate (1.5% agar) and allowed to solidify for approximately 5 minutes. Once the soft agar was solidified, 10 µL of each sample was taken separately with an automatic micropipette and spotted into the solidified agar. Once the samples were dry, the plates were incubated. The agar plates were incubated overnight at 37 ºC for *E. faecalis* 710C and 25 ºC for *L. lactis* subsp. *cremoris* HP. A positive result was considered when a halo (zone of inhibition) was formed.

6.2.3 Guided bacteriocin purification

An APT agar plate was streaked over under sterile conditions (laminar-flow cabinet) with an inoculum of *E. canintestini* 49 obtained from a glycerol stock. The agar plate was incubated O/N at 37 ºC.

One grown colony was inoculated in 50 mL of APT broth and then incubated at the same conditions as the agar plate, no shaking. After growth, 10 mL of the previous broth was added into 1 L of APT broth. After incubation (O/N, 37 ºC), the culture was centrifuged at 4000 x *g*, 4 ºC, for 10 minutes. The antimicrobial substances were purified from the supernatant.

40 g of Amberlite XAD 16N resin (Sigma Aldrich) was used per L of broth. The Amberlite resin was prepared by soaking 40 g of resin in 100 mL of 2-propanol for 30 minutes. The resin was then poured into a glass column. The column resin was kept wet to avoid bubble formation since bubbles can cause problems in the extraction procedure. The resin was then washed with 250 mL of 0.1% trifluoroacetic acid (TFA) at 10 mL/min. Once the resin was activated, the sample was passed over the column, and the flow through was collected. Once the flow through was collected, the solvents mentioned in Table 6.2 were pumped through the resin and fractions were collected separately.

Table 6.2 Order of solvents used in amberlite resin purification of bacteriocins.

Order	Quantity/Solvent	Flow
1	250 mL/H_2O	15 mL/min
2	250 mL/20% (v/v) 2-Propanol	15 mL/min
3	250 mL/40% (v/v)2- Propanol	15 mL/min
4	250 mL/ 80% (v/v) 2-Propanol + 0.1% TFA	15 mL/min

After the fractions were collected, 200 µL of each fraction was taken for a spot-on-lawn assay. Each sample was concentrated to ~100 µL for approximately 30 minutes in a SpeedVac concentrator. From the spot-on-lawn results, active antimicrobial fractions were identified, and these fractions were concentrated using a rotary evaporator. A Phenomenex Strata C8 reverse phase column was then prepared by washing with 50 mL of methanol and then 100 mL of water. The solvents that were used to elute compounds from the column are mentioned in Table 6.3.

Table 6.3 Order of solvents used in Strata C8 column purification of bacteriocins**.**

Order	Quantity/Solvent
1	50 mL/30%(v/v) Ethanol
2	50 mL/20% (v/v) 2-Propanol
3	50 mL/40% (v/v) 2-Propanol
4	50 mL/80% (v/v) 2-Propanol + 0.1% TFA

After each fraction was collected, a 200 µL sample was taken and concentrated for a spot-on-lawn assay to check antimicrobial activity of each fraction. Active fractions were then concentrated using a rotary evaporator, and then purified using analytical HPLC. The column used was a Phenomenex column, Jupiter type 5u C18 300 Å; 160 x 4.60 mm. The flow rate was 1 mL/min. The HPLC program used is shown in Table 6.4.

Table 6.4 HPLC program. Solvent A: water with 0.1% TFA, Solvent B: acetonitrile with 0.1% TFA.

Time (min)	Value (%B)
0-5	20
5-45	60
45-50	95
50-55	95
55-56	30
55-60	30
60	0

6.3 Experimental procedures used in Chapter 3

6.3.1 Purification of carnocyclin A

An APT agar plate was streaked under sterile conditions (laminar-flow cabinet) with an inoculum of *C. maltaromaticum* UAL307 obtained from a glycerol stock. The agar plate was incubated for 48 h at 25 ºC.

One grown colony was inoculated in 50 mL of APT broth and then incubated at 25 ºC for 24 hour. After growth, 10 mL of the initial broth were taken into 1 L of APT broth. After incubation, at the same conditions as the previous culture, the media was centrifuged at 4000 x *g*, 4 ºC, 10 minutes. The antimicrobial substances were purified from the supernatant. The supernatant was passed through an Amberlite XAD-16 resin (Sigma) at a flow rate of 15 mL/min at 4 ºC.

