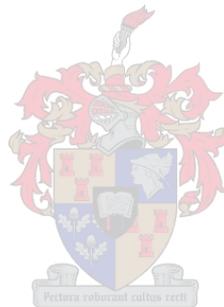


Investigating the mode of transcriptional regulation controlling plantaricin 423 expression in *Lactobacillus plantarum* 423

by

Ross Rayne Vermeulen



Thesis presented in partial fulfilment of the requirements for the degree

Master of Science at Stellenbosch University

Supervisor: Prof. Leon Milner Theodore Dicks

Co-supervisors: Prof. Johann Rohwer, Dr Anton Du Preez van Staden and Dr Shelly Deane

April 2019

DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Abstract

The discovery and use of antibiotic therapies was one of the most significant achievements of the twentieth century. However, the current rate at which antibiotic resistance develops heavily outweighs the rate that novel treatments are introduced. Without a doubt, antibiotic resistance is one of the biggest challenges scientists of the twenty-first century are facing.

With a global realization of finite resource availability and continuous climate change, the transition toward developing more sustainable systems is becoming a focal point for science, technology, and society as a whole. Such an approach also must be applied in the fight against antibiotic resistance and is already being observed through the emergence of novel fields such as biotherapeutics. Antibiotics have only recently been employed as a therapy, yet antimicrobial compounds and mechanisms for resistance have existed for millennia. Therefore, systems must already exist to ensure the sustainability of an antimicrobial compound's use by a microorganism within its environment. Such systems are likely to use a diverse array of approaches within a microbiota, with the chemical diversity of antimicrobials being one. While researching the role and effect of antimicrobials within a microbiota may elucidate new approaches and schemes to manage antimicrobial resistance, a diverse group of antimicrobials, known as bacteriocins, has already been discovered. In the past, these antimicrobial peptides have received considerably less attention than antibiotics, however due to the urgent need for alternatives they warrant serious consideration.

This study concerns the native transcriptional regulation of a subclass IIa bacteriocin, plantaricin 423, produced by *Lactobacillus plantarum* 423, one of the strains in the probiotic Entiro™ developed by our research group. The mode of transcriptional regulation for class IIa bacteriocins in the absence of local regulatory genes, as observed for plantaricin 423, is unknown. Through the development of a fluorescent promoter-reporter system, it was observed that the transcriptional regulation of plantaricin 423 responded to manganese-limiting conditions. During this research, significant progress was made for methods concerning bacteriocin classification, heterologous expression, and real-time *in vivo* transcriptional monitoring. Based on findings obtained using a fluorescent promoter-reporter system and the fact that *L. plantarum* 423 requires high intracellular Manganese concentrations for aerobic respiration, plantaricin 423 might aid in Manganese acquisition from target cells via cell wall poration.

This research represents the first steps towards understanding how *L. plantarum* 423 and *Enterococcus mundtii* ST4SA, the other strain in the Entiro™, interact with each other, the gastrointestinal microbiota and the host. Future research in this direction will be done with the hope of discovering sustainable alternatives to current problems, such as antibiotic resistance.

Opsomming

Die ontdekking van antibiotika was een van die belangrikste deurbreke van die twintigste eeu. Die tempo waarteen weerstandigheid teen antibiotika huidige tyd onwikkel is egter vinniger as wat nuwe behandelings geïmplementeer kan word. Dus is antibiotika-weerstandigheid 'n uitdaging wat in die een-en-twintigste eeu voorkom moet word.

Met 'n wêreldwye besef van klimaatsverandering en die beperktheid van natuurlike hulpbronne, word die ontwikkeling en oorgang na meer volhoubare stelsels 'n fokuspunt vir die wetenskap en die samelewing as geheel. So 'n benadering is ook toepaslik in die stryd teen antibiotika-weerstandigheid en word reeds waargeneem deur die opkoms van nuwe velde soos bio-terapeutika. Alhoewel antibiotika eers relatief onlangs as 'n terapie ingespan is, bestaan die antimikrobiese verbindings en meganismes vir weerstandigheid alreeds vir millennia. Daarom moet daar reeds stelsels in mikroorganismes bestaan wat die volhoubaarheid van hul eie antimikrobiese middels in hulle omgewing verseker. Sulke stelsels sluit waarskynlik 'n verskeidenheid meganismes in, onder andere die chemiese diversiteit van antimikrobiese middels. Terwyl verdere ondersoek tot die rol en effek van antimikrobiese middels binne die gastro-intestinale mikrobiota nuwe meganismes kan ontbloot wat antimikrobiese weerstandigheid mag help bestuur, is daar reeds 'n diverse groep antimikrobiese peptiede, bekend as bakteriosiene, beskryf. Hierdie antimikrobiese peptiede het in die verlede aansienlik minder aandag ontvang as antibiotika, maar in die lig van die noodsaaklikheid vir alternatiewe, verdien hulle verdere ondersoek.

Hierdie proefskrif het betrekking op die inheemse transkripsie regulasie van 'n subklas IIa bakteriosien, plantarisien 423, wat vervaardig word deur *Lactobacillus plantarum* 423, een van die stamme in die probiotikum Entiro™ wat deur ons navorsingsgroep ontwikkel is. Die modus van transkripsionele regulering vir klas IIa bakteriosiene, in die afwesigheid van plaaslike regulatoriese gene soos waargeneem vir plantarisien 423, is onbekend. Met die ontwikkeling van 'n fluoresserende promotor-verslaggewerstelsel is daar waargeneem dat plantarisien 423 regulasie reageer op Mangaan-beperkende toestande. Tydens hierdie navorsing is beduidende vordering gemaak tot metodologiee rakende bakteriosien klassifikasie, heterologiese uitdrukking en monitering van *in vivo* translasie in “real-time”. In die lig van resultate verkry met die gebruik van 'n fluoresserende promotor-verslaggewerstelsel en die bevinding dat *Lactobacillus plantarum* 423 hoë intra-sellulêre mangaan konsentrasies vir aërobiese respirasie benodig, kan plantarisien 423 moontlik help met die verkryging van mangaan vanuit teiken selle deur middel van selwand porie formasie.

Hierdie navorsing verteenwoordig die eerste stappe om die interaksie te verstaan tussen *L. plantarum* 423 en *Enterococcus mundtii* ST4SA (die ander stam in die probiotika Entiro™), asook interaksies met

die gastro-intestinale mikrobiota en die gasheer. Verdere navorsing in die rugting sal gemik wees om volhoubare alternatiewe oplossings vir hedendaagse probleme te vind, soos vir antibiotika-weerstandigheid.

Acknowledgements

I would like to thank the following people and organizations:

Prof. Leon Dicks (Department of Microbiology, Stellenbosch University), for his guidance and allowing creative latitude,

Prof. Johann Rohwer (Department of Biochemistry, University of Stellenbosch), for his patience, support and knowledge,

To my friend Dr. Anton Du Preez van Staden, for his valuable insight, support and mentorship,

To Dr Shelly Deane for helping me develop my skillset and intuition,

To Elzaan Booysen and the rest of my co-workers in the Microbiology department,

To my family, especially my loving mother, for their unwavering support, love and belief in me,

To my friend Jerad Gibbon, for his camaraderie and invaluable input to my research and also my friend Francesco Salini,

To Cipla Medpro (Pty) Ltd and the National Research Foundation (NRF) of South Africa for financial support and funding of the research.

This thesis is dedicated to my late grandfather, Reginald Bonthuys, who played an instrumental role in culturing my passion for the natural world and subsequently my fascination with the field of biological sciences. You are sorely missed, Pops.

“The more I learn, the less I know about before
The less I know, the more I want to look around
Digging deep for clues on higher ground”
-UB40

Contents

		Page
Chapter 1:	Introduction	1
Chapter 2:	Literature Review	5
	Introduction	6
	Lactic acid bacteria as probiotic supplements	8
	Bacteriocin classes	10
	Bacteriocin regulation in Gram-positive bacteria	13
	Quorum sensing	14
	Bacteriocin autoinduction	16
	Autoinducer-2 (AI-2) based interspecies bacteriocin regulation	18
	Co-culture induced bacteriocin transcription	19
	Anomalous bacteriocin regulation in LAB	20
	Environmental physico-chemical induction cues for class IIa expression	21
	Regulation of virulence genes in <i>Listeria monocytogenes</i>	22
	Class IIa bacteriocin expression systems	23
	Conclusion	25
	References	28
Chapter 3:	In-silico sub-grouping class IIa bacteriocins by their immunity protein sequence homology	40
Chapter 4:	Heterologous expression and purification of active plantaricin 423 and mundticin ST4SA using a novel GFP-fusion expression system	68
Chapter 5:	Investigating the mode of plantaricin 423 regulation using a fluorescent promoter-reporter system in <i>Lactobacillus plantarum</i> 423	97
Chapter 6:	General Conclusions	136
Addendum		141

Chapter 1

Introduction

Lactic acid bacteria (LAB) are Gram-positive bacteria that natively inhabit the human gastrointestinal tract (GIT) and are thus often used in probiotic supplements ^{1,2}. Probiotic supplements confer a wide range of health benefits to the host using various mechanisms ^{2,3}. However, one of their most beneficial characteristics is their ability to produce antimicrobial peptides, including bacteriocins. Due to the looming spread of antibiotic resistance and slow discovery of novel antibiotics, bacteriocins have recently gained more attention as alternatives or adjuvants to current antibiotics ⁴. In addition, researching bacteriocin regulation, mechanism of activity, and bacteriocin evolution may provide scientists with novel tools and approaches to manage and combat the spread of antimicrobial resistance ⁵.

Lactobacillus plantarum 423 and *Enterococcus mundtii* ST4SA are included in the probiotic Entiro™, commercialised by Cipla Medpro (Pty) Ltd, a South African-based pharmaceutical company owned by Cipla India. During administration, the two species most probably engage in an ecological relationship which, like many microbial interactions amongst species in the GIT, is yet to be systematically defined ⁶. Strain 423 produces the class IIa bacteriocin plantaricin 423 and strain ST4SA produces mundticin ST4SA. To better understand the ecological relationship between *L. plantarum* 423 and *E. mundtii* ST4SA, it would be prudent to understand the full effect their bacteriocins have on each other. However, the regulatory mechanisms for plantaricin 423 and mundticin ST4SA within their respective producers is not known. Therefore, hypothesizing about bacteriocin-mediated interactions between *L. plantarum* 423 and *E. mundtii* ST4SA is difficult, especially not knowing when, and where, these bacteriocins may be expressed within the GIT. Currently, the stimuli for plantaricin 423 transcription are unknown, which renders mechanistic elucidation via proteomics challenging and costly. This study aimed to elucidate the mode of transcriptional regulation of *plaA*, the gene encoding plantaricin 423, via the construction and monitoring of a fluorescent reporter system.

Chapter 2 provides a brief review of bacteriocins produced by Gram-positive bacteria and focuses on the available literature for regulatory systems pertaining to class IIa peptides. Little is known about class IIa bacteriocin regulation, especially in the absence of operon-encoded regulatory elements, as in the case of plantaricin 423. Furthermore, it was reported on two occasions that manganese, for unclear reasons, increased the anti-listerial effect of class IIa bacteriocin-producing *L. plantarum* strains ^{7,8}.

Phylogenetically subgrouping class IIa bacteriocins was the main objective in Chapter 3 as more phylogenetically related peptides may have similar mechanisms of regulation. Class IIa bacteriocins which had their structural and immunity protein genes sequenced were sub-grouped according to the amino acid sequence similarity of the immunity proteins. This approach divided class IIa bacteriocins

into three groups according to the type of immunity protein they had and therefore, the producer's mechanism of immunity⁹. Our research has shown that plantaricin 423 and its subgroup of class IIa bacteriocins (IPT2) do not have classical bacteriocin regulatory elements in their operons. The exact mechanisms involved in the regulation of these peptides is unknown. Operon elements belonging to the IPT2-subgroup of bacteriocins showed associations with cationic metal regulatory and transport motifs or operons. Such information guided the research direction and experimental design for subsequent experiments

Based on the findings discussed in Chapters 2 and 3, we hypothesise that the transcriptional regulation of plantaricin 423 is not dependent on a classical operon-encoded three-component bacteriocin regulatory system or autoinduction but may be regulated by divalent metal cations. To disprove Nisin-type autoinduction of plantaricin 423, a novel GFP-fusion system was developed in Chapter 4 and used for the heterologous expression and purification of active plantaricin 423 and mundticin ST4SA. Chapter 5 aimed to define the transcriptional response of plantaricin 423 to various cationic metals via the construction and deployment of a red fluorescent reporter system in *L. plantarum* 423.

Another outcome of this study was the development of an approach to heterologously express unmodified cationic peptides and monitor their transcriptional effect on a single gene *in vivo* and in real-time *L. plantarum* 423. Future research can use this approach to disprove plantaricin 423 autoinduction, and potentially elucidate any peptide mediated interactions between strains 423 and ST4SA. Researching bacteriocins, their transcriptional regulation and their exact role within the complex network of the GIT microbiome may lead to new fundamental findings, or biotechnical tools and approaches. These outcomes may prove invaluable in current problems like antibiotic resistance and the search for alternatives, or new fields within biotechnology such as biotherapeutics.

References

1. World health organization. Guidelines for the Evaluation of Probiotics in Food. World Heal. Organ. 1–11 (2002). at <<ftp://ftp.fao.org/es/esn/food/wgreport2.pdf>>
2. Klein, G., Pack, A., Bonaparte, C. & Reuter, G. Taxonomy and physiology of probiotic lactic acid bacteria. *Int. J. Food Microbiol.* 41, 103–125 (1998).
3. Brown, M. Modes of action of probiotics: recent developments. *J. Anim. Vet. Adv.* 10, 1895–1900 (2011).
4. Cotter, P. D., Ross, R. P. & Hill, C. Bacteriocins — a viable alternative to antibiotics? *Nat. Rev. Microbiol.* 11, 95–105 (2013).
5. Egan, K., Ross, R. P. & Hill, C. Bacteriocins: antibiotics in the age of the microbiome. *Emerg. Top. Life Sci.* 1, 55–63 (2017).
6. Bäckhed, F. et al. Defining a healthy human gut microbiome: current concepts, future directions, and clinical applications. *Cell Host Microbe* 12, 611–22 (2012).
7. Hugas, M., Garriga, M., Pascual, M., Aymerich, M. T. & Monfort, J. M. Enhancement of sakacin K activity against *Listeria monocytogenes* in fermented sausages with pepper or manganese as ingredients. *Food Microbiol.* 19, 519–528 (2002).
8. van der Veen, S. & Abee, T. Mixed species biofilms of *Listeria monocytogenes* and *Lactobacillus plantarum* show enhanced resistance to benzalkonium chloride and peracetic acid. *Int. J. Food Microbiol.* 144, 421–431 (2011).
9. Willey, J. M. & van der Donk, W. A. Lantibiotics: peptides of diverse structure and function. *Annu. Rev. Microbiol.* 61, 477–501 (2007).

Chapter 2

Literature review

Introduction

Biotechnology is the manipulation of biological processes, organisms or systems to create or enhance products intended for human benefit ¹. The application of biotechnology can be identified far back in human history, to a time where crop cultivation or selective breeding were modern techniques that shaped future political, economic, and social circumstances ¹. Modern biotechnology focuses on directing the manipulation of organisms or their products, to perform desired functions, via genetic engineering strategies and tools ¹. These approaches are becoming increasingly popular as seen in new fields such as biotherapeutics. Currently, biotechnology is branching in ten directions which have the following colour designations: green for agriculture, yellow for nutritional, red for medicine or health, white for industrial, grey for environmental, blue for aquatic and marine, brown for desert or arid regions, gold for bioinformatics, violet for law, ethical and philosophic issues, and dark for biological weapons ². The development of antibiotic therapies falls within red biotechnology and is undoubtedly one of the most significant scientific achievements of the twentieth century ³. Antibiotic therapies have had an unparalleled impact on human morbidity/mortality and are a cornerstone for the existence modern society ³. Therefore, the rise of antimicrobial resistance is a major global public health concern that must be addressed during the twenty-first century. According to the World Health Organisation (WHO), most of the drugs in use are modifications of existing antibiotic classes that only provide short-term solutions to resistance. Furthermore, the WHO reports a fast depleting pool of tools to combat antimicrobial resistance for current antibiotics ⁴.

Antibiotics have been used by microbes for millennia to modulate the growth of bacteria, but their benefits to humans have only recently been exploited. Yet, for just as long as antibiotics, antimicrobial peptides have been used by all kingdoms of life to modulate the growth of microbes, but have received considerably less attention from biotechnologists ⁵. Bacteriocins are antimicrobial peptides that some bacteria secrete into their environment to modulate the growth of other bacteria ³. These peptides and their mechanisms of action may significantly expand the arsenal of antibiotic therapies available to humans ³. While many challenges surround the use of bacteriocins as antibiotics, like their proteolytic cleavage susceptibility and toxicity, they offer many benefits ^{3,5,6}. Bacteriocins can have broad or highly specific activity spectra, rapid killing effect, and synergistic effect with antibiotics. In addition, their structures are easily manipulated due to their proteinaceous nature ^{3,5,6}. Antimicrobial resistance is an ancient phenomenon, researching bacteriocins may not only increase our arsenal of antimicrobials, but may elucidate how producer organisms have managed the development of resistance over time. Understanding bacteriocin deployment strategies and their role within the environment may also elucidate new approaches to manage and detect microbial infections.

Sustainability is a central theme in the development and continuation of any technology. The emergence of antibiotic resistance is a direct threat to the sustainability of antibiotic therapies, but one that biotechnology must overcome. Researching antimicrobial peptides and their role in the environment may provide some urgently needed solutions to this problem and promote the development of novel antibiotic-related tools. This study focuses on the class IIa antilisterial bacteriocins, plantaricin 423 and mundticin ST4SA, their role and regulation within the environment, and their production and purification.

Listeriosis is a bacterial infection, caused by *Listeria monocytogenes*, which can induce sepsis, meningitis, or encephalitis that may result in death. Upon ingestion, *L. monocytogenes* can enter the epithelial cells of the gastrointestinal tract (GIT) via interaction with the host's E-cadherin and hepatocyte growth factor receptor tyrosine kinase c-Met, using its surface proteins Internalin A and B⁷. Although the human body combats this infection via phagosomal compartmentalisation, *L. monocytogenes* escapes by expression of the pore-forming toxin listeriolysin O and phospholipases, PlcA and PlcB^{7,8}. Upon its escape into the host cell cytosol, *L. monocytogenes* quickly multiplies^{7,8}. Actin is then polymerized on the bacterial surface via the actin assembly –inducing protein, ActA. This process drives the mobile phase of *L. monocytogenes* and allows it to spread to other host cells, resulting in dissemination of the infection⁷. Between 1 January 2017 and 17 July 2018, the largest outbreak of listeriosis, that has ever been recorded, occurred in South Africa. During this period, 1060 cases were reported of which 216 people died⁹. Tragically, 93 of these deaths occurred in neonates who were less than 28 days old¹⁰. Moreover, the genome sequencing of *L. monocytogenes* isolates sampled during the epidemic from production facilities, food, human throats and human blood samples indicated a dominating presence of *L. monocytogenes* sequence type 6 (ST6)⁸. Italian and Chinese epidemiologic studies reported that *L. monocytogenes* ST6 was responsible for less than 1% and 0.5% of foodborne isolates in 2010 and 2012, respectively^{9,11,12}. The emergence of *L. monocytogenes* ST6 increased the rate of mortality in *L. monocytogenes* meningitis from 27% in 1998-2002 to 61% in 2006-2012⁹. While the recent listeriosis outbreak in South Africa has not been represented as a percentage of the total foodborne disease isolates in South Africa, it is likely to be higher than 1 and 0.5% as previously recorded in Italy and China in 2010 and 2012 respectively⁹⁻¹². This is a serious point of concern, especially since ST6 had such an impact on morbidity at low levels.

Understanding the nature of class IIa antilisterial peptides may lead to the development of early listerial detection systems, the discovery of improved (or novel) antibiotics or treatments, and management strategies to prevent the spreading of *Listeria*. In this review the known modes of Gram-positive bacteriocin regulation will be discussed, with specific relevance to plantaricin 423 and

mundticin ST4SA. The concept of a keystone probiotic is also considered, this is a microbial species that facilitates, but is not limited to, the chemical signalling within the GIT on an intraspecies, interspecies and interkingdom level. In addition, this keystone probiotic would fulfil a role synonymous with any ecological keystone species. The possibility of a keystone probiotic facilitating chemical signalling via the expression of bacteriocins will also be considered in this review.

Lactic acid bacteria as probiotic supplements

The human body contains approximately 100 trillion co-existing microorganisms. This microbiome provides a metagenome which encodes genes for functions imperative to the survival of the human host¹³. The majority of microorganisms within the human microbiome are commensals, although some function as “microbial peacekeepers” striving to ensure host health and wellbeing¹⁴. These “peacekeepers” provide multiple means to inhibit the colonisation by pathogens while stimulating the host’s immune system. These microorganisms play an especially important role in the functioning of the GIT^{14,15}. The GIT microbiome differs greatly between individuals because microbial colonisation is a dynamic process. However, every individual’s microbiome must contain the genes necessary to fulfil a fixed set of core functions^{14,16}. Some beneficial bacteria, or “peacekeepers”, can serve as probiotics and can be used as a supplement to help achieve core functions. The WHO defines probiotics as “live microorganisms which, when administered in adequate amounts confer a health benefit to the host”¹⁷. The WHO stipulates that a microorganism must fulfil specific criteria before it may be administered as a probiotic supplement^{16,17} (Table 1.1).

Lactic acid bacteria (LAB) are native inhabitants of the GIT, making them inherently resistant to low pH, bile tolerant, able to adhere to the colonic mucosa, and grow at body temperature^{15,18–21}. Therefore, LAB regularly fulfil the WHO’s probiotic prerequisites¹⁷. All species in this phylum are Gram-positive, catalase negative, microaerophilic to anaerobic, asporogenous and low in GC content¹⁸. Lactic acid bacteria have been found to confer a range of health benefits to the host when they are administered as probiotics^{15,18}. These benefits include immune modulation by developing the host’s humoral immune system, anticarcinogenic and antitumor activity, blood cholesterol reduction, alleviation of lactose intolerance, normalisation of stool transit and lowering of blood ammonia levels in patients with hepatic encephalopathy^{19,20,22,23}.

Probiotics have multiple mechanisms by which they help the body to resist pathogenic infection within the GIT. These mechanisms may result in scramble- and contested-competition. Scramble competition involves the rapid utilization of a finite resource such as space or nutrients²⁴. Space in the GIT can

Table 1.1 – WHO stipulated probiotic requirements

1.	Be identified to strain level
2.	Be safe for food and clinical use
3.	Survive the GIT environment
4.	Adhere to mucosal surfaces
5.	Produce antimicrobial substances
6.	Antagonise pathogenic bacteria
7.	Possess clinically documented and validated health effects
8.	Be stable during processing and storage

refer to the number of available enterocytic receptor binding sites. Probiotics may adhere to these binding sites and sterically inhibit binding of pathogens¹⁹. By consuming available nutrients and space, probiotic cells lower the chance of pathogens colonising the GIT, thereby causing scramble competition^{19,24}. Contested competition is a result of direct antagonistic interactions between organisms within the same environment. When LAB function as probiotics, their metabolic by-products change the chemical characteristics of their environment, resulting in contested competition¹⁹. Lactic acid, for example, is a metabolic by-product which is tolerated by LAB but affects the intercellular pH of yeasts and Gram-negative bacteria¹⁹. With specific relevance to this thesis, LAB may also inhibit the growth of some bacteria via the production of potent bacteriocins^{19,25}.

Lactobacillus plantarum 423 and *E. mundtii* ST4SA are included in the probiotic with the trade name Entiro™, marketed by Cipla Medpro. Both strains adhere to the prerequisites as stipulated by the WHO^{26–28}. *Lactobacillus plantarum* 423 was isolated from sorghum beer, resists pH values as low as 3 and produces the bacteriocin plantaricin 423^{29,30}. Plantaricin 423 inhibits the growth of pathogens such as *Bacillus cereus*, *Clostridium sporogenes*, *Enterococcus faecalis*, *Listeria* spp. and *Staphylococcus* spp.³⁰. *Enterococcus mundtii* ST4SA was isolated from soybeans and produces the bacteriocin mundticin ST4SA. The peptide is active against *E. faecalis*, *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Listeria* spp.³¹.

Bacteriocin classes

Bacteriocins are defined as antimicrobial peptides that are ribosomally synthesised and secreted into their environment to modulate the growth of other similar, or closely related, species^{32,33}. Some of these antimicrobial peptides produced by LAB have been used as food preservatives^{34,35}. However, some of these bacteriocins may also play a central role in a probiotic supplement. Bacteriocins are primarily grouped according to their heat tolerance. Peptides of classes I and II are heat-stable, while class III peptides are thermo-labile. Furthermore, a size cut-off of 10 kDa has been placed on classes I and II bacteriocins, while class III bacteriocins can be larger than 10 kDa^{36,37}. Bacteriocins from classes I and II are divided according to the presence of post-translational modifications. Class I bacteriocins are enzymatically modified after transcription, while class II peptides are unmodified³⁷.

Class I

The ribosomally produced post-translationally modified peptides (RiPPs) of class I are sub-grouped according to the type of post-translational modification found in their mature peptide. These modifications include lanthionine residues, head-to-tail cyclization, sulphur linkages, heterocycles, glycosylation and macrolactam rings. The sub-classes are Ia (lanthipeptides), Ib (cyclized peptides), Ic (sactibiotics), Id (azol(in)e-containing), Ie (glycocins) and If (lasso peptides)^{36–38}. Only lanthipeptides are within the scope of this review. Readers are referred to Acedo *et al.*³⁹, Alvarez-Sieiro *et al.*³⁷, and Arniston *et al.*³⁶ for information on the other sub-classes of class I bacteriocins.

Lanthipeptides contain the unusual thioether-containing amino acids lanthionine and β -methyl-lanthionine. Lantibiotics, which are lanthipeptides with antimicrobial activity, were previously sub-classified as type A or type B according to their structural features and antimicrobial activity³⁴. In this categorization scheme, type A causes poration of the cytoplasmic membrane and inhibits peptidoglycan synthesis, while type B peptides disrupt enzyme functions^{34,40,41}. However, as more lanthionine-containing peptides were discovered, such as SapB from *Streptomyces coelicolor*, it became apparent that not all lanthipeptides have antimicrobial activity⁴². These findings, along with inconsistent definitions of type A and type B peptides between authors, complicated the classification of lanthipeptides. Therefore, Willey *et al.*⁴³ established the current classification scheme, whereby lantibiotics are grouped according to their post-translational modification (PTM) enzymes. Bacteria produce a wide range of lanthipeptides that are divided into four types by the current classification scheme. Types I (LanBC-modified) and II (LanM-modified) have antimicrobial activity, while types III and IV do not^{37,44}. Phylogenetic analysis of the enzymes responsible for lanthipeptide PTM

demonstrated the advantage of convergent evolution. This mechanism provides high chemical diversity at a low genetic cost within the lanthipeptide group⁴⁴. This categorization approach has also provided an entry point for understanding the evolution of other ribosomally synthesised products such as class IIa bacteriocins⁴⁵.

Class II

Bacteriocins of this class are ribosomally synthesised and have limited or no post-transcriptional modifications, and never exceed a mass of 10 kDa. Class II is further divided into sub-classes depending on the bacteriocin's mode of action, genetic and biochemical characteristics. The categorization of sub-classes is liable to change as more is discovered about class II bacteriocins. Therefore, only the most established sub-classes will be presented in this review.

Subclass IIa

Class IIa, or pediocin-like bacteriocins, are defined by their high anti-*Listeria* activity and their conserved N-terminal YGNGV motif, also known as the pediocin box^{37,46-49}. Bacteriocins in this subclass have a broad range of activity, killing related LAB species and different strains of *Staphylococcus* and *Listeria*. These peptides are stabilized by disulphide bridges, are cationic, and consist of 25 to 48 amino acid residues^{33,49}. Most bacteriocins in this class have an N-terminal double-glycine leader peptide which is involved in bacteriocin processing; however, some contain a Sec-translocase signal peptide⁵⁰. The biosynthetic genes, biochemical characteristics and mode of action for subclass IIa bacteriocins are discussed in Chapter 4. The organization of class IIa bacteriocin biosynthetic genes within their operons is variable. Despite this, these genes encode a similar core set of functions and are involved in producer immunity, peptide maturation and extracellular translocation^{49,51}. Compared to the biosynthetic genes the class IIa bacteriocin regulatory genes and their operon structure are more variable, and often absent. Class IIa bacteriocins with known operon structures is presented in the Addendum, Figure 7.1.

Plantaricin 423 and mundticin ST4SA are bacteriocins that belong to subclass IIa³³. The *pla* operon encodes plantaricin 423 and its biosynthetic genes that are located on an 8 188 bp plasmid designated pPLA4⁵². The operon consists of *plaA* (structural gene), *plaB* (immunity protein), *plaC* (accessory protein) and *plaD* (ABC transporter protein). Two additional reading frames, for RepB and Mob_Pre proteins, are also present on plasmid pPLA4. These proteins are likely responsible for initiating plasmid replication and plasmid recombination, respectively. The mundticin ST4SA mature peptide is identical to mundticin KS/Enterocin CRL35. However, the pre-bacteriocin amino acid sequence differs in the N-

terminal leader by two amino acids. The description and naming of the mundtacin ST4SA operon is presented in Chapter 3.

Although class IIa bacteriocins have a highly conserved N-terminal domain, their C-terminus is variable. Nissen-Meyer *et al.*⁵³ divided class IIa bacteriocins into four sub-groups using the bacteriocins' C-terminal sequence similarity. More recently, Cui *et al.*⁴⁹ subgrouped class IIa bacteriocins into 8 groups based on their hypothetical 3D structures. According to Nissen-Meyer *et al.*⁵³, plantaricin 423 falls within sub-group 2 and mundtacin ST4SA (mundtacin KS) into sub-group 1⁵³. However, according to Cui *et al.*⁴⁹ plantaricin 423 falls into sub-group III-1, and mundtacin ST4SA (Enterocin CRL35) into sub-group I-1. As seen with early lanthipeptide classification schemes, different names are used for groups of bacteriocins with similar characteristics within the class. This is because outlying bacteriocins are interpreted and managed differently between authors. Properties of the sub-groups of class IIa, as described by Nissen-Meyer *et al.*⁵³ and Cui *et al.*⁴⁹ are further discussed in Chapter 3.

Subclass IIb

Bacteriocins of this subclass share many characteristics with subclass IIa; however, they require the formation of a heterodimer to induce antimicrobial activity in target organisms. These peptides are non-modified, less than 10 kDa (monomer), heat stable, and are exported via a dedicated ABC-transporter or Sec-translocase⁵⁴.

Other subclasses within class II bacteriocins

The definitions of other sub-classes vary between authors and online databases. These groups were not considered relevant for the current review and will not be discussed; the reader is referred to Acedo *et al.*³⁹ and Alvarez-Sieiro *et al.*³⁷ for more information.

Class III

Class III bacteriocins have relatively higher molecular weights (>10 kDa) and are heat labile. The regulatory mechanisms of this class were not considered relevant to plantaricin 423 and mundtacin ST4SA. For more information on this class, the reader is referred to Acedo *et al.*³⁹ and Alvarez-Sieiro *et al.*³⁷.

Bacteriocin regulation in Gram-positive bacteria

Bacteriocin production can be metabolically straining, and so to compensate for the additional energy expenditure, bacteriocin production is often highly regulated. Transcriptional co-ordination within a population via an environmental stimulus is a common mode of bacteriocin regulation²⁵. The synchronization of bacteriocin expression produces a spike in the environmental bacteriocin concentration. Regulated expression lowers the metabolic production cost by limiting production to necessary or favourable conditions only. Therefore, transcriptional regulation provides a means to increase the value of bacteriocin production by lowering metabolic costs while enhancing the bacteriocin's effectivity.

When administered as a probiotic, *L. plantarum* 423 and *E. mundtii* ST4SA form part of a dynamic and complex microbiome, living within a complex GIT environment. Due to the symbiotic nature of the GIT microbiome and the human host, it is clear that some level of communication must take place between them. The gut-blood barrier (GBB) is an epithelial layer that separates the blood stream from the microbiome-containing lumen, while regulating the absorption of nutrients, toxins, electrolytes and water. Dreyer⁵⁵ demonstrated that nisin A, plantaricin 423 and mundtacin ST4SA can migrate across epithelial (Caco-2) and endothelial (HUVEC) cells *in vitro*. The GIT and its microbiome probably monitor and respond to many of the same stimuli. However, the work presented by Dreyer⁵⁵ provides evidence of a host-microbiome monitoring avenue, using bacteriocins as a means of signal transduction. This avenue would allow the GIT to surveil bacteriocin-mediated interactions within the microbiota, but also respond to bacteriocin-inducing factors via the expression of bacteriocins themselves.

The human host and its GIT microbiome are locked in a symbiotic relationship which appears to be mediated by a complex signal network. However, three obvious types of relationships must occur: those between the same microbial species (intraspecies), those between different microbial species (interspecies), and those between the microbiome and the human host (interkingdom). Since *L. plantarum* 423 and *E. mundtii* ST4SA are administered as a dual strain probiotic, it is of great interest to determine if these organisms participate in co-operative behaviours. This includes the possibility of interspecies transcriptional co-ordination for bacteriocins produced by *L. plantarum* 423 and *E. mundtii* ST4SA, respectively. The known mechanisms of interspecies bacteriocin regulation will be discussed in subsequent sections.

Quorum sensing

Transcriptional regulation via quorum sensing is dependent on producer cell density, whereby a minimum number of cells must be present at a location before gene expression occurs. Bacteria determine cell density by monitoring the concentration of signal molecules which increase as a function of the producer cells' density^{25,56}. While Gram-negative organisms make use of homoserine lactone (HSL) signalling molecules in a generic manner, Gram-positive organisms secrete operon dedicated autoinducing peptides⁵⁶. By synchronising bacteriocin expression, via quorum sensing in a large population, producer cells can cause a sharp spike in bacteriocin concentration. The high bacteriocin concentration increases its effectiveness against target cells, while decreasing the metabolic burden per producer cell⁴⁸. Quorum sensing has been reported to modulate a range of physiological responses⁵⁶. It is widely reported by literature to be the main mode of class IIa bacteriocin regulation^{25,33,49,51,56,57}.

Bacteriocin quorum sensing circuits are generally comprised of three dedicated elements; a histidine protein kinase (HPK), a response regulator (RR), and an autoinducing peptide (AP)^{33,49,51,57}. The HPK is a transmembrane receptor that monitors the extracellular concentration of an AP. Once the AP concentration surpasses a predetermined threshold, autophosphorylation occurs within the HPK; this subsequently causes genetic regulation via a phosphorylation cascade^{33,49,51,57}. Generally, the activated HPK transphosphorylates its cognate RR by transferring a phosphate group from its histidine residue to the RR's conserved arginine residue^{51,56,58}. The activated RR then acts as a cytoplasmic trans-inducing transcription factor which upregulates the promoters of genes responsible for bacteriocin biosynthesis, immunity and secretion^{33,49,51,57}. Although the mechanism of quorum sensing is well described, this phenomenon has only been demonstrated to control the expression of a few class IIa bacteriocins⁵⁹⁻⁶³.

In Chapter 3, the known regulatory elements of class IIa bacteriocins are compared to the potential bacteriocin regulatory elements observed within the plantaricin 423 and mundticin ST4SA operons. For many of the class IIa bacteriocin operons, including that of *E. mundtii* ST4SA, genes encoding HPKs, RRs and APs are observed near the bacteriocin's biosynthetic operon. The presence of these genes provides a potential means for quorum sensing-based regulation to occur as described before^{25,32,48,49,51}. However, the *L. plantarum* 423 pPla4 plasmid does not contain genes encoding an AP, HPK and RR. Their absence indicates that plantaricin 423 expression may not be of an inducible nature, or at least not regulated by a classical Gram-positive bacteriocin quorum sensing system. The absence of regulatory genes within other class IIa bacteriocin operons has previously been noted for enterocin P,

mesentericin Y105, pediocin PA-1, pediocin AcH, and leucocin A, ^{33,64–69}. For example, *Lactococcus lactis* secretes enterocin P in an unusual manner via the Sec-translocase ⁵⁰. Whether or not this mode of secretion has a different mode of transcriptional bacteriocin regulation is still to be determined ⁶⁴. While it is possible that mesentericin Y105, pediocin PA-1, pediocin AcH and leucocin A operons are constitutively transcribed, little research has been done to substantiate this ^{37,66}. Literature addressing the regulation of class IIa bacteriocins in terms of transcription is limited and requires further investigation ^{60,61,63,70}.

Diep *et al.* ⁶¹ and Gursky *et al.* ⁶⁰ demonstrated that *Lactobacillus sakei* Lb706 and *Carnobacterium maltaromaticum* UAL26 regulated their class IIa bacteriocin transcription using three-component regulatory systems. The non-bacteriocinogenic (bacteriocin negative) phenotype was induced for *L. sakei* Lb706 and *C. maltaromaticum* UAL26 by using very dilute inocula, followed by incubation above 33.5 °C and 25 °C, respectively. These regulatory systems were affected by incubation temperature and not just cell density ^{60,61}. *Lactobacillus sakei* Lb706 produces Sakacin A from the *sap* operon, while *C. maltaromaticum* UAL26 produces piscicolin 126 from the *pis* operon. Within the *sap* and *pis* operons, three-component regulatory units are found encompassing the HPKs (*sapK* and *pisK*), RRs (*SapR* and *pisR*), and APs (*Sap-Ph* and *pisN*). Non-bacteriocinogenic phenotypes of *L. sakei* Lb706 and *C. maltaromaticum* UAL26 could be reverted to bacteriocinogenic phenotypes via the addition of their chemically synthesized APs, Sap-Ph and PisN, respectively. The bacteriocinogenic phenotype in *L. sakei* Lb706 could also be re-induced via incubation below 33.5 °C ⁶¹. *Carnobacterium maltaromaticum* UAL26 required 1000 times less PisN to induce piscicolin 126 expression at an incubation temperature of 15°C compared to 25°C ⁶⁰. These results indicated that *L. sakei* Lb706 and *C. maltaromaticum* UAL26 do not induce bacteriocin expression in the absence of their APs. Expression of the respective APs, and therefore initiation of quorum sensing, appears to be controlled by incubation temperature. Therefore, *L. sakei* Lb706 and *C. maltaromaticum* UAL26 only appear to perform quorum sensing below specific incubation temperatures. However, bacteriocin expression will occur in the presence of enough AP (e.g. external addition of AP) irrespective of incubation temperature. Similar results were recorded by Brurberg *et al.* ⁶³ for Sakacin P regulation in *L. sakei* LTH673. Sakacin P is encoded by the *sppA* gene within the *spp* operon which contains the genes necessary for biosynthesis, immunity and regulation ⁶³. Brurberg *et al.* ⁶³ identified that the non-bacteriocinogenic phenotype, like that of *L. sakei* Lb706 and *C. maltaromaticum* UAL26, could be reverted to a bacteriocinogenic phenotype by the addition of the AP encoded by *sppIP* ⁶³.

Although these studies have significantly increased the understanding of quorum sensing-regulated class IIa bacteriocins, they do not shed light on regulation in the apparent absence of regulatory genes,

as seen in the plantaricin 423 operon (*pla* operon). The mechanics behind other reported modes of bacteriocin regulation will now be reviewed to understand what mode of regulation *L. plantarum* 423 could be exerting over the *plaA* gene.

Bacteriocin autoinduction

In some cases, a bacteriocin has a dual-functional role whereby it acts as the AP for its own regulation. This phenomenon may account for the lack of an AP gene in the *pla* operon. One of the best-described examples of this is that of nisin regulation^{71,72}. Nisin A is a broad-spectrum class I type I lantibiotic that is used worldwide for food preservation^{72,73}. The regulatory genes, *nisR* and *nisK*, are situated downstream of the nisin structural- (*nisA*), biosynthetic- (*nisBTCP*) and immunity- (*nisI*) genes^{71,72}. NisK is an HPK and NisR a cognate RR, which has been shown to upregulate the biosynthesis of NisA. In this regulatory system, NisA acts as the AP as well as the bacteriocin, and due to this dual functionality, NisA regulation is described as being auto-inducible^{71,72}. Elucidation of nisin's tight regulatory mechanism has led to the development and use of the NICE expression system for heterologous protein expression^{32,71,74}.

Saucier *et al.*⁷⁵ reported that the antimicrobial activity of *Carnobacterium piscicola* LV17 could be induced via the addition of its chromatographically purified bacteriocins. The bacteriocinogenic phenotype was induced in *C. piscicola* LV17 by the addition of its class II bacteriocins: carnobacteriocin A (sulphoxylated derivative), purified by Worobo *et al.*⁷⁶, carnobacteriocin BM1 (sulphoxylated derivative) and carnobacteriocin B2 (both purified by Quadri *et al.*⁷⁷). Later, Saucier *et al.*⁶² claimed that the bacteriocins produced by *C. piscicola* LV17 were transcriptionally autoregulating their own expression, as seen in Nisin A regulation via the addition of spent supernatant. However, it is impossible to identify what regulatory role these peptides had on a transcriptional level via the measurement of total antimicrobial activity. Rather than a direct measurement of gene transcription for example using reporter genes.

Later, Franz *et al.*⁷⁸ demonstrated that the transcriptional regulation of carnobacteriocin A was dependant on the AP, CbnX. The operon encoding *cbnA* was sequenced on a 10 kb fragment extracted from *C. piscicola* LV17A. After identification of the putative AP gene, *cbnX*, the CbnX peptide was chemically synthesised. CbnX induced transcription of *cbnA* in a non-bacteriocinogenic culture of *C. piscicola* LV17A at a concentration as low as 10^{-11} M⁷⁸ as detected by Northern blotting.

The carnobacteriocin B2 (*cbnB2*) containing operon in *C. piscicola* LV17B was sequenced by Quadri *et al.*⁷⁰. Within this operon, the *cbnS* gene was identified as a putative AP which was likely responsible

for the induction of carnobacteriocin B2 expression in *C. piscicola* LV17B. The *cbnS* gene along with *cbnK* and *cbnR*, encoding an HPK and RR respectively, were responsible for the transcription of carnobacteriocin BM1 and B2 in *C. piscicola* LV17B⁷⁰. Two additional open reading frames within the *cbn* operon, *cbnX* and *cbnY*, were identified. However, the function of these proteins was not fully understood until Acedo *et al.*⁷⁹ reported that they produce the class IIb bacteriocin, carnobacteriocin XY⁷⁹. Re-evaluation of the results presented by Quadri *et al.*⁷⁰ with this information indicated that *cbnS* may also be responsible for regulating carnobacteriocin XY transcription. Furthermore, Quadri *et al.*⁷⁰ found that the promoter sequences of *cbnB2*, *cbnBM1* and *cbnX*, which is polycistronic with *cbnY*, were almost identical. The *cbn* operon, encoding carnobacteriocins B and XY, is harboured on a megaplasmid, while the *cbnBM1* gene occurs on the chromosome. Therefore, CbnS regulates the transcription of class IIa and class IIb bacteriocins whose operons are found chromosomally and extra-chromosomally, respectively.

In summary, *C. piscicola* LV17 produces carnobacteriocin A (*cbnA*), carnobacteriocin B2 (*cbnB2*), carnobacteriocin BM1 (*cbnBM1*), and carnobacteriocin XY (*cbnX* and *cbnY*). Transcription of these bacteriocins depends on their operon-encoded AP extracellular concentration^{70,78}. Although the results presented by Franz *et al.*⁷⁸ and Quadri *et al.*⁷⁰ seem to contradict Saucier *et al.*⁷⁵, this phenomenon may be explained^{70,75,78}. Quadri *et al.*⁸⁰ demonstrated that a bacteriocinogenic phenotype may be induced in *C. piscicola* LV17B using heterologously expressed carnobacteriocin B2 and derivatives thereof. When supplemented into the growth media at 0.3 µg/mL carnobacteriocin B2 induced bacteriocin production in *C. piscicola* LV17B⁸⁰. Therefore, it appears that bacteriocin transcription in *C. piscicola* LV17B is regulated by the AP, CbnS, as well as carnobacteriocin B2⁸¹. However, it should be stated that Quadri *et al.*⁸⁰ measured bacteriocin expression for induced cultures using the spot on lawn technique. The addition of heterologously purified carnobacteriocin B2 resulted in background activity which needed to be accounted for⁸⁰. Furthermore, direct interaction between carnobacteriocin B2 and the operon-encoded HPK, CbnK, has not been proven⁸¹. Alternatively, bacteriocin regulation in *C. piscicola* LV17 may be controlled by many factors, some of which may not yet be understood. For instance, the addition of highly concentrated bacteriocin may have caused a stress response in *C. piscicola* LV17. More recently, Gursky *et al.*⁶⁰ demonstrated that the transcription of carnobacteriocin BM1 was downregulated in *C. maltaromaticum* UAL26 at incubation temperatures above 25 °C⁶⁰. Bacteriocin expression in *C. piscicola* LV17 could also be dependent on temperature, which may have had an effect during experimentation at that time.

Classical Nisin-type autoinduction may yet be discovered in class IIa bacteriocins, or it may be a strategy that is not employed. Class IIa bacteriocins cause membrane poration, and therefore,

producers may opt for dedicated APs instead of bacteriocin autoinduction. By avoiding autoinduction type regulation, class IIa producers may prevent the stimulation of resistance in environmental target organisms when low concentrations of bacteriocin also act as the AP. Nisin has been used for over 40 years in the food preservation industry, and during this time no significant antimicrobial resistance has been reported⁷³. The low levels of resistance are likely due to nisin's dual mode of action, whereby peptidoglycan synthesis is inhibited and pores are formed in target cells' membranes⁸². Nisin may act as the AP due to the difficulty target organisms may have developing resistance to both of nisin's mechanisms of antibacterial action. However, the chance that plantaricin 423 may have some effect on transcriptional regulation cannot be excluded, this is expanded on in subsequent chapters.

Autoinducer-2 (AI-2) based interspecies bacteriocin regulation

Using AI-2 as the signal molecule, quorum sensing-based regulation has been reported to coordinate cellular functions within and between bacterial species^{56,83}. While Gram-positive bacteria use operon-dedicated quorum sensing systems, Gram-negative bacteria use the LuxI/LuxR quorum sensing circuit⁵⁶. The LuxI protein synthesises the HSL molecule that is secreted into the immediate environment. Once it exceeds a threshold concentration, HSL diffuses back into other local producer cells and binds to their LuxR protein⁵⁶. The LuxR-HSL complex then triggers the upregulation of target genes for physiological responses like motility, expression of virulence factors or bacteriocins, and plasmid conjugal transfer, amongst other genes^{56,83-85}.

