The Bacteriostatic Spectrum and Inhibitory Mechanism of Glycocin F, a Bacteriocin from *Lactobacillus plantarum* KW30

A thesis presented in partial fulfilment of the requirements for the degree of

Master of Science

in

Microbiology

at Massey University, Palmerston North,
New Zealand

Andrew Philip Kerr

2013

Acknowledgements

Thank you to the following people:

Dr Mark Patchett

Dr Gillian Norris

For support, direction and encouragement.

Patrick Main

For killing time when there was time to be killed.

Soyeon Ahn

Trevor Loo

For technical expertise and experimental assistance.

The Rest of X-Lab

For advice and allowing me to 'commandeer' resources.

The Institute formerly known as IMBS

For making it an experience.

Abstract

Bacteriocins have been deemed the "microbial weapon of choice". The ability to ribosomally synthesise these toxins means that their peptide scaffolds can be rapidly adapted to optimise stability, potency and specificity, allowing producers to outgrow closely related strains and become dominant. In some cases, a bacteriocin may inhibit a broader spectrum of microbes than just its species/genus of origin. Recently, the bacteriocin glycocin F (GccF), produced by *Lactobacillus plantarum* KW30, was biochemically and structurally characterised. GccF is unique, as it has two covalently linked N-acetylglucosamine (GlcNAc) moieties, one *O*-linked and one *S*-linked, that are critical for the inhibition of target cell growth.

How GccF causes bacteriostasis in sensitive *Lactobacillus* cells was unknown. Experiments were developed and conducted to probe the antimicrobial spectrum of GccF and how this spectrum is affected by free GlcNAc. It was found that a variety of species and strains, not just those closely related to *L. plantarum* KW30, were inhibited by the addition of GccF to cultures in solid or liquid media. Susceptible strains were identified in the genera *Streptococcus*, *Enterococcus*, and *Bacillus*. Interestingly, assays indicated that free GlcNAc plays a more dynamic role in modulating GccF activity than previously thought. The protective effect of high concentrations of GlcNAc, including the reversal of GccF-induced bacteriostasis, was confirmed for susceptible *L. plantarum* strains, but surprisingly addition of relatively low concentrations of GlcNAc prior to GccF led to a concentration-dependent increase in bacteriostasis for some other species including *Enterococcus faecalis*. GccF's mechanism of action was found to be different to the bactericidal membrane-permeabilising effect of the lantibiotic nisin, as *L. plantarum* cells treated with GccF did not die, and there was no substantial release of ATP from cells upon GccF-induced bacteriostasis.

It was also found that for Gram-negative bacteria, which are generally resistant to GccF, growth inhibition was greatly enhanced if the integrity of the outer membrane was compromised by treatment with polymyxin, or by expression of a 'leaky' mutant of the outer membrane secretin PulD. Thus GccF-mediated inhibition of growth is limited to Gram-positive bacteria mainly because of the barrier function of the Gram-negative outer membrane.

Experiments to identify changes in *E. faecalis* V583 gene expression or the levels of specific proteins in response to free GlcNAc were inconclusive due to time constraints. Further research is needed to determine GccF's exact mechanism of action.

The results of experiments with GccF, with and without added GlcNAc, on a range of bacterial species led to a hypothetical model for the mechanism of action of GccF, specifically that GccF may be ‘hijacking’ GlcNAc-specific phosphotransferase system signalling pathways. This could disrupt normal GlcNAc metabolism, perhaps resulting in UDP-GlcNAc becoming limiting for peptidoglycan synthesis, thus preventing cell wall expansion, and normal cell growth and division.

Table of Contents

List of Figures

Introduction:

Results and Discussion:

List of Tables

List of Abbreviations

ABC	ATP binding cassette
Abs	Absorbance (values)
ACN	Acetonitrile
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BLiS	Bacteriocin-like substance
BP (bp)	Base pairs
cDNA	Copied DNA
Cfu	Colony forming units
Da	Dalton
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ESR	Institute of Environmental Science and Research
EtBr	Ethidium bromide
G/Gram –ve	Gram-negative
G/Gram +ve	Gram-positive
GccF	Glycocin F
GcnA	N-acetyl-β-D-glucosaminidase
GlcN	Glucosamine
GlcNAc	N-acetylglucosamine
GlcNAc-1/6-P	N-acetlyglucosamine-1/6-phosphate
GRAS	Generally regarded as safe
IM	Inner membrane
kDa	kilodalton
KEGG	Kyoto Encyclopaedia of Genes and Genomes
LAB	Lactic acid bacteria
LB	Luria broth