Subsequently, the column was washed with 750 mL of 30% ethanol, and the active peptide was eluted with 1 L of 70% 2-propanol, pH 2 (acidified with TFA). The eluted fraction was concentrated to ~25 mL and then loaded onto a C18 cartridge which was washed at a flow rate of 5 mL/min with the solvents mentioned in Table 6.5.

Table 6.5 Order of solvents used in Strata C18 column purification of carnocyclin A.

Order	Quantity/Solvent
1	60 mL/H_2O
2	60 mL/30% (v/v) Ethanol
3	60 mL/20% (v/v) 2-Propanol
4	30 mL/40% (v/v) 2-Propanol
5	100 mL/70% (v/v) 2-Propanol, pH 2 (acidified with TFA)

The active peptide was eluted in solvent number 5, which was concentrated using a rotary evaporator to 5 mL and then lyophilized. The lyophilized sample was then dissolved in 5 mL of distilled water to be purified through HPLC (Beckman System Gold, Beckman Coulter, Inc., Mississauga, ON, Canada). Samples of 1 mL were injected onto a C8 column (5 µm particle size, 10 mm × 250 mm, Vydac 208TP510, Hichrom, Berkshire, United Kingdom). The HPLC mobile phase consisted of (A) 2-Propanol and (B) H_2O, each containing 0.1% TFA. A gradient HPLC method was used, consisting of 8 min hold at 20% A; 30 min increase of A from 20 to 86%; 30 min hold at 86% A; and a 3 min decrease from 86 to 20% A, with a flow rate of 1 mL/min and ultraviolet detection set at 220 nm. Carnocyclin A eluted as a broad peak (retention time 40 min).

6.3.2 Growth of *L. monocytogenes* up to 60 generations

An agar plate was streaked under sterile conditions (laminar-flow cabinet) with an inoculum of the corresponding *L. monocytogenes* strain from a glycerol stock. The agar plate was incubated for 24 h at 32 ºC. 10 mL of APT media were then inoculated with one colony of the agar plate and incubated for 8 hours/150 rpm. Knowing that bacteria have an exponential cell division, serial dilutions were prepared with 10 µL of the previous culture added into a test tube with 990 µL of sterile APT media. This was repeated five times and then incubated on the same conditions as previously mentioned.

6.3.3 Purification of leucocin A by HPLC

After confirming the cleavage of the of the peptide (Section 4.2) was successful by running a SDS-PAGE gel. The solution containing the peptide was then purified using analytical HPLC (Table 6.4). The sample was loaded onto a C18 column (GraceVydac Protein and Peptide 100 mm column, 10 micron) at a flow rate of 5 mL/min, UV = 220 nm).

Table 6.6 HPLC program. Solvent A: water with 0.1% TFA, Solvent B: acetonitrile with 0.1% TFA.

Time (min)	Value (%B)
0-5	20
5-45	85
45-50	85
50-55	5
55-56	30
55-60	30
60	0

6.4 Experimental procedures used in Chapter 4.3

6.4.1 Isolation of lacticin 3147 from culture supernatant

An agar plate was streaked under sterile conditions (laminar-flow cabinet) with an inoculum of *L. lactis* subs *lactis DPC3147* obtained from a glycerol stock. The agar plate was incubated for 24 h at 30 ºC. One of the grown colonies was picked with an inoculation loop and inoculated in 10 mL of M17 broth which was incubated under the same conditions.

One litre of media was prepared as follows: The media contained 800 mL: 2.5g of tryptone, 5.0 g of yeast extract, 1.5 g of D/L-methionine, 50 mg of $MnSO_4 \cdot 4H_2O$, and 125 mg of $MgSO_4$.

Before sterilization, the media was passed through an Amberlite XAD-16 column (75 g of resin) to remove hydrophobic components that could interfere with the isolation of the peptide. Solutions of 100 mL of D-glucose (100 g/mL) and β-glycerophosphate (19.0 g/L) were separately prepared and sterilized before they were added to the media to prevent caramelization. One litre of media was inoculated with 10 mL of initial culture, and it was incubated for 24 h at 30 ºC without aeration.