The AI-2 dependent quorum sensing system is a hybrid of the Gram-positive two-component regulatory system and the Gram-negative LuxI/LuxR system. In this system, LuxS synthesises AI-2 that is then secreted from the cell and extracellularly detected by LuxQ. However, AI-2 does not diffuse back into producer cells like HSL but rather upregulates target genes through a phosphorylation cascade as seen in Gram-positive two-component regulation. The reader is referred to McNab *et al.*⁸³ for more information about the HSL and AI-2 signalling pathway via LuxQ, LuxU and LuxO.

AI-2 quorum sensing circuits may be found in many Gram-negative and -positive species, which includes *L. plantarum* 423^{56,83}. The AI-2 system up- and down-regulates the transcription of a variety of different genes within a single bacterium⁸³. Due to the circuit's wide distribution amongst species, AI-2 based regulation is thought to function as a mode of interspecies quorum sensing. For example, the oral pathogen *Streptococcus mutans* uses the luxS system to transcriptionally regulate the lantibiotic mutacin I (*mutA*)⁸⁶. Transcription of *mutA* is dependent on the expression of the positive regulator gene *mutR*^{86,87}. AI-2 downregulates the transcriptional repressor *irvA*, which is responsible for repression of the *mutR* gene. Therefore, *mutA* expression is indirectly induced by AI-2 due to its

downregulation of *irvA*. To this end, it is feasible that *mutA* transcription can be upregulated via species non-specific AI-2 production. The mechanism by which AI-2 down-regulates *irvA* is most likely a phosphorylation cascade; however, the genes encoding this regulatory pathway are yet to be elucidated in *S. mutans*⁸⁶.

While the *mutR* regulator gene is within the mutacin I operon there are no local AP or HPK genes, as seen in the *pla* operon. Therefore, it is possible that plantaricin 423 is being regulated by a distant phosphorylation cascade which is depended on AI-2 concentrations.

Co-culture induced bacteriocin transcription

Organisms rarely occur as pure cultures within the environment, especially within the GIT where a plethora of extracellular metabolites forms a complex signalling network. Therefore, it is not surprising that co-culturing bacteria often results in altered gene regulation, as seen in some bacteriocinogenic LAB⁸⁸⁻⁹⁰. While interspecies AI-2 signalling is likely to play a role in these interspecies regulations, some *L. plantarum* strains have been reported to partake in inter- and intra-species peptide-mediated crosstalk^{88,91-93}.

In addition to the *pla* operon (plantaricin 423), *L. plantarum* 423 also harbours the *pln* locus which encodes genes for the production, immunity and regulation of two class IIb bacteriocins, plantaricin EF and plantaricin JK^{94,95}. The PlnA protein, encoded by the *plnABCD* regulatory unit, is an AP which is responsible for quorum sensing-based regulation of the *pln* locus⁹². Within the regulatory unit, *plnB* encodes an HPK and *plnCD* encodes two RRs, PlnC and PlnD, respectively. Diep *et al.*⁹² reported that PlnA induced the upregulation of 17 open reading frames (ORFs) in *L. plantarum* C11. These 17 ORFs make up four different operon structures designated *plnEFI*, *plnGHSTUV*, *plnJKLR*, and *plnMNOP*. The *plnGHSTUV* encodes a bacteriocin ABC-transporter (PlnG) and accessory protein (PlnH); However, the exact function of proteins encoded by *plnSTUV* is unclear. The *plnEFI* and *plnJKLR* encode the class IIb bacteriocins plantaricin EF and plantaricin JK, along with their cognate immunity proteins. The *plnMNOP* operon encodes a bacteriocin-like peptide and presumably its immunity protein. However, the exact function of the PlnM peptide is unknown. The promoter regions for each one of these four open reading frames (*plnEFI*, *plnGHSTUV*, *plnJKLR*, and *plnMNOP*) and *plnABCD* show high similarity, further indicating that their transcription is controlled by one AP, PlnA^{92,96,97}.

The *pln* gene cluster is common in many *L. plantarum* species and occurs in different arrangements. *Lactobacillus plantarum* 423 shows a similar organization to those of *L. plantarum* WCFS1, 37A, LPT44/1, LPT57/1 and CECT4185 as presented by Maldonado-Barragán *et al.*⁹¹. Bacteriocinogenic

phenotypes in *L. plantarum* strains were variously induced by co-culture with *Bacillus cereus* ATCC9139, *Bifidobacterium longum* H1542, *Clostridium sporogenes* C22/10, *Enterococcus faecium* LP6T1a-20, *Lactococcus lactis* MG1363, *L. lactis* IL1403, *Listeria innocua* BL86/26, *Pediococcus pentosaceus* FBB63, *Propionibacterium acidipropionici* NCDO563, *Staphylococcus aureus* CECT239, and *Streptococcus thermophilus* ST20. Furthermore, the induction spectrum in *L. plantarum* spp. is strain specific, and not operon organisation dependent⁹¹. Due to this promiscuous nature of bacteriocin induction in *L. plantarum*, the *pln* operon is thought to play a central role in the regulation of interspecies microbial interactions^{88,93}.

Di Cagno *et al.*⁸⁸ demonstrated that *L. plantarum* DC400 induced a significant stress response in other sourdough LAB during co-culture. It was found that *L. plantarum* DC400 increased the synthesis of PlnA during co-culture. However, in response to PlnA, native or synthetic, co-inhabiting species increased the expression of proteins involved in stress response, amino acid metabolism, membrane transport, nucleotide metabolism, transcriptional regulation, energy metabolism, and cell redox homeostasis. *Lactobacillus plantarum* DC400 also increased the viability of Caco-2/TC7 cells (human colon carcinoma), and induced differentiation into enterocytes containing tight junctions during co-culture⁸⁸. Due to these intraspecies, interspecies and interkingdom effects, *L. plantarum* is thought to play a central role in GIT health during colonisation, via the modulatory and antimicrobial effects of proteins from the *pln* locus⁸⁸.

While class IIa bacteriocin APs have a specificity for their dedicated HPKs, the *pln* locus is sensitive to the presence of many different bacterial species⁹¹. Therefore, co-culturing *L. plantarum* 423 and *E. mundtii* ST4SA may stimulate expression of the *pln* operon in *L. plantarum* 423. The increased levels of PlnA may create a *quid pro quo* relationship between *L. plantarum* 423 and *E. mundtii* ST4SA resulting in an altered expression profile of *E. mundtii* ST4SA similar to that demonstrated by Di Cagno *et al.*⁸⁸.

Anomalous bacteriocin regulation in LAB

Some class I and II bacteriocin regulatory operons provide evidence for regulation via a non-quorum sensing environmental stimulus. Epidermin is a class I, type I lantibiotic and like Nisin, the epidermin structural gene, *epiA*, is encoded by the *epi* operon. The *epi* operon encodes Lan-BCD type PTM enzymes but does not contain a dedicated HPK as seen in the Nisin operon. However, the *epi* operon does contain the *epiQ* gene which produces a protein that resembles an RR and is imperative for the expression of EpiA⁹⁸. EpiQ does not resemble the RR in the Nisin operon, NisR, but shares C-terminal homology with PhoB and OmpR⁹⁸. In *Escherichia coli*, the PhoB and OmpR regulators respond to the

changing concentrations of environmental phosphate⁹⁹. Although Peschel *et al.*⁹⁸ attempted to determine the effect different media constituents had on EpiA expression, the exact trigger for EpiQ phosphorylation and subsequent *epiA* transcription remains unknown.

PhoB- and OmpR-like RRs can also be found within the class IIa bacteriocin group, as seen in the case of divercin which is encoded by the *dvn* operon⁸¹. The *dvn* operon is interesting because in addition to the *dvnV41* (structural gene), and *dvnT1* (ABC-transporter), the *dvn* operon harbours two genes, *dvnT2* and *dvnI*, encoding putative immunity proteins. From a regulatory perspective, the *dvn* operon encodes the HPK (*dvnK*) and RR (*dvnR*), but an AP gene is absent¹⁰⁰. DvnR shows high similarity to the OmpR regulators, while DvnK has C-terminal homology to the *cztS_silS_copS* conserved domain of the heavy metal sensor kinase family. This conserved sensor domain is reportedly involved in heavy metal resistance efflux systems for copper, silver, cadmium, and/or zinc¹⁰¹.

Environmental physico-chemical induction cues for class IIa expression

Growth temperature has been demonstrated to play a significant role in the modulation of class IIa bacteriocin quorum sensing as seen previously in the case of *L. sakei* Lb706 and *C. maltaromaticum* UAL26^{51,60,61}. However, other environmental factors like pH and ionic strength have been reported to affect class IIa bacteriocin production in starter cultures for fermented foods⁵¹. The growth of *L. sakei* CTC494 and the expression of its class IIa bacteriocin, sakacin K, was affected by the presence of sodium chloride and sodium nitrate. Sodium chloride was reported to decrease biomass and specific bacteriocin production, while sodium nitrate only decreased biomass and therefore total bacteriocin production¹⁰². Hugas *et al.*³⁵ noted that *L. sakei* CTC494 was able to inhibit *Listeria* growth better in Spanish-style fermented sausages compared to German-style fermented sausages. Ingredients in Spanish-style fermented sausages influenced class IIa bacteriocin activity by *L. sakei* CTC494. Pepper, more specifically the manganese in pepper, was found to be responsible for increased anti-listerial activity in model-fermented sausage experiments using *L. sakei* CTC494. Fermented sausage formulations, containing *L. sakei* CTC494 and *L. monocytogenes*, supplemented with pepper or manganese sulphate showed a three-log cycle decrease in listeria after 8 days, respectively³⁵.

These studies measured bacteriocin activity against *L. monocytogenes* and not the transcriptional rate of specific bacteriocin genes. It is difficult to determine the cause of an increased anti-listerial activity by only measuring bacteriocin activity. Increased activity may be due to a synergistic effect between manganese and sakacin K or the improved growth of *L. sakei* CTC494 due to manganese supplementation. It can also be a result of transcriptional upregulation of genes within the sakacin K operon via the stimulation by environmental manganese.

Regulation of virulence genes in *Listeria monocytogenes*

Listeria monocytogenes is a ubiquitous organism that can exist as a peaceful saprophyte or intracellular pathogen which infects humans¹⁰³. This is due to the arsenal of virulence genes harboured by *Listeria* which allow it to grow within mammalian cells^{103,104}. As mentioned earlier, these genes include internalin A and B, listeriolysin O, phospholipases (PlcA and PlcB) and ActA. The reader is referred to Gray *et al.*¹⁰³ for more information on these genes. The expression of these and other virulence genes in *L. monocytogenes* is controlled on a transcriptional and post-transcriptional level in response to multiple stimuli^{103,104}.

The transcription of listerial virulence genes is generally controlled by the PrfA positive regulator^{103,104}. However, the transcription of *prfA* itself is controlled by three promoters, two of which, *PprfA_{P1}* and *PprfA_{P2}*, are directly upstream of *prfA*. Further upstream from the *prfA* gene, the *plcA* gene is found under the transcriptional control of the third promoter *PplcA*. This promoter directs the monocistronic transcription of *plcA* and the polycistronic transcription of *plcA-prfA*.

Transcription of *prfA* from *PprfA_{P1}* is induced by the regulatory factor sigma A (σ^A) during the growth of *L. monocytogenes* in broth. This RNA transcript forms a thermosensitive secondary-structure which inhibits its own translation at temperatures below 30°C. Monocistronic *prfA* transcription from *PprfA_{P1}* induced by σ^A is temperature independent. However, polycistronic transcription of *plcA-prfA* from *PplcA*, and transcription of other virulence genes, is directed by PrfA and is, therefore, temperature dependent via the *PprfA₁* RNA thermosensitive structure¹⁰³.

The *PprfA_{P2}* promoter contains a PrfA binding sequence, allowing for autoinduction of PrfA, and a sigma B (σ^B) binding domain. The σ^B induction factor induces the transcription of genes that respond to environmental stress such as low pH, high osmolarity, oxidative stress, and carbon starvation. Some of these stressful environments can be found within the human body; σ^B transcription of PrfA has been linked to the expression of virulence genes involved in bile tolerance, resistance to acid shock, resistance to osmotic and ethanol stress^{103,104}. The PrfA regulator may also be controlled at a post-transcriptional level; however, the reader is referred to Gray *et al.*¹⁰³ for more information. Other environmental stimuli such as low concentrations of free iron have been shown to regulate the expression of LLO and ActA, a protein responsible for intracellular bacterial mobility¹⁰³. Corbett *et al.*¹⁰⁵ demonstrated that two zinc-sensitive uptake systems, ZurAM and ZinABC, play a role in intracellular growth and ActA-based motility of *L. monocytogenes*. When *L. monocytogenes* mutants defective in ZurAM and ZinABC, or both, were evaluated in an oral mouse model, the mutants had reduced pathogenicity. The concentration of divalent cations is tightly regulated by the human body in the

phenomenon known as nutritional immunity. Divalent transition metals like iron, zinc, manganese, and copper are essential for the activity of many enzymes ⁷. It is widely reported that the body sequesters iron and thereby inhibits pathogenic invasion via nutrient availability. Therefore, a change in the concentration of environmental iron or zinc provides a good stimulus for *L. monocytogenes* to assess its environmental location ^{103,105}.

In addition to iron, the body tightly sequesters both inter- and extra-cellular manganese and zinc to resist pathogenic infection ¹⁰⁶. The effect of iron and zinc on listerial virulence gene regulation and pathogenicity has been reported, yet studies assessing the effect of manganese are rare ^{7,105}. Sword ¹⁰⁷ demonstrated that the LD₅₀ intraperitoneal dose of *L. monocytogenes* in mice was significantly lowered when co-administered with iron or manganese. While results suggest that manganese might regulate virulence genes in *L. monocytogenes*, manganese is known to regulate virulence in other human pathogens such as *S. aureus*, *Mycobacterium tuberculosis*, *B. subtilis*, *Salmonella typhimurium*, *Yersinia pestis*, *Shigella flexneri*, *E. faecalis*, and *B. anthracis* ^{7,108}. Although there is limited experimental data, the potential regulatory role of manganese on *L. monocytogenes* pathogenicity has been excellently outlined in a review by Jesse *et al.* ⁷.

It is interesting to note that manganese appears to effect co-cultures of *L. monocytogenes* and *L. plantarum*. Van der Veen *et al.* ¹⁰⁹ observed the effect manganese has on mixed biofilms of *L. monocytogenes* and *L. plantarum*. Van der Veen *et al.* (2010) agreed with the observations made by Hugas *et al.* (2002) in fermented sausages. In the absence of manganese, the number of *L. monocytogenes* CFU was 10-100 times more than the number of *L. plantarum* in mixed biofilms. However, manganese supplementation resulted in equal amounts of CFUs for *L. monocytogenes* and *L. plantarum* in mixed biofilms. When manganese and glucose were supplemented into the biofilm together, there were approximately 1-2 log₁₀ more *L. plantarum* than *L. monocytogenes* CFUs. In monocultures, manganese did not appear to significantly affect the growth of *L. monocytogenes*.

Due to limited literature, it is difficult to make an accurate hypothesis of the effect manganese might have on the relationship between *L. plantarum* and *L. monocytogenes* within the GIT. This is especially difficult when the effect of manganese on class IIa bacteriocins, or their transcriptional regulation, is unknown.

Class IIa bacteriocin expression systems

To understand the specific effect extracellular plantaricin 423 and mundticin ST4SA may have on bacteriocin transcription in *L. plantarum* 423, these bacteriocins needed to be purified. Like many

other class IIa bacteriocins, plantaricin 423 and mundtacin ST4SA have previously been purified from producer strain supernatant³⁰. Bacteriocins are usually chromatographically purified by a multi-step process taking advantage of their cationic and hydrophobic properties⁴⁶. Not only is this process tedious, but often results in poor/variable yields due to the inducible nature of bacteriocins in their native producer, which often not fully understood^{46,110,111}. Results from the application of bacteriocins purified by this approach may be further complicated due to co-purification of other proteins or contaminants. Bacteriocins with similar characteristics, or potent autoinducing peptides, can be co-purified which results in a trade-off between the purity and yield of a sample.

Many class IIa bacteriocins have been produced using heterologous expression systems^{46,112–116}. These systems aim to improve the large-scale production and rapid purification of bacteriocins. These systems also allow for the specific production of putative bacteriocins from genes identified by genome mining. Furthermore, by using heterologous expression systems, transcriptional regulation can be investigated with a lowered likelihood of co-purified native producer APs.

Improvements in class IIa bacteriocin heterologous expression systems may allow them to have a greater application in research as well as the food preservation- and pharmaceutical-industry. Purification of N-terminus His-tagged Pediocin PA-01 expressed in *E. coli* resulted in low concentrations of biologically active bacteriocin¹¹⁷. The majority of the His-tagged pediocin was found in the insoluble fraction; this is most likely due to the bacteriocin's low solubility, size and the toxic effect on recombinant *E. coli*¹¹⁷.

Fortunately, the yields of heterologously expressed class IIa bacteriocins can be significantly improved by fusing the mature peptide to a larger and more soluble protein. Class IIa bacteriocins have been heterologously expressed with a variety of fusion proteins. Pediocin PA-1, carnobacteriocins BM1 and B2, divercin V41, enterocin P and pisciolin 124 have been expressed as fusion proteins with thioredoxin^{46,112–116}. Carnobacteriocin B2 and pediocin AcH have been expressed as secreted fusion proteins with the maltose-binding protein^{80,118}. Divercin V41, enterocin P, pediocin PA-1 and piscicolin 126 were also expressed as secreted fusion proteins with intein–chitin-binding domain^{80,119}. Pediocin PA-01 has also been expressed as a fusion protein with His-tagged mouse dihydrofolate reductase (DHFR)¹²⁰. Although class IIa bacteriocins fused to thioredoxin are not secreted, this system produces the highest recorded yields ranging from 20-320 mg pure bacteriocin per litre of culture^{112,114}. Thioredoxin is an 11 675 kDa highly soluble protein, which has a rigid solvent-accessible α -helix and can accumulate to up to 40% of the total cellular protein in *E. coli*^{121–123}. Due to its size, solubility and cellular localization, thioredoxin aids in the expression and purification of class IIa bacteriocins

^{112,121,122}. Thioredoxin-bacteriocin fusion proteins were found in the soluble fraction of *E. coli* which indicates lower toxicity and results in easier extraction, higher yields and better protein folding ^{112,114}.

Although heterologous bacteriocin expression solves many purification problems, it is by no means a silver bullet. The optimization of expression can only be assessed post purification, which is a multi-step process that introduces variability between treatments. That is, heterologous expression cannot be easily measured *in vivo* nor in real time. Furthermore, the bacteriocin peptide needs to be liberated from its fusion partner after purification to yield active peptide. Bacteriocin liberation can be achieved by incorporating a unique amino acid sequence between the fusion carrier protein and bacteriocin, which may be cleaved by a specific protease ¹²⁴. Commercial proteases are expensive which limits high scale production, they often leave residual N-terminal amino acids, and may result in non-specific cleavage of the bacteriocin peptide. Alternatively, cleavage can be induced by certain chemicals: cyanogen bromide (methionine residue cleavage), BNPS-skatole (tryptophan cleavage), formic acid (aspartic acid-proline bond cleavage), hydroxylamine (asparagine-glycine bond cleavage), 2-nitro-5-thiocyanobenzoic acid (cysteine residue cleavage) ¹²⁵. However, the addition of these chemicals can result in toxic samples or non-specific cleavage. Finally, heterologous expression systems for more complex peptides, like the class I RiPPs, has also been shown. However, class I RiPP expression is complicated by the requirement for leader-peptide-directed post-translational modification by their respective postribosomal peptide synthesis (PRPS) enzymes.

Conclusion

To fully understand the relationship between *L. plantarum* 423 and *E. mundtii* ST4SA within the GIT, the full effect of their bacteriocins in both an intra- and inter-species capacity needs to be researched. Currently the mechanism of intraspecies regulation for plantaricin 423 and mundtacin ST4SA is undefined. This makes hypothesising their exact interspecies class IIa bacteriocin-mediated relationship difficult, especially during administration within the GIT.

However, literature presents an alternative, or additional, means by which these microbes might be interacting during co-culture. Plantaricin 423 harbours the *pln* locus which is co-culture inducible. The *plaA* protein, a product of *pln*, has been shown to induce transcriptional regulation of a variety of genes in other species, and to modulate human GIT cells. Omics (proteomics or transcriptomics) should be performed on *L. plantarum* 423 and *E. mundtii* ST4SA, stimulated with PlnA or AI-2, both in pure and co-culture conditions. These data will provide insight into the regulatory effect these two microbes have on each other during co-culture conditions. More importantly, it may provide evidence for *L. plantarum* 423 acting as a keystone species within the GIT microbiome by facilitating chemical

signalling on an intraspecies, interspecies and interkingdom level. This discovery would further motivate use of *L. plantarum* 423 as a probiotic supplement.

This review has demonstrated that little is known about class IIa bacteriocin regulation in general, but especially in the absence of local regulatory operons. The first step to understanding plantaricin 423 and mundticin ST4SA regulation is to update the subclassification of known class IIa bacteriocins and allocate the known modes of regulation to these subclasses. Subclassification of class IIa bacteriocins is mostly consistent between Nissen-Meyer *et al.*⁵³ and Cui *et al.*⁴⁹ who used two different classification schemes. Willey *et al.*⁴³ has demonstrated the benefits of lanthipeptide subclassification using the amino acid sequence of the lanthipeptide's PTM enzymes. Although class IIa bacteriocins do not have PTM enzymes, the smallest conserved operon units always contain an immunity protein, which can be used for classification as seen in Willey *et al.*⁴³. These classifications schemes will be fully discussed in subsequent chapters.

Literature paints a very complex picture in terms of the potential regulatory mechanisms *L. plantarum* 423 may be using to control the expression of plantaricin 423. This high level of complexity does, however, provide a clear experimental approach. Many studies have used Northern blotting to monitor transcription, but in these cases a non-bacteriocinogenic phenotype may be easily induced and probes could be designed to putative regulatory operons. Proteomics may also be used, but this approach is subject to similar problems especially if a bacteriocinogenic phenotype is unobtainable. Furthermore, monitoring expression in both cases requires a protein or RNA extraction step which results in variability. Therefore, a reporter gene assay is a sound choice as measurements can be made easily and provide both qualitative and quantitative data.

In this way, expression can be fluorometrically monitored in the presence or absence of plantaricin 423 on a genetic and protein level, and under different growth conditions. Using a fluorescent reporter protein like Green or Red Fluorescent Protein (GFP and RFP) will allow for *in vivo* measurements. Although using a reporter gene does not provide much information about the biochemical mechanism of regulation, it can be useful to elucidate the mode, providing a starting point for techniques such as northern blotting, transcriptomics, and proteomics. The reader will find that chapter 4 presents the development of a novel heterologous expression system for plantaricin 423 and mundticin ST4SA. Chapter 5 covers the construction and employment of a fluorescent reporter system to monitor plantaricin 423 transcription in *L. plantarum* 423, in the presence and absence of *plaA* and under various growth conditions.

References

1. Bhatia, S. Introduction to Pharmaceutical Biotechnology. Volume 11–61 (IOP Publishing, 2018). doi:10.1088/978-0-7503-1299-8ch1
2. Barcelos, M. C. S., Lupki, F. B., Campolina, G. A., Nelson, D. L. & Molina, G. The colors of biotechnology: General overview and developments of white, green and blue areas. *FEMS Microbiol. Lett.* 365, 1-11 (2018).
3. Cotter, P. D., Ross, R. P. & Hill, C. Bacteriocins — a viable alternative to antibiotics? *Nat. Rev. Microbiol.* 11, 95–105 (2013).
4. Tacconelli, E. et al. Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. *Lancet Infect. Dis.* 18, 318–327 (2018).
5. Wang, G. et al. Antimicrobial peptides in 2014. *Pharmaceuticals (Basel)*. 8, 123–150 (2015).
6. Bahar, A. A. & Ren, D. Antimicrobial peptides. *Pharmaceuticals (Basel)*. 6, 1543–1575 (2013).
7. Jesse, H. E., Roberts, I. S. & Cavet, J. S. in *Advances in Bacterial Pathogen Biology* 65, 83–123 (Elsevier Ltd., 2014).
8. Allam, M. et al. Whole-genome sequences of *Listeria monocytogenes* sequence type 6 isolates associated with a Large Foodborne Outbreak in South Africa, 2017 to 2018. *Genome Announc.* 6, 543–553 (2018).
9. Koopmans, M. M. et al. *Listeria monocytogenes* sequence type 6 and increased rate of unfavorable outcome in meningitis: epidemiologic cohort study. *Clin. Infect. Dis.* 57, 247–253 (2013).
10. National Listeria Incident Management Team, Situation report. 1–5 (2018). at https://foodpolitics.com/wp-content/uploads/Listeriosis-outbreak-situation-report-_26July2018_fordistribution.pdf
11. Parisi, A. et al. Amplified Fragment Length Polymorphism and Multi-Locus Sequence Typing for high-resolution genotyping of *Listeria monocytogenes* from foods and the environment. *Food Microbiol.* 27, 101–108 (2010).

12. Wang, Y. et al. Genetic diversity and molecular typing of *Listeria monocytogenes* in China. *BMC Microbiol.* 12, 119 (2012).
13. Qin, J. et al. A human gut microbial gene catalogue established by metagenomic sequencing. *Nature.* 464, 59–65 (2010).
14. Velasquez-Manoff, M. Gut microbiome: the peacekeepers. *Nature.* 518, S3–S11 (2015).
15. Brown, M. Modes of action of probiotics: recent developments. *J. Anim. Vet. Adv.* 10, 1895–1900 (2011).
16. Borchers, A. T., Selmi, C., Meyers, F. J., Keen, C. L. & Gershwin, M. E. Probiotics and immunity. *J. Gastroenterol.* 44, 26–46 (2009).
17. World health organization. Guidelines for the Evaluation of Probiotics in Food. *World Heal. Organ.* 1–11 (2002). at <<ftp://ftp.fao.org/es/esn/food/wgreport2.pdf>>
18. Klein, G., Pack, A., Bonaparte, C. & Reuter, G. Taxonomy and physiology of probiotic lactic acid bacteria. *Int. J. Food Microbiol.* 41, 103–125 (1998).
19. Dicks, L. M. T. & Botes, M. Probiotic lactic acid bacteria in the gastro-intestinal tract: health benefits, safety and mode of action. *Benef. Microbes* 1, 11–29 (2010).
20. Rupa, P. & Mine, Y. Recent advances in the role of probiotics in human inflammation and gut health. *J. Agric. Food Chem.* 60, 8249–8256 (2012).
21. van Reenen, C. A. & Dicks, L. M. T. Horizontal gene transfer amongst probiotic lactic acid bacteria and other intestinal microbiota: what are the possibilities? A review. *Arch. Microbiol.* 193, 157–168 (2011).
22. Gilliland, S. E. & Walker, D. K. Factors to consider when selecting a culture of *Lactobacillus acidophilus* as a dietary adjunct to produce a hypocholesterolemic effect in humans. *J. Dairy Sci.* 73, 905–911 (1990).
23. Dugas, B. et al. Immunity and probiotics. *Immunol. Today.* 20, 387–390 (1999).
24. Hibbing, M. E., Fuqua, C., Parsek, M. R. & Peterson, S. B. Bacterial competition: surviving and thriving in the microbial jungle. *Nat. Rev. Microbiol.* 8, 15–25 (2010).

25. Kuipers, O. P., de Ruyter, P. G. G., Kleerebezem, M. & de Vos, W. M. Quorum sensing-controlled gene expression in lactic acid bacteria. *J. Biotechnol.* 64, 15–21 (1998).
26. Ramiah, K., van Reenen, C. A. & Dicks, L. M. T. Expression of the mucus adhesion gene *Mub*, surface layer protein *slp* and adhesion-like factor EF-TU of *Lactobacillus acidophilus* ATCC 4356 under digestive stress conditions, as monitored with real-time PCR. *Probiotics Antimicrob. Proteins.* 1, 91–95 (2009).
27. Ramiah, K., van Reenen, C. A. & Dicks, L. M. T. Surface-bound proteins of *Lactobacillus plantarum* 423 that contribute to adhesion of Caco-2 cells and their role in competitive exclusion and displacement of *Clostridium sporogenes* and *Enterococcus faecalis*. *Res. Microbiol.* 159, 470–475 (2008).
28. Botes, M., Loos, B., van Reenen, C. A. & Dicks, L. M. T. Adhesion of the probiotic strains *Enterococcus mundtii* ST4SA and *Lactobacillus plantarum* 423 to Caco-2 cells under conditions simulating the intestinal tract, and in the presence of antibiotics and anti-inflammatory medicaments. *Arch. Microbiol.* 190, 573–584 (2008).
29. Maré, L., Wolfaardt, G. M. & Dicks, L. M. T. Adhesion of *Lactobacillus plantarum* 423 and *Lactobacillus salivarius* 241 to the intestinal tract of piglets, as recorded with fluorescent in situ hybridization (FISH), and production of plantaricin 423 by cells colonized to the ileum. *J. Appl. Microbiol.* 100, 838–845 (2006).
30. van Reenen, C. a, Dicks, L. M. & Chikindas, M. L. Isolation, purification and partial characterization of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum*. *J. Appl. Microbiol.* 84, 1131–1137 (1998).
31. Granger, M., van Reenen, C. A. a & Dicks, L. M. T. M. T. Effect of gastro-intestinal conditions on the growth of *Enterococcus mundtii* ST4SA, and production of bacteriocin ST4SA recorded by real-time PCR. *Int. J. Food Microbiol.* 123, 277–280 (2008).
32. Kotel'nikova, E. A. & Gel'fand, M. S. Production of bacteriocins by gram-positive bacteria and the mechanisms of transcriptional regulation. *Genetika.* 38, 758–772 (2002).
33. Ennahar, S., Sashihara, T., Sonomoto, K. & Ishizaki, A. Class IIa bacteriocins: biosynthesis, structure and activity. *FEMS Microbiol. Rev.* 24, 85–106 (2000).

34. Jung, G. Lantibiotics—ribosomally synthesized biologically active polypeptides containing sulfide bridges and α,β -didehydroamino acids. *Angew. Chemie Int. Ed. English.* 30, 1051–1068 (1991).
35. Hugas, M., Garriga, M., Pascual, M., Aymerich, M. T. & Monfort, J. M. Enhancement of sakacin K activity against *Listeria monocytogenes* in fermented sausages with pepper or manganese as ingredients. *Food Microbiol.* 19, 519–528 (2002).
36. Arnison, P. G. et al. Ribosomally synthesized and post-translationally modified peptide natural products: overview and recommendations for a universal nomenclature. *Nat. Prod. Rep.* 30, 108–160 (2013).
37. Alvarez-Sieiro, P., Montalbán-López, M., Mu, D. & Kuipers, O. P. Bacteriocins of lactic acid bacteria: extending the family. *Appl. Microbiol. Biotechnol.* 100, 2939–2951 (2016).
38. Hegemann, J. D., Zimmermann, M., Xie, X. & Marahiel, M. A. Lasso peptides: an intriguing class of bacterial natural products. *Acc. Chem. Res.* 48, 1909–1919 (2015).
39. Acedo, J. Z., Chiorean, S., Vederas, J. C. & van Belkum, M. J. The expanding structural variety among bacteriocins from Gram-positive bacteria. *FEMS Microbiol. Rev.* 42, 805–828 (2018).
40. van Kraaij, C., de Vos, W. M., Siezen, R. J. & Kuipers, O. P. Lantibiotics: biosynthesis, mode of action and applications. *Nat. Prod. Rep.* 16, 575–587 (1999).
41. McAuliffe, O., Ross, R. P. & Hill, C. Lantibiotics: structure, biosynthesis and mode of action. *FEMS Microbiol. Rev.* 25, 285–308 (2001).
42. Kodani, S. et al. The SapB morphogen is a lantibiotic-like peptide derived from the product of the developmental gene *ramS* in *Streptomyces coelicolor*. *Proc. Natl. Acad. Sci. U. S. A.* 101, 11448–11453 (2004).
43. Willey, J. M. & van der Donk, W. A. Lantibiotics: peptides of diverse structure and function. *Annu. Rev. Microbiol.* 61, 477–501 (2007).
44. Knerr, P. J. & van der Donk, W. A. Discovery, biosynthesis, and engineering of lantipeptides. *Annu. Rev. Biochem.* 81, 479–505 (2012).
45. Zhang, Q., Yu, Y., Vélasquez, J. E. & van der Donk, W. A. Evolution of lanthipeptide synthetases. *Proc. Natl. Acad. Sci. U. S. A.* 109, 18361–18366 (2012).

46. Lohans, C. T. & Vederas, J. C. Development of class IIa bacteriocins as therapeutic agents. *Int. J. Microbiol.* 2012, Add Page Numbers (2012).
47. Kjos, M. et al. Target recognition, resistance, immunity and genome mining of class II bacteriocins from Gram-positive bacteria. *Microbiology.* 157, 3256–3267 (2011).
48. Eijsink, V. G. H. et al. Production of class II bacteriocins by lactic acid bacteria; an example of biological warfare and communication. *Antonie Van Leeuwenhoek.* 81, 639–654 (2002).
49. Cui, Y. et al. Class IIa bacteriocins: diversity and new developments. *Int. J. Mol. Sci.* 13, 16668–16707 (2012).
50. Herranz, C. & Driessen, A. J. M. Sec-mediated secretion of bacteriocin enterocin P by *Lactococcus lactis*. *Appl. Environ. Microbiol.* 71, 1959–1963 (2005).
51. Drider, D., Fimland, G., Hechard, Y., McMullen, L. M. & Prevost, H. The continuing story of class IIa bacteriocins. *Microbiol. Mol. Biol. Rev.* 70, 564–582 (2006).
52. Van Reenen, C. A., Chikindas, M. L., Van Zyl, W. H. & Dicks, L. M. T. Characterization and heterologous expression of a class IIa bacteriocin, plantaricin 423 from *Lactobacillus plantarum* 423, in *Saccharomyces cerevisiae*. *Int. J. Food Microbiol.* 81, 29–40 (2003).
53. Nissen-Meyer, J., Rogne, P., Oppegard, C., Haugen, H. & Kristiansen, P. Structure-function relationships of the non-lanthionine-containing peptide (class II) bacteriocins produced by Gram-positive bacteria. *Curr. Pharm. Biotechnol.* 10, 19–37 (2009).
54. Garneau, S., Martin, N. I. & Vederas, J. C. Two-peptide bacteriocins produced by lactic acid bacteria. *Biochimie.* 84, 577–592 (1992).
55. Dreyer, L. The ability of antimicrobial peptides to migrate across the gastrointestinal epithelial and vascular endothelial barriers. (2018).
56. Miller, M. B. & Bassler, B. L. Quorum sensing in bacteria. *Annu. Rev. Microbiol.* 55, 165–199 (2001).
57. Sturme, M. H. J. et al. Cell to cell communication by autoinducing peptides in gram-positive bacteria. *Antonie Van Leeuwenhoek.* 81, 233–243 (2002).
58. Cho, H. S., Pelton, J. G., Yan, D., Kustu, S. & Wemmer, D. E. Phosphoaspartates in bacterial signal transduction. *Curr. Opin. Struct. Biol.* 11, 679–684 (2001).

59. Eijsink, V. G., Brurberg, M. B., Middelhoven, P. H. & Nes, I. F. Induction of bacteriocin production in *Lactobacillus sake* by a secreted peptide. These include: induction of bacteriocin production in *Lactobacillus sake* by a secreted peptide. 178, 2232–2237 (1996).
60. Gursky, L. J. et al. Production of piscicolin 126 by *Carnobacterium maltaromaticum* UAL26 is controlled by temperature and induction peptide concentration. Arch. Microbiol. 186, 317–325 (2006).
61. Diep, D. B., Axelsson, L., Grefsli, C. & Nes, I. F. The synthesis of the bacteriocin sakacin A is a temperature-sensitive process regulated by a pheromone peptide through a three-component regulatory system. Microbiology. 146, 2155–2160 (2000).
62. Saucier, L., Paradkar, A. S., Frost, L. S., Jensen, S. E. & Stiles, M. E. Transcriptional analysis and regulation of carnobacteriocin production in *Carnobacterium piscicola* LV17. Gene. 188, 271–277 (1997).
63. Brurberg, M. B., Nes, I. F. & Eijsink, V. G. Pheromone-induced production of antimicrobial peptides in *Lactobacillus*. Mol. Microbiol. 26, 347–360 (1997).
64. Cintas, L. M., Casaus, P., Håvarstein, L. S., Hernández, P. E. & Nes, I. F. Biochemical and genetic characterization of enterocin P, a novel sec-dependent bacteriocin from *Enterococcus faecium* P13 with a broad antimicrobial spectrum. Appl. Environ. Microbiol. 63, 4321–30 (1997).
65. Fremaux, C., Yann, H. & Cenatiempo, Y. Mesentericin Y105 gene clusters in *Leuconostoc mesenteroides* Y105. Microbiology. 141, 1637–1645 (1995).
66. Marugg, J. D. et al. Cloning, expression, and nucleotide sequence of genes involved in production of pediocin PA-1, a bacteriocin from *Pediococcus acidilactici* PAC1.0. Appl. Environ. Microbiol. 58, 2360–2367 (1992).
67. Bukhtiyarova, M., Yang, R. & Ray, B. Analysis of the pediocin Ach gene cluster from plasmid pSMB74 and its expression in a pediocin-negative *Pediococcus acidilactici* strain. Appl. Environ. Microbiol. 60, 3405–3408 (1994).
68. Fregeau Gallagher, N. L. et al. Three-dimensional structure of leucocin a in trifluoroethanol and dodecylphosphocholine micelles: Spatial location of residues critical for biological activity in type IIa bacteriocins from lactic acid bacteria. Biochemistry. 36, 15062–15072 (1997).

69. van Belkum, M. J. & Stiles, M. E. Molecular characterization of genes involved in the production of the bacteriocin leucocin A from *Leuconostoc gelidum*. *Appl. Environ. Microbiol.* 61, 3573–3579 (1995).
70. Quadri, L. E. et al. Characterization of a locus from *Carnobacterium piscicola* LV17B involved in bacteriocin production and immunity: evidence for global inducer-mediated transcriptional regulation. *J. Bacteriol.* 179, 6163–6171 (1997).
71. Kuipers, O. P., Beerthuyzen, M. M., de Ruyter, P. G., Luesink, E. J. & de Vos, W. M. Autoregulation of nisin biosynthesis in *Lactococcus lactis* by signal transduction. *J. Biol. Chem.* 270, 27299–27304 (1995).
72. Engelke, G. et al. Regulation of nisin biosynthesis and immunity in *Lactococcus lactis* 6F3. *Appl. Environ. Microbiol.* 60, 814–825 (1994).
73. Ortega, M. A. et al. Structure and mechanism of the tRNA-dependent lantibiotic dehydratase NisB. *Nature.* 517, 509–512 (2015).
74. Mierau, I. & Kleerebezem, M. 10 Years of the nisin-controlled gene expression system (NICE) in *Lactococcus lactis*. *Appl. Microbiol. Biotechnol.* 68, 705–717 (2005).
75. Saucier, L., Poon, A. & Stiles, M. E. Induction of bacteriocin in *Carnobacterium piscicola* LV17. *J. Appl. Bacteriol.* 78, 684–690 (1995).
76. Worobo, R. W. et al. Characteristics and genetic determinant of a hydrophobic peptide bacteriocin, carnobacteriocin A, produced by *Carnobacterium piscicola* LV17A. *Microbiology.* 140, 517–526 (1994).
77. Quadri, L. E., Sailer, M., Roy, K. L., Vederas, J. C. & Stiles, M. E. Chemical and genetic characterization of bacteriocins produced by *Carnobacterium piscicola* LV17B. *J. Biol. Chem.* 269, 12204–12211 (1994).
78. Franz, C. M. A. P., Van Belkum, M. J., Worobo, R. W., Vederas, J. C. & Stiles, M. E. Characterization of the genetic locus responsible for production and immunity of carnobacteriocin A: The immunity gene confers cross-protection to enterocin B. *Microbiology.* 146, 621–631 (2000).
79. Acedo, J. Z. et al. Identification and three-dimensional structure of carnobacteriocin XY, a class IIb bacteriocin produced by *Carnobacteria*. *FEBS Lett.* 591, 1349–1359 (2017).

80. Quadri, L. E. N., Yan, L. Z., Stiles, M. E. & Vederas, J. C. Effect of amino acid substitutions on the activity of Carnobacteriocin B2. *J. Biol. Chem.* 272, 3384–3388 (1997).
81. Kleerebezem, M. & Quadri, L. E. Peptide pheromone-dependent regulation of antimicrobial peptide production in Gram-positive bacteria: a case of multicellular behavior. *Peptides.* 22, 1579–1596 (2001).
82. Wiedemann, I. et al. Specific binding of nisin to the peptidoglycan precursor lipid II combines pore formation and inhibition of cell wall biosynthesis for potent antibiotic activity. *J. Biol. Chem.* 276, 1772–1779 (2001).
83. McNab, R. Microbial dinner-party conversations: the role of LuxS in interspecies communication. *J. Med. Microbiol.* 52, 541–545 (2003).
84. Fuqua, C., Parsek, M. R. & Greenberg, E. P. Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. *Annu. Rev. Genet.* 35, 439–468 (2001).
85. Schauder, S. & Bassler, B. L. The languages of bacteria. *Genes Dev.* 15, 1468–1480 (2001).
86. Merritt, J., Kreth, J., Shi, W. & Qi, F. LuxS controls bacteriocin production in *Streptococcus mutans* through a novel regulatory component. *Mol. Microbiol.* 57, 960–969 (2005).
87. Qi, F., Chen, P. & Caufield, P. W. Purification and biochemical characterization of Mutacin I from the group I strain of *Streptococcus mutans*, CH43, and genetic analysis of Mutacin I Biosynthesis Genes. *Appl. Environ. Microbiol.* 66, 3221–3229 (2000).
88. Di Cagno, R. et al. Quorum sensing in sourdough *Lactobacillus plantarum* DC400: Induction of plantaricin A (PlnA) under co-cultivation with other lactic acid bacteria and effect of PlnA on bacterial and Caco-2 cells. *Proteomics.* 10, 2175–2190 (2010).
89. Domínguez-Manzano, J. & Jiménez-Díaz, R. Suppression of bacteriocin production in mixed-species cultures of lactic acid bacteria. *Food Control.* 30, 474–479 (2013).
90. Goers, L., Freemont, P. & Polizzi, K. M. Co-culture systems and technologies: taking synthetic biology to the next level. *J. R. Soc. Interface* 11, (2014).
91. Maldonado-Barragán, A., Caballero-Guerrero, B., Lucena-Padrós, H. & Ruiz-Barba, J. L. Induction of bacteriocin production by coculture is widespread among plantaricin-producing

- Lactobacillus plantarum* strains with different regulatory operons. *Food Microbiol.* 33, 40–47 (2013).
92. Diep, D. B., Håvarstein, L. S. & Nes, I. F. Characterization of the locus responsible for the bacteriocin production in *Lactobacillus plantarum* C11. *J. Bacteriol.* 178, 4472–4483 (1996).
 93. Di Cagno, R., De Angelis, M., Calasso, M. & Gobbetti, M. Proteomics of the bacterial cross-talk by quorum sensing. *J. Proteomics.* 74, 19–34 (2011).
 94. Ekblad, B. et al. Structure-Function analysis of the two-peptide bacteriocin plantaricin EF. *Biochemistry.* 55, 5106–5116 (2016).
 95. Haugen, H. S., Fimland, G., Nissen-Meyer, J. & Kristiansen, P. E. Three-dimensional structure in lipid micelles of the pediocin-like antimicrobial peptide curvacin A. *Biochemistry.* 44, 16149–16157 (2005).
 96. Risøen, P. A., Brurberg, M. B., Eijsink, V. G. & Nes, I. F. Functional analysis of promoters involved in quorum sensing-based regulation of bacteriocin production in *Lactobacillus*. *Mol. Microbiol.* 37, 619–628 (2000).
 97. Risøen, P. A. et al. Regulation of bacteriocin production in *Lactobacillus plantarum* depends on a conserved promoter arrangement with consensus binding sequence. *Mol. Genet. Genomics.* 265, 198–206 (2001).
 98. Peschel, A., Augustin, J., Kupke, T., Stevanovic, S. & Götz, F. Regulation of epidermin biosynthetic genes by EpiQ. *Mol. Microbiol.* 9, 31–39 (1993).
 99. Kou, X., Liu, Y., Li, C., Liu, M. & Jiang, L. Dimerization and conformational exchanges of the receiver domain of response regulator phoB from *Escherichia coli*. *J. Phys. Chem. B.* 122, 5749–5757 (2018).
 100. Métivier, A. et al. Divercin V41, a new bacteriocin with two disulphide bonds produced by *Carnobacterium divergens* V41: primary structure and genomic organization. *Microbiology.* 144 Pt 1, 2837–2844 (1998).
 101. Marchler-Bauer, A. et al. CDD/SPARCLE: functional classification of proteins via subfamily domain architectures. *Nucleic Acids Res.* 45, D200–D203 (2017).

102. Leroy, F. & de Vuyst, L. The presence of salt and a curing agent reduces bacteriocin production by *Lactobacillus sakei* CTC 494, a potential starter culture for sausage fermentation. *Appl. Environ. Microbiol.* 65, 5350–5356 (1999).
103. Gray, M. J., Freitag, N. E. & Boor, K. J. How the bacterial pathogen *Listeria monocytogenes* mediates the switch from environmental Dr. Jekyll to pathogenic Mr. Hyde. *Infect. Immun.* 74, 2505–2512 (2006).
104. Kreft, J. & Vázquez-Boland, J. A. Regulation of virulence genes in *Listeria*. *Int. J. Med. Microbiol.* 291, 145–157 (2001).
105. Corbett, D. et al. Two zinc uptake systems contribute to the full virulence of *Listeria monocytogenes* during growth in vitro and in vivo. *Infect. Immun.* 80, 14–21 (2012).
106. Kehl-Fie, T. E. & Skaar, E. P. Nutritional immunity beyond iron: a role for manganese and zinc. *Curr. Opin. Chem. Biol.* 14, 218–224 (2010).
107. Sword, C. P. Mechanisms of pathogenesis in *Listeria monocytogenes* infection. I. Influence of iron. *J. Bacteriol.* 92, 536–542 (1966).
108. Papp-Wallace, K. M. & Maguire, M. E. Manganese transport and the role of manganese in virulence. *Annu. Rev. Microbiol.* 60, 187–209 (2006).
109. van der Veen, S. & Abee, T. Mixed species biofilms of *Listeria monocytogenes* and *Lactobacillus plantarum* show enhanced resistance to benzalkonium chloride and peracetic acid. *Int. J. Food Microbiol.* 144, 421–431 (2011).
110. Guyonnet, D., Fremaux, C., Cenatiempo, Y. & Berjeaud, J. M. Method for rapid purification of class IIa bacteriocins and comparison of their activities. *Appl. Environ. Microbiol.* 66, 1744–1748 (2000).
111. Carolissen-Mackay, V., Arendse, G. & Hastings, J. W. Purification of bacteriocins of lactic acid bacteria: Problems and pointers. *Int. J. Food Microbiol.* 34, 1–16 (1997).
112. Liu, S., Han, Y. & Zhou, Z. Fusion expression of pedA gene to obtain biologically active pediocin PA-1 in *Escherichia coli*. *J. Zhejiang Univ. Sci. B* 12, 65–71 (2011).

113. Gibbs, G. M., Davidson, B. E. & Hillier, A. J. Novel Expression System for large-scale Production and Purification of Recombinant Class IIa Bacteriocins and Its Application to Piscicolin 126. *Appl. Environ. Microbiol.* 70, 3292–3297 (2004).
114. Jasniewski, J., Cailliez-Grimal, C., Gelhaye, E. & Revol-Junelles, A. M. Optimization of the production and purification processes of carnobacteriocins Cbn BM1 and Cbn B2 from *Carnobacterium maltaromaticum* CP5 by heterologous expression in *Escherichia coli*. *J. Microbiol. Methods* 73, 41–48 (2008).
115. Richard, C., Drider, D., Elmorjani, K., Prévost, H. & Marion, D. Heterologous expression and purification of active divercin v41, a class IIa bacteriocin encoded by a synthetic gene in *Escherichia coli* heterologous expression and purification of active divercin v41 , a class IIa bacteriocin encoded by a synthetic gene. *Society.* 186, 4276–4284 (2004).
116. Cuozzo, S., Calvez, S., Prévost, H. & Drider, D. Improvement of enterocin P purification process. *Folia Microbiol. (Praha).* 51, 401–405 (2006).
117. Moon, G. S., Pyun, Y. R. & Kim, W. J. Characterization of the pediocin operon of *Pediococcus acidilactici* K10 and expression of his-tagged recombinant pediocin PA-1 in *Escherichia coli*. *J. Microbiol. Biotechnol.* 15, 403–411 (2005).
118. Austin, C. Novel approach to obtain biologically active recombinant heterodimeric proteins in *Escherichia coli*. *J. Chromatogr. B.* 786, 93–107 (2003).
119. Ingham, A. B., Sproat, K. W., Tizard, M. L. V & Moore, R. J. A versatile system for the expression of nonmodified bacteriocins in *Escherichia coli*. *J. Appl. Microbiol.* 98, 676–683 (2005).
120. Moon, G. S., Pyun, Y. R. & Kim, W. J. Expression and purification of a fusion-typed pediocin PA-1 in *Escherichia coli* and recovery of biologically active pediocin PA-1. *Int. J. Food Microbiol.* 108, 136–140 (2006).
121. Kimple, M. E. & Sondek, J. *Current Protocols in Protein Science* (eds. Coligan, J. E., Dunn, B. M., Speicher, D. W. & Wingfield, P. T.) 9.9.1-9.9.19 (John Wiley & Sons, Inc., 2004). doi:10.1002/0471140864.ps0909s36
122. Bell, M. R., Engleka, M. J., Malik, A. & Strickler, J. E. To fuse or not to fuse: What is your purpose? *Protein Sci.* 22, 1466–1477 (2013).

123. LaVallie, E. R. et al. A thioredoxin gene fusion expression system that circumvents inclusion body formation in the *E. coli* cytoplasm. *Nat. Biotechnol.* 11, 187–193 (1993).
124. Jenny, R. J., Mann, K. G. & Lundblad, R. L. A critical review of the methods for cleavage of fusion proteins with thrombin and factor Xa. *Protein Expr. Purif.* 31, 1–11 (2003).
125. Crimmins, D. L., Mische, S. M. & Denslow, N. D. Chemical cleavage of proteins in solution. *Curr. Protoc. protein Sci.* Chapter 11, Unit 11.4 (2005).

Chapter 3

In-silico sub-grouping class IIa bacteriocins by their immunity protein sequence homology

Abstract

Aims: This study aimed to subgroup class IIa bacteriocins using their immunity protein amino acid sequence homology and cross-referencing known modes, or mechanisms of transcriptional regulation with established groupings. Defining subgroup regulation may help elucidate the mode of plantaricin 423 regulation in the absence of local regulatory genes on the *pla4* plasmid. Furthermore, understanding how *Enterococcus mundtii* ST4SA and *Lactobacillus plantarum* 423 regulate their class IIa bacteriocins may explain part of their ecological relationship within the gastrointestinal tract.

Methods and Results: Southern hybridisation and colony blotting were used to elucidate the complete sequence of the pST4SA315 plasmid harbouring the mundticin ST4SA biosynthetic operon, *munST4*. Multiple sequence alignments were performed using the UniProt multiple sequence alignment tool (<http://www.uniprot.org/>). Maximum likelihood phylogenetic trees were generated using CLC main workbench. BLAST analysis was performed (<https://blast.ncbi.nlm.nih.gov/Blast>) to generate information for cross-referencing groupings against proteins with known functions.

Conclusion: Three distinct types of immunity proteins were observed within known class IIa bacteriocin operons. Plantaricin 423 has a type 2 immunity protein. Cross-referenced functional information on operons of Type 2 indicates a potential regulatory response to cationic metals.

Introduction

The class IIa bacteriocin family has previously been sub-classified on two independent occasions by Nissen-Meyer *et al.*¹ and Cui *et al.*² respectively, with each author using different classification schemes. Nissen-Meyer *et al.*¹ performed a multiple sequence alignment on the more diverse C-terminal of all the known class II bacteriocins and divided them into four subgroups (Appendix Table 1 and Figure 3.5A). Peptides of subgroups 1, 3, and 4 are longer than those of subgroup 2, due to subgroup 2's shorter C-terminal half¹. Most of the peptides in subgroup 1, 2, and 4 have hairpin-stabilizing C-terminal tryptophan or cysteine residues, unlike members of subgroup 3¹. According to Nissen-Meyer *et al.*¹ enterocin SE-K4 and carnobacteriocin B2 are members of subgroup 4, with no hairpin- or cysteine residues in their C-terminus. Cui *et al.*² later classified known class IIa bacteriocins into eight groups according to their conserved primary structures, predicted 3D structures and mode of action (Appendix Table 1 and Figure 3.5B)². The reader is referred to Cui and co-workers² for a full list of grouped bacteriocins, and their resulting consensus sequences. Only the differences between

the classifications by Nissen-Meyer *et al.*¹ and Cui *et al.*² will be discussed further. Inconsistent numbering and use of the terms “grouping” and “subgrouping” have been used in different publications. The respective terminologies have been maintained in this discussion.

Cui *et al.*² further divided subgroup 1, defined by Nissen-Meyer *et al.*¹, into three subgroups, i.e. subgroup I-1, I-2, and I-3. Cui *et al.*² agreed with the grouping of bacteriocins in subgroup 4 as proposed by Nissen-Meyer *et al.*¹, however they referred to the group as group II (Figure 3.5B). Cui *et al.*² renamed and split subgroup 2, defined by Nissen-Meyer *et al.*¹, into two additional subgroups (groups III-1 and III-2) according to consensus sequence similarities (Figure 3.5B). Similar to subgroup 3 defined by Nissen-Meyer *et al.*¹, members of subgroup IV, defined by Cui *et al.*², lack hairpin-stabilizing tryptophan or cysteine residues in the C-terminal¹. Ubericin A is a 49 amino acid peptide that has high sequence similarity with subgroup I-3, but has a tertiary structure similar to curvacin A grouped in subgroup IV (determined by SWISS-MODEL Workspace²). According to Cui *et al.*², group VI-VIII are groups populated by few bacteriocins that are different from one another, and other larger groups of class IIa bacteriocins.

Plantaricin 423 has been placed in subgroup 2 by Nissen-Meyer, which has a characteristic shorter C-terminus¹. Mundticin ST4SA and mundticin KS have identical mature peptide sequences, however, their leader sequences differ by two amino acids. The mature mundticin KS peptide has been placed in subgroup 1 by Nissen-Meyer and co-workers¹. Plantaricin 423 has two C-terminal cysteine residues, while mundticin ST4SA has a C-terminal tryptophan.

Class I bacteriocins (lantibiotics) consists of a much more populated group of bacteriocins, which in the past, has been subjected to a variety of classification schemes. Willey and co-workers³ described the benefits of classifying class I lantipeptides according to each peptide’s post-translational modification (PTM) enzymes’ amino acid sequence homologies. Lantipeptides contain the unusual thioether-containing amino acids lanthionine and β -methyl-lanthionine. These peptides are produced from various operons by Firmicutes, Actinobacteria, Proteobacteria, Bacteroides and Cyanobacteria⁴. The approach presented by Willey *et al.*³ assumes that peptides with similar functions have similar structures and therefore similar sequences. Phylogenetic analysis based on amino acid sequence similarity of these peptides can, therefore, return distorted relationships between producers. This is especially evident in lantipeptides where the unusual thioether amino acids have properties that are independent of the peptide’s function. For example, class I and II lantipeptides have antimicrobial activity while class III does not, but all classes have thioether-containing amino acids. Willey *et al.*³

observed the phylogenetic relationships between classes of lantipeptides by comparing how they are post-translationally processed using multiple sequence alignment of the PTM enzymes. Class I is processed by separate dehydratases (LanB) and cyclases (LanC), while class II is dehydrated and cyclized by a single LanM-type enzyme. Class III peptides have little to no antimicrobial activity and are processed by LanKC. The classification scheme described by Willey *et al.*³ is currently used in the phylogenetic analyses of lantipeptides. The reader is referred Arnison and co-workers⁵ for the current nomenclature and grouping of lantibiotics. The classification used by Willey *et al.*³ can be used for subclassifying other ribosomally produced proteins, like class IIa bacteriocins, to better understand their phylogenetic relationships.

The class IIa bacteriocins considered in this study are all produced by Firmicutes and show relatively high sequence similarity to one another as they fulfill a similar function^{1,2}. The similarities/dissimilarities in bacteriocin and immunity protein sequence can be phylogenetically analyzed to better understand their evolutionary relationships. More phylogenetically related groups may use similar mechanisms of regulation. This allows the limited knowledge of class IIa bacteriocin regulation to be applied to entire subgroupings. Other information like motifs with known function identification, operon location, structure and producer strain may be assigned to the phylogenetically determined subgroups. This information may provide insight into the environmental role each bacteriocin plays and other potential evolutionary relationships within the class IIa bacteriocins.

According to Bactibase (<http://bactibase.hammamilab.org/main.php>), an integrated online open-access Gram-positive and -negative bacteriocin database, there are approximately 39 peptides with the conserved N-terminal YGNGV motif which are grouped as class IIa bacteriocins. The class IIa bacteriocin family is growing and therefore, every time a novel class IIa bacteriocin is discovered the consensus sequence of this family changes slightly. From this group of 39 class IIa bacteriocins, 25 peptides were selected for this study on the premise that their structural and immunity genes had been sequenced.

This study presents the subclassification of class IIa bacteriocins by their mechanism of immunity, elucidated via sequence similarity of each bacteriocin's immunity protein. *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA are included in the probiotic with the trade name Entiro™, marketed by Cipla Medpro. These two lactic acid bacteria produce plantaricin 423 and mundtacin ST4SA, respectively. Sub-classification of the class IIa bacteriocins and cross-referencing of these groups with the known modes of bacteriocin regulation is important for hypothesizing how plantaricin 423 and

mundticin ST4SA are regulated. Understanding plantaricin 423 and mundticin ST4SA regulation will ultimately help elucidate the type of ecological relationship these two microbes engage in within the gastrointestinal tract during administration.

Materials and Methods

Bacterial strains, culture conditions and molecular cloning

Bacterial strains and culture conditions

Lactobacillus plantarum 423 and *E. mundtii* ST4SA were cultured in De Man, Rogosa and Sharpe (MRS) broth at 37 °C, without agitation. All growth media were supplied by Merck-Millipore (USA). Subcloning experiments were performed in *Escherichia coli* DH5 α , which was cultured on Luria-Bertani (LB) medium at 37 °C with constant agitation. For the selection and maintenance of pBR322-derived plasmids in *Escherichia coli* DH5 α , transformants were cultured with LB broth containing 150 μ g/mL ampicillin.

Molecular techniques

DNA analysis and extraction, manipulation, plasmid cloning, and Southern hybridization/colony blotting was performed according to Sambrook *et al.* ⁶. Plasmid isolations from *E. coli* DH5 α were performed using the PureYield™ plasmid miniprep system (Promega, Madison, WI, USA).

T4 DNA ligase and restriction enzymes (RE) were purchased from New England Biolabs (NEB, Ipswich, MA, USA) and used according to the manufacturer's instructions. Polymerase chain reaction (PCR) amplifications were performed using Q5 high-fidelity PCR DNA polymerase (NEB) according to manufacturer's instructions in a GeneAmp PCR system 9700 (ABI, Foster City, CA). Oligonucleotides were designed using the CLC main workbench program (CLC bio, Aarhus, Denmark) and purchased from Inqaba Biotechnical Industries (Pretoria, South Africa). DNA sequencing was performed by the Central Analytical Facilities (CAF) at the University of Stellenbosch, South Africa.

Agarose gel electrophoresis was used for the analysis and/or purification of RE mapped DNA fragments in TBE buffer at 10V/cm using the Ephortec™ 3000V (Triad Scientific, Manasquan United States) power supply ⁷. Excised gel DNA fragments were purified using the Zymoclean™ gel DNA recovery kit (Zymo Research Corporation, Irvine, CA, USA).

Southern hybridization was used to identify, and then shotgun clone an EcoRV/HindIII fragment into pBR322 which proved that one of the nodes (Node 29) from the unassembled *E. mundtii* ST4SA genome was circular. Colony blot Southern hybridization was used to screen for clones containing the joining fragment of Node 29. Southern hybridization was performed using a 198 bp PCR fragment of the mundticin ST4SA gene, amplified from the 317F primer (5'-TGAGGCGGCCGCAAGTTTTGAAGAAATTAACAGC-3') and 302R primer (5'-AAGCTTAACTTTTCCAACCAGCTGCTCC-3') as a probe. The PCR fragment was randomly DIG-labelled according to the manufacturer's instructions (Roche). Sequencing of captured fragments was performed at CAF using the Node_29_SeqR primer (5'-AAGTAAAACAAATACGGCGG-3').

Multiple sequence alignments and maximum-likelihood phylogenetic tree generation

The *Lactobacillus plantarum* 423 and *E. mundtii* ST4SA genomes have been sequenced on Ion proton and Illumina platforms, reads were assembled into contigs using SPAdes v3.10.1. These genomes are currently unassembled, but contigs containing the complete plantaricin 423 and mundticin ST4SA bacteriocin and immunity protein sequences were identified.

The amino acid sequences of the class IIa bacteriocins and their immunity proteins from Table 1 (Appendix) were aligned using the UniProt multiple sequence alignment tool (<http://www.uniprot.org/>). Maximum likelihood phylogenetic analysis was performed on the aligned sequences using CLC main workbench with the WAG substitution model, and 1000 bootstrap replicates.

BLAST analysis

Nucleotide and protein sequences of interest from established groupings were aligned using the NCBI BLAST tool for cross-referencing sequences with genes and proteins or motifs of known function (<https://www.ncbi.nlm.nih.gov/>).

Results

Southern blotting and colony hybridizations

From the autoradiograph of the EcoRV/HindIII-digested *E. mundtii* ST4SA genome a fragment of approximately 2100 bp containing the mundtacin ST4SA gene was identified (Figure 3.1B, Lane 3). The fragments in the autoradiograph (lane 3) corresponding to the size of approximately 2.1 kb as

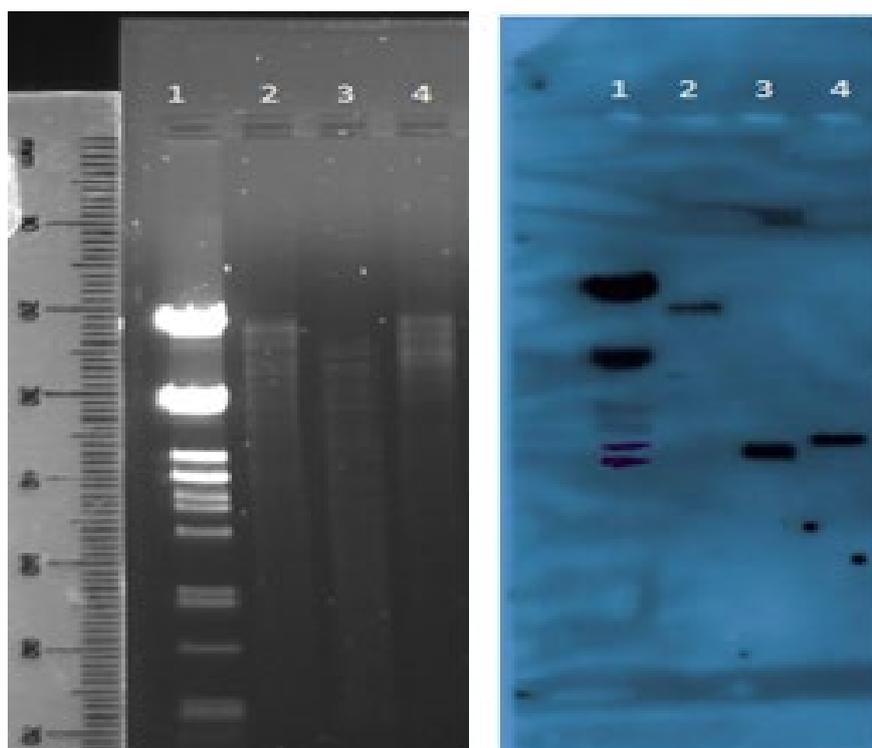


Figure 3.1 – A, electrophoretic separation of the *E. mundtii* ST4 gDNA digestions. B, corresponding autoradiograph after southern hybridisation using the DIG labelled probe for the mundtacin ST4SA gene. Lanes: 1 - Lambda/pst ladder, 2 – EcoRV digestion, 3 – EcoRV/HindIII digestion 4 – HindIII digestion. The band in lane 3(right) corresponds to approximately 2100bp.

identified by the probe, were shotgun cloned. Clones harboring the correct fragment were then identified using colony hybridization, and plasmid DNA was extracted and sequenced.



Figure 3.2 - The mundtacin ST4SA operon: mundtacin ST4SA structural gene (*munST4-A*), ABC transporter (*munST4-T*), and immunity protein (*munST4-I*). The fragment PCR amplified using the 317F and 302R primer set (blue arrows) was used as a probe in Southern hybridization.

From the sequence, it was confirmed that Node 29 from the *E. mundtii* ST4SA unassembled genome was indeed circular and therefore a megaplasmid, designated pST4SA315 (Appendix, Figure 3.9). Putative coding sequences found on pST4SA315 were identified and annotated, with the mundtacin ST4SA operon, designated *munST4* (Figure 3.2).

Multiple sequence alignments

Class IIa bacteriocin alignment

The bacteriocins of class IIa used in this study aligned with high homology especially in the N-terminal pediocin box, or YGNGV motif, as expected (Figure 3.3).

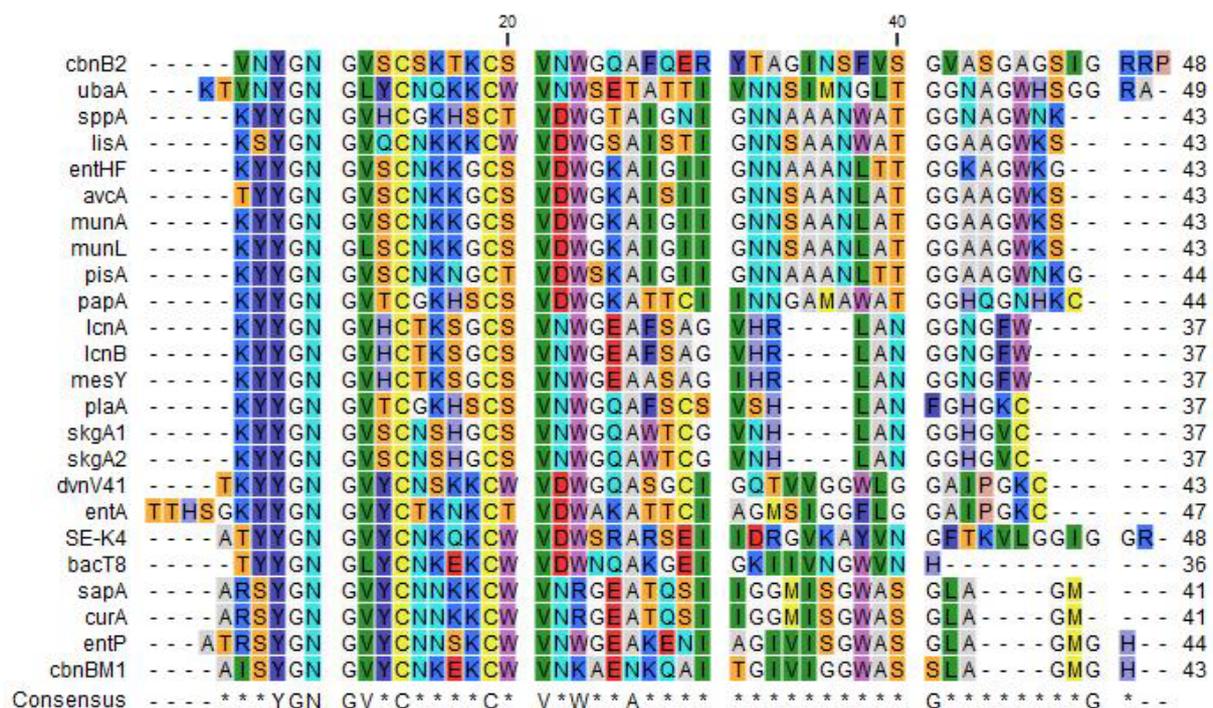


Figure 3.3 – UniProt multiple sequence alignment for the mature class IIa bacteriocin amino acid sequences specified in Appendix Table 1. The pediocin box with the YGNGV motif may be seen in the consensus sequence at the bottom of the alignment.

Immunity protein alignment

The immunity proteins for class IIa bacteriocins did not group as seen for the mature bacteriocin sequence alignments in Figure 3.3. Alignment of the immunity proteins produced a minimum of three distinctly different sub-groupings (Figure 3.4).

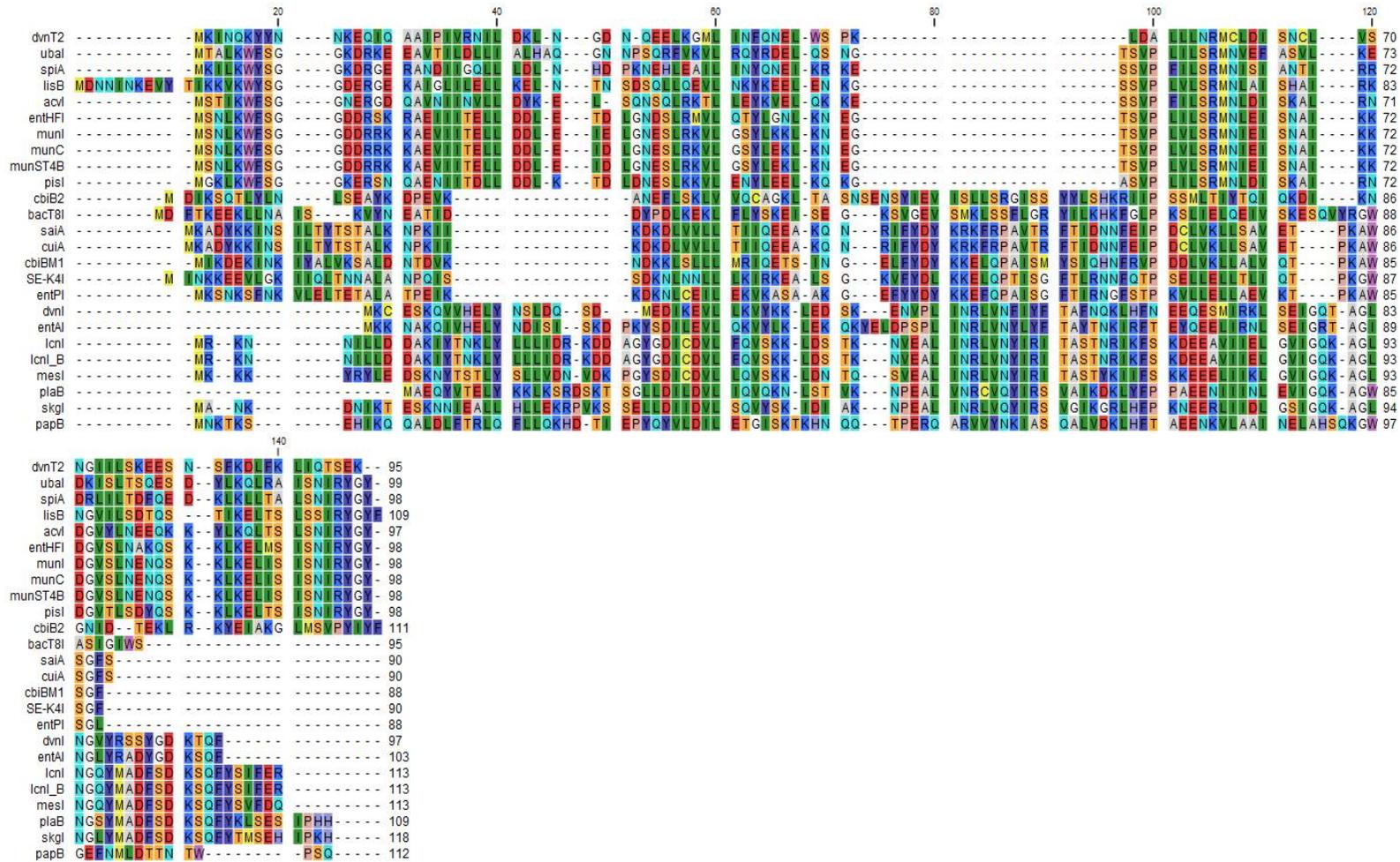


Figure 3.4 - UniProt multiple sequence alignment for the class IIa bacteriocin immunity protein amino acid sequences of Appendix Table 1 sorted according to similarity.

Maximum likelihood phylogenetic tree analysis

The previous subgroupings of class IIa according to Nissen-Meyer *et al.*¹ and Cui *et al.*² have been superimposed on the class IIa bacteriocin phylogenetic tree in Figure 3.5A and B, respectively. Ungrouped bacteriocins seen in Figure 3.5A had not been discovered at the time of classification.

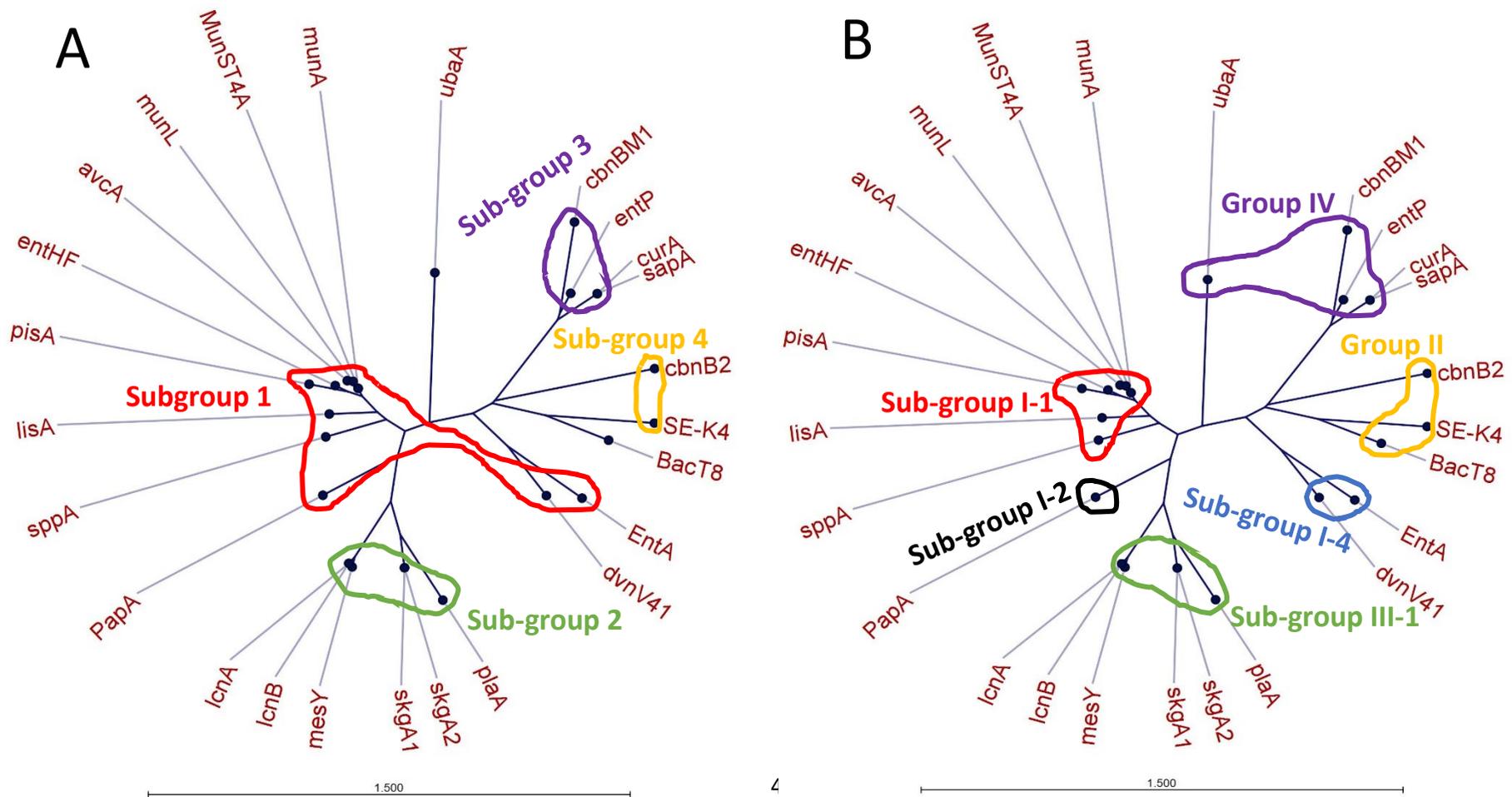


Figure 3.5 - Superimposition of the previous groupings by Nissen-Meyer *et al.*¹ (A) and Cui *et al.*² (B) on a class IIa bacteriocin maximum likelihood phylogenetic tree, generated from the bacteriocin amino acid sequence alignment from Figure 3.3.

Immunity protein phylogenetic analysis

Three distinct types of immunity proteins showing little homology to one another were observed and used for the class IIa bacteriocin grouping in this study (Figure 3.6). The CbiB2 and BacT8I immunity proteins are dissimilar to all other types and show low similarity to one another. These proteins may represent other types of immunity proteins that are yet to be characterized or may be outliers. For this study, they were considered an out-group of type 3 immunity proteins (Figure 3.6).

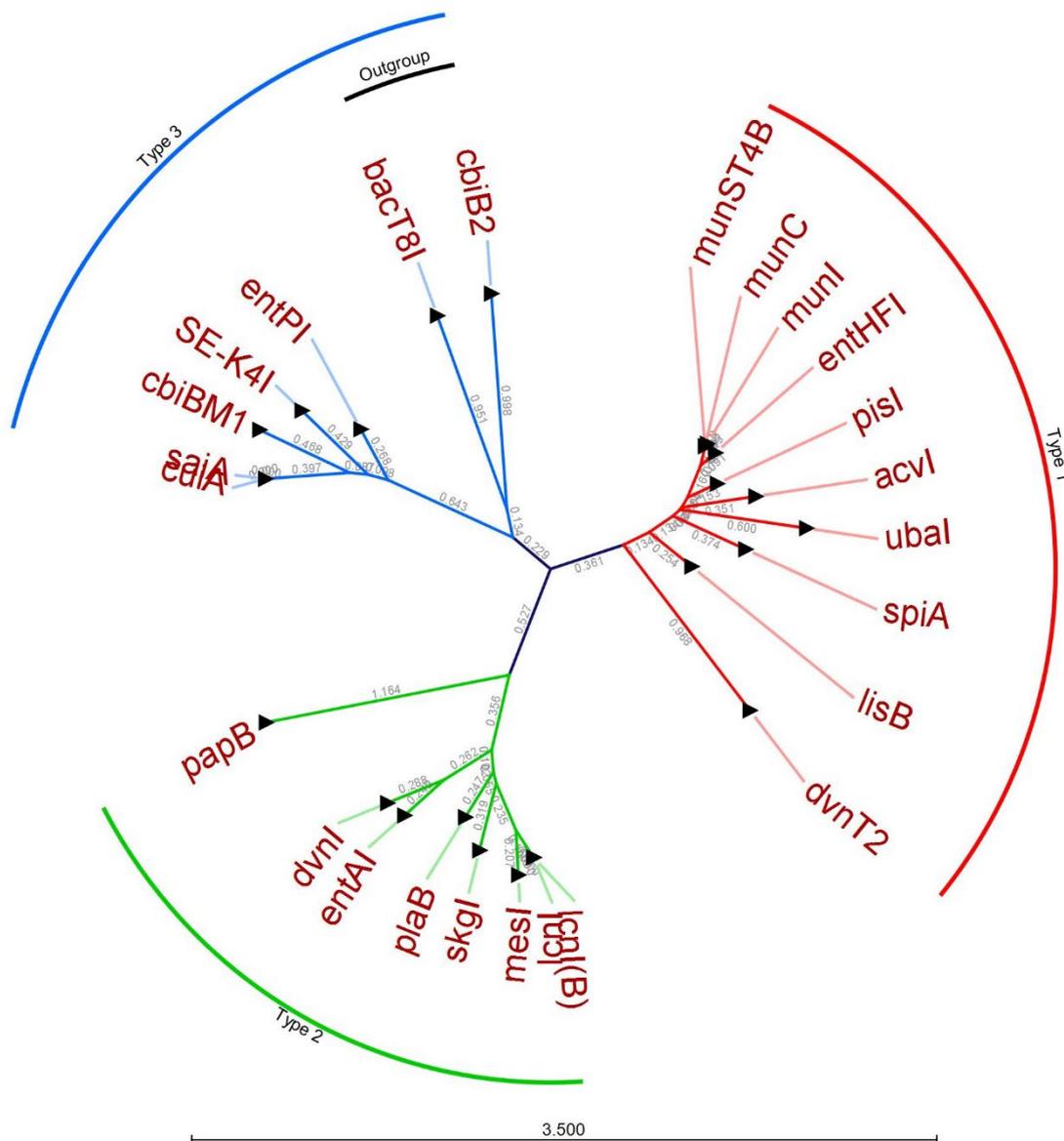


Figure 3.6 - Maximum likelihood phylogenetic tree representing the sequence similarity of class IIa bacteriocin immunity proteins considered in this study.

Class IIa bacteriocin classification

Figure 3.7 depicts the grouping proposed in this study as per the distinct types of immunity proteins observed in Figure 3.6. Type 3 immunity proteins can most likely be further divided into two or three more types, due to dissimilarities seen in the carnobacteriocin immunity proteins, CbiB2 and BacT8I. However, this should be undertaken by a future study as it falls outside the scope of this research.

Analysis of bacteriocin operons with type 2 immunity proteins

The BLAST was used to analyze the coding sequences from operons with type 2 immunity proteins on a nucleotide and protein level to detect motifs of known function. Alignment of sequences to known motifs may provide evidence for regulation within the class, and particularly for plantaricin 423. The structural organization of all class IIa bacteriocin operons discussed in this section may be found in the Addendum, Figure 7.1.

The enterocin A biosynthetic operon is flanked by two operons containing a ferrous iron uptake transporter, FeoB (domain architecture ID: 11417566), and a sensor histidine kinase BaeS (domain architecture ID 11428264), respectively.

The divercin V41 operon presents a fascinating case because it harbors two immunity proteins within its operon, *dvnl* and *dvn2*, an HPK (*dvnK*) and the RR (*dvnR*). According to the BLASTp alignment, within the sensor histidine kinase BaeS conserved domain there is a “non-specific hit” to the CztS_silS_copS motif (NCBI ID: TIGR01386)⁸. The CztS_silS_copS motif is associated with heavy-metal resistance efflux systems for copper, silver, cadmium, and/or zinc (NCBI ID: TIGR01386).

The mesentericin Y105 operon is located on a megaplasmid, however, it does not contain an HPK or RR as seen in divercin V41 and enterocin A, but does harbor an HTH_XRE-like induction factor, MesF^{8,9}. The HTH_XRE is a zinc-finger-containing DNA binding response element involved in stress response to xenobiotics (domain architecture ID 10650117).

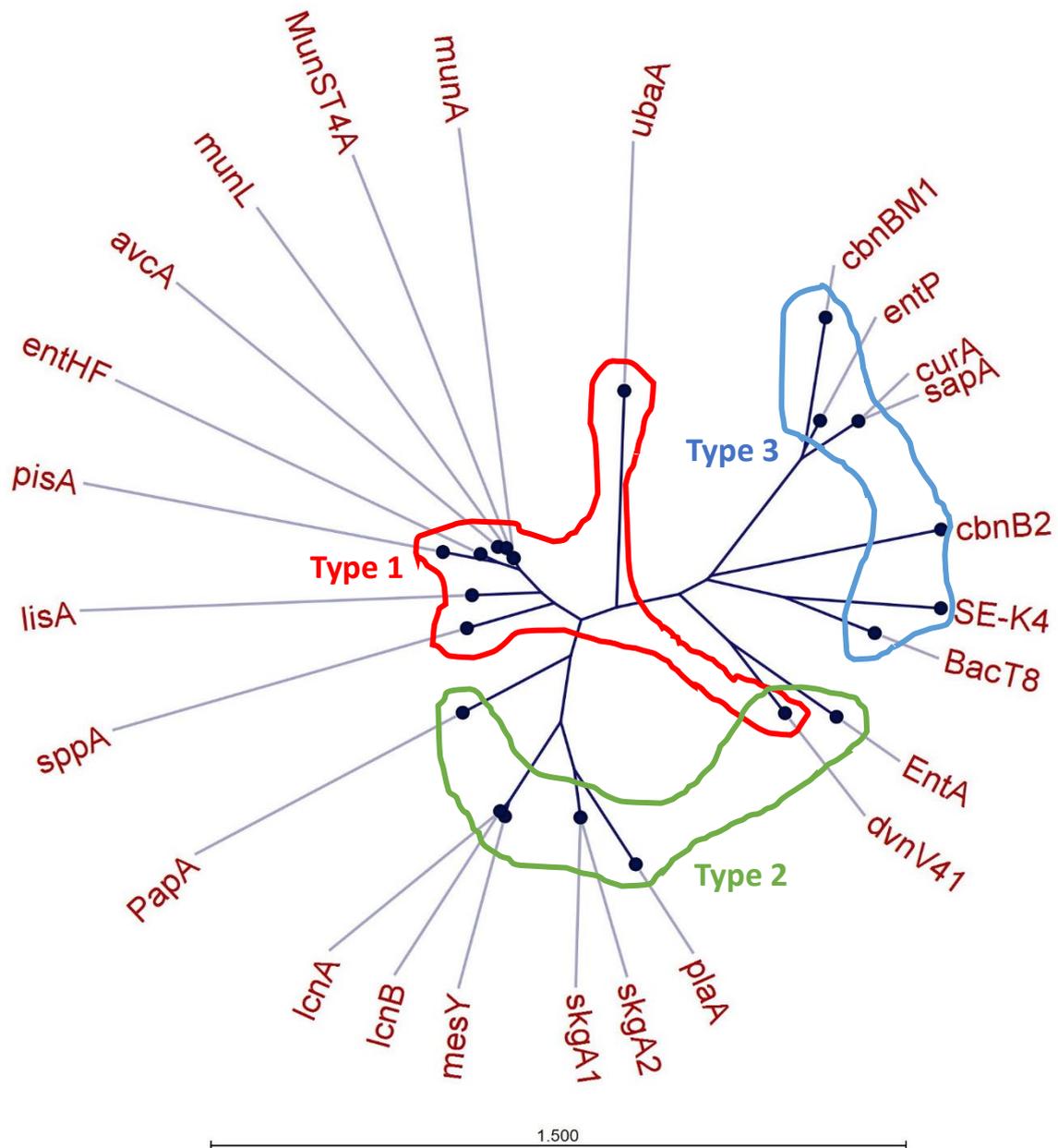


Figure 3.7 – Superimposition of the class IIa immunity protein grouping from Figure 3.5 on a class IIa bacteriocin phylogenetic tree, generated from bacteriocin amino acid sequence similarity.

The pediocin PA-1 operon does not contain regulatory elements but analysis of the immunity protein, PapB, indicates a relatedness to the PbsX transcriptional regulator. The PapB protein has a 71% identity (E value = $3e^{-58}$) to a multispecies PbsX transcriptional regulator from *Lactobacillus* (NCBI Reference Sequence: WP_096641391.1). BLASTn analysis of the *papB* gene aligned with an 84% identity (E value = $6e^{-83}$) to a *PbsX* regulator coding sequence on the *Lactobacillus pentosus* strain BGM48 chromosome (NCBI Sequence ID: CP016491.1, Gene: BB562_15595). Neighboring the *pbsX*

regulator gene (BB562_15595) occurs a copper-translocating P-type ATPase coding sequence (protein domain architecture NCBI ID: 11550563), which contains the ZntA motif associated with transport of zinc, cadmium, mercury, and lead (conserved domain NCBI ID: PRK11033).

The plantaricin 423 immunity gene, *plaB* (GenBank: AF304384.2), aligns with a 70% identity (E value = $1e^{-23}$) to a second coding sequence within the *Lactobacillus plantarum* 423 unassembled genome. Alignment of the protein sequence against the translated genome coding sequence indicated a 57% identity to the second potential protein (E value = $5e^{-39}$, coverage of 99%, 75% positive matches) as seen in Figure 3.8.

The coding sequences with known function surrounding this second *plaB*-like sequence on Node_9 within the un-assembled *L. plantarum* 423 genome may be found in the Appendix, Figure 3.10. Notable are the downstream iron- or zinc-uptake regulator and upstream metallo-phosphoesterase.

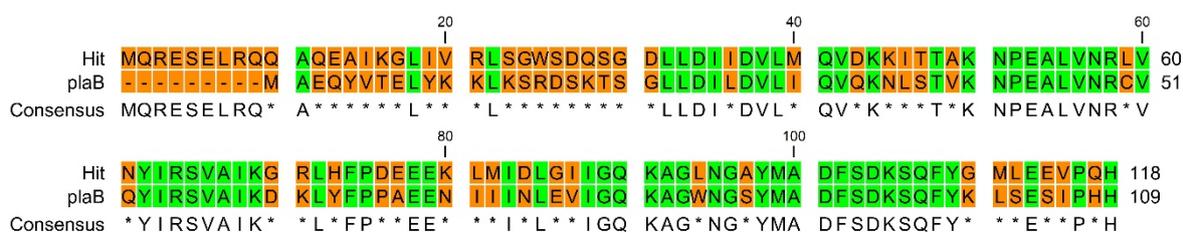


Figure 3.8 – Amino acid sequence alignment of the PlaB peptide with the translated coding sequence of the second *plaB*-like sequence “Hit” within the unassembled *L. plantarum* 423 genome.

Discussion

The approach used in this study, whereby class IIa bacteriocins are grouped according to their producers mechanism of immunity, is mostly in agreement with Nissen-Meyer *et al.*¹ and Cui *et al.*². However, there are a few notable differences, alignment of the immunity proteins presented 3 distinct types, which were then used to subgroup the class IIa bacteriocins considered in this study (Figure 3.7). Nodes with tighter groupings within a subgroup are observed. However, due to the limited number of sequenced immunity proteins and scope of this research, a high stringency approach as seen in Cui *et al.*² was not applied but could be in future studies. Additional genes such as the ABC-transporter could also be employed to increase the confidence of high stringency grouping.

General observations

Unlike the grouping by Nissen-Meyer *et al.*¹ and Cui *et al.*², enterocin A is not grouped as 1 or I-4, respectively, this bacteriocin’s immunity protein has been grouped as a Type 2 (Table 1). Divercin V41

has two immunity proteins and therefore falls into Type 1 and Type 2 according to the system presented in this study (Table 1). Pediocin PA-1 has also been moved to Type 2, based on its immunity protein group (Table 1). With this rearrangement, the bacteriocins with Type 2 immunity proteins are still characteristically shorter than the other Types/groups/sub-groups (Table 1 and Figure 3.7)^{1,2}. However, it has become apparent that many bacteriocins within the Type 2 grouping have two disulfide bonds, and an accessory protein gene within their operon to assist in correct bond formation¹⁰. This trait is not observed in leucocin-A and -B (identical mature peptides) and mesentericin Y105 (Table 1 and Figure 3.7). The immunity type 3 grouping presents a concatenation of sub-groups 3 and 4 (Nissen-Meyer *et al.*¹) and various subgroups of Cui *et al.*² (Table 1). The current grouping is in disagreement with the grouping by Cui *et al.*² of ubericin A as it has a Type 1 immunity protein.

The class IIa bacteriocins which have had their regulatory mechanisms elucidated have either type-1 or -3 immunity proteins. Regulatory studies on bacteriocins belonging to immunity type 2 have previously not been performed.