After growth, the cells from the media were removed by centrifugation (20 min, 4000 x *g*). The supernatant was then passed through an Amberlite XAD-16 column (60 g of resin) at a flow rate of 15 mL/min. The column was prepared as described in general procedures. The column was first washed with 1.0 L of 30% ethanol, and then the active peptide was eluted with 1.0 L of 70% isopropanol (0.1% TFA). This fraction was concentrated to a volume of 100 mL using a rotary evaporator. The concentrated solution was loaded onto a C18 cartridge and washed with the solutions mentioned in Table 6.7.

Table 6.7 Order of solvents used in Strata C18 column purification of lacticin 3147.

Order	Quantity/Solvent
1	60 mL/H_2O
2	60 mL/30% (v/v) Ethanol
3	40 mL/25%(v/v) 2-Propanol
4	100 mL/70% (v/v) 2-Propanol, 0.1% TFA

The active peptide was eluted in solvent number 4, which was reduced to a 20 mL volume by rotary evaporation and then lyophilized. The lyophilized sample was then dissolved in 5 mL of H_2O for reverse-phase high-performance liquid chromatography. The sample was loaded onto a C18 column (GraceVydac Protein and Peptide 100 mm column, 10 micron) at a flow rate of 10 mL/min, UV = 220 nm, Gradient = water/acetonitrile, 0.1% TFA (70:30) for 5 min. Then the acetonitrile ratio was ramped up to 95% over 45 minutes and kept at this ratio for 5 minutes. Next, the acetonitrile ratio was lowered to 30 % over 3 min and maintained this percentage for 5 min.

6.4.2 Isolation of lacticin 3147 from cells

The initial culture growth was done as mentioned in the isolation of lacticin 3147 from the supernatant. The cells from the media were collected by centrifugation (20 min, 4000 x *g*, 4 ºC) and then resuspended in 250 mL of 70% (v/v) isopropanol, 0.1% TFA. The solution was stirred for 3 h at 4 ºC and then centrifuged for 20 min at 8000 rpm. The supernatant was concentrated to ~20 mL using a rotary evaporator, and the peptide purification was carried out as has been previously mentioned.

6.4.3 Yeastolate preparation

One litre of unlabeled yeast medium was prepared with 100 mM potassium phosphate, 2.5 g/L glucose, 2 mg/L biotin and 6.7 g/L of yeast nitrogen base media without amino acids (Invitrogen, Thermo Fisher). The medium was adjusted to pH 6 and then sterilized through filtration. 100 mL of feeding solution for the fed-batch phase was also prepared following the same recipe, but instead of 2.5 g/L of glucose, 25 g/L of glucose were used.

An agar plate of LB was streaked under sterile conditions (laminar-flow cabinet) with an inoculum of *P. pastoris* GS115 obtained from the corresponding glycerol stock. The agar plate was incubated overnight at 30 ºC. One grown colony was picked with a sterile inoculation loop to inoculate10 mL of yeast medium incubated overnight at 30 ºC, 200 rpm. Under sterile conditions, the cells were harvested by centrifugation at 5000 × *g* for 15 min. The cells were resuspended in 1 L of yeast medium and incubated at 30 ºC, 500 rpm in a 4 L flask. After 8 h, 100 mL of feeding solution was added to the medium and incubated with shaking at 225 rpm for 4 hours. The cells were harvested by centrifugation (5000 × g for 15 min).

The cell pellet was washed with MilliQ water and centrifugated at 5000 × g for 30 min. A 30% (w/v) yeast slurry was prepared with the cell pellet and 1 M NaOH. The pH of the solution was adjusted to 7.5. Zymolyase 20T (USBiological, 0.5% (w/w)) was added to the yeast slurry and incubated at 35 ºC in a water bath for 6 h (maintaining pH at 7.5). Zymolyase 20T was inactivated at 65 ºC for 20 min. The pH was adjusted to 6, and 0.5% (w/w) of papain powder (Sigma-Aldrich) was added. The slurry was incubated for five days at 50 ºC. The solution was centrifuged at 8000

× g for 50 min, and the supernatant was collected. The supernatant was filtered through a 10 kDa MWCO filtration cell (Millipore). The flow-through was collected and lyophilized.

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