Type 1 immunity protein subgroup (T1IP)

All class IIa bacteriocins within T1IP, occur on the chromosome or a megaplasmid, except for listeriocin 1743A which occurs on a cryptic plasmid (partially sequenced operons not included)¹¹. Listeriocin 1743A is produced by *Listeria innocua* and is the only bacteriocin in this group that is produced by an organism which is outside of the Lactobacillales order. Maturation and translocation of listeriocin 1743A is also different from general class IIa processing as it is mediated by the Sec translocase. This *sec* complex is responsible for the majority of transmembrane protein trafficking in prokaryotes¹². Sec-dependent processing is also observed for enterocin P and bacteriocin T8, which have Type 3 immunity proteins (Appendix: Table 1 and Figure 3.7). Enterocin P is associated with a transposon and occurs on a megaplasmid while bacteriocin T8 is located on a plasmid; both bacteriocins are produced by organisms belonging to the order Lactobacillales (Appendix: Table 1). Whether *sec* mediated class IIa bacteriocins have a unique mode of regulation is currently unknown, but they appear to occur as concise operons associated with more mobile elements (Table 1). Therefore, a more generic mode of regulation can be anticipated as these concise operons only contain a structural and an immunity gene. It is interesting to note that while listeriocin 1743A, enterocin P, and bacteriocin T8 are all associated with mobile elements and are processed by their hosts' Sec translocase; their bacteriocin and immunity protein amino acid sequences are all dissimilar (Figure 3.3, 3.4 and 3.7). This might be due to the convergent evolution of a mobile, Sec-translocase-processed class IIa bacteriocins within *Listeria innocua* (listeriocin 1743A), and *Enterococcus faecium* (enterocin P, and bacteriocin T8).

Sakacin A is produced by the *sap* operon which encodes an HPK (*sapK*), RR (*SapR*) and AP (*Sap-Ph*)¹³. Piscicolin 126 is produced from the *pis* operon which encodes an HPK (*pisK*), RR (*pisR*) and AP (*pisN*)¹⁴. These classical three-component regulatory operons are responsible for temperature sensitive quorum-sensing-based induction of sakacin A and piscicolin 126, which have Type 1 immunity proteins^{13,14}.

The majority of T1IP class IIa bacteriocins which harbor three-component regulatory systems are likely regulated by a quorum sensing mechanism which is temperature dependent as seen in sakacin A and piscicolin 126. However, there are exceptions within the T1IP like divercin V41 and listeriocin 1743A. The *dvn* operon encodes two immunity proteins, which will be critically discussed at a later stage. Transcriptional regulation of avicin A (*avcA*), another member of the Type 1 group has not been studied. However, a classical three-component regulatory operon is found neighboring the *avc* biosynthetic genes (Table 1).

Type 3 immunity protein subgroup (T3IP)

Transcriptional studies on T3IP bacteriocins present a more unconventional mode of quorum-sensing-based regulation. *Carnobacterium piscicola* LV17B produces two class IIa bacteriocins and one class IIb bacteriocin. The *cbnB2* operon is located on a megaplasmid and contains the biosynthetic and three-component regulatory genes to produce carnobacteriocin B2 and carnobacteriocin XY (class IIb). The *cbnB2* operon harbours a regulatory element containing an HPK (*cbnK*), RR (*cbnR*) and AP (*cbnS*). The *cbnBM1* is a concise operon located on the chromosome and encodes the carnobacteriocin BM1 structural gene and immunity protein. Quadri *et al.*¹⁵ demonstrated that the CbnS AP was responsible for the induction of carnobacteriocin B2, XY and BM1 as these genes for these proteins share homologous repeats in their promoter regions¹⁶. However, it was also demonstrated that heterologously purified carnobacteriocin B2 was able to induce a bacteriocin-positive phenotype in *C. piscicola* LV17B^{17,18}. These results point to nisin-type autoinduction capabilities in addition to AP induction for bacteriocin expression in *C. piscicola* LV17B and therefore the immunity protein Type 3 grouping as a whole¹⁵. However, this mechanism of induction needs to be confirmed on a transcriptional level for carnobacteriocin B2, XY, and BM1⁴⁰.

Type 2 immunity protein subgroup (T2IP)

Although there are no transcriptional studies on any T2IP class IIa bacteriocins, motifs associated with proteins of known regulatory function were observed. It should be noted that the operons for type 2

bacteriocins such as pediocin PA-1, sakacin G and plantaricin 423 are associated with mobile elements and lack any local regulatory genes (Table 1). While less mobile operons like those for divercin V41 and enterocin A appear to contain two-component regulatory elements devoid of APs (Table 1). Therefore, two different mechanisms of regulation may occur in T2IP even though they appear to both respond to divalent cations in some way.

BLAST analysis indicated that almost all bacteriocin operons within the Type 2 immunity grouping show some link to a regulatory system which depends on environmental metal cation response. The divercin V41 operon presents the strongest evidence that the T2IP bacteriocin regulation is somehow dependent on metal cations. The Dvn2 immunity protein shares high similarity to other Type 1 immunity proteins, but Dvn1 aligns with Type 2 immunity proteins, while there is only one structural gene, *dvnV41*. The operon-encoded sensor histidine kinase, DvnK, contains the heavy-metal-sensing motif CztS_silS_copS and not an AP recognition motif. The enterocin A operon is found neighboring an iron uptake system (Addendum, Figure 7.1 : SAP083A_004 and SAP083A_005). Therefore, transcription of the type 2 bacteriocin divercin V41 and enterocin A operons appears to be stimulated by a cationic metal, like iron, copper, silver, cadmium, zinc or combinations of these.

While the plantaricin 423, pediocin PA-1 and mesentericin Y105 operons do not contain any classical regulatory genes, a link to cation metal regulation can still be found. The induction factor for mesentericin Y105, MesF, contains a zinc finger and is thought to respond to xenobiotics. It has previously been demonstrated that induction factors with zinc fingers require zinc for DNA binding¹⁹. Furthermore, metals such as zinc and cadmium which are cationic, can be considered xenobiotic and induce a transcriptional response in prokaryotes²⁰.

The plantaricin 423 and pediocin PA-1 operons are both found on plasmids which are less than 10 Kb, devoid of any known regulatory protein genes, and contain genes for proteins which promote conjugal plasmid transfer. BLAST analysis of the pediocin immunity gene, *papB*, indicates high similarity to *pbsX* induction factors of other lactobacilli, which are chromosomally associated with cationic metal transport. Similarly, the *plaB* gene shows high homology to another coding sequence within the *L. plantarum* 423 unassembled genome. Upstream of the second coding sequences showing high homology to *plaB*, a coding sequence for a putative ferric uptake regulator protein is observed (conserved domain ID: cd07153) as seen in Appendix, Figure 3.10.

Using this sub-grouping approach and cross-referencing to motifs of known function, a hypothesis was generated for stimulating the transcriptional regulation of plantaricin 423: Transcriptional regulation

of plantaricin 423 is not dependant on a classical operon-encoded three-component bacteriocin regulatory system or autoinduction, but rather an environmental stimulus like the presence of certain cationic metals. To test this hypothesis a heterologous expression system and a reporter system for plantaricin 423 was developed in Chapter 4 and 5 respectively.

Appendix

Table 1 – Class IIa bacteriocins and their corresponding immunity proteins

Bacteriocin	Protein	Accession number	Immunity protein	Accession number	Operon location	Regulatory operon nucleotide accession	Immunity protein grouping	Ref ¹	Ref ²	Producer	Ref
Avicin A	AvcA	ACZ36002.1	AvcI	ACZ36003.1	Chromosome	FJ851402.1	1		I-1	<i>Enterococcus avium</i>	²¹
Divercin V41 ¹	DvnV41	CAA11804.1	Dvn2	CAA11807.1	Chromosome	AJ224003.1	1	1	I-4	<i>Carnobacterium divergens</i>	²²
Enterocin CRL35 (Mundticin KS)	MunA	AAQ95741.1	MunC	AAQ95743.1	Megaplasmid		1	1	I-1	<i>Enterococcus mundtii</i>	²³
Enterocin HF	EentHF	AHW46186.1	^N EntHFI	AHW46188.1	Fragment only		1		I-1	<i>Enterococcus faecium</i>	²⁴
Listeriocin 743A	LisA	AAK19401.1	LisB	AAK19402.1	cryptic plasmid		1	1	I-1	<i>Listeria innocua</i>	¹¹

Mundtacin L	MunL	ACQ77507.1	MunI	ACQ77509.1	Plasmid (Fragment only)		1		I-1	<i>Enterococcus mundtii</i>	²⁵
Mundtacin ST4SA	^N MunST4A		^N MunST4I		Megaplasmid		1	1	I-1	<i>Enterococcus mundtii</i>	This study
Piscicolin 126	PisA	AAK69419.1	PisI	AAK69418.1	Unknown	AF275938.1	1	1	I-1	<i>Carnobacterium piscicola</i>	²⁶
Sakacin-P (Sakacin 674)	SppA	CAA88428.1	SpiA	CAA88429.1	Chromosome	Z48542.1	1	1	I-1	<i>Lactobacillus sakei</i>	²⁷
Ubericin A	UbaA	ABQ23939.1	UbaI	ABQ23940.1	Fragment only		1		IV	<i>Streptococcus uberis</i>	²⁸
Divercin V41 ¹	DvnV41	CAA11804.1	DvnI	CAA11807.1	Chromosome	AJ224003.1	2	1	I-4	<i>Carnobacterium divergens</i>	²²

Enterocin A	EntA	AVJ44360.1	^N EntAI	AVJ44361.1	Chromosome Transposon	CP025392.1	2	1	I-4	<i>Enterococcus faecium</i>	²⁹
Leucocin-A (Leucocin A-UAL 187)	LcnA	AAA68003.1	LcnI	AAA68004.1	Megaplasmid (Partial only)		2	2	III-1	<i>Leuconostoc gelidum</i>	³⁰
Leucocin-B (Leucocin B-Ta11a)	LcnB	AAC60488.1	^N LcnI(B)	AAC60489.1	Megaplasmid Transposon		2		III-1	<i>Leuconostoc gelidum</i>	³¹
Mesentericin Y105	MesY	AAP37395.1	MesI	AAP37394.1	Megaplasmid (Partial only)	AY286003.1	2	2	III-1	<i>Leuconostoc mesenteroides</i>	³²
Pediocin PA-1 (Pediocin ACH)	PapA	ATL64002.1	PapB	ATL64003.1	Plasmid		2	1	I-2	<i>Pediococcus acidilactici</i>	³³
Plantaricin 423	PlaA	AAL09346.1	PlaB	AAL09347.2	Plasmid		2	2	III-1	<i>Lactobacillus plantarum</i>	³⁴

Sakacin G	SkgA1	ACB72725.1	SkgI	ACB72723.1	Megaplasmid Transposon		2	2	III-1	<i>Lactobacillus sakei</i>	³⁵
	SkgA2	ACB72724.1	SkgI		Megaplasmid Transposon		2	2	III-1	<i>Lactobacillus sakei</i>	³⁵
Bacteriocin T8	^N BacT8	ABD60751.1	^N BacT8I	ABD60752.1	Plasmid		3		III-1	<i>Enterococcus faecium</i>	³⁶
Carnobacteriocin B2	CbnB2	AAA72431.1	CbiB2	AAA72432.1	Megaplasmid	L47121.1	3	4	II	<i>Carnobacterium piscicola</i>	³⁷
Carnobacteriocin BM1	CbnBM1	AAA23014.1	^N CbiBM1	AAA23015.1	Chromosome	L47121.1	3	3	IV	<i>Carnobacterium piscicola</i>	³⁷
Curvacin-A	CurA	AAB28845.1	CuiA	AAB28846.2	Megaplasmid		3	3	IV	<i>Lactobacillus curvatus</i>	³⁸

Enterocin P	EntP	AAC45870.1	^N EntPI	AAC45871.1	Megaplasmid		3	3	IV	<i>Enterococcus faecium</i>	³⁹
Enterocin SE-K4	^N SE-K4	BAC20326.1	^N SE-K4I	BAC20329.1	Plasmid (Partial only)		3	4	II	<i>Enterococcus faecalis</i>	⁴⁰
Sakacin-A	SapA	CAA86942.1	SaiA	CAA86941.1	Chromosome Fragment	Z46867.1	3			<i>Lactobacillus sakei</i>	⁴¹

^A Divercin appears twice in the chart because it has two immunity proteins, *dvnl* and *dvnl2*.

^N Names of genes adopted for this study only and are not found in literature or online databases.

Fragment only – Partially sequenced bacteriocin operon

Ref¹ Nissen-Meyer *et al*¹

Ref² Cui *et al*²

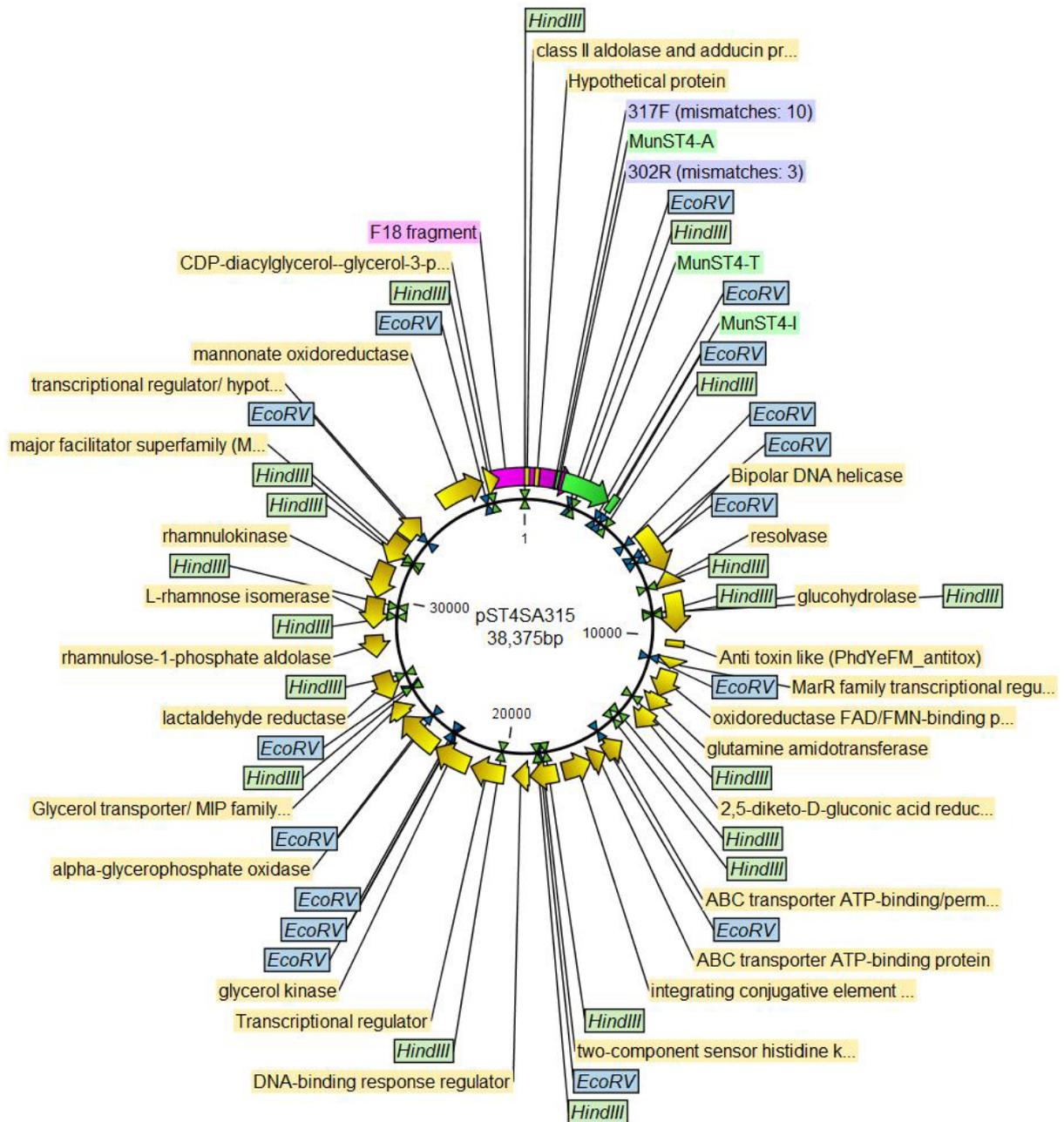


Figure 3.9 – The pST4SA315 plasmid harboring the *munST4* operon and putative annotations of coding sequences.

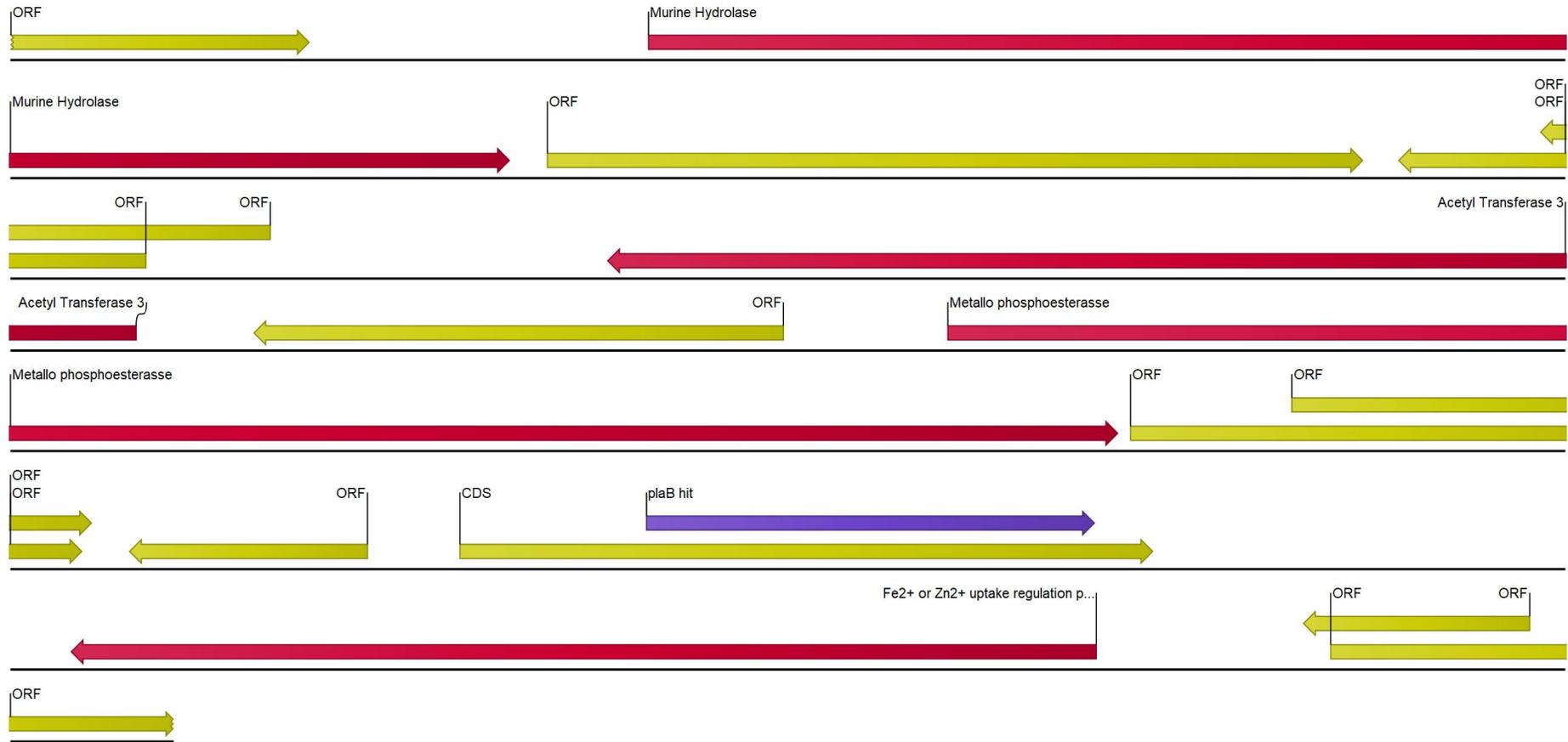


Figure 3.10 -Alignment of the plaB immunity protein for plantaricin 423, to a second location within the unassembled genome of *L. plantarum* 423. Upstream of the second region of homology, metallo phosphoesterase and downstream an iron or zinc uptake regulatory protein.

References

1. Nissen-Meyer, J., Rogne, P., Oppedgaard, C., Haugen, H. & Kristiansen, P. Structure-function relationships of the non-lanthionine-containing peptide (class II) bacteriocins produced by Gram-positive bacteria. *Curr. Pharm. Biotechnol.* 10, 19–37 (2009).
2. Cui, Y. et al. Class IIa bacteriocins: diversity and new developments. *Int. J. Mol. Sci.* 13, 16668–16707 (2012).
3. Willey, J. M. & van der Donk, W. A. Lantibiotics: peptides of diverse structure and function. *Annu. Rev. Microbiol.* 61, 477–501 (2007).
4. Zhang, Q., Yu, Y., Vélasquez, J. E. & van der Donk, W. A. Evolution of lanthipeptide synthetases. *Proc. Natl. Acad. Sci. U. S. A.* 109, 18361–18366 (2012).
5. Arnison, P. G. et al. Ribosomally synthesized and post-translationally modified peptide natural products: overview and recommendations for a universal nomenclature. *Nat. Prod. Rep.* 30, 108–160 (2013).
6. Sambrook, J., Fritsch, E. F. & Maniatis, T. *Molecular cloning: a laboratory manual*. Mol. cloning a Lab. manual. (1989). at <<https://www.cabdirect.org/cabdirect/abstract/19901616061>>
7. Ausubel, F. M. et al. *Current Protocols in Molecular Biology*. Current protocols in molecular biology / edited by Frederick M. Ausubel ... [et al.] Chapter 2, (John Wiley & Sons, Inc., 2003).
8. Marchler-Bauer, A. et al. CDD/SPARCLE: functional classification of proteins via subfamily domain architectures. *Nucleic Acids Res.* 45, D200–D203 (2017).
9. Aucher, W. et al. Differences in mesentericin secretion systems from two *Leuconostoc* strains. *FEMS Microbiol. Lett.* 232, 15–22 (2004).
10. Oppedgård, C., Fimland, G., Anonsen, J. H. & Nissen-Meyer, J. The pediocin PA-1 accessory protein ensures correct disulfide bond formation in the antimicrobial peptide pediocin PA-1. *Biochemistry.* 54, 2967–2974 (2015).
11. Kalmokoff, M. L., Banerjee, S. K., Cyr, T., Hefford, M. A. & Gleeson, T. Identification of a new plasmid-encoded sec-dependent bacteriocin produced by *Listeria innocua* 743. *Appl. Environ. Microbiol.* 67, 4041–4047 (2001).
12. du Plessis, D. J. F., Nouwen, N. & Driessen, A. J. M. The Sec translocase. *Biochim. Biophys. Acta* 1808, 851–865 (2011).
13. Diep, D. B., Axelsson, L., Grefslis, C. & Nes, I. F. The synthesis of the bacteriocin sakacin A is a temperature-sensitive process regulated by a pheromone peptide through a three-component regulatory system. *Microbiology.* 146, 2155–2160 (2000).

14. Gursky, L. J. et al. Production of piscicolin 126 by *Carnobacterium maltaromaticum* UAL26 is controlled by temperature and induction peptide concentration. *Arch. Microbiol.* 186, 317–325 (2006).
15. Quadri, L. E. et al. Characterization of a locus from *Carnobacterium piscicola* LV17B involved in bacteriocin production and immunity: evidence for global inducer-mediated transcriptional regulation. *J. Bacteriol.* 179, 6163–6171 (1997).
16. Saucier, L., Paradkar, A. S., Frost, L. S., Jensen, S. E. & Stiles, M. E. Transcriptional analysis and regulation of carnobacteriocin production in *Carnobacterium piscicola* LV17. *Gene* 188, 271–277 (1997).
17. Quadri, L. E. N., Yan, L. Z., Stiles, M. E. & Vederas, J. C. Effect of amino acid substitutions on the activity of Carnobacteriocin B2. *J. Biol. Chem.* 272, 3384–3388 (1997).
18. Quadri, L. E., Yan, L. Z., Stiles, M. E. & Vederas, J. C. Effect of amino acid substitutions on the activity of carnobacteriocin B2. Overproduction of the antimicrobial peptide, its engineered variants, and its precursor in *Escherichia coli*. *J. Biol. Chem.* 272, 3384–3388 (1997).
19. Hanas, J. S., Hazuda, D. J., Bogenhagen, D. F., Wu, F. Y. & Wu, C. W. Xenopus transcription factor A requires zinc for binding to the 5 S RNA gene. *J. Biol. Chem.* 258, 14120–14125 (1983).
20. Malgieri, G. et al. Zinc to cadmium replacement in the prokaryotic zinc-finger domain. *Metalomics.* 6, 96–104 (2014).
21. Kanatani, K., Oshimura, M. & Sano, K. Isolation and characterization of acidocin A and cloning of the bacteriocin gene from *Lactobacillus acidophilus*. *Appl. Environ. Microbiol.* 61, 1061–1067 (1995).
22. Métivier, A. et al. Divercin V41, a new bacteriocin with two disulphide bonds produced by *Carnobacterium divergens* V41: primary structure and genomic organization. *Microbiology.* 144 Pt 1, 2837–2844 (1998).
23. Farías, M. E., Farías, R. N., De Ruiz Holgado, A. P. & Sesma, F. Purification and N-terminal amino acid sequence of enterocin CRL 35, a ‘pediocin-like’ bacteriocin produced by *Enterococcus faecium* CRL 35. *Lett. Appl. Microbiol.* 22, 417–419 (1996).
24. Arbulu, S. et al. Solution Structure of enterocin HF, an antilisterial bacteriocin produced by *Enterococcus faecium* M3K31. *J. Agric. Food Chem.* 63, 10689–10695 (2015).
25. Feng, G., Guron, G. K. P., Churey, J. J. & Worobo, R. W. Characterization of mundticin L, a class IIa anti-*Listeria* bacteriocin from *Enterococcus mundtii* CUGF08. *Appl. Environ. Microbiol.* 75, 5708–5713 (2009).
26. Jack, R. W. et al. Characterization of the chemical and antimicrobial properties of piscicolin 126, a bacteriocin produced by *Carnobacterium piscicola* JG126. *Appl. Environ. Microbiol.* 62, 2897–2903 (1996).

27. Tichaczek, P. S., Vogel, R. F. & Hammes, W. P. Cloning and sequencing of *sakP* encoding sakacin P, the bacteriocin produced by *Lactobacillus sake* LTH 673. *Microbiology*. 140 Pt 2, 361–7 (1994).
28. Heng, N. C. K., Burtenshaw, G. A., Jack, R. W. & Tagg, J. R. Ubericin A, a class IIa bacteriocin produced by *Streptococcus uberis*. *Appl. Environ. Microbiol.* 73, 7763–7766 (2007).
29. Aymerich, T. et al. Biochemical and genetic characterization of enterocin A from *Enterococcus faecium*, a new antilisterial bacteriocin in the pediocin family of bacteriocins. *Appl. Environ. Microbiol.* 62, 1676–1682 (1996).
30. Hastings, J. W. et al. Characterization of leucocin A-UAL 187 and cloning of the bacteriocin gene from *Leuconostoc gelidum*. *J. Bacteriol.* 173, 7491–7500 (1991).
31. Makhloufi, K. M. et al. Characterization of leucocin B-KM432Bz from *Leuconostoc pseudomesenteroides* isolated from boza, and comparison of its efficiency to pediocin PA-1. *PLoS One*. 8, e70484 (2013).
32. Fremaux, C., Yann, H. & Cenatiempo, Y. Mesentericin Y105 gene clusters in *Leuconostoc mesenteroides* Y105. *Microbiology*. 141, 1637–1645 (1995).
33. Marugg, J. D. et al. Cloning, expression, and nucleotide sequence of genes involved in production of pediocin PA-1, a bacteriocin from *Pediococcus acidilactici* PAC1.0. *Appl. Environ. Microbiol.* 58, 2360–2367 (1992).
34. Van Reenen, C. A., Chikindas, M. L., Van Zyl, W. H. & Dicks, L. M. T. Characterization and heterologous expression of a class IIa bacteriocin, plantaricin 423 from *Lactobacillus plantarum* 423, in *Saccharomyces cerevisiae*. *Int. J. Food Microbiol.* 81, 29–40 (2003).
35. Simon, L., Fremaux, C., Cenatiempo, Y. & Berjeaud, J. M. Sakacin G, a new type of antilisterial bacteriocin. *Appl. Environ. Microbiol.* 68, 6416–6420 (2002).
36. De Kwaadsteniet, M., Fraser, T., Van Reenen, C. A. & Dicks, L. M. T. Bacteriocin T8, a novel class IIa sec-dependent bacteriocin produced by *Enterococcus faecium* T8, isolated from vaginal secretions of children infected with human immunodeficiency virus. *Appl. Environ. Microbiol.* 72, 4761–4766 (2006).
37. Quadri, L. E., Sailer, M., Roy, K. L., Vederas, J. C. & Stiles, M. E. Chemical and genetic characterization of bacteriocins produced by *Carnobacterium piscicola* LV17B. *J. Biol. Chem.* 269, 12204–12211 (1994).
38. Haugen, H. S., Kristiansen, P. E., Fimland, G. & Nissen-Meyer, J. Mutational analysis of the class IIa bacteriocin curvacin A and its orientation in target cell membranes. *Appl. Environ. Microbiol.* 74, 6766–73 (2008).
39. Herranz, C. & Driessen, A. J. M. Sec-mediated secretion of bacteriocin enterocin P by *Lactococcus lactis*. *Appl. Environ. Microbiol.* 71, 1959–63 (2005).

40. Eguchi, T. et al. Isolation and characterization of enterocin SE-K4 produced by thermophilic enterococci, *Enterococcus faecalis* K-4. Biosci. Biotechnol. Biochem. 65, 247–253 (2001).
41. Holck, A., Axelsson, L., Birkeland, S. E., Aukrust, T. & Blom, H. Purification and amino acid sequence of sakacin A, a bacteriocin from *Lactobacillus sake* Lb706. J. Gen. Microbiol. 138, 2715–2720 (1992).

Chapter 4

Heterologous expression and purification of active plantaricin 423 and mundticin ST4SA using a novel GFP-fusion expression system

Abstract

Aim: This study aimed to heterologously expression and purification of mature plantaricin 423 and mundtacin ST4SA for application in transcriptional regulation analysis of the *plaA* gene.

Methods: Mature plantaricin 423 and mundtacin ST4SA were fused to the C-terminal of green fluorescent protein (GFP) and heterologously overexpressed in *E. coli* BL21 (DE3) using the inducible T7 promoter. Fermentation of the fusion constructs PlantEx, and MunEx were optimized, extracted and purified using Immobilised Metal Affinity Chromatography. Semi-purified PlantEx and MunEx were weighed off after lyophilization, purity was then estimated with SDS-PAGE to estimate concentrations. Liberated plantaricin 423 and mundtacin ST4SA were spot-tested against *Listeria monocytogenes*.

Conclusion: Two class IIa bacteriocins, plantaricin 423 and mundtacin ST4SA, were successfully expressed using the GFP-fusion-based expression system. Furthermore, GFP-bacteriocin concentration could be measured in a semi-quantifiable manner showing comparable production to previously used expression systems for class IIa bacteriocins to previous class IIa expression systems.

Introduction

Class IIa bacteriocins are made up of three domains; the double-glycine-type leader, N-terminal pediocin box and pore-forming C-terminus (Figure 4.1). Each domain is responsible for a different step in achieving antimicrobial activity via membrane poration. The bacteriocin structural gene encodes a double-glycine-type leader fused to the N-terminus of the core peptide to produce a pre-bacteriocin. The double-glycine-type leader functions as a secretory signal that is recognized by the operon-encoded ABC transporter. Cleavage of the leader peptide during bacteriocin transmembrane translocation is mediated by the ABC transporter, which results in mature extracellular bacteriocin¹⁻³. Although the peptide is termed mature, it is unstructured in aqueous solution^{1,4}. Only upon interaction with a membrane does the N-terminal pediocin box form a cationic three-stranded anti-parallel β -sheet-like structure, stabilized by a conserved disulfide bond⁴⁻⁹. The structured N-terminus mediates bacteriocin binding to the target cell wall via docking to an extracellular loop of the IIC protein (MptC) from the sugar transporter mannose phosphotransferase system (Man-PTS)^{4,10,11}. The C-terminus then forms a hairpin-like domain that penetrates the hydrophobic core of the target cell membrane^{1,2,4}. Between the N- and C-termini there is a flexible hinge which allows the C-terminus to transverse the cell membrane while the N-terminus associates with the MptC^{3,4,12,13}. Aggregation of

the class IIa bacteriocin within a target cell wall results in pore formation and subsequent cell death^{14–16}. These pores cause leakage of essential metabolites, protons (H⁺) and charged ions which dissipates the target cell's transmembrane potential ($\Delta \psi$) and pH gradient (ΔpH)^{14–16}. Pediocin PA-1 also causes the efflux of 2- α -aminoisobutyric acid (AIB) a nonmetabolizable analog of alanine, and L-glutamate¹⁴. Therefore, cell death occurs via the depletion of intracellular ATP in an attempt to restore the proton motive force, and metabolite starvation.

Lactobacillus plantarum 423 and *Enterococcus mundtii* ST4SA produce the class IIa bacteriocins plantaricin 423 and mundticin ST4SA, respectively (Table 4.1). *Lactobacillus plantarum* 423 was isolated from sorghum beer and harbors the plantaricin 423 pre-bacteriocin gene, *plaA*, on the pPLA4 plasmid (Addendum, Figure 7.1)^{17,18}. Plantaricin 423 shows inhibition activity toward food-borne pathogens such as *Bacillus cereus*, *Clostridium sporogenes*, *E. faecalis*, *Listeria* spp. and *Staphylococcus* spp.¹⁸. *Enterococcus mundtii* ST4SA was isolated from soybeans and harbors the Mundticin ST4 pre-bacteriocin gene *munST4SA*, found on the pST4SA315 plasmid (Chapter 3, Figure 3.). Mundticin ST4 is active against *E. faecalis*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *L. monocytogenes*¹⁹.

Table 4.1 – Biochemical properties of plantaricin 423 and mundticin ST4SA

Bacteriocin (Producer)	Nucleotide Accession no.	Protein Accession no.	Peptide size (aa)	MP* size (aa)	MP Mass# (Da)	Ref
Plantaricin 423 (<i>L. plantarum</i> 423)	AF304384.2	AAL09346.1	56	37	3928.74	¹⁸
Mundticin ST4SA (<i>E. mundtii</i> ST4SA)	Not available	Not available	59	43	4285.10	¹⁹
*MP = Mature peptide # Theoretical mass (including the formation of disulfide bridges)						

Plantaricin 423 has two C-terminal cysteines while mundtacin ST4SA/KS has a C-terminal tryptophan (Figure 4.1). Therefore, plantaricin 423 has the potential to form two disulfide bonds while mundtacin ST4SA can only form one.

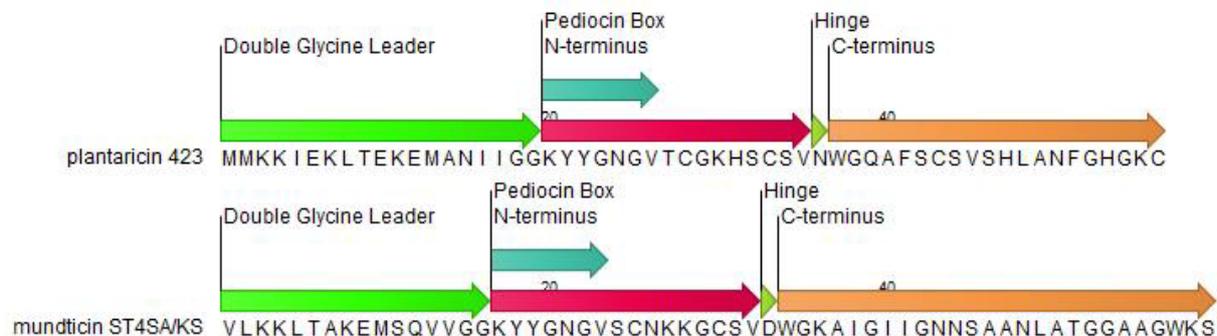


Figure 4.1. Pre-bacteriocin amino acid sequences for Plantaricin 423 and Mundtacin ST4. The following domains are seen; double glycine leader (Green), N-terminus (Maroon), conserved YGNGV motif or Pediocin Box (Light Blue), Flexible hinge (Light green) and the pore forming C-terminus (orange).

Like many other class IIa bacteriocins, plantaricin 423 and mundtacin ST4SA have previously been purified from the native producer's supernatant via a multi-step purification process¹⁸. The peptides are usually chromatographically purified by taking advantage of their cationic and hydrophobic properties²⁰. Not only is this process long, but it often results in poor or variable yields due to the inducible nature of bacteriocins²⁰⁻²². Results from this approach may be further complicated by the co-purification of contaminants such as APs, which are especially problematic when investigating inducible bacteriocin promoters.

Many class IIa bacteriocins have been produced using heterologous expression systems. These systems aim to improve the large-scale production and rapid purification of class IIa bacteriocins. Improvements in heterologous bacteriocin production may allow them to have more significant application in research as well as the food preservation and pharmaceutical industry. Purification of N-terminal His-tagged Pediocin PA-01 expressed in *E. coli* resulted in low concentrations of biologically active bacteriocin²³. The majority of the His-tagged pediocin was found in the insoluble fraction, most likely due to the bacteriocin's solubility, size and the toxic effect on recombinant *E. coli*²³. Similar results were obtained when N-terminal His-tagged plantaricin 423 and mundtacin ST4SA were expressed in *E. coli* BL21 (DE3) (data not shown).

The yields of heterologously expressed class IIa bacteriocins are significantly improved when the mature peptide is fused to a larger, more soluble protein²⁴⁻²⁹. Although class IIa bacteriocins fused to thioredoxin are not secreted, this system produces the highest recorded yields ranging from 20-320

mg pure bacteriocin per liter of culture^{28,29}. Thioredoxin is an 11 675 Da highly soluble protein, which has a rigid solvent-accessible α -helix and can accumulate up to 40% of the total cellular protein in *E. coli*^{30–32}. Due to its size, solubility and cellular localization, thioredoxin aids in the expression and purification of class IIa bacteriocins^{29–31}. Thioredoxin-bacteriocin fusion proteins were found in the soluble fraction of *E. coli* which indicates lower toxicity and results in simpler extraction, higher yields and better protein folding^{28,29}.

The Green Fluorescent Protein (GFP) gene, *mgfp5*, encodes a 26 kDa protein that forms a β -cannylindrical structure made up of 11 β -sheets surrounding a central axial-like α -helix producing fluorophore^{33,34}. Posttranslational folding of GFP and chromophore formation is autocatalytic and does not require any cofactors except oxygen^{33,34}. The folded protein is stable, non-toxic, soluble and has an excitation-emission autofluorescence at 488 and 509 nm, respectively. These characteristics make GFP applicable in molecular biology as a cell marker, reporter gene or fusion tag^{33,34}. The GFP gene *mgfp5* can be fused to class IIa bacteriocins in the same manner as the thioredoxin gene. Coupling class IIa bacteriocins to GFP may provide many of the same benefits as thioredoxin; with additional benefits such as monitoring protein expression fluorometrically *in vivo* and in real time. Monitorable fluorescence produced by GFP may have significant benefits during industrial production of heterologously expressed proteins. Here the development of a novel GFP-fusion system for the expression of two class IIa bacteriocins, plantaricin 423 and mundticin ST4SA, is presented.

Materials and Methods

Bacterial strain, culture conditions, and molecular cloning

Bacterial strains and culture conditions

The lactic acid bacteria (LAB), *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA were cultured on De Man, Rogosa and Sharpe (MRS) media, at 37 °C without agitation. Luria Bertani medium, supplemented with 1.2% agar for solid medium, was used to culture *Escherichia coli* BL21 (DE3) during molecular cloning protocols. Terrific broth was used for the expression of heterologous proteins in recombinant *E. coli* BL21 (DE3)³⁵. For the selection and maintenance of pRSF-Duet derived plasmids, growth media were supplemented with 50 μ g/mL kanamycin. All growth media were supplied by Merck-Millipore (USA).

Molecular techniques

DNA analysis, manipulation, and plasmid cloning were performed according to Sambrook et al.³⁶. Genomic DNA and plasmid DNA isolations from *L. plantarum* 423 and *E. mundtii* ST4SA were performed according to Moore et al.¹⁹ and O'Sullivan et al.²⁰, respectively. Plasmid DNA extractions were performed using the PureYield™ Plasmid Miniprep System (Promega, Madison, WI, USA) according to the manufacturer's instructions.

T4 DNA ligase and restriction enzymes (RE) were purchased from New England Biolabs (NEB, Ipswich, MA, USA) and used according to the manufacturer's instructions. Polymerase chain reaction (PCR) amplifications were performed using Q5 high-fidelity PCR DNA polymerase (NEB) according to manufacturer's instructions in a GeneAmp PCR system 9700 (ABI, Foster City, CA).

Oligonucleotides were designed using the CLC main workbench program (CLC bio, Aarhus, Denmark) and purchased from Inqaba Biotechnical Industries (Pretoria, South Africa). DNA sequencing was performed by the Central Analytical Facilities (CAF) at the University of Stellenbosch, South Africa.

Agarose gel electrophoresis was used for the analysis and purification of restriction enzyme (RE) digested DNA fragments in TBE buffer at 10V/cm using the Ephortec™ 3000V (Triad Scientific, Manassan United States) power supply³⁷. Excised gel DNA fragments were purified using the Zymoclean™ gel DNA recovery kit (Zymo Research Corporation, Irvine, CA, USA).

Construction of pRSF-PlantEX and pRSF-MunEX expression vectors

GFP-bacteriocin fusion plasmid *in silico* map construction and primer design

The GFP-bacteriocin expression system constructed in this study is represented in Figure 4.2. The pRSFDuet-1 (Novagen) vector was used for the β -D-1-thiogalactopyranoside (IPTG) induction of the heterologous expression of the His-tagged GFP-bacteriocin fusion proteins in this study³⁵. The N-terminal of *mgfp5* (GFP) was fused to a hexahistidine tag in pRSFDuet which was under the transcriptional control of the T7 promoter (Figure 4.2). The double-glycine leader sequences from mundtacin ST4SA and plantaricin 423 were excluded so that the mature peptide's N-terminus was fused to the C-terminus of GFP. The WELQut protease (Thermo-Fisher Scientific, USA) amino acid cleavage sequence was introduced between GFP and the respective mature peptide sequence (Figure 4.2). This site allowed for posttranslational cleavage of the mature bacteriocins using the WELQut protease.

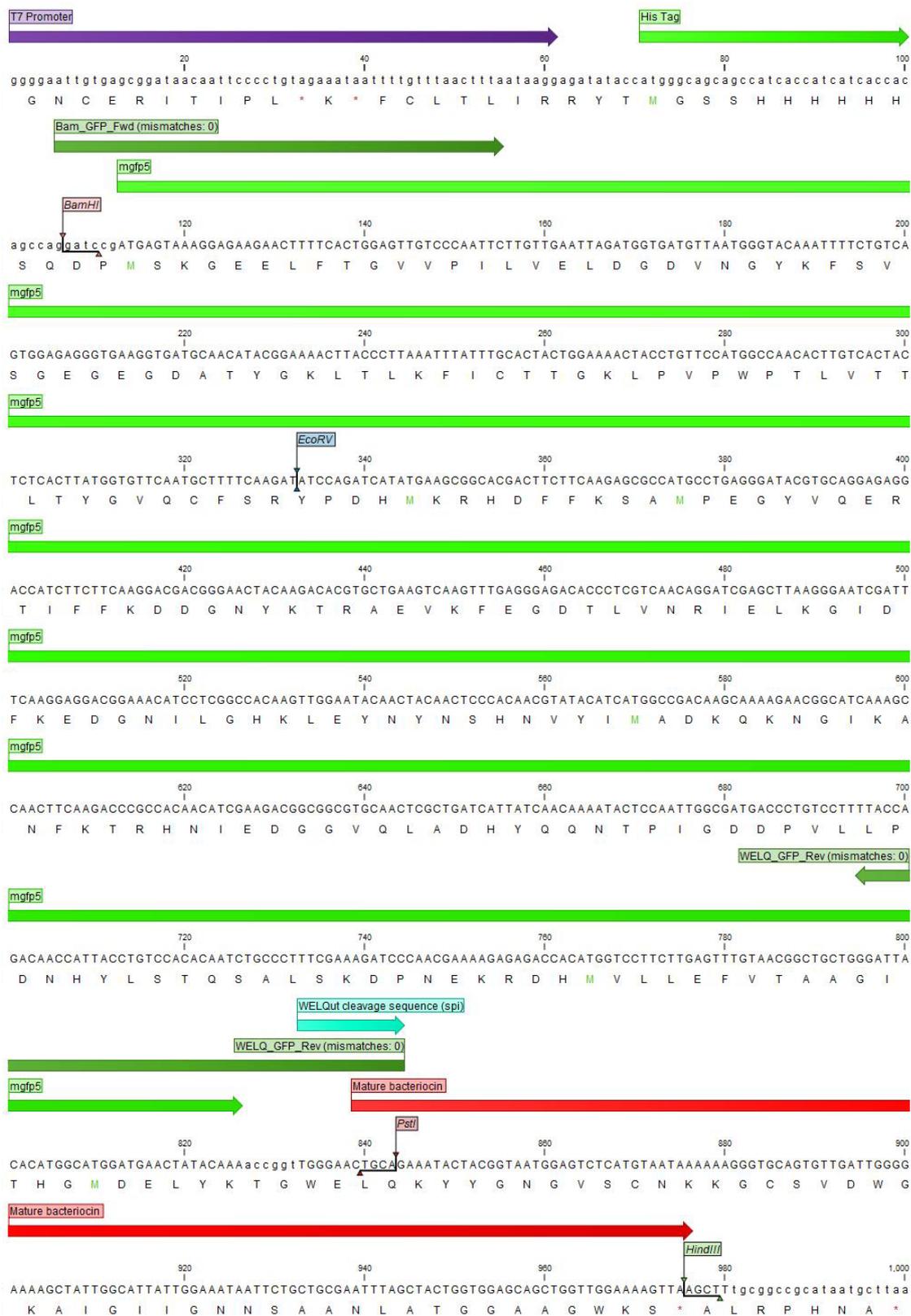


Figure. 4.2 *In silico* template for the his-tagged GFP expression system with WELQ amino acid sequence for post translational liberation of the bacteriocin of interest using the WELQut protease.

The GFP_Bam_Fwd forward primer installed a *Bam*HI site 5' of *mgfp5* (GFP gene) for cloning into pRSFDuet (Table 4.4). The GFP_WELQ_Rev reverse primer extended the *mgfp5* sequence with an *Age*I restriction site followed by the DNA sequence encoding the WELQ amino acid sequence (recognition sequence for WELQ protease), followed by a *Pst*I site (Figure 4.2). The PlantEX_Pst_Fwd/PlantEx_Hind_Rev and MunEx_Pst_Fwd/MunEx_Hind_Rev primer sets annealed to the mature plantaricin 423 (*plaA*) and mundtacin ST4 (*munST4SA*) gene sequences, respectively (Table 4.4). These primer sets added 5' *Pst*I and 3' *Hind*III restriction sites for cloning mature plantaricin 423 (*plaA*) or mundtacin ST4SA (*munST4SA*) genes as GFP fusions in pRSFDuet, represented as the "mature bacteriocin" in Figure 4.2.

Construction of the plantaricin 423 and mundtacin ST4SA GFP-bacteriocin expression vectors

The pTRKH3p15A-ErmGFP plasmid was used as a template for the PCR amplification of *mgfp5* using the GFP_Bam_Fwd/ GFP_WELQ_Rev primer set (Table 4.4). The *mgfp5* amplicon was cloned using the cloneJET PCR cloning system according to the manufacturer's instructions (Thermo Scientific, USA). Plasmid DNA was extracted from ampicillin resistant transformants and sequenced using the pJET1.2_Fwd and pJET1.2_Rev primer set (Table 4.4).

Genomic DNA from *L. plantarum* 423 and *E. mundtii* ST4SA was used as templates to amplify mature plantaricin 423 and mundtacin ST4SA bacteriocin genes using the PlantEX_Pst_Fwd/PlantEx_Hind_Rev and MunEx_Pst_Fwd/MunEx_Hind_Rev primer sets respectively (Table 4.4). The amplified bacteriocin genes were purified using the GeneJet PCR purification kit (Thermo Scientific) and digested with *Pst*I and *Hind*III. The digestion mixtures were purified again using the GeneJet PCR purification kit according to the manufacturer's instructions and used in subsequent cloning experiments.

The pJET-GFP plasmid was digested with *Bam*HI/*Pst*I; the pRSFDuet vector was digested with *Bam*HI/*Hind*III. The linear pRSFDuet vector and digested GFP fragment were purified using agarose gel electrophoresis, gel-excised and recovered using the Zymoclean Gel DNA Recovery kit (Zymo Research Corporation, Irvine, CA, USA). In one single ligation reaction, the *Bam*HI/*Hind*III GFP fragment and respective *Pst*I/*Hind*III bacteriocin fragment was ligated into the linear pRSFDuet vector. The fragments were ligated using a V:l:l molar end ratio of 1:3:3. The resulting constructs, pRSF-PlantEX, and pRSF-MunEX, respectively, were used to transform chemically competent *E. coli* BL21 (DE3) cells. The pRSF-PlantEX and pRSF-MunEX plasmids were extracted from phenotypically green fluorescent, kanamycin resistant colonies of *E. coli* BL21 (DE3). The mature plantaricin 423 and mundtacin ST4SA genes were sequenced in pRSF-PlantEx and pRSF-MunEx plasmids using the MCS1_Rev primer and

confirmed to be correct (Table 4.4). Plantaricin 423 or mundticin ST4SA fused to the C-terminus of His-tagged GFP will be referred to as the PlantEx and MunEx proteins, respectively, from here on.

Expression and purification protocols

Overexpression of GFP-bacteriocin fusion proteins in *E. coli* BL21 (DE3)

Starter cultures of 30 mL LB broth containing 50 µg/mL kanamycin were inoculated with respective *E. coli* BL21 (DE3) transformants containing pRSF-PlantEx or pRSF-MunEx constructs. The starter cultures were incubated at 37 °C for 12 h with constant agitation. Starter cultures were used as an inoculum for the expression of PlantEx and MunEx respectively (1% v/v). At an OD₆₀₀ of 0.6-0.65, expression of the respective GFP fusion proteins was induced using 0.1 mM IPTG.

Ni-NTA Purification of MunEx and PlantEx proteins

Extraction of the overexpressed GFP-bacteriocin proteins, PlantEx and MunEx, was performed according to a modified protocol from Shi *et al.*³⁵. Induced cells were harvested by centrifugation at 8 000 g for 20 min at 4 °C. The supernatant was discarded, and the cell pellet was resuspended in 15 mL/g wet weight SB buffer supplemented with 1 mg/mL lysozyme and incubated with agitation at 8 °C for 45 min (Table 4.3). After incubation, the lysed cells were subjected to sonication (50% amplitude, 2s pulse, 2s pause, 6min) using the Omni Ruptor 400 (Ultrasonic Homogenizer, Omni International Inc, Kennesaw, GA). RNaseI and DNaseI were added to a final concentration of 10 µg/mL and 5 µg/mL, respectively, and the lysate incubated at room temperature for 15 min. The cell lysate was then centrifuged for 90 min at 20 000 g at 4 °C; the cell-free supernatant was collected. Imidazole was added to the cell-free supernatant to a final concentration of 10 mM.

The His-tagged GFP-bacteriocin fusion proteins, PlantEx and MunEx, were purified with immobilized metal affinity chromatography (IMAC) using the Ni-NTA superflow resin (Qiagen, Germany), according to the Qiagen expressionist handbook's instructions for batch purification. His-tagged proteins were purified using 25 mL of Qiagen Ni-NTA superflow resin in combination with a 26 mm diameter adjustable length flash column (Glasschem, Stellenbosch, South Africa). The Ni-NTA superflow resin was equilibrated in SB10 (SB buffer containing 10 mM imidazole) buffer and then added directly to the cell-free supernatant. The slurry was gently agitated for 2 h at 8 °C using a shaker, after which the Ni-NTA superflow resin slurry was packed into the column. The ÄKTA purifier system (Amersham, Biosciences) was used for IMAC purification according to the following program; 5 column volumes

(CV) SB10 (2% B buffer where A is SB and B is SB500), washed with 10 CV of SB20 (4% B buffer), elution occurred in approximately 40 mL of SB500 (100% B buffer) (Table 4.3). Eluted proteins were detected at 254 nm and 280 nm respectively. Eluted His-tagged proteins were desalted using size exclusion chromatography. The ÄKTA purifier system was used in conjunction with Sephadex G25 resin packed into a chromatography column (GE Healthcare Technologies) for exchanging the sample from SB500 to WELQut cut buffer (Table 4.3) at a flow rate of 1 mL/min.

Incubation temperature optimization for MunEx expression

Due to the high cost of the WELQut protease, only the MunEx fermentation was temperature optimized as cleaved MunEx had a higher volume-specific activity. The MunEx expression temperature optimization was performed at 18, 26 and 37 °C with three biological repeats of *E. coli* BL21 (DE3) harboring the pRSF-MunEx plasmid. The three biological repeats of *E. coli* pRSF-MunEx were used to inoculate three 400 mL flasks of terrific broth containing 50 µg/mL kanamycin. The cultures were grown at 37 °C until induction using 0.1 mM IPTG at an OD_{600nm} of 0.6-0.65. Each 400 mL culture was split into three 100 mL cultures that were incubated at 18, 26 and 37 °C for 48 h, respectively.

To measure *in vivo* MunEx expression, 1 mL samples from each flask were collected in triplicate at the time of induction; samples were collected again at 24 and 48 h and frozen at -80 °C. After collection, samples were thawed, centrifuged and washed twice with potassium phosphate buffer (pH 7.4). The *in vivo* expression of MunEx was fluorometrically measured in relative fluorescent units (RFUs) at 488 nm (excitation) and 509 nm (emission) using a Tecan Spark M10™ (Tecan Group Ltd., Austria).

After 48 h, the total MunEx RFU production for each sample was calculated by harvesting the induced cells from 80 mL of culture (centrifugation 8000 x g for 20 min at 4 °C). The mass of each cell pellet was then measured before resuspension in 15 mL/g wet weight SB buffer (Table 4.3). The MunEx in each sample was extracted and purified using IMAC as described previously. Briefly, 5 mL from each cell-free lysate was purified using 5 ml Ni-NTA Superflow cartridges (Qiagen). The RFUs of each Ni-NTA purified MunEx sample was measured using the Tecan M10 Spark™ (Tecan Group Ltd., Austria). The RFUs/g were then calculated for each sample by dividing the measured RFUs by the equivalent wet weight (g) of cells purified (i.e., in 5mL). The total RFUs produced for each sample was calculated by multiplying the RFUs/g by the total wet weight of cells in each sample.

Upscaled production of PlantEx and MunEx

Upscaled heterologous expression of the plantaricin 423 and mundticin ST4SA GFP fusion proteins was performed using a 5 L fermenter (Minifors, Infors AG, CH - 4103 Bottmingen/Basel, Switzerland). Terrific broth (2.7L), containing 0.005% antifoam 204 (Sigma-Aldrich), was prepared and autoclaved. Once cool, 300 mL of sterile 10x TB buffer and kanamycin (50 µg/mL final concentration) was aseptically added (Table 4.3). The broth was heated to 37 °C, aerated at 1 L/min with sterile compressed air and stirred at 300 RPM. The pH and dissolved oxygen levels were not controlled.

The starter cultures of pRSF-PlantEx and pRSF-MunEx *E. coli* BL21 (DE3) were used as an inoculum at 1% v/v, for respective expressions. At an OD_{600nm} of 0.6 – 0.65, expression of the respective GFP fusion proteins was induced using 0.1 mM IPTG (Sigma-Aldrich). Respective fermentations were then cooled to 18 °C and incubated for 48 h. PlantEx and MunEx proteins were extracted and purified as previously described.

Concentration estimation

Three 1 mL samples from the Ni-NTA purified PlantEx and MunEx were lyophilized, and the residual mass weighed off in triplicate to estimate total protein mass. The purified PlantEx and MunEx samples were electrophoretically separated using tricine SDS-PAGE to estimate sample purity³⁸. To avoid saturation during Coomassie staining the 10 x dilutions of PlantEx and MunEx were used to estimate protein purity (Appendix, Figure 4.6). Gel analyzer 2010a (<http://www.gelanalyzer.com/>) was used to determine the pixel density of each stained band in respective lanes and used to estimate sample purity³⁹.

WELQut cleavage optimization

Cleavage parameters were optimized using a modification of the method supplied with the WELQut protease by Thermo Scientific. The WELQut-to-sample ratios were set to 1:100, 1:50, 1:25, 1:5 (v/v) for 50 µL samples of MunEx and PlantEx respectively and diluted to a final volume of 250 µL in WELQut cut buffer (Table 4.3). Cleavage reactions were incubated at 28 °C, and 50 µL samples were collected at 2, 4, 8, and 16 h, respectively. Cleavage was assessed by the spot plate method using BHI solid medium seeded with *Listeria monocytogenes* (Ref). Samples from 16 h cleavage reactions were also analyzed using tricine SDS-PAGE electrophoresis as previously described.

Liquid chromatography-mass spectrometry (LCMS)

The Ni-NTA purified PlantEx and MunEx samples, respectively, were manually injected onto a Discovery BIO Wide Pore C18 High Pressure Liquid Chromatography (HPLC) column (10 µm, 250 x 10 mm, Sigma-Aldrich). The chromatographic system comprised out of two Waters 510 pumps, controlled by MAXIMA software. The Waters 440 detector recorded column effluent at 254 nm during hydrophobic separation over a linear gradient of 10% to 100% solution B in solution A over 12 min (Solution A: de-ionized water containing 0.1% TFA, v/v, and solution B: acetonitrile containing 0.1% TFA, v/v).

Statistical analysis

Analysis of variance (ANOVA) to determine the statistical significance of sample data was performed using Graph Prism Version 3.0 (Graph Pad Software, San Diego, CA).

Results

Incubation temperature optimization for MunEx expression

Significantly higher fluorescence was measured at 18, and 26 °C compared to 37 °C, while RFUs were comparable between 24 and 48 h for all three incubation temperatures as determined by ANOVA (Figure 4.3). However, these *in vivo* measurements do not consider total wet cell weight and therefore do not represent total RFU produced at each temperature. For measurement of total RFUs, the following calculation was used; Total RFU = (RFUs of Ni-NTA purified eluent/wet cell weight used for purification) x (total wet cell weight) (Table 4.2). Total RFU production for MunEx is represented in Figure 4.4 (Appendix), where the highest recorded RFUs were produced at 18 °C, confirmed using ANOVA.

Green fluorescent bands were observed after SDS-PAGE separation, before sample fixing in the gel (Appendix, Figure 4.5A). Fluorescent images were captured using the Minibus Pro DNR camera (Bio-Imaging System Ltd, Israel) after excitation at 302 nm. Stained SDS-PAGE samples are presented in Figure 4.5B (Appendix); bands 1 and 2 both have a fluorescent property while band 3 represents the WELQut enzyme. No stained bands correlating to the size of mundtacin ST4SA were observed in Figure 4.5B (Appendix). However, inhibition zones were observed when the SDS-PAGE gel was overlaid with *L. monocytogenes* seeded medium (Appendix, Figure 4.5C). The biological triplicates grown at 18 and

26 °C all had a fluorescent banding pattern and inhibition zones at a location that correlated with the mature mundtacin ST4SA predicted mass (Appendix, Figure 4.5D). Therefore, fluorescent intensities presented in Figure 4.4 (Appendix) are proportional to the concentration of the MunEx protein.

Upscaled production of PlantEx and MunEx with yield approximation

The *E. coli* pRSF-PlantEx and pRSF-MunEx fermentations were incubated at 18 °C after IPTG induction for 48 h in the 5 L fermenter. After extraction, Ni-NTA purification and buffer exchange of the PlantEx and MunEx proteins, 39 mL of PlantEx and 42 mL of MunEx was obtained. After lyophilization of 1 mL PlantEx and MunEx, 12.96 mg and 17.96 mg residual mass was measured, respectively. PlantEx and MunEx are approximately 72 and 61 % pure (Gel analyzer), with approximate concentrations of 9.33 mg/mL and 10.95 mg/mL, respectively. At these purities, the total yield of PlantEx and MunEx are approximately 121 mg/L of culture and 153 mg/L of culture, respectively.

WELQut cleavage optimization

After cleavage of the mature peptides plantaricin 423 and mundtacin ST4SA, from PlantEx and MunEx respectively, High-Pressure Liquid Chromatography (HPLC) was used to further purify the liberated bacteriocins. Although peaks with anti-listerial activity were obtained from the HPLC separation, all activity was lost after lyophilization and resuspension in 100mM Tris pH 7.5 (data not shown). Whether this is a resuspension problem or an actual loss in the activity must still be determined. Therefore, the Ni-NTA purified eluents of PlantEx and MunEx provided the highest possible concentrations of plantaricin 423 and mundtacin ST4SA for application in Chapter 4. However, HPLC separation was sufficient for use in LC-MS analysis, as discussed below.

The WELQut cleavage reaction was optimized according to the stock concentrations of PlantEx and MunEx respectively. In Figure 4.7 (Appendix), PlantEx and MunEx cleavage reactions were sampled at time intervals of 2, 4, 8, and 16 h for a range of sample: WELQut ($\mu\text{L}/\mu\text{L}$) ratios. PlantEx showed maximal activity at a ratio of 1:25, while the volume of mundtacin spotted was too high, resulting in indifferentiable clear zones (Appendix, Figure 4.7). The optimal cleavage ratio at 28 °C for PlantEx and MunEx cleavage after 16 h was confirmed at a WELQut to sample ratio of 1:10 and 1:25 (v/v) respectively (Appendix, Figure 4.8). Mundtacin ST4SA (10 μL spotted) appears to have higher activity compared to plantaricin 423 (100 μL spotted) as seen in Figure 4.8 (Appendix).

The same samples spotted in Figure 4.8 (Appendix) were analyzed using SDS-PAGE electrophoresis in Figure 4.9. (Appendix) The fluorescent band in Figure 4.9 (Appendix) A and C in column 1 represents uncleaved PlantEx and MunEx respectively. Figure 4.9 B and D represent the PlantEx and MunEx electrophoretic separations after Coomassie staining. Figure 4.9 (Appendix), rows 2 – 5 represent an increasing ratio of the WELQut protease to sample. Figures 4.9 (Appendix) A and C (rows 2 – 5) indicated that cleavage caused a size increase for the fluorescent property of PlantEx and MunEx bands from column 1. The fluorescent intensity of the larger fluorescent band (2 and 5 for PlantEx and MunEx cleavages, respectively) increased as the ratio of WELQut to sample increased (Appendix, Figure 4.8).

LC-MS

Electrospray ionization-MS performed on HPLC-purified samples confirmed the presence of products with masses corresponding to plantaricin 423 and mundticin ST4SA. The observed mass for plantaricin 423 was 3928.91 Da that is in close agreement with the theoretical monoisotopic mass of 3928.74 Da (equivalent to the formation of two disulfide bridges). The observed mass for mundticin ST4SA was 4285.49 Da that is in close agreement with the theoretical monoisotopic mass of 4285.10 Da (equivalent to the formation of one disulfide bridge).

Discussion

In this study we evaluated the efficacy of a GFP-fusion-based expression system for the high yield production of two class IIa bacteriocins belonging to two different subgroups of class IIa (Chapter 3). We have shown successful expression of active bacteriocin using this system and present data on the ease of optimization of expression approaches.

While studies have demonstrated that incubation temperature influences heterologous protein expression levels, no studies, to our knowledge, have considered this for heterologous class IIa bacteriocin expression⁴⁰. Bacteriocins which have been expressed as fusion partners with thioredoxin include pediocin PA-1, carnobacteriocins BM1, and B2, divercin V41, enterocin P and piscicolin 124, with pediocin PA-1 also fused to mouse dihydrofolate reductase and enterocin A fused to cellulose-binding domain^{20,24,26–29,41–43}. These studies performed their expressions at 37 °C or did not specify temperature, while in this study higher expression temperatures were shown to influence the final GFP-bacteriocin yield negatively in terms of RFU output.

Although expression levels of other fusion partners may be adequate at 37 °C, these systems are not as easily or quickly optimizable as the GFP-fusion partner system. Furthermore, when determining the optimal expression temperature, the total wet cell weight must be taken into consideration. *In vivo* measurements of MunEx production indicate that there is no significant difference between the fermentations at 18 and 26 °C (Appendix, Figure 4.3). However, when the total RFUs produced were calculated, nearly 2 times higher yields were obtained at 18 °C as compared to 26 °C, and more than 5 times the fermentation at 37 °C (Appendix, Figure 4.4 and Table 4.2).

PlantEx and MunEx were produced at approximately 121 mg/L of culture and 153 mg/L of culture, respectively. This would theoretically produce 15.08 mg/L and 20,56 mg/L mature plantaricin 423 and mundticin ST4SA upon complete cleavage with the WELQut protease. However, cleavage using the WELQut enzyme at the optimal ratios, of 10:1 and 25:1 for PlantEx and MunEx respectively, is costly and is currently a limiting factor for high scale production. The highest yields of class IIa bacteriocin from heterologous expression were reported using thioredoxin as the bacteriocin fusion partner to carnobacteriocin B2 and BM1 at 320 mg/L of culture ²⁸. However, cyanogen bromide was used to liberate carnobacteriocin B2 at these concentrations and cleavage with cyanogen bromide produces toxic samples that may limit downstream applications.

Thioredoxin can accumulate up to 40% of the total cellular protein in *E. coli* and is highly soluble which promotes correct tertiary structure folding by circumventing inclusion body packaging ³². Furthermore, expressing class IIa bacteriocins with a fusion partner has been shown to reduce toxicity to the *E. coli* expression host ²⁹. The benefits provided by thioredoxin fusion in *E. coli* are also provided by GFP as a fusion partner. As seen with class IIa-thioredoxin fusion, PlantEx and MunEx were purified from the soluble fraction due to the solubility and size of GFP. The larger size of GFP likely quenched the bacteriocins' antimicrobial activity, lowering the bacteriocins' toxic effect and metabolic interference. However, the benefits of GFP include, but are not limited to, real time and *in vivo* visualization during fermentation. Clear visualization was possible throughout the extraction, purification, and analysis processes. The GFP-fused class IIa bacteriocin proteins were visualized during SDS-PAGE electrophoretic separation without staining (Appendix, Figure 4.5 and 4.9). Visualization indicates not only the location of the heterologously expressed proteins but also trends that occur during cleavage and liberation of the bacteriocin using WELQut protease.

Furthermore, loss of the product or its intensity at any stage of the extraction or purification process is immediately noticed. This robust fluorometric property of GFP is due to the isolation of its

fluorescent chromophore by the compact β -can cylindrical structure³³. This cylindrical structure provides resistance to denaturation from heat, sodium dodecyl sulfate (SDS), urea, beta-mercaptoethanol (BME) and dithiothreitol (DTT). However, its integrity is sensitive to pH^{44,45}.

High volume ratios of the WELQut:GFP-bacteriocin sample may be required as the WELQut protease appears to associate with GFP post cleavage (Appendix, Figure 4.5 and 4.9). Optimal spot activity was observed for PlantEx and MunEx at WELQut:sample ratios of 1:10 and 1:25 respectively (Appendix, Figure 4.8). In Figure 4.9A (Appendix), the intensity of the uncleaved PlantEx fluorescent band decreases as the WELQut:sample ratio increases. Lane 6 in Figure 4.9A (Appendix), has no uncleaved PlantEx and the highest spot activity in Figure 4.8. Figure 4.9C (Appendix) indicates that complete cleavage was not achieved for MunEx as lane 6 has a fluorescent band at the location of uncut MunEx. The maximal spotted activity of MunEx was equal for ratios 1:10 and 1:25 in Figure 4.8, the size of these zones is likely due to the mundtacin ST4SA diffusion limit during spot testing.

Although the inhibition zones produced by plantaricin 423 and mundtacin ST4SA should not be directly compared, cleaved MunEx displayed considerably higher activity against *L. monocytogenes* per volume. Although PlantEx was at a lower concentration compared to MunEx, incorrect folding of plantaricin 423 is more likely responsible for the observed difference in activity. Mundtacin ST4SA has two cysteine residues while plantaricin 423 has four (Appendix, Figure 4.1). Mundtacin ST4SA can only form one disulfide bond, and therefore it only has one disulfide bond-mediated tertiary structure conformation. Plantaricin 423, like pediocin PA-1, has four cysteine residues which can form two disulfide bonds and therefore produce three different tertiary structure conformations⁹. The pedC accessory protein is found in the pediocin PA-1 operon and functions as a chaperone-like protein that ensures correct disulfide bond formation in pediocin PA-1⁹. The accessory protein from the *pla* operon, *plaC*, is currently being co-expressed with the PlantEx protein to assess its influence on plantaricin 423 disulfide bond formation and the effect on specific activity. Heterologous co-expression may also be used to elucidate the mechanism that *plaC* uses to ensure correct bond formation.

Green fluorescent protein provides sample visualization as a fusion partner during fermentation, extraction, purification, and analysis will undoubtedly be invaluable in both future research and industrial applications concerning heterologous class IIa bacteriocin expression. However, the extent to which GFP can function as a fusion partner for other cationic peptides should be explored.

Appendix

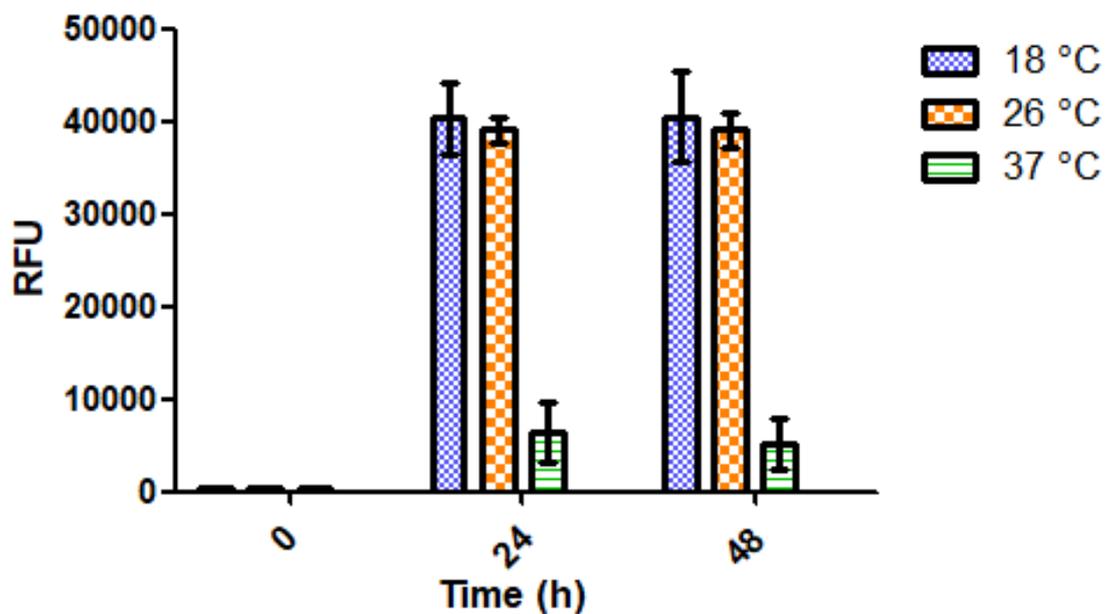


Figure 4.3. Total *in vivo* RFU of *E. coli* pRSF-MunEx expressing MunEx at 18 °C, 26 °C, and 37 °C after 0, 24 and 48 h expression. RFU = Relative fluorescent units.

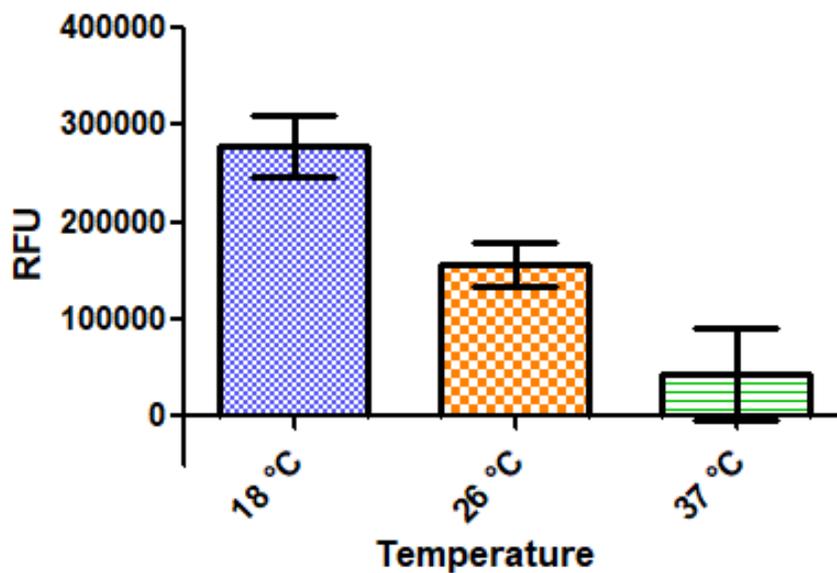


Figure 4.4. Total RFU of Ni-NTA purified samples from fermentation reactions of *E. coli* pRSF-MunEx at 18 °C, 26 °C and 37 °C, respectively, after 48 h. RFU = Relative fluorescent units.

Table 4.2 – Total RFU produced from Ni-NTA purified MunEx temperature optimization fermentations

Sample	Temperature (°C)	WCM* (g)	Resuspension volume	Ni-NTA Eluent fluorescence (RFU)	RFU#/g	Total RFU# produced	Average per temperature	Standard deviation
G18	18	2.47	37.05	33728	101286.2	250177.1	277693.5	31773.05
O18		2.39	35.85	37680	113153.1	270436		
Y18		2.5	37.05	41620	124986.9	312467.5		
G26	26	1.22	18.3	47975	144070.0	175765.5	156734.1	22064.16
O26		1.15	17.25	38381	115260.2	132549.3		
Y26		1.41	21.15	38233	114813.8	161887.5		
G37	37	1.38	11.4	23436	70378.3	97122.16	44340.56	46817.4
O37		0.8	20.7	11684	35089.1	28071.28		
Y37		0.76	12	3430	10300.3	7828.22		

*WCM = Wet cell mass

RFU = Relative fluorescent units

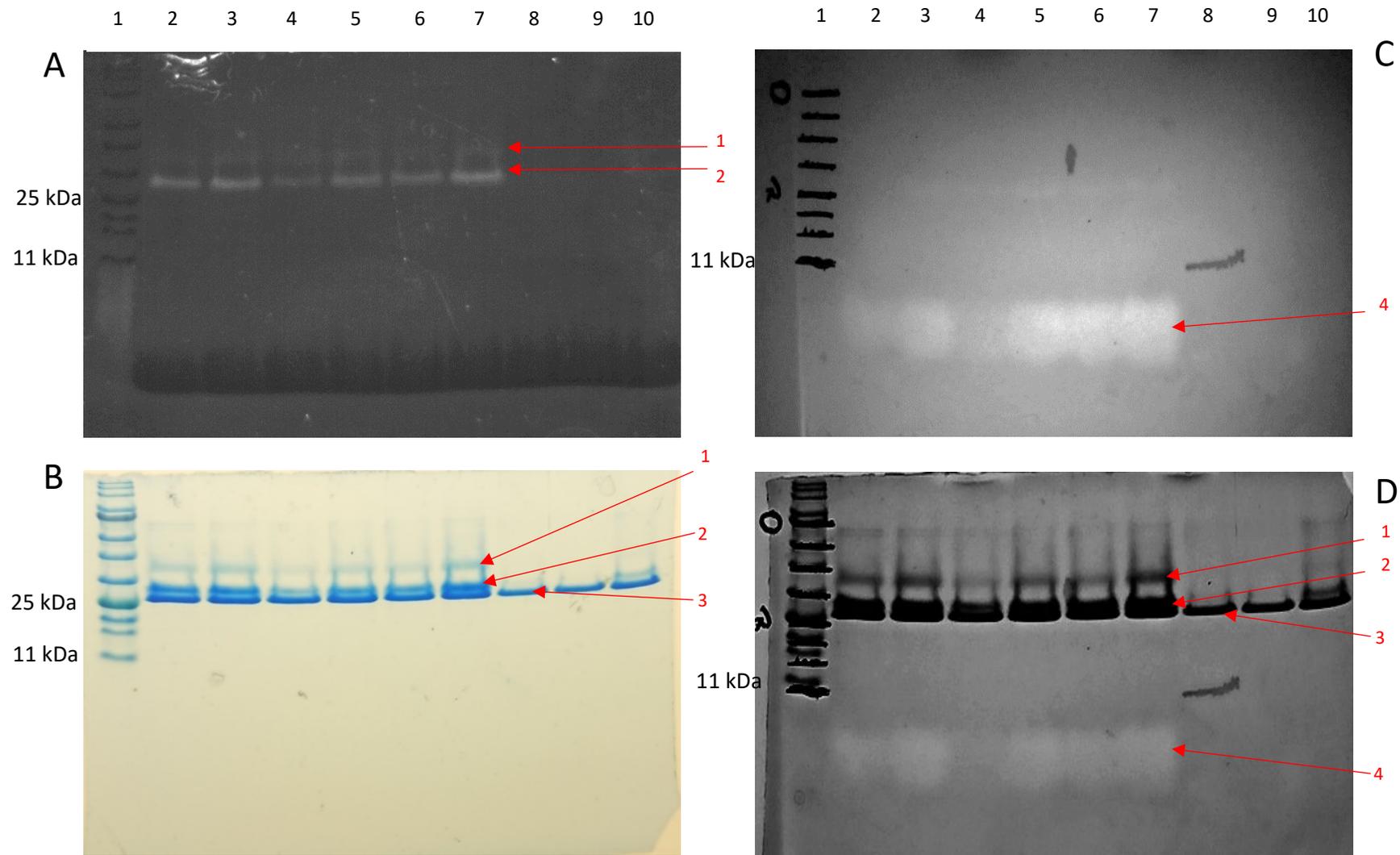


Figure 4.5. SDS-PAGE analysis of Ni-NTA purified MunEx from *E. coli* pRSF-MunEx fermentations at 18, 26 and 37 °C in triplicate. Lane: 1 – Ladder, 2 to 4 – biological triplicate fermentations at 18 °C, 5 to 7 – 26 °C, 8 to 10 – 37 °C. (A) Fluorometrically photographed gel where GFP fluorescence is shown as white bands. (B) Coomassie stained gel. (C) Overlay of gel with agar seeded with *L. monocytogenes* (D) Superimposition of image B on C. Bands: 1 – putative WELQut and GFP complex, 2 – uncleaved MunEx, 3 – WELQut, 4 – clear zone void of *L. monocytogenes*.

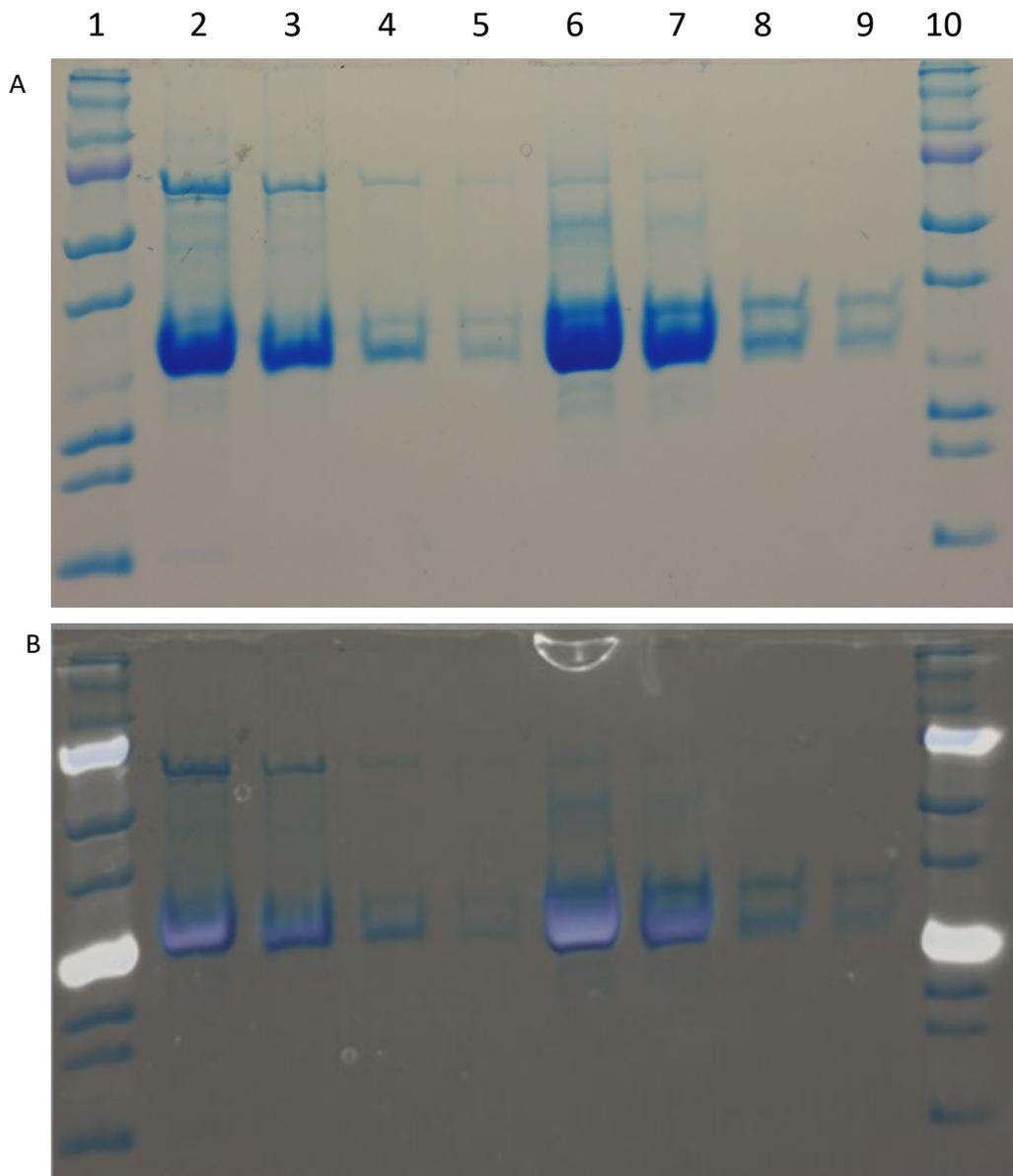


Figure 4.6. (A) Upscale expression and Ni-NTA purification of PlantEx and MunEx electrophoretically separated at various dilutions for purity estimation using Gel analyser. Lane: 1 - Ladder, 2 - PlantEx undiluted, 3 - PlantEx 2x diluted, 4 - PlantEx 10x diluted, 5 - PlantEx 20x diluted, 6 - MunEx undiluted, 7 - MunEx 2x diluted, 8 - MunEx 10x diluted, 9 - MunEx 20x diluted, 10 - Ladder. (B) Gel fluorometrically photographed and image super imposed on the stained gel. The florescence of GFP is observed as white bands in lane 2 – 9.

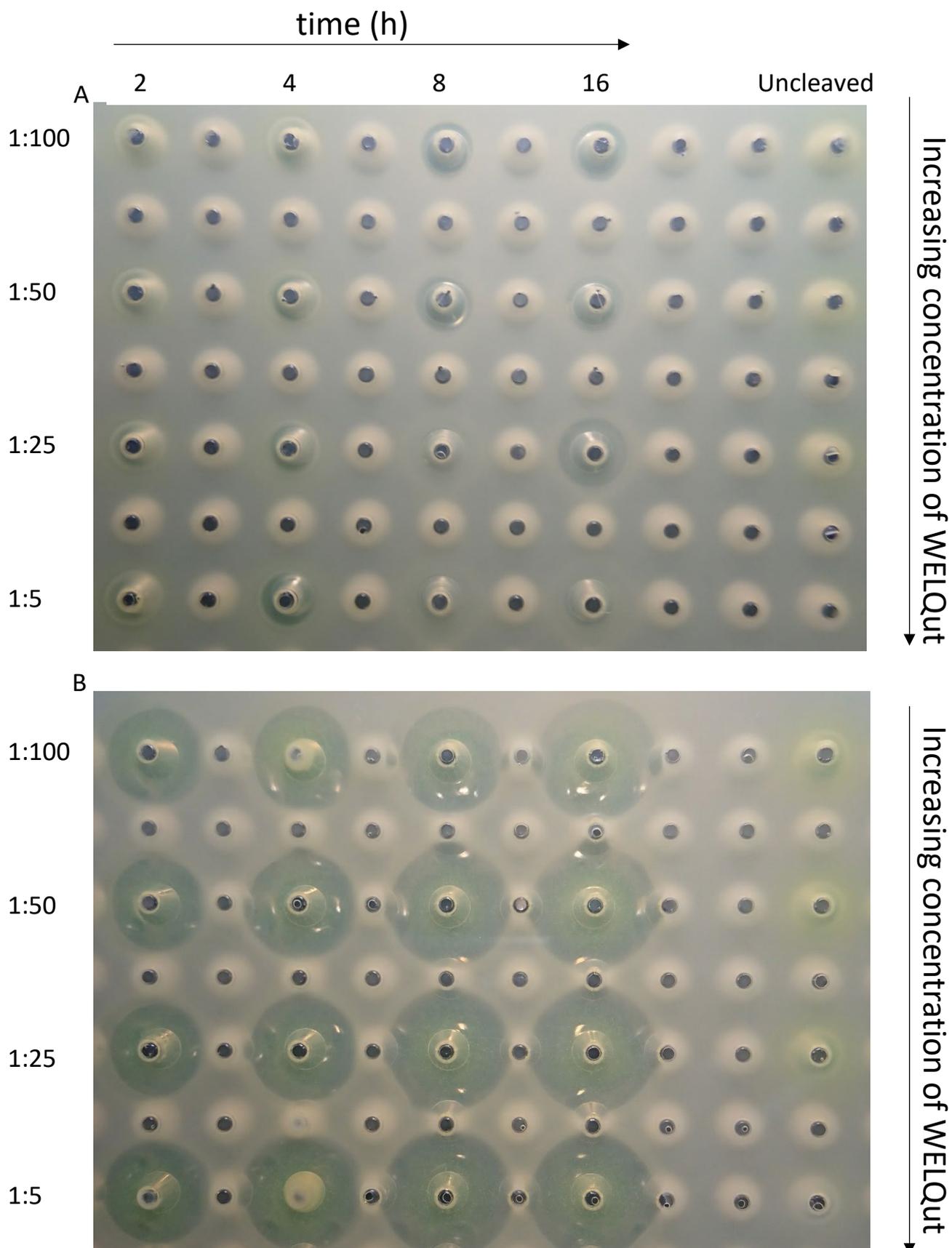


Figure 4.7. WELQut cleavage optimisation of incubation time and WELQut : sample ratio for maximal liberation of plantaricin 423 and mundtacin ST4SA from Ni-NTA purified PlantEx and MunEx, respectively. Cleavage assessed using the spot plate technique against *L. monocytogenes*. (A) PlantEx cleaved at 1:100, 1:50, 1:25, 1:5 WELQut : sample ratios over time at 28 °C. (B) MunEx cleaved at 1:100, 1:50, 1:25, 1:5 WELQut : sample ratios over time at 28 °C. Uncleaved PlantEx (A) and MunEx (B) did not show antilisterial activity.

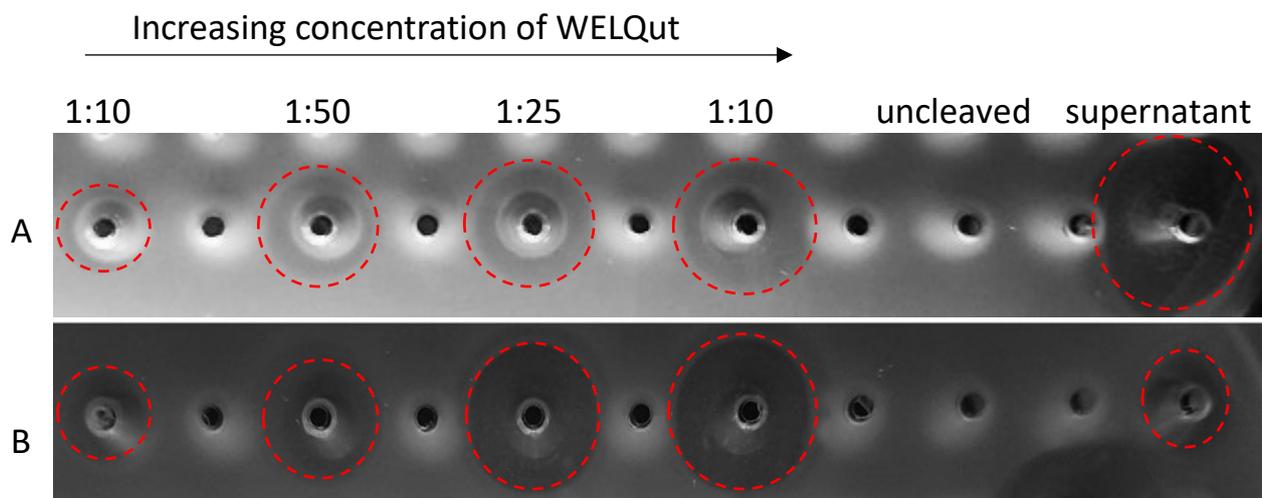


Figure 4.8. Confirmation of WELQut : sample ratio for liberation of plantaricin 423 (A) and mundticin ST4SA (B) from Ni-NTA purified PlantEx and MunEx, respectively. Cleavage assessed using the spot plate technique against *L. monocytogenes*. Cleavage ratios indicated on top of panel. Cleavage was performed at 28 °C for 16 hours. Post cleavage, 100 µL of PlantEx (A) and 10 µL MunEx (B) was spotted from each cleavage reaction. Spent supernatant (Right) was spotted from *L. plantarum* 423 (A) and *E. mundtii* ST4SA (B) at equal volumes to PlantEx and MunEx respectively. Uncleaved PlantEx (A) and MunEx (B) did not show antilisterial activity. Zones are indicated with dashed red lines.

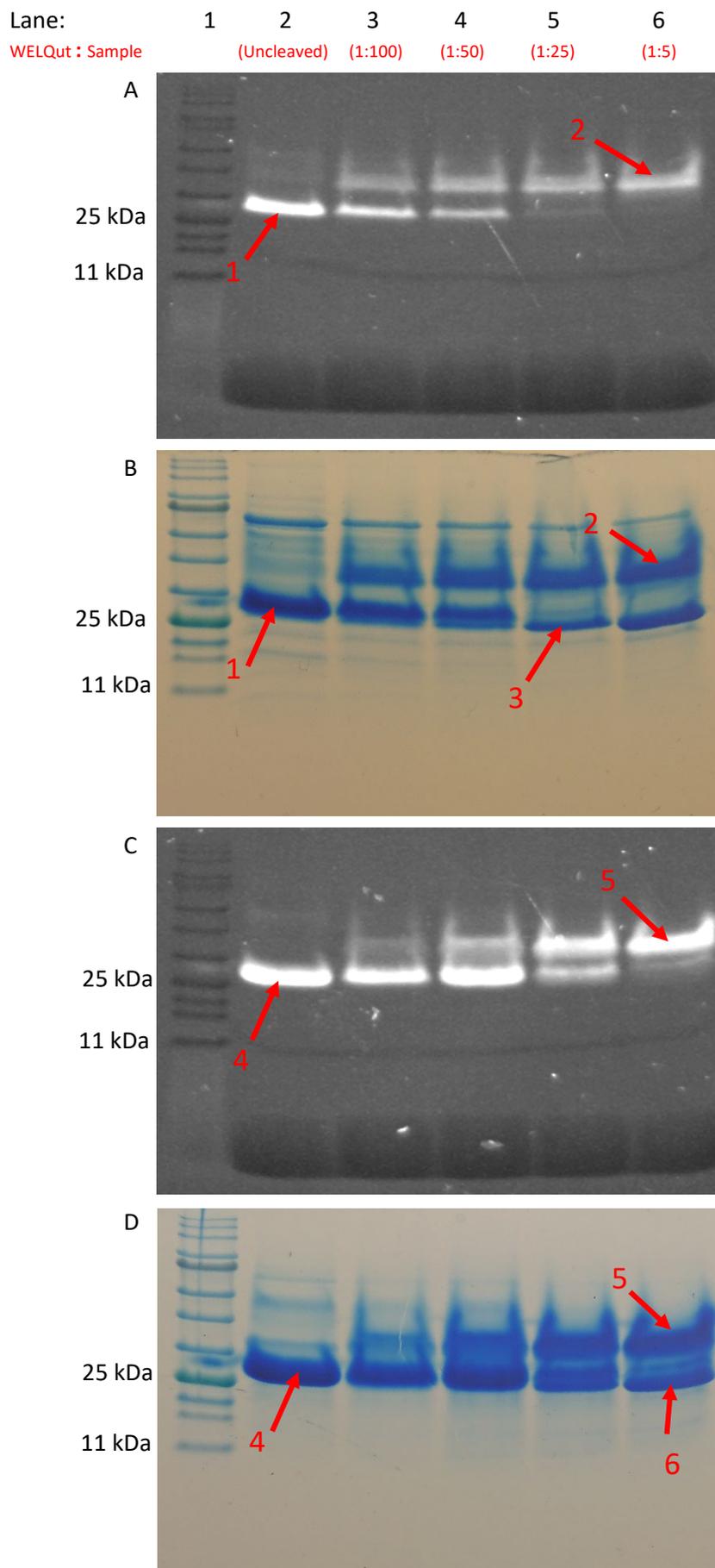


Figure 4.9. SDS-PAGE analysis of WELQut cleaved PlantEx (A and B) and MunEx (C and D). A and C represent unstained SDS-PAGE gels fluorometrically photographed. B and D represent stained gels of A and C. Lane: 1 – Ladder, 2 – uncleaved sample, 3 to 6 WELQut cleavage samples, sample ratios as indicated. Band 1 - Uncleaved PlantEx, 2 – putative WELQut and GFP complex, 3 - WELQut, 4 – Uncleaved MunEx, 5 – putative WELQut and GFP complex, 6 – WELQut.

Table 4.3 – Buffers used in IMAC purification and WELQut cleavage

SB	50 mM Tris, 500 mM NaCl, 10% glycerol (v/v) pH8.0
SB500	50 mM Tris, 500 mM NaCl, 500 mM imidazole, 10% glycerol (v/v) pH8.0
WELQut cut buffer	100 mM Tris, pH 8.0

Table 4.4 – Primers

GFP_Bam_Fwd	5'-GGATCCGATGAGTAAAGGAGAAGAAGTCTTCTCACTGGAGTTGTCCAATTC-3'
GFP_WELQ_Rev	5'- CTGCAGTTCCCAACCGGTTTTGTATAGTTCATCCATGCCATGTGTAATCC-3'
PlantEX_Pst_Fwd	5'- TAAGGGATCCGTGGGAAGTGCAGAAATACTATG-3'
PlantEx_Hind_Rev	5'- TATTAAGCTTAGCACTTTCCATGACCGAAGTTAGCTAAATG-3'
MunEx_Pst_Fwd	5'- ATCGCTGCAGAAATACTACGGTAATGGAGTCTCATGTAATAAAAAAG-3'
MunEx_Hind_Rev	5'- ACGCAAGCTTAACTTTTCCAACCAGCTGC-3'
pJET1.2_Fwd	5'- CGACTCACTATAGGGAGAGCGGC-3'
pJET1.2_Rev	5'- AAGAACATCGATTTTCCATGGCAG-3'
MCS1_Rev	5'-GATTATGCGGCCGTGTACAA-3'

References

1. Ennahar, S., Sashihara, T., Sonomoto, K. & Ishizaki, A. Class IIa bacteriocins: biosynthesis, structure and activity. *FEMS Microbiol. Rev.* 24, 85–106 (2000).
2. Drider, D., Fimland, G., Hechard, Y., McMullen, L. M. & Prevost, H. The continuing story of Class IIa bacteriocins. *Microbiol. Mol. Biol. Rev.* 70, 564–582 (2006).
3. Cui, Y. et al. Class IIa bacteriocins: diversity and new developments. *Int. J. Mol. Sci.* 13, 16668–16707 (2012).
4. Nissen-Meyer, J., Rogne, P., Oppegard, C., Haugen, H. & Kristiansen, P. Structure-function relationships of the non-lanthionine-containing peptide (class II) Bacteriocins Produced by Gram-positive bacteria. *Curr. Pharm. Biotechnol.* 10, 19–37 (2009).
5. Fregeau Gallagher, N. L. et al. Three-dimensional structure of leucocin a in trifluoroethanol and dodecylphosphocholine micelles: Spatial location of residues critical for biological activity in type IIa bacteriocins from lactic acid bacteria. *Biochemistry.* 36, 15062–15072 (1997).
6. Haugen, H. S., Fimland, G., Nissen-Meyer, J. & Kristiansen, P. E. Three-dimensional structure in lipid micelles of the pediocin-like antimicrobial peptide curvacin A. *Biochemistry.* 44, 16149–16157 (2005).
7. Uteng, M. et al. Three-dimensional structure in lipid micelles of the pediocin-like antimicrobial peptide sakacin P and a sakacin P variant that is structurally stabilized by an inserted C-terminal disulfide bridge. *Biochemistry.* 42, 11417–11426 (2003).
8. Wang, Y. et al. Solution structure of carnobacteriocin B2 and implications for structure - Activity relationships among type IIa bacteriocins from lactic acid bacteria. *Biochemistry.* 38, 15438–15447 (1999).
9. Oppegård, C., Fimland, G., Anonsen, J. H. & Nissen-Meyer, J. The pediocin PA-1 accessory protein ensures correct disulfide bond formation in the antimicrobial peptide pediocin PA-1. *Biochemistry.* 54, 2967–2974 (2015).

10. Ramnath, M., Beukes, M., Tamura, K. & Hastings, J. W. Absence of a putative mannose-specific phosphotransferase system enzyme IIAB component in a leucocin a-resistant strain of *Listeria monocytogenes*, as shown by two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Appl. Environ. Microbiol.* 66, 3098–3101 (2000).
11. Kjos, M., Salehian, Z., Nes, I. F. & Diep, D. B. An extracellular loop of the mannose phosphotransferase system component IIC is responsible for specific targeting by class IIa bacteriocins. *J. Bacteriol.* 192, 5906–5913 (2010).
12. Eijsink, V. G. H. et al. Production of class II bacteriocins by lactic acid bacteria; an example of biological warfare and communication. *Antonie Van Leeuwenhoek.* 81, 639–654 (2002).
13. Kjos, M. et al. Target recognition, resistance, immunity and genome mining of class II bacteriocins from Gram-positive bacteria. *Microbiology.* 157, 3256–3267 (2011).
14. Chikindas, M. L. et al. Pediocin PA-1, a bacteriocin from *Pediococcus acidilactici* PAC1.0, forms hydrophilic pores in the cytoplasmic membrane of target cells. *Appl. Environ. Microbiol.* 59, 3577–3584 (1993).
15. Montville, T. J. & Chen, Y. Mechanistic action of pediocin and nisin: Recent progress and unresolved questions. *Appl. Microbiol. Biotechnol.* 50, 511–519 (1998).
16. Bennik, M. H. J., Vanloo, B., Brasseur, R., Gorris, L. G. M. & Smid, E. J. A novel bacteriocin with a YGNGV motif from vegetable-associated *Enterococcus mundtii*: Full characterization and interaction with target organisms. *Biochim. Biophys. Acta - Biomembr.* 1373, 47–58 (1998).
17. Maré, L., Wolfaardt, G. M. & Dicks, L. M. T. Adhesion of *Lactobacillus plantarum* 423 and *Lactobacillus salivarius* 241 to the intestinal tract of piglets, as recorded with fluorescent in situ hybridization (FISH), and production of plantaricin 423 by cells colonized to the ileum. *J. Appl. Microbiol.* 100, 838–845 (2006).
18. van Reenen, C. a, Dicks, L. M. & Chikindas, M. L. Isolation, purification and partial characterization of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum*. *J. Appl. Microbiol.* 84, 1131–1137 (1998).

19. Granger, M., van Reenen, C. A. & Dicks, L. M. T. Effect of gastro-intestinal conditions on the growth of *Enterococcus mundtii* ST4SA, and production of bacteriocin ST4SA recorded by real-time PCR. *Int. J. Food Microbiol.* 123, 277–280 (2008).
20. Lohans, C. T. & Vederas, J. C. Development of class IIa bacteriocins as therapeutic agents. *Int. J. Microbiol.* 2012, 1–13 (2012).
21. Guyonnet, D., Fremaux, C., Cenatiempo, Y. & Berjeaud, J. M. Method for rapid purification of class IIa bacteriocins and comparison of their activities. *Appl. Environ. Microbiol.* 66, 1744–1748 (2000).
22. Carolissen-Mackay, V., Arendse, G. & Hastings, J. W. Purification of bacteriocins of lactic acid bacteria: Problems and pointers. *Int. J. Food Microbiol.* 34, 1–16 (1997).
23. Moon, G. S., Pyun, Y. R. & Kim, W. J. Characterization of the pediocin operon of *Pediococcus acidilactici* K10 and expression of his-tagged recombinant pediocin PA-1 in *Escherichia coli*. *J. Microbiol. Biotechnol.* 15, 403–411 (2005).
24. Klocke, M., Mundt, K., Idler, F., Jung, S. & Backhausen, J. E. Heterologous expression of enterocin A, a bacteriocin from *Enterococcus faecium*, fused to a cellulose-binding domain in *Escherichia coli* results in a functional protein with inhibitory activity against *Listeria*. *Appl. Microbiol. Biotechnol.* 67, 532–538 (2005).
25. Miller, K. W., Schamber, R., Chen, Y. & Ray, B. Production of active chimeric pediocin AcH in *Escherichia coli* in the absence of processing and secretion genes from the *Pediococcus pap* operon. *Appl. Environ. Microbiol.* 64, 14–20 (1998).
26. Moon, G. S., Pyun, Y. R. & Kim, W. J. Expression and purification of a fusion-typed pediocin PA-1 in *Escherichia coli* and recovery of biologically active pediocin PA-1. *Int. J. Food Microbiol.* 108, 136–140 (2006).
27. Beaulieu, L., Tolkatchev, D., Jette, J. F., Groleau, D. & Subirade, M. Production of active pediocin PA-1 in *Escherichia coli* using a thioredoxin gene fusion expression approach: cloning, expression, purification, and characterization. *Can. J. Microbiol.* 53, 1246–1258 (2007).

28. Jasniewski, J., Cailliez-Grimal, C., Gelhaye, E. & Revol-Junelles, A. M. Optimization of the production and purification processes of carnobacteriocins Cbn BM1 and Cbn B2 from *Carnobacterium maltaromaticum* CP5 by heterologous expression in *Escherichia coli*. *J. Microbiol. Methods* 73, 41–48 (2008).
29. Liu, S., Han, Y. & Zhou, Z. Fusion expression of *pedA* gene to obtain biologically active pediocin PA-1 in *Escherichia coli*. *J. Zhejiang Univ. Sci. B.* 12, 65–71 (2011).
30. Kimple, M. E. & Sondek, J. in *Current Protocols in Protein Science* (eds. Coligan, J. E., Dunn, B. M., Speicher, D. W. & Wingfield, P. T.) Unit 9.9.1-9.9.19 (John Wiley & Sons, Inc., 2004). doi:10.1002/0471140864.ps0909s36
31. Bell, M. R., Engleka, M. J., Malik, A. & Strickler, J. E. To fuse or not to fuse: What is your purpose? *Protein Sci.* 22, 1466–1477 (2013).
32. LaVallie, E. R. et al. A thioredoxin gene fusion expression system that circumvents inclusion body formation in the *E. coli* cytoplasm. *Nat. Biotechnol.* 11, 187–193 (1993).
33. Zimmer, M. Green Fluorescent Protein (GFP): applications, structure, and related photophysical behavior. *Chem. Rev.* 102, 759–782 (2002).
34. Tsien, R. Y. The green fluorescent protein. *Annu. Rev. Biochem.* 67, 509–544 (1998).
35. Shi, Y., Yang, X., Garg, N. & Van Der Donk, W. A. Production of lantipeptides in *Escherichia coli*. *J. Am. Chem. Soc.* 133, 2338–2341 (2011).
36. Sambrook, J., Fritsch, E. F. & Maniatis, T. *Molecular cloning: a laboratory manual. Mol. cloning a Lab. manual.* (1989). at <<https://www.cabdirect.org/cabdirect/abstract/19901616061>>
37. Ausubel, F. M. et al. *Current Protocols in Molecular Biology. Current protocols in molecular biology / edited by Frederick M. Ausubel ... [et al.] Chapter 2,* (John Wiley & Sons, Inc., 2003).
38. Schägger, H. & von Jagow, G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* 166, 368–379 (1987).
39. Lazer, I. & GelAnalyzer, L. I. Freeware 1D gel electrophoresis image analysis software. (2010).

40. Sivashanmugam, A. et al. Practical protocols for production of very high yields of recombinant proteins using *Escherichia coli*. *Protein Sci.* 18, 936–948 (2009).
41. Gibbs, G. M., Davidson, B. E. & Hillier, A. J. Novel Expression System for Large-Scale Production and Purification of Recombinant Class IIa Bacteriocins and Its Application to Piscicolin 126. *Appl. Environ. Microbiol.* 70, 3292–3297 (2004).
42. Richard, C., Drider, D., Elmorjani, K., Prévost, H. & Marion, D. Heterologous expression and purification of active divercin V41, a class IIa bacteriocin encoded by a synthetic gene in *Escherichia coli* heterologous expression and purification of active divercin V41, a class IIa bacteriocin encoded by a synthetic gene. *Society.* 186, 4276–4284 (2004).
43. Cuozzo, S., Calvez, S., Prévost, H. & Drider, D. Improvement of enterocin P purification process. *Folia Microbiol. (Praha).* 51, 401–405 (2006).
44. Saeed, I. A. & Ashraf, S. S. Denaturation studies reveal significant differences between GFP and blue fluorescent protein. *Int. J. Biol. Macromol.* 45, 236–241 (2009).
45. Krasowska, J., Olasek, M., Bzowska, A., Clark, P. L. & Wielgus-Kutrowska, B. The comparison of aggregation and folding of Enhanced Green Fluorescent Protein (EGFP) by spectroscopic studies. *J. Spectrosc.* 24, 343–348 (2010).

Chapter 5

Investigating the mode of plantaricin 423
regulation using a fluorescent promoter-
reporter system in *Lactobacillus plantarum*
423

Abstract

Aim: This study aimed to construct and deploy a fluorescent reporter system to monitor and thereby elucidate the mode of transcriptional regulation of plantaricin 423 in *Lactobacillus plantarum* 423.

Methods and Results: The *mCherry* gene was fused to the plantaricin 423 promoter which allowed for fluorometric reporting on plantaricin 423 expression during growth. Divalent cations were removed from the growth media of *L. plantarum* 423 using Chelex 100 ion exchange resin. The transcriptional response of plantaricin 423 to manganese was measured in real time and *in vivo*.

Conclusion: The mCherry-based reporter system indicated that the *plaA* gene is upregulated in response to manganese limitation.

Introduction

As described in Chapter 2, transcriptional regulation of bacteriocin genes in Gam-positive bacteria is a complex process that can be performed using a range of mechanisms and is dependent on a variety of signals. Some variables that control Gram-positive bacteriocin expression are acidity, nutrient competition/composition, synthesis of diacetyl- or LuxS-mediated compounds, and AP concentrations¹⁻⁴. These variables are typically monitored by Gram-positive organisms using two-component regulatory systems. In these systems, a sensor kinase monitors the environmental stimulus and elicits a transcriptional response via its cognate RR. When these two components monitor and respond to the concentration of an operon-encoded AP, the regulatory element is said to be a three-component regulatory system^{1,5}.

Three-component regulatory systems are often responsible for quorum sensing based transcriptional regulation of bacteriocin expression⁴⁻⁸. However, within the available literature on Gram-positive bacteriocin regulatory mechanisms, it is common to find the HPK, RR or AP missing from regulatory operons. Nisin's quorum sensing operon does not contain an AP which results in autoinduction type regulation, while the epidermin operon only contains the RR, EpiQ^{9,10}. Nisin and Epidermin are both type I lantibiotics, however, there is evidence that suggests the expression of epidermin is dependent on an environmental stimulus like phosphate concentration¹⁰. On the other hand, the mutacin I operon contains an RR whose transcription is controlled by a distant AI-2 system³.

Although the examples above are all lantibiotics, similar inconsistencies are observed in subclass IIa bacteriocin regulatory operons. For example, the carnobacteriocin BM1 operon does not appear to contain any regulatory elements but is instead regulated by a distant operon encoding carnobacteriocin B2 which harbors an HPK, RR and AP¹¹. Carnobacteriocin BM1 transcription has also been linked to incubation temperature in *C. maltaromaticum* UAL26, indicating that the same gene may be controlled differently in different organisms¹².

Lactobacillus plantarum 423 harbors the genes for an AI-2 type regulatory system; However, *L. plantarum* 423 also harbors the chromosomal *pln* locus which contains a full three-component regulatory operon for subclass IIb bacteriocin expression¹³. The presence of these elements could potentially result in delocalized regulation of plantaricin 423 by an AI-2 regulatory system or the *pln* locus RRs, *plnC* and *plnD*, as seen for mutacin I or carnobacteriocin BM1 respectively. It has been demonstrated that the *pln* locus is induced by the co-culturing of *L. plantarum* strains with other bacterial species, thus presenting an indirect avenue for interspecies bacteriocin-mediated crosstalk^{14,15}. Finally, plantaricin 423 may also have autoinduction capabilities in the absence of local regulatory elements, as seen in the case of carnobacteriocin B2.

Literature does not yet report any quorum sensing based transcriptional regulation of subclass IIa bacteriocins in *L. plantarum* species. However, environmental manganese has been shown to increase the antilisterial activity of subclass IIa bacteriocin producing *L. plantarum* species^{16,17}. Furthermore, Chapter 3 presents evidence which suggests that subclass IIa bacteriocins with type 2 immunity proteins transcriptionally regulate their bacteriocin operons in response to cationic metals. This chapter presents the construction of a fluorescent reporter system to monitor the transcriptional regulation of plantaricin 423 (*plaA*) in *L. plantarum* 423 *in vivo*. This system was deployed in the wild-type *L. plantarum* 423 and a cured *L. plantarum* 423 devoid of the pPla4 plasmid harbouring the native *pla* operon.

Materials and Methods

Bacterial strains, culture conditions, and molecular techniques

Bacterial strains and culture conditions

The LAB, *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA were cultured on De Man, Rogosa and Sharpe (MRS) media, at 37 °C without agitation. For solid media, 1.2% (w/v) agar was added to the respective growth media. All growth media were supplied by Merck-Millipore (USA).

For the selection and maintenance of pGKV223D-derived plasmids in *Lactobacillus plantarum* 423, transformants were cultured on MRS supplemented with 7.5 µg/mL erythromycin. Subcloning experiments were performed in *Escherichia coli* MC1061 that was cultured on either Luria-Bertani (LB) or Brain Heart Infusion (BHI) medium at 37 °C with constant agitation. For the selection and maintenance of pJET1.2-derived plasmids in *Escherichia coli* MC1061, transformants were cultured with LB medium containing 150 µg/mL ampicillin. For the selection and maintenance of pGKV223D-derived plasmids in *Escherichia coli* MC1061, transformants were cultured with BHI medium containing 200 µg/mL erythromycin.

Divalent cations were removed from MRS broth using Chelex 100 ion exchange resin (Bio Rad, Watford, UK) according to the manufacturer's instructions. The MRS medium was passed over regenerated Chelex 100 resin three times. The before and after concentrations of Mg, Mn, Zn, Ca, and Fe was not measured.

Molecular techniques

DNA analysis, manipulation, and cloning were performed according to Sambrook¹⁸. Genomic DNA and plasmid DNA isolations from *L. plantarum* 423 were performed according to Moore *et al.*¹⁹ and O'Sullivan *et al.*²⁰ respectively. Plasmid isolations from *E. coli* MC1061 were either performed using the PureYield™ plasmid miniprep system (Promega, Madison, WI, USA) when nucleotide sequencing was required or according to Birnboim *et al.*¹⁹ for plasmid mapping.

Electrocompetent *L. plantarum* 423 cells were prepared according to Van Zyl *et al.*²⁰. Competent *L. plantarum* 423 cells were electrotransformed with 1 µg of pDNA using the Gene Pulsar (BioRad Laboratories, Hercules, CA) set under the following conditions; voltage: 1.5 KV/0.2 cm, resistance: 200 Ω, and capacitance: 25 µFD²⁰.

T4 DNA ligase and restriction enzymes (RE) were purchased from New England Biolabs (NEB, Ipswich, MA, USA) and used according to the manufacturer's instructions. Polymerase chain reaction (PCR) amplifications were performed using Q5 high-fidelity PCR DNA polymerase (NEB) according to the manufacturer's instructions in a GeneAmp PCR system 9700 (ABI, Foster City, CA). Oligonucleotides were designed using the CLC main workbench program (CLC bio, Aarhus, Denmark) and purchased from Inqaba Biotechnical Industries (Pretoria, South Africa). DNA sequencing was performed by Central Analytical Facilities (CAF) at the University of Stellenbosch, South Africa.

Agarose gel electrophoresis was used for the analysis and purification of RE mapped DNA fragments in TBE buffer at 10V/cm using the Ephortec™ 3000V (Triad Scientific, Manassas United States) power supply²¹. Excised gel DNA fragments were purified using the Zymoclean™ gel DNA recovery kit (Zymo Research Corporation, Irvine, CA, USA).

Construction and deployment of a plantaricin 423 fluorescent reporter system

Significant complications due to the limited information on the functional elements within the *pla* operon were encountered during the construction of a fluorescent plantaricin 423 reporter system for use in *L. plantarum* 423. The initial cloning approaches and their results will be more fully presented in the Appendix.

The reader is referred to the Appendix, Table 5.3 for a comprehensive overview of all reporter plasmids constructed and deployed in *L. plantarum* 423. The pGKV223D plasmid was used for the construction of the reporter system that would mirror the transcriptional activity of the native *plaA* bacteriocin gene in *L. plantarum* 423 (Appendix, Figure 5.16). The pGKV223D plasmid contains the *repA* gene for replication in *E. coli* MC1061 and LAB, the chloramphenicol resistance marker *cat86*, the erythromycin resistance marker *ermC*, and the *P23* promoter (Appendix, Figure 5.16).

Van Zyl *et al.*²⁰ previously cloned the lactate dehydrogenase promoter from *L. plantarum* 423 in transcriptional control of the Red Fluorescent Protein (RFP) *mCherry* (Figure 5.1 and Appendix, Figure 5.17). This construct pGKVCherry, was used as a positive control for fluorescence in *L. plantarum* 423.

Reconstruction of the *plaA* reporter plasmid pGKV-plaAPrV2mChB

The first version of the reporter system cloned *mCherry* into the position of *plaA*, starting at the location of the first methionine coded for by *plaA*, however fluorescence in *L. plantarum* 423 was not observed (Appendix, Figure 5.15). The second configuration of the *plaA* promoter-*mCherry* reporter

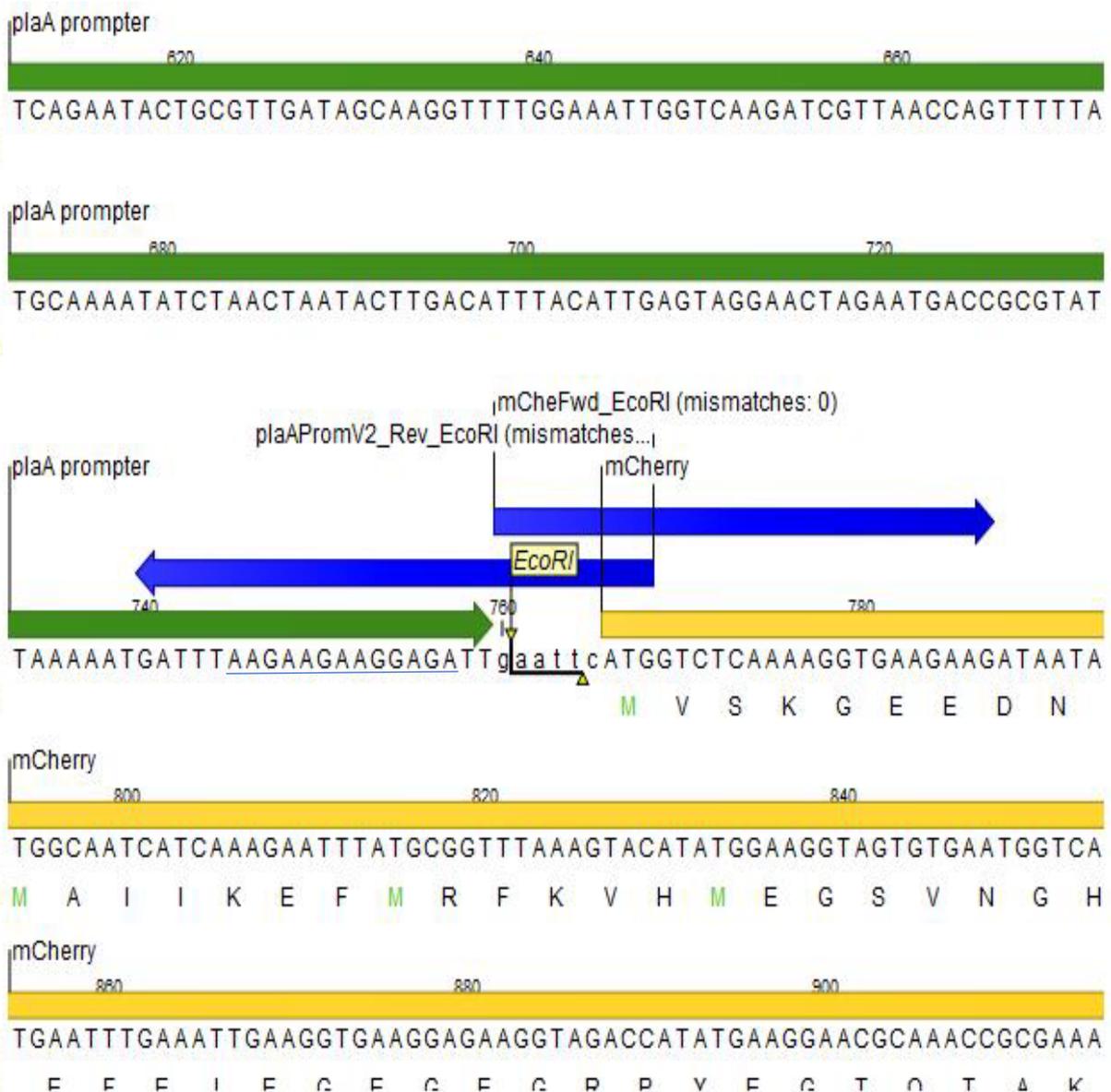


Figure 5.1 - The second version of the *plaA* promoter-*mCherry* reporter system set the start codon of *mCherry* in the position of the second methionine of *plaA* in the native *plaA* operon.

fragment cloned *mCherry*'s start methionine into the second methionine position coded for by *plaA* (Appendix, Figure 5.14). The length of the upstream region was also extended from 267bp to 732bp to include any unidentified regulatory elements within the *plaA* promoter region.

The second configuration of the *plaA* promoter, *plaAPrV2*, was PCR amplified from *L. plantarum* 423 gDNA using the *plaAPr2_Nsil_Fwd/plaAPr2_EcoRI_Rev* primer set (Appendix, Table 5.2). The *plaB* gene was PCR amplified from *L. plantarum* 423 gDNA using the *plaB_BamHI_Fwd/plaB_PstI_Rev* primer set (Appendix, Table 5.2). The *plaAPrV2* and *plaB* amplicons were cloned using the CloneJET kit, sequenced and confirmed to be correct as previously described. In a four-way ligation, the *plaAPrV2* (*Nsil/EcoRI* fragment), *mCherry* (*EcoRI/BamHI* fragment) and *plaB* (*BamHI/PstI* fragment)

genes were cloned into the pGKV223D plasmid at NsiI/PstI. The resulting pGKV-plaAPrV2mChB plasmid was extracted from *E. coli* transformants expressing a red fluorescent, erythromycin resistant phenotype (Appendix, Figure 5.22).

Electrotransformation of *L. plantarum* 423 with the pGKV-plaAPrV2mChB plasmid

Electrocompetent *L. plantarum* 423 cells were transformed with pGKV-plaAV2mCh plasmid as previously described. Genomic DNA was extracted from erythromycin resistant *L. plantarum* 423 transformants. Transformants were confirmed to be *L. plantarum* 423 by PCR amplification of the *JDM1_2142_79* gene using the 2142Fwd/2142Rev primer set (Appendix, Table 5.2)²². The presence of pGKV-plaAPrV2mCh was confirmed via PCR amplification using the plaAPrV2_NsiI_Fwd/mChe_BamHI_Rev primer set. Ten *L. plantarum* 423 pGKV-plaAPrV2mCh transformants that expressed a red fluorescent phenotype were recovered and cryopreserved (Appendix, Table 5.2).

Growth media for RFP quantification

Full strength MRS broth had high background fluorescence which rendered *mCherry* expression by *L. plantarum* 423 pGKVCherry undetectable in the Tecan Spark M10™ (Tecan Group Ltd., Austria). The broth also supported a microbial load far greater than the linear range for spectroscopic measurements made by the Tecan Spark M10™ (OD₅₅₀ limit of 1). For lowering background fluorescence and limiting *L. plantarum* 423 growth to an OD₅₅₀ of 1, transformants were cultured in filter-sterilized 25% MRS broth containing 5 µg/mL erythromycin.

The Tecan Spark M10™ was used to incubate and simultaneously make *in vivo* fluorometric and spectroscopic measurements of *L. plantarum* 423 pGKV-plaAPrV2mChB reporter clones during their growth cycle. The wells of a black flat-bottom 96-well microtiter plate (Greiner bio-one, Monroe, NC) were filled with 250 µL MRS broth inoculated with three *L. plantarum* 423 pGKV-plaAPrV2mCh transformants in triplicate, one *L. plantarum* 423 pGKVCherry (LDH promoter) transformant in triplicate, and one *L. plantarum* 423 pGKV-mCh(promoterless) transformant in triplicate. The Tecan Spark M10™ was programmed to execute the following kinetic incubation loop at 37°C for 24 hours on 96-well black microtiter plates within the large incubation chamber: Incubate for 30min, shake at 300 RPM for 10s, settle for 10s, measure optical density at 600 nm, measure fluorescent output with excitation at 560 nm and emission at 610 nm.

Elucidation of unidentified regulatory elements within the *pla* operon using the cured *L. plantarum* 423 strain

To elucidate potential regulatory elements within the plantaricin 423 operon (*pla*) the pGKV-plaAPrV2mCh (less *plaB*) and pGKV-plaAPrV2mChBCD (immunity, transporter, and accessory protein genes) plasmids were constructed.

Construction of pGKV-plaAPrV2mCh and pGKV-plaAPrV2mChBCD reporter plasmids

The pGKV-plaAPrV2mCh plasmid was constructed by cloning *plaAPrV2-mCherry* into pGKV223D on a NsiI/BamHI fragment from the pGKV-plaAPrV2mChB plasmid (Appendix, Figure 5.23). The pGKV-plaAPrV2mCh plasmid was extracted from phenotypically red fluorescent, erythromycin resistant *E. coli* transformants.

The *plaBCD* fragment was PCR amplified from *L. plantarum* 423 gDNA using the *plaB_BamHI_Fwd/plaD_PstI_Rev* primer set (Appendix, Table 5.2). The *plaBCD* amplicon was cloned using the CloneJET kit, sequenced using the pJET1.2 Fwd/Rev primers and the *plaBCD* sequencing primers (Appendix, Table 5.2). The *plaBCD* fragment was cloned into pGKV-plaAPrV2mCh on a BamHI/PstI fragment to construct the pGKV-plaAPrV2mChBCD plasmid (Appendix, Figure 5.24). The pGKV-plaAPrV2mCh and pGKV-plaAPrV2mChBCD plasmids, respectively, were extracted from erythromycin resistant *E. coli* MC1061 transformants.

Transformation of the pPla4 cured *L. plantarum* 423 with pGKV-plaAPrV2mCh, pGKV-plaAPrV2mChB and pGKV-plaAPrV2mChBCD

L. plantarum 423 has previously been cured of its plasmids by Van Reenen *et al.*²³ to generate a pPla4 negative strain (*L. plantarum* 423-neg). Electrocompetent *L. plantarum* 423-neg were prepared and transformed with pGKV-plaAPrV2mCh, pGKV-plaAPrV2mChB, and pGKV-plaAPrV2mChBCD as previously described²⁰. Genomic DNA was extracted from erythromycin resistant transformants of each plasmid. Transformants were confirmed to be *L. plantarum* 423 via PCR amplification using the 2142Fwd/2142Rev primer set. The presence of the reporter plasmids was confirmed via PCR amplification using the *plaAPrV2_NsiI_Fwd/mChe_BamHI_Rev* primer set.

The transcriptional effect of zinc, iron, magnesium, and manganese on plantaricin 423 expression

The pPla4-cured and -uncured *L. plantarum* 423 pGKV-plaAPrV2mChB transformants were cultured on MRS solid medium pre-treated with Chelex 100, to lower the concentration of divalent cations (MRS-Div), for two rounds at 36 °C. Thereafter, *L. plantarum* 423 pGKV-plaAPrV2mChB transformants were streaked onto MRS-Div media supplemented with 10 mM zinc sulfate, 5 mM iron sulfate, 5 g/L magnesium sulfate or 0.5 g/L manganese sulfate respectively (Merck). The concentrations of magnesium- and manganese-sulphate supplemented here are the same as in untreated MRS broth, and are equivalent to 2.28 and 0.2241 mM respectively.

In a 96-well microtiter plate, the effect of manganese sulfate on cured and uncured *L. plantarum* 423 pGKV-plaAPrV2mChB transformants was measured at various concentrations in biological triplicates (Appendix, Table 5.1).

Measuring the effect of heterologously expressed plantaricin 423 on transcription in the cured *L. plantarum* 423 pGKV-plaAPrV2mChB transformants

The effect of WELQut-cleaved and -uncleaved PlantEx (from chapter 4) on cured *L. plantarum* 423 pGKV-plaAPrV2mChB was measured in the MRS-Div medium using three biological triplicates. For each treatment, 25 µL of cleaved and uncleaved PlantEx (filter sterilized) was added to 225 µL inoculated MRS-Div media. For the liberation of mature plantaricin 423 from PlantEx, 50 µL of Ni-NTA purified PlantEx was cleaved using 5 µL of the WELQut protease and diluted to a final volume of 70 µL with sterile Milli-Q purified water. For the uncleaved treatment, 50 µL of PlantEx was made up to a total volume of 70 µL with sterile Milli-Q purified water.

Specific mCherry production rate calculations

The specific RFU production rate for mCherry acting as a proxy for plantaricin 423 was calculated at each sample point by using that point plus the previous and following points to estimate the gradient of each point's tangent by liner regression.

Specific production rate was represented as:

$$q_p = \frac{dp}{dt} \cdot \frac{1}{x}$$

where the term p was measured in the relative fluorescent units (RFU) produced by mCherry, and biomass (x) was measured as the OD_{550} . Transformants grew at different rates through time and had different lag phases, therefore to compare promoter activity between treatments, the % Max growth was calculated for each treatment as each OD_{550} relative to the maximal OD_{550} in that treatment. The specific production rate was also calculated relative to the maximum observed in each treatment as % Max q_p for comparison of promoter activity.

Results

In vivo quantification of mCherry expression in uncured *L. plantarum* 423 reporter strains

Plantaricin 423 was expressed at adequate levels for *in vivo* fluorometric detection of mCherry by the Tecan Spark M10™ in the *L. plantarum* 423 pGKV-plaAPrV2mChB transformant. Due to the 25% strength MRS broth, *L. plantarum* 423 pGKV-plaAPrV2mChB transformants completed their growth cycle without exceeding an OD_{550} of 1. The *L. plantarum* 423 pGKVCherry (positive control) transformant produced measurable fluorescence throughout growth as expected (data not shown). Only background fluorescence was detected from the pGKV-mCh(negative control) transformant (data not shown).

Expression of mCherry in the *L. plantarum* 423-neg transformed with the pGKV-plaAPrV2mCh, pGKV-plaAPrV2mChB, and pGKV-plaAPrV2mChBCD plasmids.

Upon first transformation, only the *L. plantarum* 423-neg pGKV-plaAPrV2mChB transformants had a red fluorescent phenotype, while pGKV-plaAPrV2mCh and pGKV-plaAPrV2mChBCD transformants did not. Yet the supernatant of each transformant had comparable amounts of anti-listerial activity when spot tested, indicating transcriptional firing of the native *plaA* promoter in all cases (data not shown). Upon sequencing the promoter region for the pGKV-plaAPrV2mCh and pGKV-plaAPrV2mChBCD plasmids, a point mutation was observed 62 bp upstream from the mCherry start codon (Figure 5.2).

When a unmutated *plaAPrV2* promoter fragment was cloned in pGKV-plaAPrV2mCh, pGKV-plaAPrV2mChB and pGKV-plaAPrV2mChBCD, transformants produced comparable fluorescent output profiles in *L. plantarum* 423-neg during growth, as confirmed by the Tecan Spark M10™ (data not shown). This indicated that transcriptional regulation of the *plaA* gene only required the *plaA* promoter region and not any regulatory elements on the pPla4 plasmid.

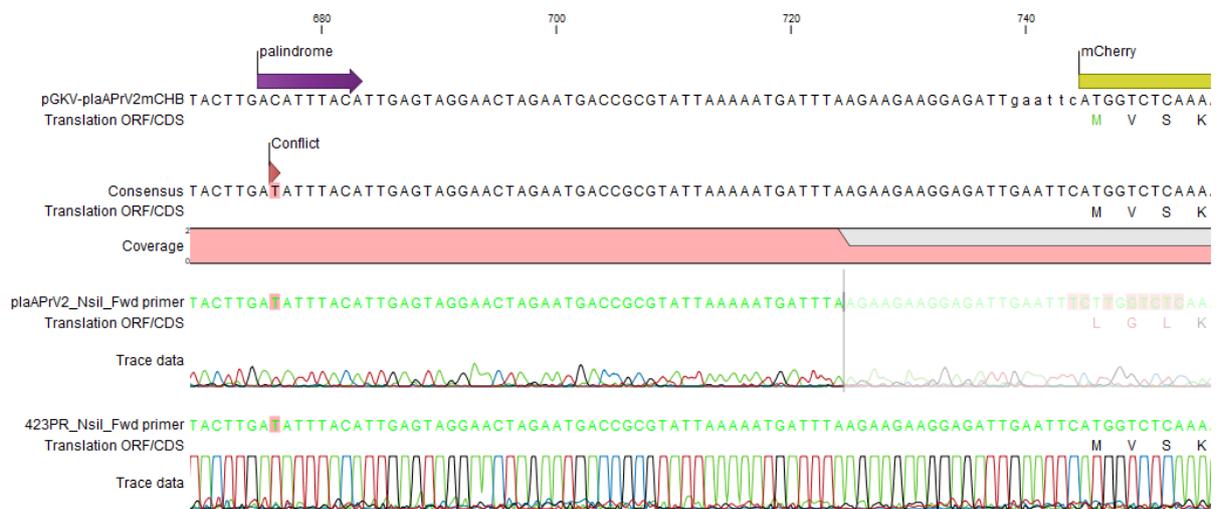


Figure 5.2 – Nucleotide sequence of the pGKV-plaAPrV2mCh and pGKV-plaAPrV2mChBCD plasmids deployed in *L. plantarum* 423 which did not produce a fluorescent phenotype. Upstream of the *mCherry* start codon, a point mutation was observed in a palindromic sequence “ACATTTACA”, so it read “ATATTTACA”. In the second position, a cytosine was substituted by a thymine. Correcting this point mutation lead to a fluorescent phenotype in *L. plantarum* 423.

The effect of manganese on mCherry transcription in the cured and uncured *L. plantarum* 423 pGKV-plaAPrV2mChB transformants

Plating the cured and uncured *L. plantarum* 423 pGKV-plaAPrV2mChB transformants on MRS-Div media for two rounds abolished the red fluorescent phenotype. Upon sub-culturing on MRS-Div media supplemented with zinc, iron, magnesium, and manganese, respectively, a red fluorescent phenotype was observed for colonies grown with iron or manganese (data not shown).

The genome for the cured *L. plantarum* 423 has not been sequenced, and while it has been confirmed that the pPla4 plasmid was removed from this *L. plantarum* 423, it is unknown what other genes may have been removed as well. The transcriptional effect of manganese on both the cured (*L. plantarum* 423-neg) and uncured *L. plantarum* 423 was therefore assessed. However, a fluorometric response was only seen when iron was added at 5 mM concentrations which interfered with optical density readings.

Lactobacillus plantarum 423-neg pGKV-plaAPrV2mChB mCherry expression

Decreasing manganese concentration caused a decrease in maximal growth of *L. plantarum* 423-neg pGKV-plaAPrV2mChB transformants rendering direct comparison of mCherry expression in each treatment difficult (Figure 5.3). Growth in none of the treatments exceeded an OD₅₅₀ of 1; however, treatments below 0.1941 μM manganese were excluded from further analysis as growth was significantly inhibited (Figure 5.3).

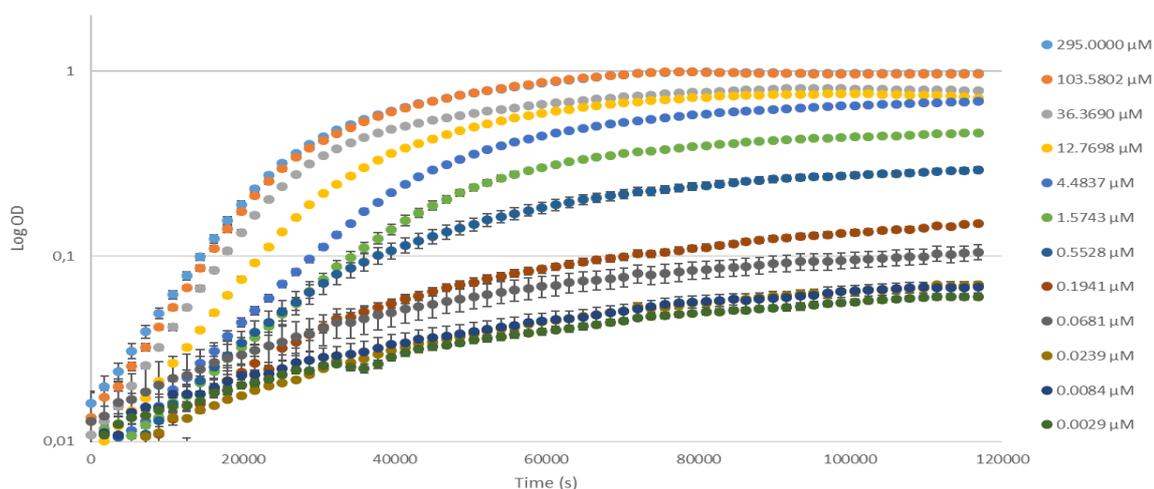


Figure 5.3 – Effect of manganese limitation on growth performance for *L. plantarum* 423-neg pGKV-plaAPrV2mChB transformants visualised as a semilogarithmic plot of OD₅₅₀ values against time. Decreasing the available manganese caused a decrease in the maximal growth.

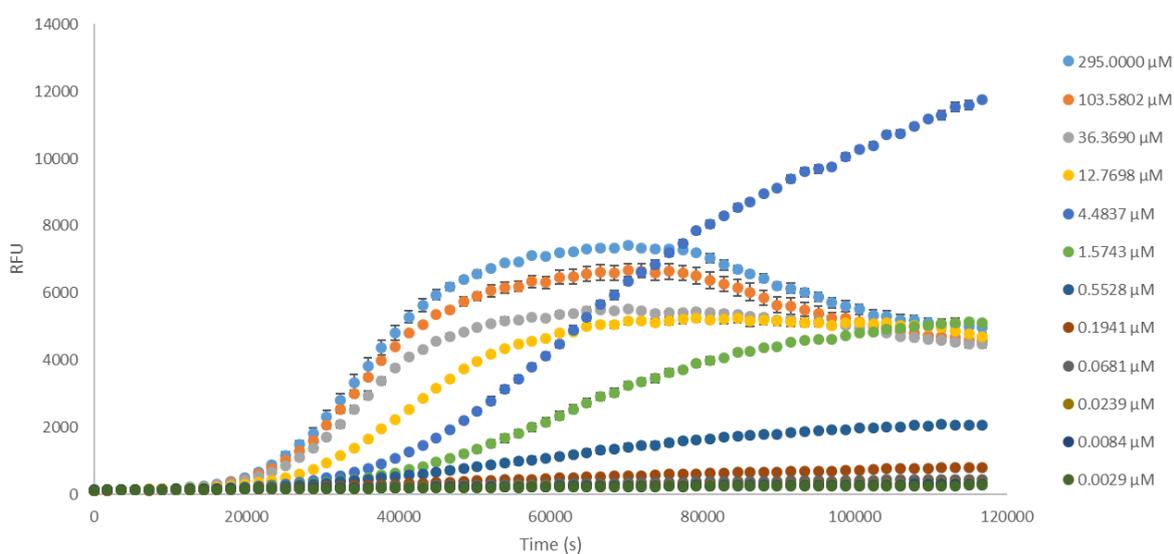


Figure 5.4 – Effect of manganese limitation on *plaA* transcription measured in RFUs produced by the mCherry protein in *L. plantarum* 423-neg pGKV-plaAPrV2mChB reporter strain. Maximal RFU output was observed at submaximal manganese supplementation (4.48 μM).

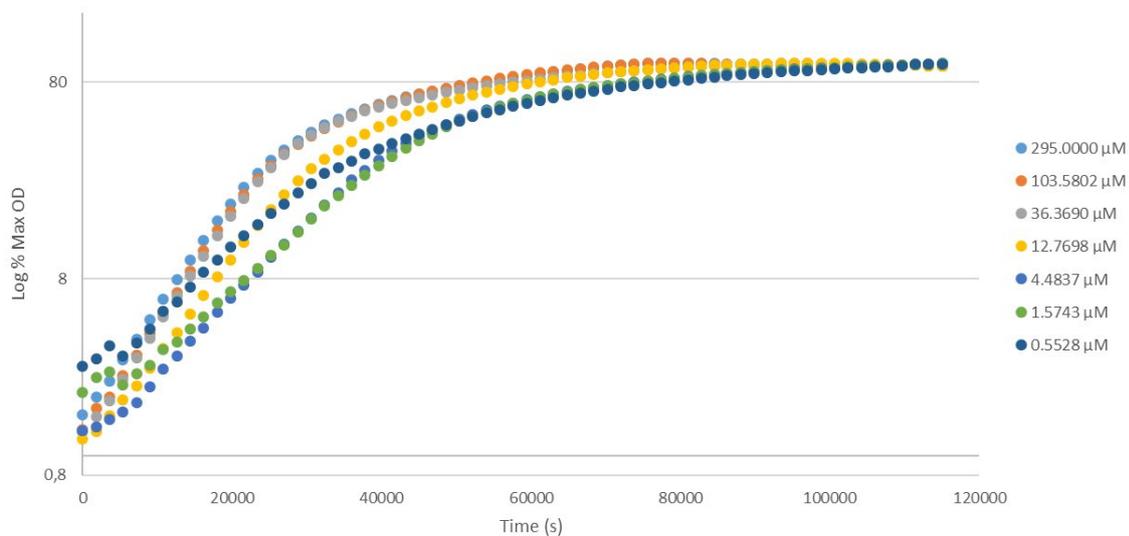


Figure 5.5 – Identification at which percentage of maximal growth all *L. plantarum* 423-neg pGKV-plaAPrV2mChB transformants had exited the log phase and entered stationary phase in each manganese treatment. At 80% of the total growth in each treatment, *L. plantarum* 423-neg pGKV-plaAPrV2mChB had exited exponential growth.

The RFU expression profile in *L. plantarum* 423-neg pGKV-plaAPrV2mChB changed during incubation at 4.4837 μM manganese or lower concentrations (Figure 5.4). Treatment with 4.48 μM manganese produced a significantly higher total RFU yield, indicating that manganese limitation may be an inducing factor for transcription of *plaA* from the *plaV2Pr* fragment (Figure 5.4). However, to confirm these results specific RFU production rates needed to be compared at the same point in the growth cycle for each treatment.

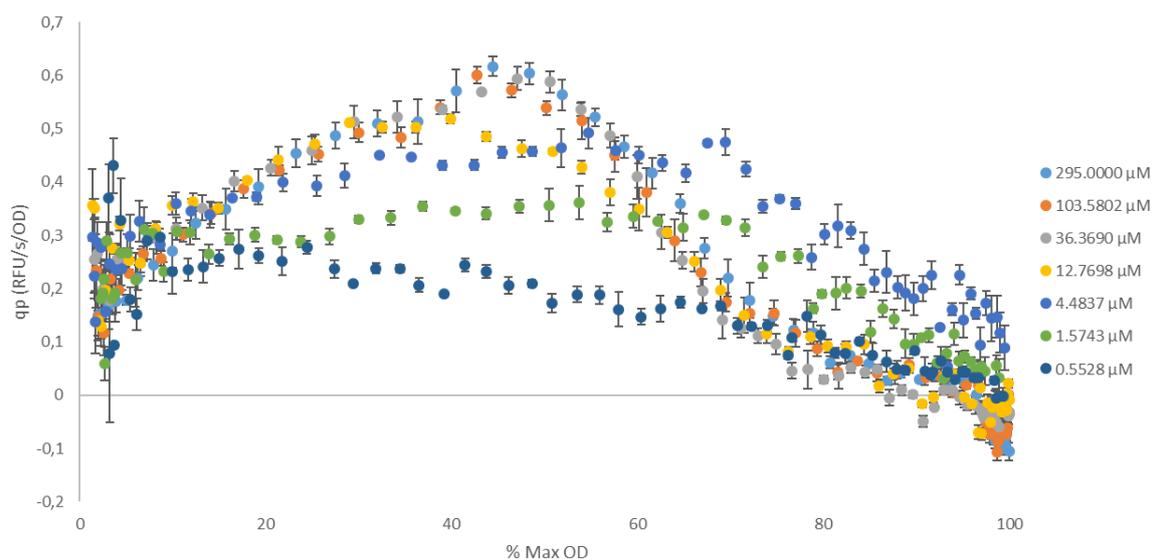


Figure 5.6 - Effect of manganese limitation on the specific RFU production rate of mCherry, representing plantaricin 423 expression, in the *L. plantarum* 423-neg pGKV-plaAPrV2mChB reporter strain. The *plaA* promoter changes its expression profile to a more constitutive response up to 80% maximal growth at 4.48 μM manganese or lower treatment concentrations.

The decreasing concentration of manganese caused *L. plantarum* 423-neg pGKV-plaAPrV2mChB transformants to grow differently over time, making a direct comparison between treatments difficult. To compare the activity of the *plaA* promoter at the same point in a growth cycle for each treatment, the percentage of maximal growth for each point was calculated and plotted against time (Figure 5.5). Although cultures in each treatment reached their maximal OD₅₅₀ at different times, it can be seen from figure 5.5 that after 80 % of total growth as measured by OD₅₅₀, *L. plantarum* 423-neg pGKV-plaAPrV2mChB had entered the stationary phase in each treatment.

The specific RFU production rate was then plotted against % Max growth to compare promoter activity directly and therefore *plaA* transcription at different manganese concentrations (Figure 5.6). The *plaA* promoter appears to have two different transcriptional profiles, where switching from one to the other is influenced by manganese concentration (Figures 5.6 and 5.7). When *L. plantarum* 423-neg pGKV-plaAPrV2mChB is cultured with a manganese concentration between 295.00 and 12.76 μM , transcription appears to reach a relatively higher specific production rate and then quickly slows down after 50% Maximal growth (Figure 5.6). In contrast, at 4.48 μM or lower manganese concentrations, the specific RFU production rate appears to be lower but more level throughout growth (Figure 5.6 and 5.7). If there are two transcriptional modes to the *plaA* promoter, representing RFU specific production for each treatment as a percentage of its maximum provides an interpretation of the transcriptional profiles for the two modes (Figures 5.6 and 5.7).

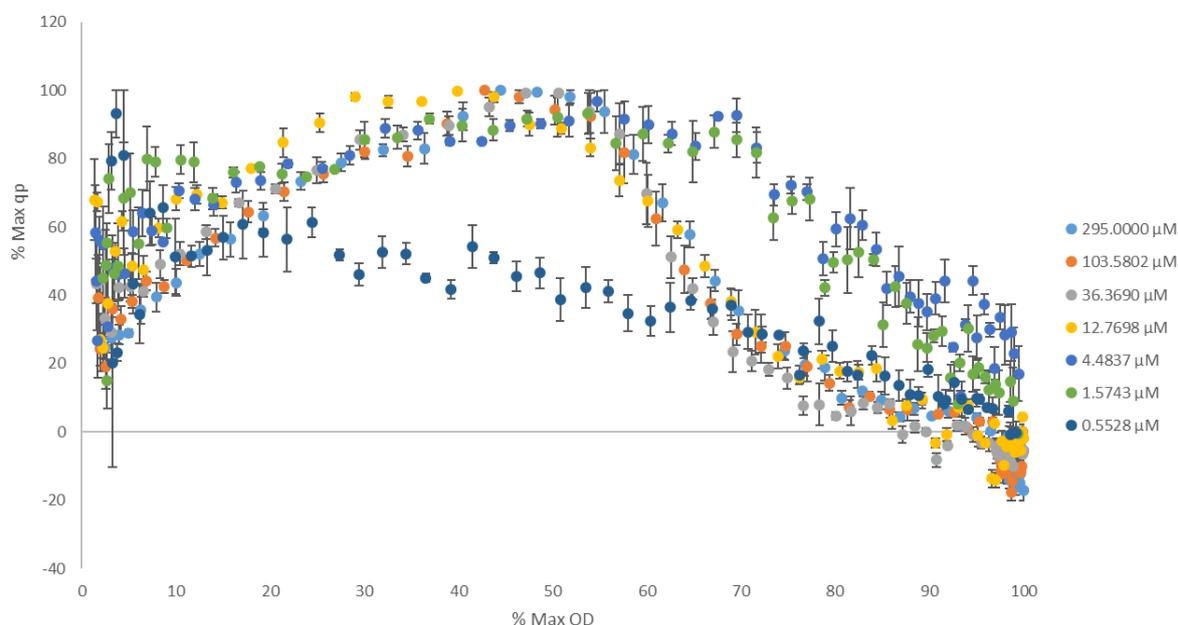


Figure 5.7 - Effect of manganese limitation on the specific RFU production rate expressed as a percentage of its maximum in each treatment. The *plaA* promoter appears to transcribe in two different modes in *L. plantarum* 423-neg pGKV-plaAPrV2mChB. This plot indicates that at a manganese concentration of 4.48 and 1.57 μM , plantaricin 423 is transcribed throughout the growth cycle.

This difference in promoter activity is especially evident between 70 – 90 % total growth for the 295.00 – 12.76 μM and 4.48 – 1.57 μM treatments relative to each treatment's maximal production rate (Figure 5.7). Manganese added at 0.55 μM results in the % Max specific production rate steadily decreasing throughout the growth cycle. This is likely due to the hindered total growth observed in Figure 5.3.

L. plantarum 423 pGKV-plaAPrV2mChB mCherry expression in the uncured strain

As seen in the cured reporter strain (*L. plantarum* 423-neg pGKV-plaAPrV2mChB), the maximal growth achieved decreased with decreasing manganese concentration supplementation (Figure 5.8).

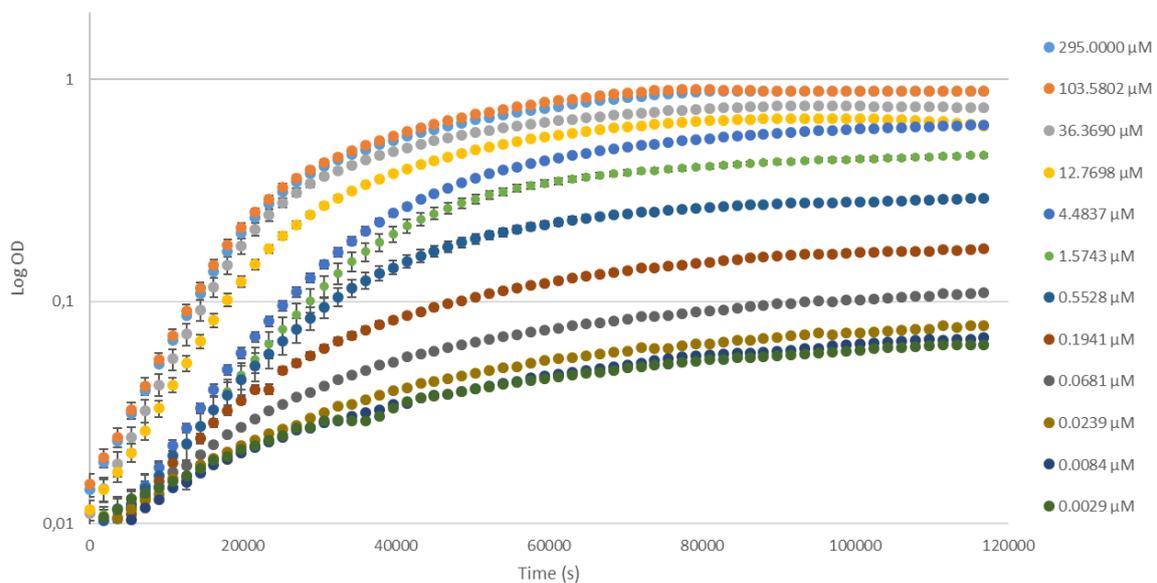


Figure 5.8 - Effect of manganese limitation on growth performance for the uncured *L. plantarum* 423 pGKV-plaAPrV2mChB transformants visualised as a semilogarithmic plot of OD values against time. Decreasing the available manganese caused a decrease in the maximal growth.

Treatments below a concentration of 0.55 μM manganese were excluded from further analysis due to the limited maximal growth (Figure 5.8). The RFU profile switching seen in Figure 5.4 for the *L. plantarum* 423-neg pGKV-plaAPrV2mChB strain was also observed in the *L. plantarum* 423 pGKV-plaAPrV2mChB reporter strain (Figure 5.9). However, the switch occurred at 12.76 μM manganese and not 4.48 μM and below (Figure 5.9). Maximal gene expression was once again seen at sub-maximal manganese supplementation treatments, although the total RFU is less intense in Figure 5.9 (Uncured) compared to Figure 5.4. (Cured). The highest RFU produced at sub-maximal manganese supplementation indicates that manganese limitation stimulates *plaA* transcription in *L. plantarum* 423 pGKV-plaAPrV2mChB as it did in the cured strain (Figure 5.9).

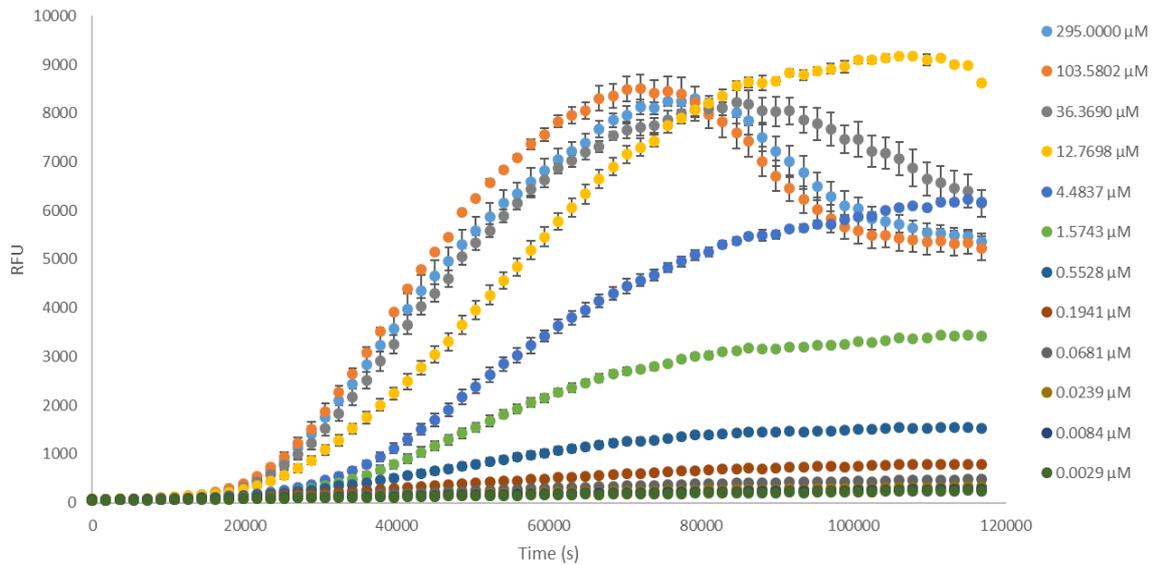


Figure 5.9 - Effect of manganese limitation on *plA* transcription measured in RFUs produced by the mCherry protein in *L. plantarum* 423 pGKV-*plA*PrV2mChB (uncured) reporter strain. Maximal RFU output was observed at submaximal manganese supplementation (12.76 μ M).

To directly compare promoter activity between treatments, the % Maximal growth was plotted against time for each treatment (Figure 5.10). After 80% of maximal growth, *L. plantarum* 423 pGKV-*plA*PrV2mChB had entered stationary phase growth in each treatment (Figure 5.10). For both the cured and uncured strain 80% growth was chosen as the point where treatments had defiantly exited log phase growth. The specific RFU production rates at each stage in growth was more comparable between all treatments for *L. plantarum* 423 pGKV-*plA*PrV2mChB (Figure 5.11). As observed for the

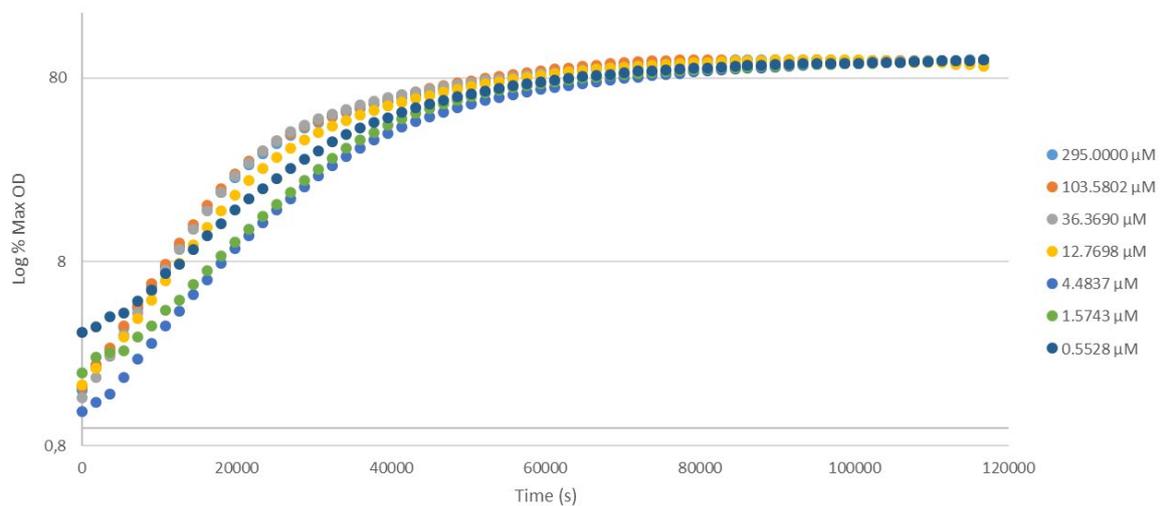


Figure 5.10 - Identification at which percentage of maximal growth all *L. plantarum* 423 pGKV-*plA*PrV2mChB transformants had exited the log phase and entered stationary phase in each manganese treatment. At 80% of the total growth in each treatment, *L. plantarum* 423 pGKV-*plA*PrV2mChB had exited exponential growth.

cured strain, the % Max specific production rate vs % Max growth profile highlighted a change in the promoter activity at 12.76 μM manganese relative to its maximal production rate (Figure 5.12).

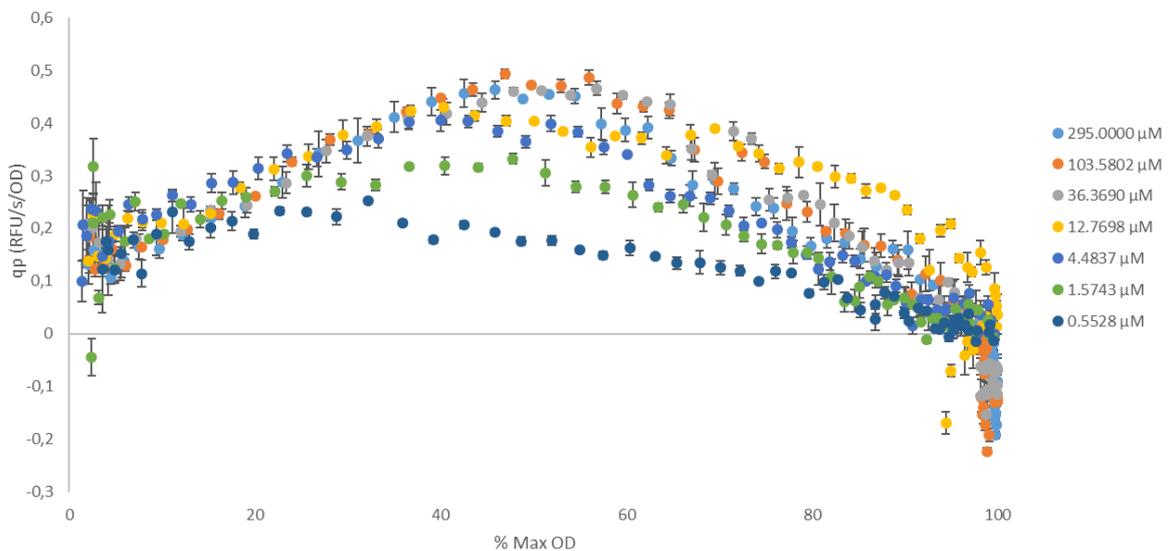


Figure 5.11 - Effect of manganese limitation on the specific RFU production rate produced by mCherry which therefore represents plantaricin 423 expression in the *L. plantarum* 423 pGKV-plaAPrV2mChB reporter strain. The *plaA* promoter profiles are comparable for all treatments above 0.55 μM .

Plotting the specific RFU production rate against time further indicated the switching of the *plaA* promoter at different manganese treatment concentrations (Figure 5.12). It can be observed that at 70 000s, *L. plantarum* 423 pGKV-plaAPrV2mChB in treatments from 295.00 – 12.769 μM MnSO_4 had all entered stationary phase, having achieved greater than 80% of their maximum growth (Figure

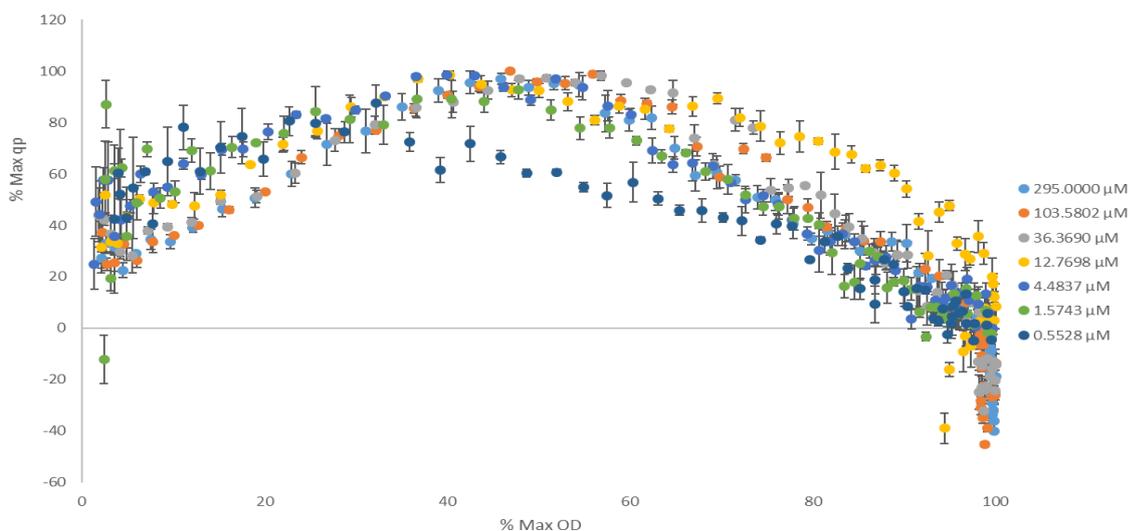


Figure 5.12 - Effect of manganese limitation on the specific RFU production rate expressed as a percentage its maximum in each treatment. At 12.76 μM manganese treatment, the *plaA* promoter is more active with respect to its maximal rate after 80 % max growth (stationary phase).

5.10). However, after 70 000s cultures in the 295.00 – 36.3 μM manganese treatments had a negative specific production rate. This indicated that the promoter was strongly repressed for a long enough time that mCherry fluorescence starts to degrade. The specific production rate then started to accelerate again after 90 000s. While in the 12.76 μM manganese treatment the specific production rate was positive throughout the growth cycle until late stationary phase.

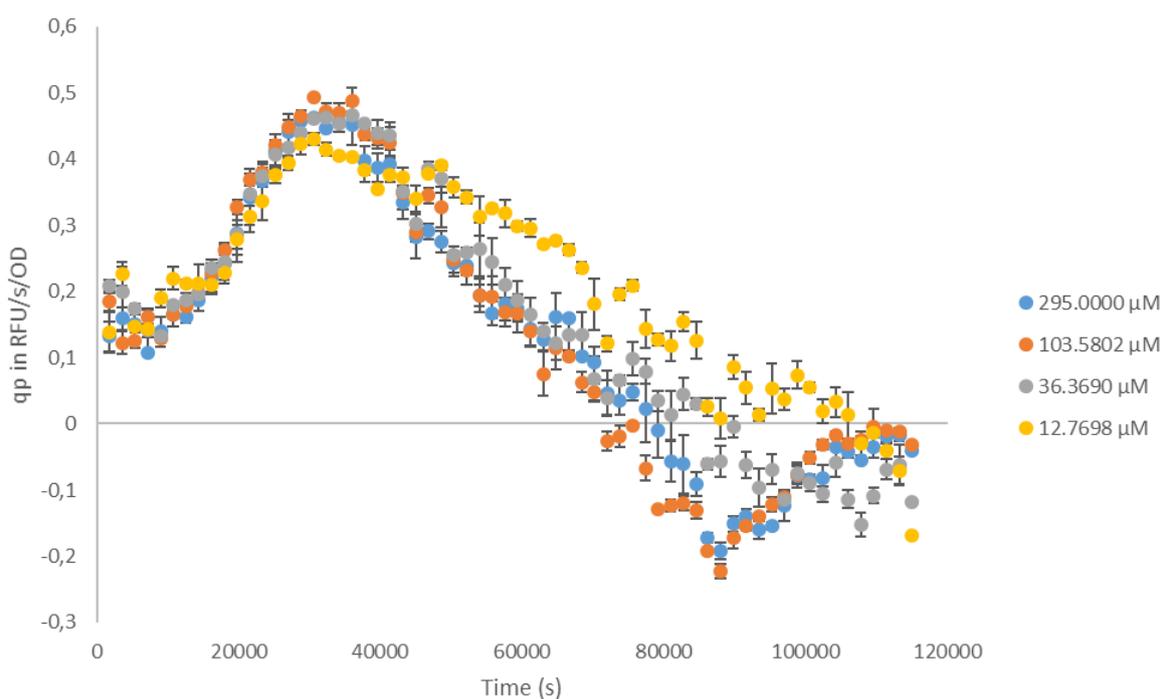


Figure 5.13 – Specific RFU production rate profile during the growth of *L. plantarum* 423 pGKV-plaAPrV2mChB over time. For the 295.00 – 36.36 μM treatments the RFU specific production rate become negative indicating strong repression of the *plaA* promoter for a long enough time that mCherry fluorescence started to dissipate. Treatment with 12.76 μM manganese produced a specific production rate that is positive until late in stationary phase

The effect of PlantEx on mCherry transcription in the *L. plantarum* 423-neg pGKV-plaAPrV2mChB transformants

The Ni-NTA purified PlantEx protein from Chapter 4 was used to ascertain if extracellular plantaricin 423 had any transcriptional effect on the *plaA* gene. WELQut cleaved PlantEx appeared to delay the onset of growth in *L. plantarum* 423-neg pGKV-plaAPrV2mChB transformant while, the RFU output

profiles were indistinguishable between un-cleaved and cleaved samples (data not shown). The effect of WELQut alone on the reporter strain was not measured.

Although cleaved and uncleaved PlantEx can be supplemented into the growth media of *L. plantarum* 423 pGKV-plaAPrV2mChB without interfering with mCherry RFU measurements, a more in-depth study needs to be conducted before conclusions can be drawn about the regulatory role of plantaricin 423 (data not shown). Such a study needs to more accurately calculate the concentration of liberated plantaricin 423 in the sample and determine if WELQut has an effect on growth.

Discussion

The mCherry-based reporter system developed in this chapter was able to successfully respond to and fluorometrically report on native plantaricin 423 transcriptional induction. The mechanism of transcriptional regulation has not been elucidated for plantaricin 423, or any other subclass IIa bacteriocins in the subgroup IPT2 (Chapter 3). This includes pediocin PA-1, which is likely the most well-known subclass IIa bacteriocin ^{1,24–26}.

Elucidating the regulatory mechanisms behind plantaricin 423 transcription requires an understanding of what factors stimulate plantaricin 423 expression. Once the stimulus is identified, an instance of high plantaricin 423 expression can be compared to an instance of low expression in *L. plantarum* 423. Then the differences between the two instances can be compared on an RNA and protein level to identify potential regulatory elements. The aim of this chapter was to investigate the mode of plantaricin 423 regulation on a transcriptional level. Manganese and iron were identified as factors which influenced the induction of mCherry transcription controlled by the *plaA* promoter. The Tecan Spark M10™ was able to monitor the effect of varying manganese concentrations on plantaricin 423 transcription, via the RFU output by mCherry, during a growth cycle. The effect of iron on plantaricin 423 regulation could not be fully elucidated by the Tecan Spark M10™ as iron supplementation had a significant impact on optical density readings.

The same reporter plasmid, pGKV-plaAPrV2mChB, was employed in both *L. plantarum* 423 which harbored the native pPla4 plasmid and a cured *L. plantarum* 423, devoid of the pPla4 plasmid. Although similar trends in the RFU profiles produced by these two systems during manganese limitation was observed, it is unknown which system best represented native *plaA* transcription. When the pPla4 plasmid is co-present with pGKV-plaAPrV2mChB, the *plaA* promoter exists in two different operon settings. One promoter directs transcription of plantaricin 423 and the other mCherry,

however, only the expression of mCherry was monitored. Therefore, any regulatory response to a stimulus is divided between these two promoters. This possible titration effect might explain why a less significant RFU response was seen in uncured *L. plantarum* 423, whereby the total RFUs produced were less, and deceleration of the RFU specific production rate happened earlier in growth under manganese limiting conditions.

The *L. plantarum* 423-neg pGKV-plaAPrV2mChB reporter transformant is arguably a simplified representation of *plaA* regulation. In this version, there is only one *plaA* promoter, and *L. plantarum* 423 has been relieved of any metabolic tax imposed by plantaricin 423 expression. This may be why a definite switch in *plaA* promoter activity was observed during manganese limitation in *L. plantarum* 423-neg pGKV-plaAPrV2mChB. However, it must be noted that the genome of *L. plantarum* 423-neg has not been sequenced, and therefore it is unknown if genes other than those on pPla4 were inadvertently lost during the plasmid curing process. Furthermore, plantaricin 423 could have a direct or indirect regulatory effect on transcription from the *plaA* promoter. If plantaricin 423 does have a self-regulatory effect, it would not be observed in this simplified version of *plaA* regulation.

Although the reporter systems in the cured and uncured *L. plantarum* 423 produced different results in terms of RFU specific production rates, both systems responded to limiting manganese conditions. Most organisms require manganese in trace amounts, yet *Lactobacillus plantarum*, a facultative anaerobe, requires significantly higher levels of intracellular manganese to manage the formation of reactive oxygen species^{27,28}. These abnormally high manganese levels are required to replace the activity of a superoxide dismutase enzyme, which *L. plantarum* 423 does not express. *Lactobacillus plantarum* requires high intracellular manganese levels in the millimolar range (30 – 35 mM) for the non-enzymatic conversion of superoxide to hydrogen peroxide^{27,28}.

Archibald *et al.*²⁷ demonstrated that manganese uptake in *L. plantarum* was dependent on a specific active transport system powered by the transmembrane proton gradient²⁷. This specific uptake system drove manganese transport until the intracellular manganese concentration accumulated to approximately 32 mM. However, this intracellular level of manganese could not be achieved when there was less than 200 μ M manganese present in the growth medium²⁷. Initial manganese uptake rates in manganese-starved cells showed first order kinetics with respect to an extracellular manganese concentration of 1 – 100 nM, and near-zero order rates at extracellular manganese concentrations of more than 1 μ M²⁷. Manganese uptake then decreased from the initial uptake rate with time, but sharply decreased with the accumulation of substantial intracellular manganese²⁷.

Herein might lie the selective pressure to maintain the pPla4 plasmid within *L. plantarum* 423. Plantaricin 423 could aid *L. plantarum* 423 in achieving sufficient intracellular manganese levels via poration of target cell walls, causing leakage of manganese into the environment²⁹. According to Watanabe *et al.*²⁸, higher manganese levels would facilitate aerobic respiration in *L. plantarum* and therefore produce higher maximal growth, providing *L. plantarum* 423 with a selective advantage. The findings of Archibald *et al.*²⁷ may also explain why the *plaA* promoter was down-regulated during growth at manganese concentrations of 295.00 – 36.3 µM. In these treatments, if sufficient intracellular manganese was accumulated, transcriptional repression of the active transport system would likely occur. If *plaA* transcriptional regulation mirrors the transcriptional regulation of the active transport system, this would explain why down-regulation was observed at higher manganese treatments.

One well-described mechanism of transcriptional repression in response to metals like iron, magnesium, zinc, and manganese is via the Fur metalloregulatory proteins. In this process, repression occurs upon metal-dependent dimerization of the Fur repressor protein when sufficient intracellular levels of metal have been achieved³⁰. As seen in Chapter 3, a gene for a Fur-like metalloregulator occurs downstream of a gene showing high homology to the *plaB* immunity protein gene within the *L. plantarum* 423 unassembled genome. In lower manganese concentration treatments (4.48 and 12.76 µM) for the cured and uncured *L. plantarum* 423 pGKV-*plaA*PrV2mChB transformants, the *plaA* promoter appeared to fire for longer and at a higher % of its maximal rate throughout the growth cycle. This might be due to manganese deepened dimerization failure at these lower concentrations.

Finally, the point mutation seen in an upstream palindromic sequence resulted in complete repression of the *plaA* promoter. This indicates that transcriptional regulation of plantaricin 423 may have another level of complexity. If such a point mutation occurred in the region of DNA that a Fur-like repressor could potentially bind to, one would anticipate alleviation of repression. In this case, transcriptional failure points to the requirement of an induction factor for the expression of plantaricin 423. In addition, the *plaA* promoter produces significantly different specific RFU production rates under high and low manganese treatments, which points to two different modes of *plaA* activity. For this reason, delocalized regulation by a distant RR or induction factor should not be excluded in future studies on plantaricin 423 regulation. Gene knock-out studies may now be performed on the Fur-like repressor and coding sequence showing high homology to *plaB*, to elucidate their role in the regulatory mechanism.

Appendix

Construction of the Green Fluorescent Protein (GFP) based *plaA* trans-reporter system

In this study the constitutive GFP reporter, pGKV-LDHM5, was constructed by substituting the *mCherry* gene on a BamHI/HindIII fragment with the *mgfp5* gene encoding GFP, in pGKVCherry (Appendix, Figure 5.19)³¹. The *mgfp5* gene was PCR amplified from the pTRKH3p15A-ErmGFP plasmid using the *mgfp_BamHI_Fwd* /*mgfp_HindIII_Rev* primer set and cloned in pJET1.2. Plasmid DNA was extracted from ampicillin resistant transformants, and the sequence confirmed using the pJET1.2_Fwd and pJET1.2_Rev primer set. The *mgfp5* gene was confirmed to be correct using the CLC assemble-to-reference function. The pGKV-LDHM5 plasmid was extracted from an erythromycin resistant *E. coli* MC1061 transformant expressing a green fluorescent phenotype.

Construction of the pGKV-M5 backbone vector

The pTRKH3p15A-ErmGFP plasmid contains the *mgfp5* gene with EcoRI/BamHI flanking RE sites. The pGKV-M5 plasmid was constructed by cloning *mgfp5* on an EcoRI/BamHI fragment from pTRKH3p15A-ErmGFP into pGKV223D (Figure 5.20). The pGKV-M5 plasmid DNA was extracted from an erythromycin resistant *E. coli* MC1061 transformant. This promoterless construct served as a negative fluorescence control and the backbone for the pGKV-*plaAM5* reporter plasmid. The pGKV-M5 plasmid was extracted from an erythromycin resistant *E. coli* MC1061 transformant.

Construction of the plantaricin 423 trans-reporter plasmid pGKV-*plaAM5*

Figure 5.14 depicts the upstream region of the *plaA* gene that was assumed to contain the plantaricin 423 promoter. This putative promoter (1st version), *plaAPr*, was used to construct the pGKV-*plaAM5* reporter plasmid (Figure 5.21). The *plaAPr* region was PCR amplified from *L. plantarum* 423 gDNA using the 423PR_NsiI_Fwd /423PR_EcoRI_Rev primer set. The amplicon was cloned using the CloneJET kit, sequenced and confirmed to be correct as previously described. The *plaAPr* was then cloned into pGKV-M5 on a NsiI/EcoRI fragment to construct pGKV-*plaAM5* (Figure 5.21). The pGKV-*plaAM5* plasmid was extracted from an erythromycin resistant *E. coli* MC1061 transformant displaying a green fluorescent phenotype.



Figure 5.14 – The native configuration of the upstream region of plantaricin 423 *plaA* in *L. plantarum* 423. Two successive Methionine codons are observed at the beginning of *plaA*, therefore the reporter gene *mCherry* may be cloned at two distances from the putative ribosomal binding site (underlined in purple) in the promoter region.

Figure 5.14 depicts the native form of the *plaA* promoter in transcriptional control of the *plaA* gene. Figure 5.15 depicts how the *plaAPr* fragment was positioned for the transcriptional control of the *mgfp5* gene in pGKV-*plaAM5*. In this cloning of the promoter (Figure 5.15), the first methionine in the *plaA* gene was assumed to be the start codon. The *mgfp5* start codon was cloned into the position of *plaA*'s first methionine codon in pGKV-*plaAM5* (Figure 5.20).

Electrotransformation of *L. plantarum* 423 with the pGKV-LDHM5, pGKV-M5, and pGKV-*plaAM5* plasmids.

Electrocompetent *L. plantarum* 423 was transformed with the pGKV-LDHM5, pGKV-M5, and pGKV-*plaAM5* plasmids respectively via electroporation. Genomic DNA was extracted from erythromycin resistant *L. plantarum* 423 transformants of pGKV-LDHM5, pGKV-M5, and pGKV-*plaAM5* plasmids respectively. The *X479* gene, native to *L. plantarum*, was PCR amplified from each gDNA extract using the 2142Fwd/2142Rev primer set. The GFP_BamHI_Fwd/GFP_HindIII_Rev, GFP_BamHI_Fwd/GFP_HindIII_Rev and 423PrNsi_Fwd/GFP_HindIII_Rev primer sets were used to confirm transformation with pGKV-LDHM5, pGKV-M5, and pGKV-*plaAM5* via PCR amplification.

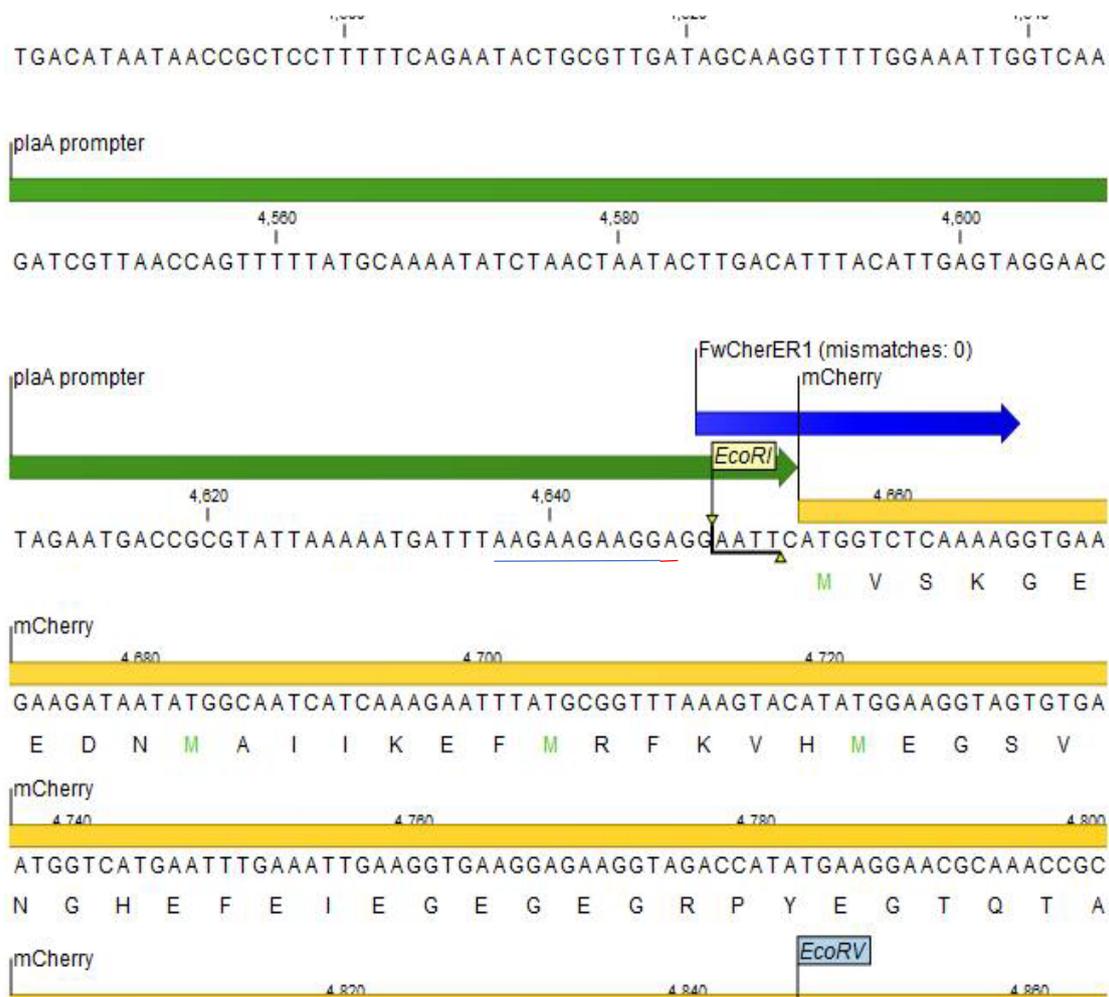


Figure 5.15 – The first version of the *plA* promoter-*mCherry* reporter system set the start codon of *mCherry* in the position of the first methionine of *plA* in the native *pln* operon.

None of the *L. plantarum* 423 transformants produced a green fluorescent phenotype. The non-fluorescent phenotype for the *L. plantarum* 423 pGKV-LDHM5 transformant agrees with the findings of Lizier *et al.*³¹ The *mgfp5* gene does not produce a fluorescent GFP under the cellular conditions of *L. plantarum* 423; therefore it cannot be used as a *in vivo* reporter in this study.

Construction of the mCherry based reporter plasmids pGKV-mCh and pGKV-plaAmCh

The previously constructed pGKVCherry plasmid was used as the positive fluorescent control in *L. plantarum* 423 as it harbors the LDH promoter in transcriptional control of the *mCherry* gene (Figure 5.17)²⁰. The *mCherry* gene was PCR amplified from the pGKVCherry plasmid using the mChe_EcoRI_Fwd/mChe_BamHI_Rev primer set²⁰. The modified *mCherry* amplicon was cloned using the CloneJET kit, sequenced and confirmed to be correct as previously described. The *mCherry* gene was then cloned on an EcoRI/BamHI fragment into pGKV-M5 and pGKV-plaAM5 to replace the *mgfp5*

sequences. This cloning generated the promoterless pGKV-mCh plasmid and the *plaA* reporter plasmid pGKV-plaAmCh.

Electrotransformation of *L. plantarum* 423 with the pGKVCherry, pGKV-mCh, and pGKV-plaAmCh plasmids.

Electrocompetent *L. plantarum* 423 cells were transformed with pGKVCherry, pGKV-mCh, and pGKV-plaAmCh plasmids as previously described. Genomic DNA was extracted from erythromycin resistant *L. plantarum* 423 transformed with pGKV-mCherry and pGKV-plaAmCh plasmids. The *JDM1_2142* gene was amplified from each gDNA extract using the 2142Fwd/2142Rev primer set ²². The mChe_EcoRI_Fwd/mChe_BamHI_Rev and 423PrNsi_Fwd/mChe_BamHI_Rev primer sets were used to confirm transformation with pGKV-mCherry and pGKV-plaAmCh, respectively, via PCR amplification.

The *L. plantarum* 423 pGKVCherry transformant had a red fluorescent phenotype, however, the pGKV-plaAmCh transformant did not. The *L. plantarum* 423 pGKV-plaAmCh transformant's supernatant was shown to be active against *Listeria* using the spot plate technique (result not shown). Therefore, the native *plaA* promoter was active and had transcribed plantaricin 423 during growth. These results indicated that the introduced *plaAPr* promoter fragment, on pGKV-plaAmCh, was not functioning.

Redesign of the promoter-reporter fragment can be found in in the main Methods & Materials section of Chapter 5.

Table 5.1 – Serial dilution of manganese sulfate to induce manganese limitation in *L. plantarum* 423 reporter strains

Treatment	Concentration μM
1	295.0000
2	103.5802
3	36.3690
4	12.7698
5	4.4837
6	1.5743
7	0.5528
8	0.1941
9	0.0681
10	0.0239
11	0.0084
12	0.0029

Table 5.2 - Oligonucleotides

Target	Primer	Sequence 5' -> 3'	Restriction sites	Product size (bp)
<i>JDM1_2142</i>	2142Fwd	ccgaattCGATACTAATACCTCGTAATACCG	EcoRI	1803
	2142Rev	gctctagaATATGAAATGGCCTATGATGG	XbaI	
<i>plaA</i> promoter (1 st version)	423PR_Nsil_Fwd	atgcatGACCAATCTTATTAGCCAGTTC	Nsil	267
	423PR_EcoRI_Rev	CATCATgaattcCTCCTTCTTCTTAAATC	EcoRI	

	mgfp_BamHI_Fwd	ggatccATGAGTAAAGGAGAAGAAC	BamHI	
<i>Mgfp5</i>				727
	mgfp_HindIII_Rev	aagcttTTATTTGTATAGTTCATCCATGCC	HindIII	
	plaAPrV2_Nsil_Fwd	atgcatTCTATTTAAAGTCGCATG	Nsil	
<i>plaA</i> promoter (2 nd version)				742
	plaAPrV2_EcoRI_Rev	CATgaattcAATCTCCTTCTTCTTAAATC	EcoRI	
	mChe_EcoRI_Fwd	gaattcATGGTCTCAAAGGTGAAGAAG	EcoRI	
<i>mCherry</i>				721
	mCh_BamHI_Rev	ggatccCTATTTATATAATTCATCCATACCAC	BamHI	
	plaB_BamHI_Fwd	ggatccAATGGGGGGGATTATCTTTGG	BamHI	
<i>plaB</i>				297
	plaB_PstI_Rev	ctgcagACCGGTCTAATGGTGTGGGATTG	PstI	
	plaB_BamHI_Fwd	ggatccAATGGGGGGGATTATCTTTGG	BamHI	
<i>plaBCD</i>				3372
	PlaD(Corrected)_PstI_Rev	ctgcagTTATTCTTGCTTATGAATTAACCGTGC	PstI	
<i>plaC</i>	PlaC(1)_seq_Fwd	TTGTCTAAGAAATTTGGTC		
<i>plaC</i>	PlaC(528)_seq_Fwd	ACCTGGTTAATATGGTTTTG		
<i>plaD</i>	PlaD(404)_seq_Fwd	AGATTGCAATTATCATAGC		<i>plaBCD</i> sequencing
<i>plaD</i>	plaD(928)_seq_Fwd	TTTTTATGCTCGTTAGTTG		
<i>plaD</i>	PlaD(1449)_seq_Fwd	TGGTGATATTGAGGTTAATC		
<i>plaD</i>	PlaD(1954)_seq_Fwd	TCAACCAGTAATTTAGACAC		

Table 5.3 – *plaA* transcriptional reporter constructs deployed in *L. plantarum* 423 strains and their role within mode elucidation

Strain	Construct	Function	Reference
<i>L. plantarum</i> 423 (uncured)			
	pGKVCherry	Positive control for functional mCherry expression measured in RFU	²⁰
	pGKV-mCh	Promoterless pGKV derivative used as a negative fluorescence control and backbone vector for the construction of reporter plasmids harbouring the <i>plaA</i> promoter	This study
	pGKV-plaAPrV2mChB	<i>plaA</i> reporter construct used to mirror the transcriptional activity of plantaricin 423 in the uncured <i>L. plantarum</i> 423. The construct harbours the <i>plaB</i> immunity protein cloned into the same position as the seen in the native operon. Transformant was used to assess the transcriptional effect of manganese on <i>plaA</i> transcription in the uncured strain.	This study
<i>L. plantarum</i> 423-neg			
	pGKV-plaAPrV2mCh	<i>plaA</i> reporter construct harbouring just the <i>plaA</i> promoter fused to <i>mCherry</i> . Construct proved minimum requirement for transcription of <i>plaA</i> from the <i>pla</i> operon is just the promoter fragment (<i>plaAPrV2</i>).	This study
	pGKV-plaAPrV2mChB	<i>plaA</i> reporter construct harbouring just the <i>plaA</i> promoter fused to <i>mCherry</i> and the downstream immunity protein gene <i>plaB</i> . Construct proved <i>plaB</i> did not affect transcription of <i>plaA</i> . The presence of PlaB was not assessed due to inherent immunity of <i>L. plantarum</i> 423 to plantaricin 423 ²³ .	This study

	Transformant was used to assess the transcriptional effect of manganese on <i>plaA</i> transcription in the cured strain.	
pGKV- plaAPrV2mChBCD	<i>plaA</i> reporter construct harbouring just the <i>plaA</i> promoter fused to <i>mCherry</i> and the downstream <i>pla</i> operon immunity protein (<i>plaB</i>), accessory protein (<i>plaC</i>), ABD-transporter (<i>plaD</i>). Construct proved <i>plaBCD</i> did not affect transcription of <i>plaA</i> . The presence of PlaB, PlaC, and PlaD transcription was not assessed.	This study

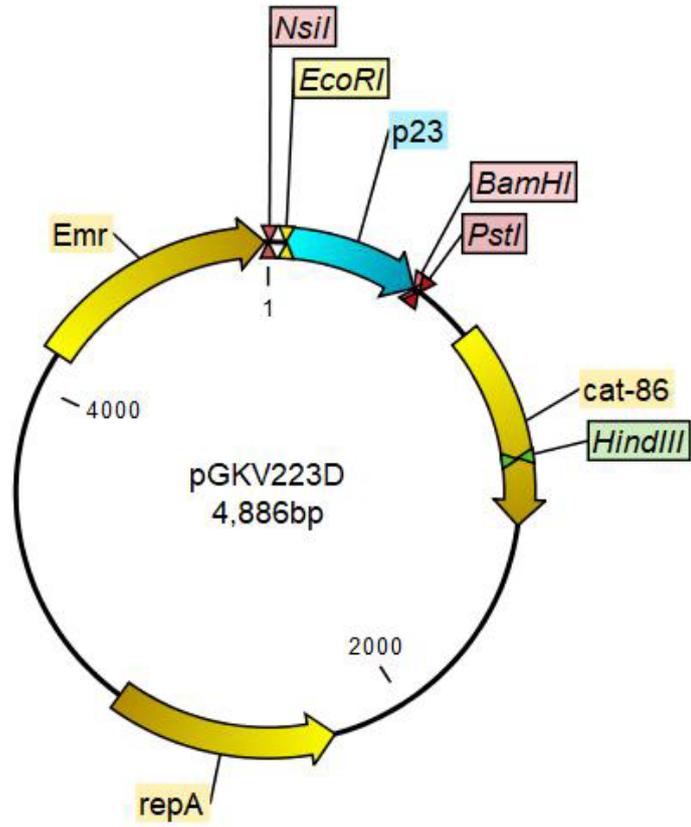


Figure 5.16 - The pGKV223D plasmid used to construct the reporter system for plantaricin 423 transcription. *repA* – replication in *E. coli*, *Cat86* – chloramphenicol resistance marker, *ErmC* – erythromycin resistance marker.

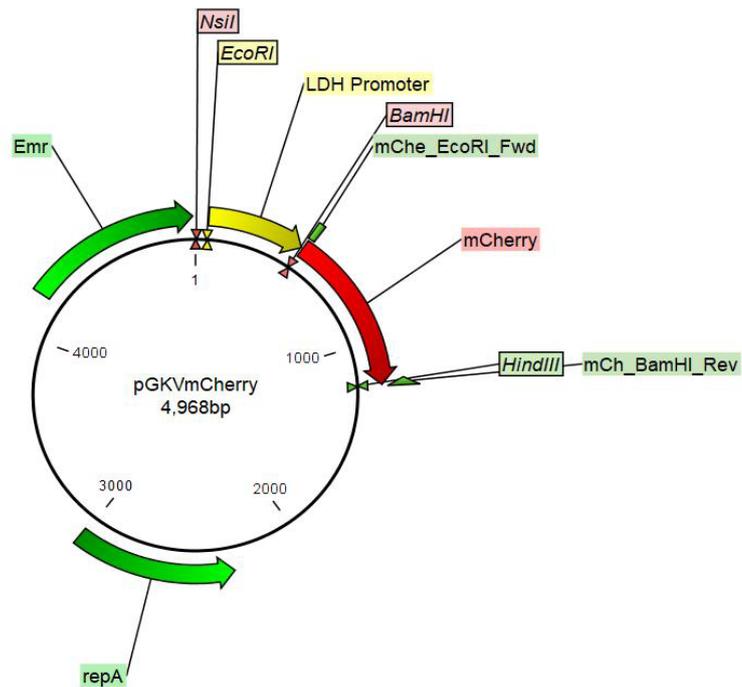


Figure 5.17 - The previously constructed pGKVCherry plasmid constructed by Van Zyl et al⁵², which constitutively transcribes mCherry from the LDH promoter in *L. plantarum* 423

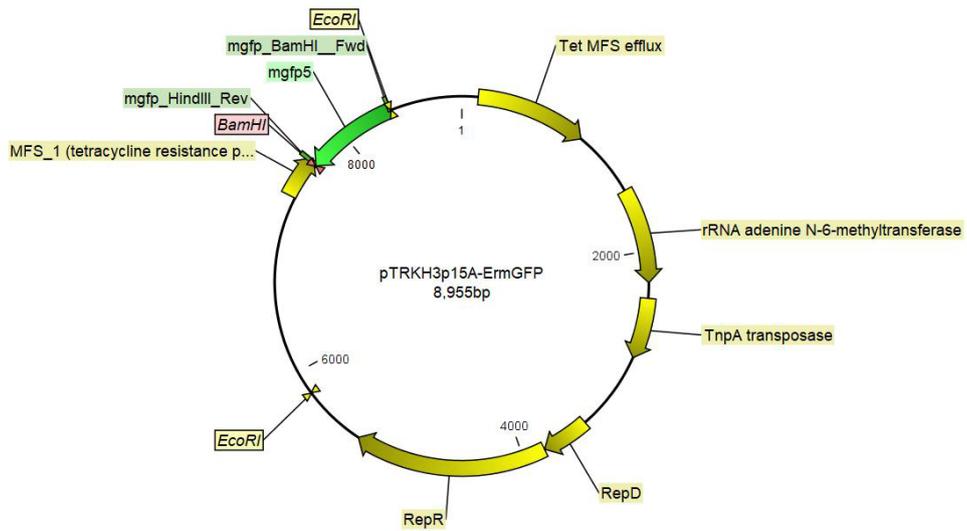


Figure 5.18 – pTRKH3p15A-ErmGFP plasmid use a template for mgfp5 PCR amplification.

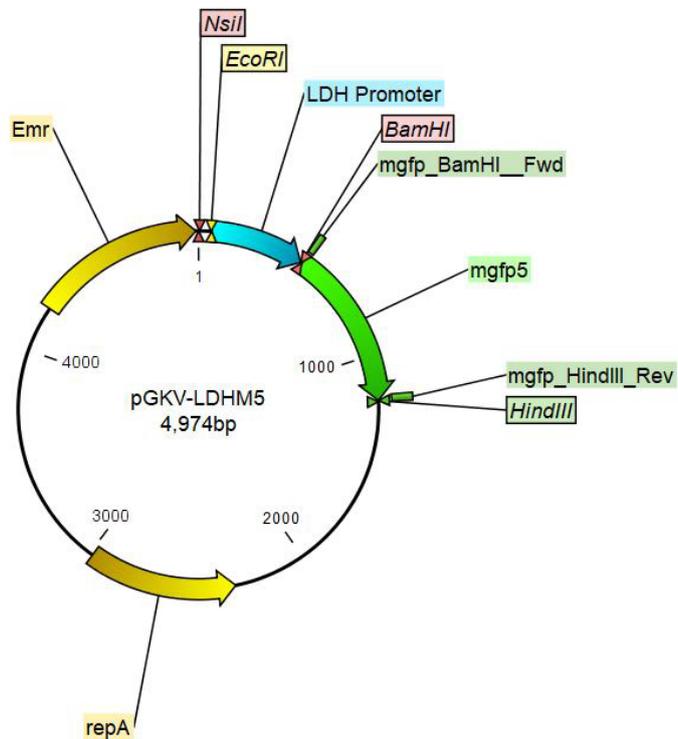


Figure 5.19 – The pGKV-LDHM5 plasmid was derived from pGKVCherry to function as a constitutive GFP reporter in *L. plantarum* 423. The LDH promoter was controlled the expression of the mgfp5 gene to produce GFP.

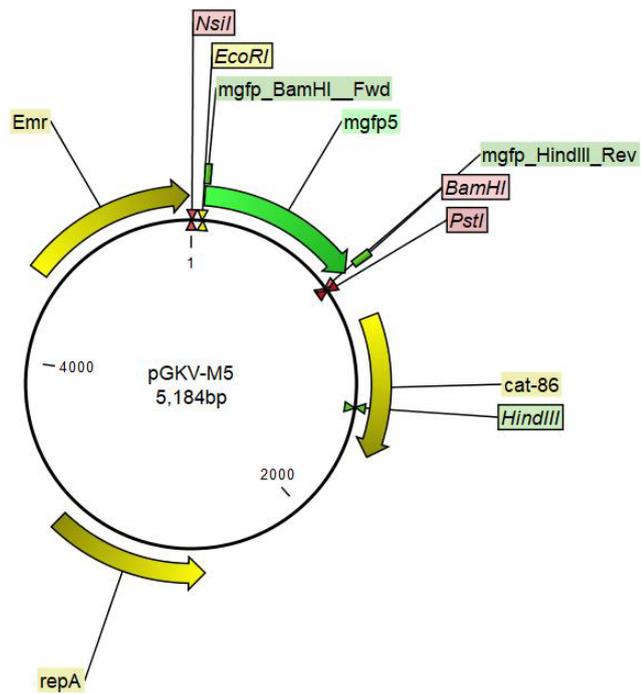


Figure 5.20 - The pGKV-M5 backbone vector was constructed without a promoter in transcriptional control of mgfp5. This vector served as a negative fluorescence control, and the backbone for the subsequent fluorescent reporter plasmids.

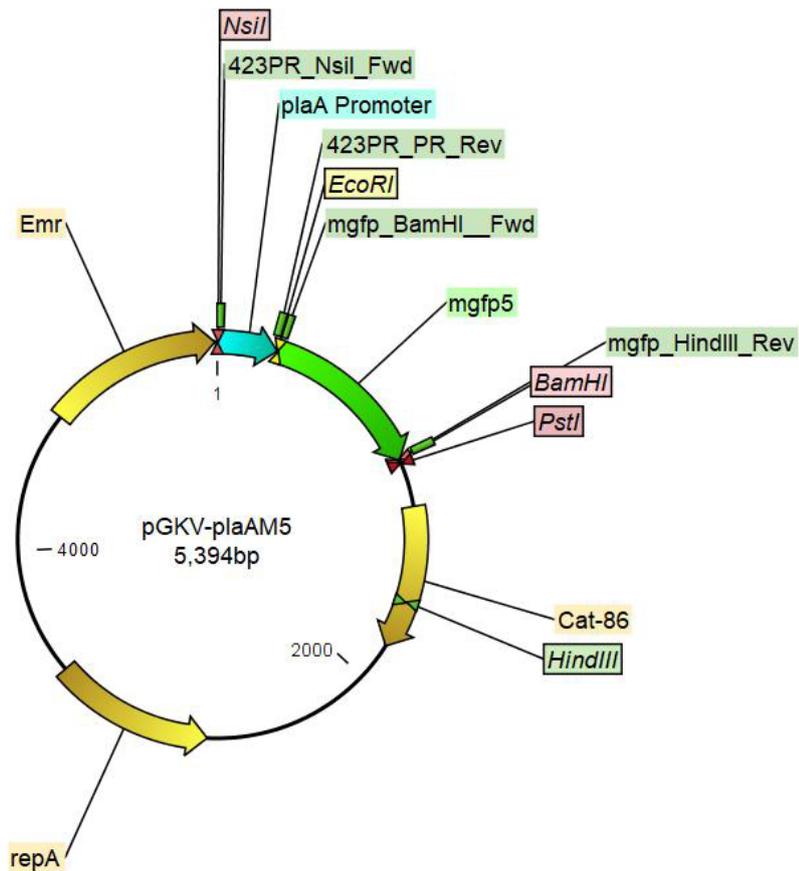


Figure 5.21 – The pGKV-plaM5 plasmid was derived from pGKV-M5 and contain the putative *plaA* promoter cloned in transcriptional control of the *mgfp5* gene. The *plaA* promoter-*mgfp5* fragment was constructed with the start codon for *mgfp5* in the position of the first methionine for *plaA* (1st version of promoter reporter fragment).

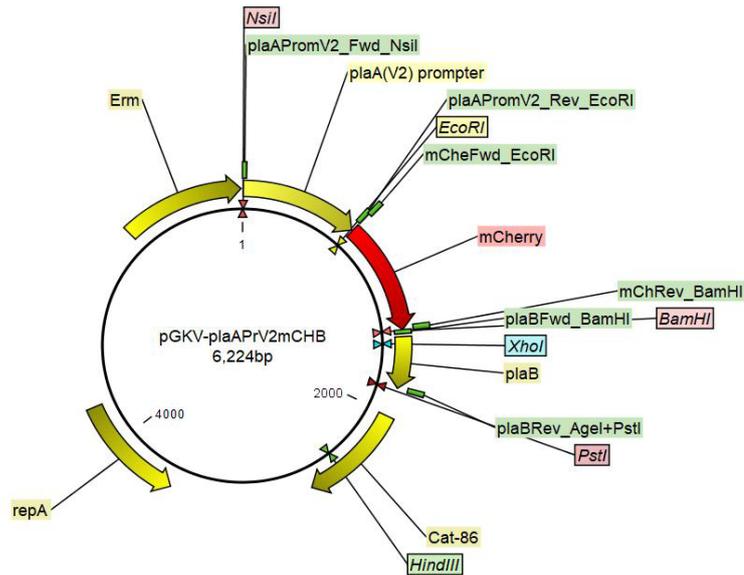


Figure 5.22 – The pGKV-plaAPrV2mChB plasmid is identical to the pGKV-plaAPrV2mCh plasmid however it also contains the *plaB* immunity protein gene from the *pla* operon.

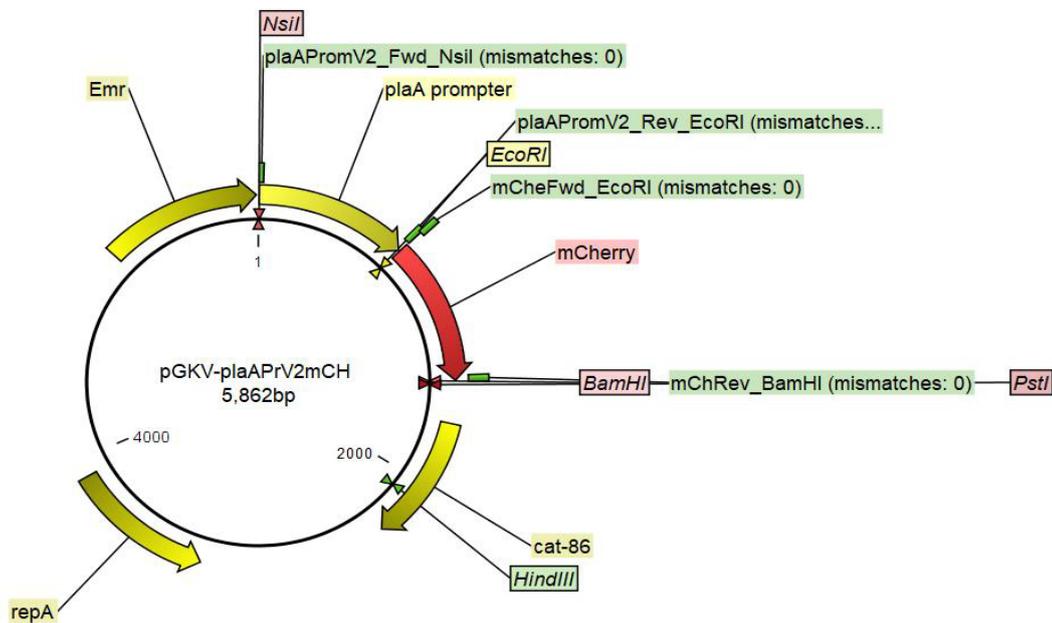


Figure 5.23 – The pGKV-plaAPrV2mCh plasmid was derived from the pGKV223D plasmid. It contains the *plaA* promoter region *plaAPrV2* fused to the *mCherry*. This plasmid mirrored the transcriptional activity of the *plaA* gene and fluorometrically reported the level of expression.

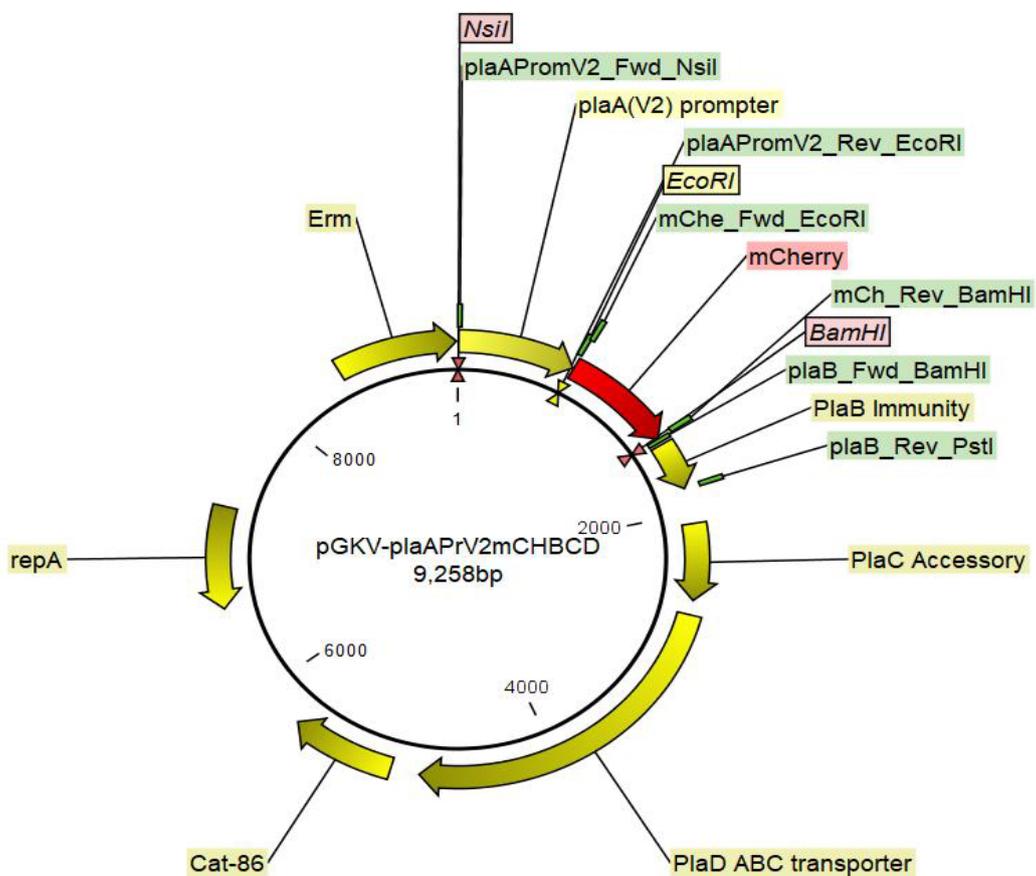


Figure 5.24 - The pGKV-plaAPrV2mChBCD plasmid is identical to the pGKV-plaAPrV2mChB plasmid however it also contains the *plaC* accessory protein and *plaD* ABC transporter genes from the *pla* operon.

References

1. Drider, D., Fimland, G., Hechard, Y., McMullen, L. M. & Prevost, H. The continuing story of Class IIa bacteriocins. *Microbiol. Mol. Biol. Rev.* 70, 564–582 (2006).
2. Kehl-Fie, T. E. & Skaar, E. P. Nutritional immunity beyond iron: a role for manganese and zinc. *Curr. Opin. Chem. Biol.* 14, 218–224 (2010).
3. Merritt, J., Kreth, J., Shi, W. & Qi, F. LuxS controls bacteriocin production in *Streptococcus mutans* through a novel regulatory component. *Mol. Microbiol.* 57, 960–969 (2005).
4. Maldonado-Barragán, A., Caballero-Guerrero, B., Lucena-Padrós, H. & Ruiz-Barba, J. L. Induction of bacteriocin production by co-culture is widespread among plantaricin-producing *Lactobacillus plantarum* strains with different regulatory operons. *Food Microbiol.* 33, 40–47 (2013).
5. Franz, C. M. A. P., Van Belkum, M. J., Worobo, R. W., Vederas, J. C. & Stiles, M. E. Characterization of the genetic locus responsible for production and immunity of carnobacteriocin A: The immunity gene confers cross-protection to enterocin B. *Microbiology.* 146, 621–631 (2000).
6. Miller, M. B. & Bassler, B. L. Quorum sensing in bacteria. *Annu. Rev. Microbiol.* 55, 165–199 (2001).
7. Kuipers, O. P., de Ruyter, P. G. G., Kleerebezem, M. & de Vos, W. M. Quorum sensing-controlled gene expression in lactic acid bacteria. *J. Biotechnol.* 64, 15–21 (1998).
8. Horinouchi, S., Ueda, K., Nakayama, J. & Ikeda, T. in *Comprehensive Natural Products II* 21, 283–337 (Elsevier, 2010).
9. Engelke, G. et al. Regulation of nisin biosynthesis and immunity in *Lactococcus lactis* 6F3. *Appl. Environ. Microbiol.* 60, 814–825 (1994).
10. Peschel, A., Augustin, J., Kupke, T., Stevanovic, S. & Götz, F. Regulation of epidermin biosynthetic genes by EpiQ. *Mol. Microbiol.* 9, 31–39 (1993).
11. Saucier, L., Paradkar, A. S., Frost, L. S., Jensen, S. E. & Stiles, M. E. Transcriptional analysis and regulation of carnobacteriocin production in *Carnobacterium piscicola* LV17. *Gene.* 188, 271–277 (1997).
12. Gursky, L. J. et al. Production of piscicolin 126 by *Carnobacterium maltaromaticum* UAL26 is controlled by temperature and induction peptide concentration. *Arch. Microbiol.* 186, 317–325 (2006).
13. Ekblad, B. et al. Structure-function analysis of the two-peptide bacteriocin plantaricin EF. *Biochemistry.* 55, 5106–5116 (2016).

14. Di Cagno, R. et al. Quorum sensing in sourdough *Lactobacillus plantarum* DC400: Induction of plantaricin A (PlnA) under co-cultivation with other lactic acid bacteria and effect of PlnA on bacterial and Caco-2 cells. *Proteomics*. 10, 2175–2190 (2010).
15. Diep, D. B., Håvarstein, L. S. & Nes, I. F. Characterization of the locus responsible for the bacteriocin production in *Lactobacillus plantarum* C11. *J. Bacteriol.* 178, 4472–4483 (1996).
16. van der Veen, S. & Abee, T. Mixed species biofilms of *Listeria monocytogenes* and *Lactobacillus plantarum* show enhanced resistance to benzalkonium chloride and peracetic acid. *Int. J. Food Microbiol.* 144, 421–431 (2011).
17. Hugas, M., Garriga, M., Pascual, M., Aymerich, M. T. & Monfort, J. M. Enhancement of sakacin K activity against *Listeria monocytogenes* in fermented sausages with pepper or manganese as ingredients. *Food Microbiol.* 19, 519–528 (2002).
18. Sambrook, J., Fritsch, E. F. & Maniatis, T. *Molecular cloning: a laboratory manual*. Mol. cloning a Lab. manual. (1989). at <<https://www.cabdirect.org/cabdirect/abstract/19901616061>>
19. Birnboim, H. C. & Doly, J. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* 7, 1513–1523 (1979).
20. van Zyl, W. F., Deane, S. M. & Dicks, L. M. T. Use of the mCherry fluorescent protein to study intestinal colonization by *Enterococcus mundtii* ST4SA and *Lactobacillus plantarum* 423 in mice. *Appl. Environ. Microbiol.* 81, 5993–6002 (2015).
21. Ausubel, F. M. et al. *Current Protocols in Molecular Biology*. Current protocols in molecular biology / edited by Frederick M. Ausubel ... [et al.] Chapter 2, (John Wiley & Sons, Inc., 2003).
22. Heunis, T., Deane, S., Smit, S. & Dicks, L. M. T. Proteomic profiling of the acid stress response in *Lactobacillus plantarum* 423. *J. Proteome Res.* 13, 4028–4039 (2014).
23. Van Reenen, C. a., Van Zyl, W. H. & Dicks, L. M. T. Expression of the immunity protein of plantaricin 423, produced by *Lactobacillus plantarum* 423, and analysis of the plasmid encoding the bacteriocin. *Appl. Environ. Microbiol.* 72, 7644–7651 (2006).
24. Uteng, M. et al. Three-dimensional structure in lipid micelles of the pediocin-like antimicrobial peptide sakacin P and a sakacin P variant that is structurally stabilized by an inserted C-terminal disulfide bridge. *Biochemistry*. 42, 11417–11426 (2003).
25. Moon, G. S., Pyun, Y. R. & Kim, W. J. Characterization of the pediocin operon of *Pediococcus acidilactici* K10 and expression of his-tagged recombinant pediocin PA-1 in *Escherichia coli*. *J. Microbiol. Biotechnol.* 15, 403–411 (2005).
26. Ramnath, M., Beukes, M., Tamura, K. & Hastings, J. W. Absence of a putative mannose-specific phosphotransferase system enzyme IIAB component in a leucocin a-resistant strain of *Listeria monocytogenes*, as shown by two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Appl. Environ. Microbiol.* 66, 3098–3101 (2000).

27. Archibald, F. S. & Duong, M. N. Manganese acquisition by *Lactobacillus plantarum*. *J. Bacteriol.* 158, 1–8 (1984).
28. Watanabe, M., van der Veen, S., Nakajima, H. & Abee, T. Effect of respiration and manganese on oxidative stress resistance of *Lactobacillus plantarum* WCFS1. *Microbiology.* 158, 293–300 (2012).
29. Nissen-Meyer, J., Rogne, P., Oppegard, C., Haugen, H. & Kristiansen, P. Structure-function relationships of the non-lanthionine-containing peptide (class II) bacteriocins produced by Gram-positive bacteria. *Curr. Pharm. Biotechnol.* 10, 19–37 (2009).
30. Lee, J.-W. & Helmann, J. D. Functional specialization within the Fur family of metalloregulators. *Biometals.* 20, 485–499 (2007).
31. Lizier, M., Sarra, P. G., Cauda, R. & Lucchini, F. Comparison of expression vectors in *Lactobacillus reuteri* strains. *FEMS Microbiol. Lett.* 308, 8–15 (2010).

Chapter 6

General Conclusions

The end goal of this research was to provide evidence that will help better understand how *L. plantarum* 423 and *E. mundtii* ST4SA function as a probiotic supplement. It has become evident that the probiotic effect of *L. plantarum* 423 and *E. mundtii* ST4SA is dependent on the intraspecies, interspecies and interkingdom relationships they engage in within the gastrointestinal tract (GIT). While the physiochemical properties of the GIT define the governing laws that these organisms must live by, communication between these organisms is likely established by some of their metabolic by-products. It has been proposed that some part of the relationship between strains 423 and ST4SA is mediated by the subclass IIa bacteriocins they produce. However, to understand if this is a direct line for chemical communication or just an indirect metabolic effect of each producer's class IIa bacteriocin, one must first fully understand the regulatory mechanism of each bacteriocin.

This study observed the transcriptional upregulation of the *plaA* gene in response to manganese limitation in *L. plantarum* 423 using a fluorescent promoter-reporter system paired with real-time *in vivo* measurements made by the Tecan Spark M10. *Lactobacillus plantarum* is a facultative anaerobe that does not contain a complete electron transport chain, Krebs cycle or superoxide dismutase^{1,2}. However, it has been demonstrated that *L. plantarum* aerobically respire using its electron transport chain (ETC) in the presence of haem, vitamin K2 (menaquinone) and manganese¹⁻³. Superoxide is a radical oxygen species which escapes from the ETC during aerobic respiration and can damage DNA and proteins. However, aerobic respiration capabilities are beneficial as they yield more energy per glucose molecule. *Lactobacillus plantarum* 423 does not express a superoxide dismutase, and therefore requires high intracellular levels of manganese to scavenge and convert the superoxide radicals into hydrogen peroxide^{1,2}. It has also been demonstrated that manganese uptake in *L. plantarum* 423 is a specific and active process that occurs at a rate which is many folds higher than in certain other bacteria¹. Therefore, plantaricin 423 may aid *L. plantarum* 423 in manganese acquisition via the poration and subsequent leakage of manganese from target organisms.

Plantaricin 423 is a class IIa bacteriocin produced from the *plaA* gene and has high potency against *Listeria* species. Class IIa bacteriocins show high sequence homology, especially in an N-terminus conserved motif known as the pediocin box. While this high level of homology within the class IIa bacteriocins may distort their phylogenetic relationships, it also indicates that these bacteriocins all fulfil a similar function. Their conserved pediocin box mediates docking to an extracellular loop of the IIC protein (MptC) from the sugar transporter mannose phosphotransferase system (Man-PTS) found in *Listeria* spp.⁴⁻⁶.

L. plantarum 423 is known to be a native inhabitant of the GIT and produces an anti-listerial peptide in response to manganese limiting conditions. Transition metals like manganese fulfil critical roles for

the functioning of biological systems. The human body employs mechanisms to limit the availability of transition metals to invading bacteria in the process known as nutritional immunity⁷. Manganese (Mn^{2+}) availability is limited at mucosal and epithelial surfaces within the host via sequestration by proteins like the S100 family^{7,8}. The mucosal and epithelial surfaces of the gut-blood barrier within the GIT present a location where *L. plantarum* 423 and *Listeria* spp. might interact, as *Listeria* must cross this barrier to invade the human host. Pathogenic organisms like *Listeria* monitor the concentration of transition metals to assess when they are within the human body. Although it is currently unknown if low concentrations of manganese stimulate the expression of virulence genes in *Listeria*, as seen with iron, literature indicates that this phenomenon is probable^{8,9}.

It may be hypothesized that *L. plantarum* 423 can inhibit the growth of *L. monocytogenes* close to the GIT epithelial layer via manganese-mediated scramble and contested competition strategies. *Lactobacillus plantarum* 423 may cause scramble competition by working with the GIT to sequester manganese using its specific, high-velocity active transport system. Even though *Listeria* may respond to these conditions via the expression of virulence genes, lower manganese levels would make survival more difficult as manganese uptake in *Listeria* is thought to be by diffusion^{7,8}. Under manganese limiting conditions *L. plantarum* 423 can induce contested competition by transcriptionally upregulating the *plaA* gene which produces the anti-listerial peptide, plantaricin 423. Furthermore, facultative anaerobes like *L. plantarum* 423 are thought to play an essential part in driving the microbiota in the GIT lumen towards anaerobiosis by consuming oxygen released from intestinal tissue¹⁰.

The research in this thesis has also presented significant secondary findings. Chapter 3 provides a testament to the classification scheme presented by Willey *et al.*¹¹, as significant evidence for IPT2 subclass IIa bacteriocin regulation could be established. Chapter 4 presents a rapidly optimizable peptide expression system that allows sample visualization throughout the fermentation, extraction, purification and optimization process. This system will undoubtedly be employed in future cationic peptide studies where high yield heterologous expression is required. This thesis, but in particular Chapter 5, presents an approach to fluorometrically monitor and generate high quality real-time *in vivo* data on the transcriptional rates of a single gene with potential autoinduction capabilities in *L. plantarum* 423. This approach should now be applied to the regulation of the *plnA* gene and its effect on an intraspecies, interspecies and interkingdom level. Such interactions may highlight *L. plantarum* 423 as a keystone probiotic, which would further motivate its use.

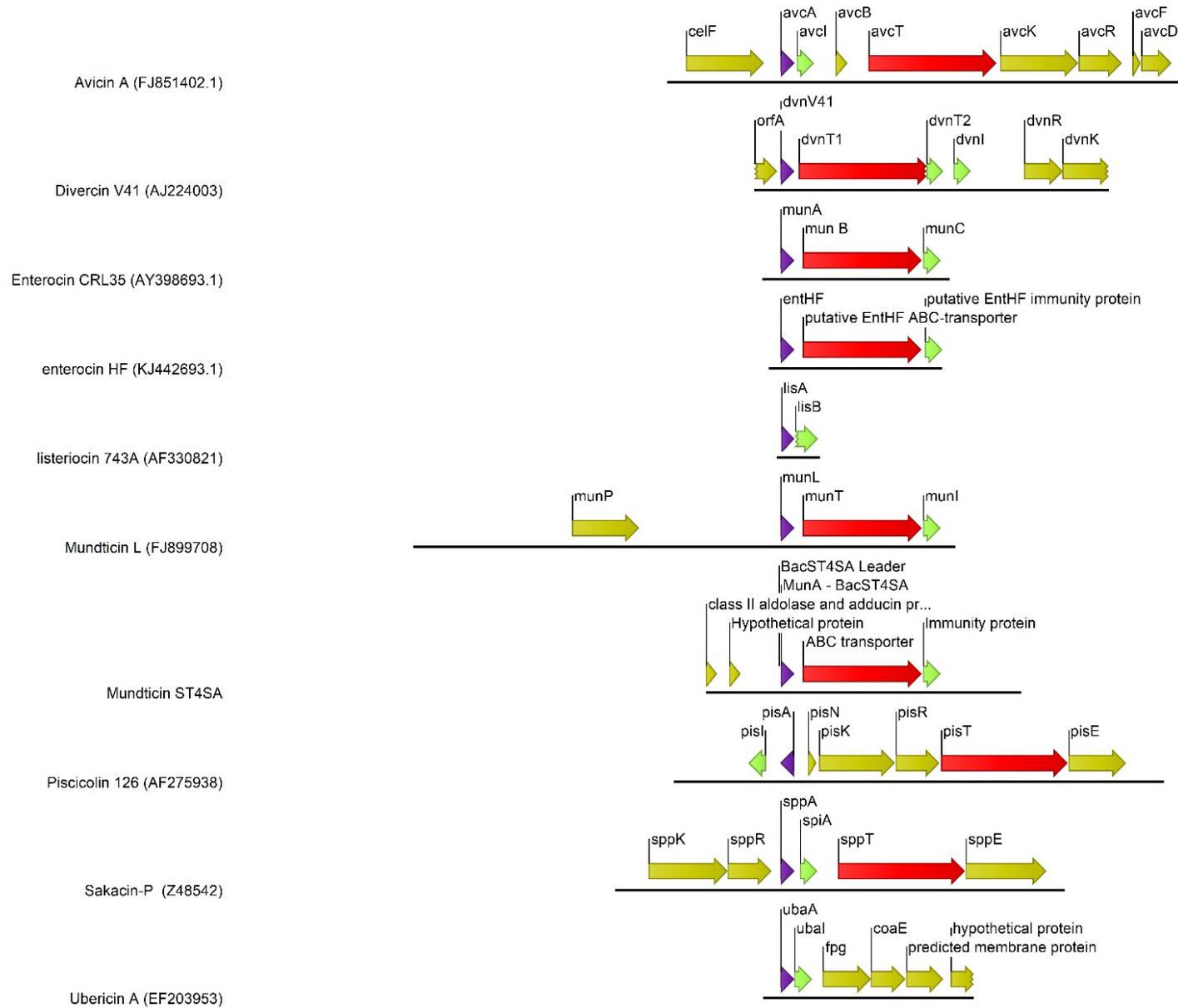
In conclusion, *L. plantarum* 423 presents an inextricably linked tripartite anti-listerial scheme, whereby strain 423 strives to respire aerobically near the epithelial layer driving the lumen into an anaerobic

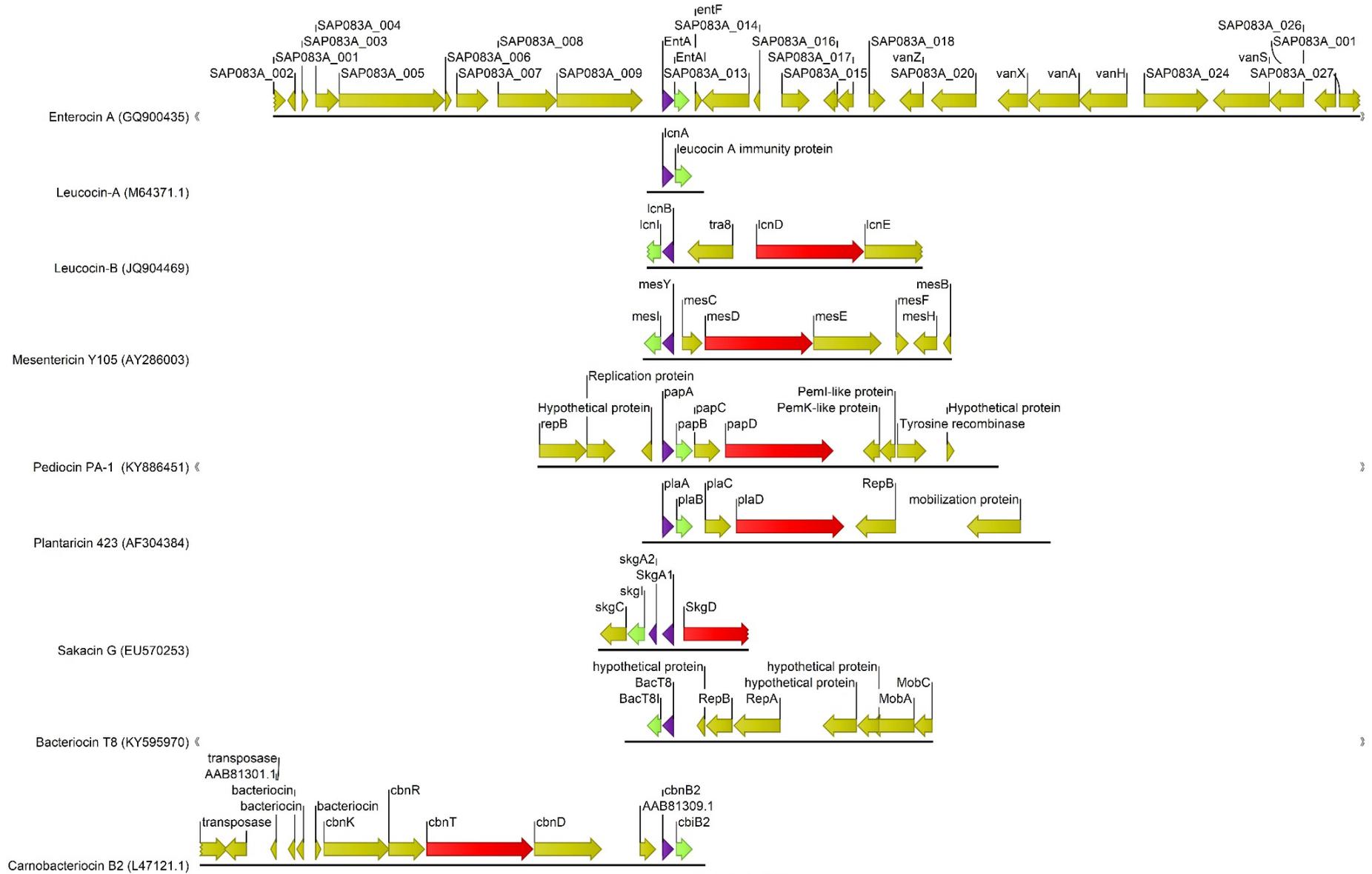
state ¹⁰. For aerobic respiration, *L. plantarum* 423 requires large amounts of intracellular manganese to cope with superoxide formation. Under manganese limiting conditions, *L. plantarum* may increase the available manganese through poration of target cell membranes, via the expression of plantaricin 423. Available manganese in the environment is quickly sequestered by *L. plantarum* 423 cells using its high-velocity specific transport system, which in turn drives aerobic respiration. Considering the recent Listeriosis outbreak in South Africa, this is a hypothetical model for antilisterial activity that deserves further research.

References

1. Archibald, F. S. & Duong, M. N. Manganese acquisition by *Lactobacillus plantarum*. *J. Bacteriol.* 158, 1–8 (1984).
2. Watanabe, M., van der Veen, S., Nakajima, H. & Abee, T. Effect of respiration and manganese on oxidative stress resistance of *Lactobacillus plantarum* WCFS1. *Microbiology.* 158, 293–300 (2012).
3. Brooijmans, R. et al. Heme and menaquinone induced electron transport in lactic acid bacteria. *Microb. Cell Fact.* 8, 1–11 (2009).
4. Ramnath, M., Beukes, M., Tamura, K. & Hastings, J. W. Absence of a putative mannose-specific phosphotransferase system enzyme IIAB component in a leucocin a-resistant strain of *Listeria monocytogenes*, as shown by two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Appl. Environ. Microbiol.* 66, 3098–3101 (2000).
5. Kjos, M., Salehian, Z., Nes, I. F. & Diep, D. B. An extracellular loop of the mannose phosphotransferase system component IIC is responsible for specific targeting by class IIa bacteriocins. *J. Bacteriol.* 192, 5906–5913 (2010).
6. Nissen-Meyer, J., Rogne, P., Oppegard, C., Haugen, H. & Kristiansen, P. Structure-function relationships of the non-lanthionine-containing peptide (class II) bacteriocins Produced by Gram-positive bacteria. *Curr. Pharm. Biotechnol.* 10, 19–37 (2009).
7. Hood, M. I. & Skaar, E. P. Nutritional immunity: transition metals at the pathogen-host interface. *Nat. Rev. Microbiol.* 10, 525–537 (2012).
8. Jesse, H. E., Roberts, I. S. & Cavet, J. S. *iAdvances in bacterial pathogen biology.* 65, 83–123 (Elsevier Ltd., 2014).
9. Papp-Wallace, K. M. & Maguire, M. E. Manganese transport and the role of manganese in virulence. *Annu. Rev. Microbiol.* 60, 187–209 (2006).
10. Friedman, E. S. et al. Microbes vs. chemistry in the origin of the anaerobic gut lumen. *Proc. Natl. Acad. Sci. U. S. A.* 115, 4170–4175 (2018).
11. Willey, J. M. & van der Donk, W. A. Lantibiotics: peptides of diverse structure and function. *Annu. Rev. Microbiol.* 61, 477–501 (2007).

Addendum





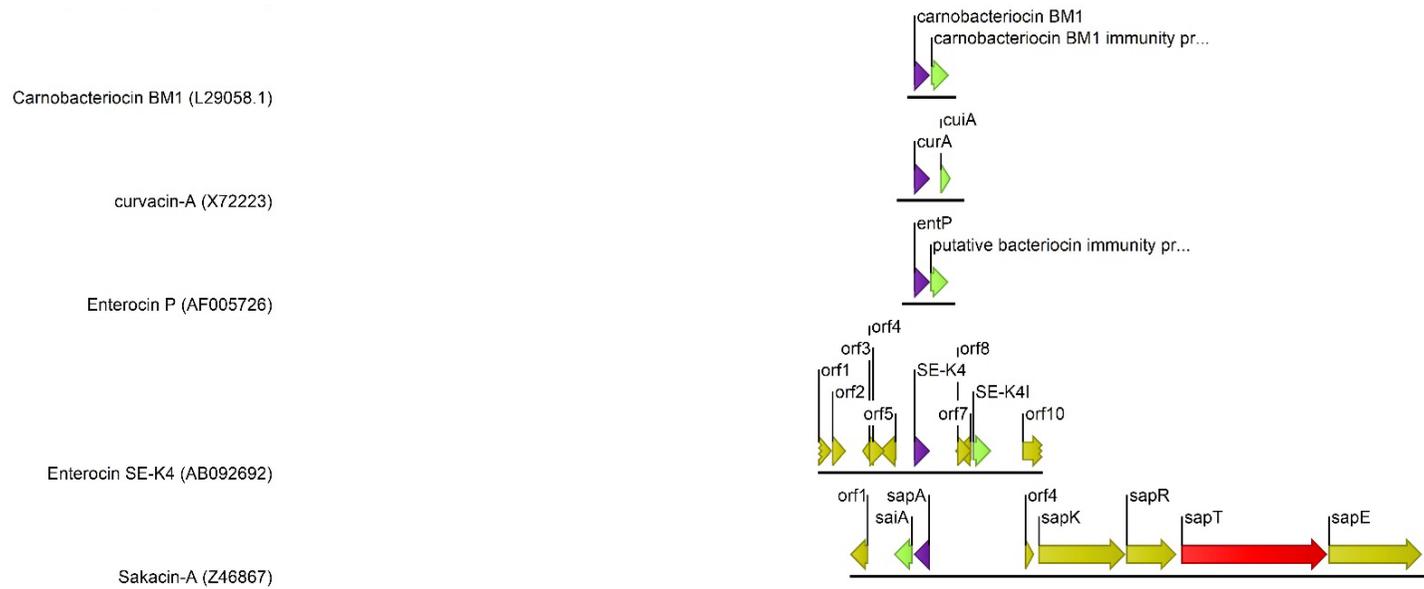


Figure 7.1 – The operon organization of class IIa bacteriocins considered in this study.