man-PTS	Mannose-phosphotransferase system
mRNA	Messenger RNA
MRS	de Man, Rogosa and Sharpe medium
MRSA	Methicillin resistant *Staphylococcus aureus*
MS	Mass Spectrometry
NCBI	National Centre of Biotechnology Information
NZRM	New Zealand Reference Culture Collection, Medical Section
OD	Optical density
OM	Outer membrane
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEP	Phosphoenolpyruvate
PTM	Post-translational modification
PTS	Phosphotransferase system
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RT	Room Temperature
RT-PCR	Real-time polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
T(x)	Time (value in minutes)
TBE	Tris/Borate/EDTA buffer
TEMED	*N,N,N′,N′*-Tetramethylethylene diamine
TSB	Tryptone soya broth
TSBgly	Tryptone soya broth with 1.2% (w/v) glycine
UDP	Uridine diphosphate
UV	Ultraviolet
VRE	Vancomycin resistant *enterococci*
w/v	Weight per volume

1.0 A BRIEF INTRODUCTION TO BACTERIOCINS

1.1 Introduction

Antimicrobial molecules are synthesised and secreted by a wide range of organisms, including bacteria, fungi, animals, and plants. In bacteria and fungi, many of these compounds, *e.g.* non-ribosomal peptides, are synthesised via multiple enzymatic reactions, while others are ribosomally synthesised. The large array of ribosomally-synthesised produced by individual bacteria enable a particular species to maintain a competitive advantage over other species and strains, ensuring their own survival. These peptides are collectively known as bacteriocins and generally have a phylogenetically narrow spectrum of antimicrobial activity, typically centred on closely-related strains or species, which enables producers to outgrow non-producer strains, and thus optimise their numbers in particular ecosystem or niche. However, despite the influence of competition for resources between strains within a species, many bacteriocins have been found to inhibit distantly related species.

Since the discovery of colicins (peptides produced by, and active against *Escherichia coli*) nearly ninety years ago, bacteriocin activity spectra, regulation and mechanisms of activity have been areas of in depth investigation. Some have a narrow spectrum of activity, targeting a single receptor in susceptible cells. Other bacteriocins, such as the model lantibiotic, nisin, have a wide spectrum of activity[6], and can act synergistically with eukaryotic host peptides[7]. Interest in bacteriocin research has recently flourished, possibly due to the widespread emergence of antibiotic resistance in pathogenic bacteria.

Due to their effectiveness, availability and spectrum of antimicrobial activities, some bacteriocins are used commercially for improved health outcomes and increased food shelf-life. The probiotic benefits of certain ‘generally regarded as safe’ (GRAS) strains of bacteria are now recognised by the nutraceutical industry, and in some cases these benefits can be directly attributed to bacteriocin production. This is particularly so for food-grade lactic acid bacteria (LAB) which have the most extensively studied bacteriocins in terms of activity, mechanism of action and immunity. LABs are ubiquitous, and are able to manipulate the environment to their advantage by fermenting hexose sugars to lactic acid, lowering environmental pH. Because of their ability to synthesise bacteriocins that can inhibit

pathogen growth, LAB have been used to prevent food and product spoilage, and for their probiotic properties.

1.2 Bacteriocins

Often referred to as the "microbial weapon of choice"[8], it has been suggested that 30-99% of *Archaea* and *Bacteria* species secrete at least one bacteriocin[9; 10]. Bacteriocins are varied in terms of structure, mechanism of action and spectrum of species/strains affected, and collectively constitute a diverse array of antimicrobial peptides. While both Gram-negative and Gram-positive bacteria have been found to produce bacteriocins, those from Gram-positive organisms appear to have a broader antimicrobial spectrum and potency[11]. Interestingly, very few Gram-positive bacteriocins are able to inhibit Gram-negative bacteria without aid from other treatments to destabilise the outer membrane[12; 13], possibly because the bacteriocins are too large to pass through the Gram-negative outer membrane, and thus are unable to reach their targets/receptors in the inner membrane.

Bacteria must possess several co-ordinating proteins in order to respond to bacteriocin-inducing stimuli, produce prepeptide(s), modify prepeptide(s) and release active bacteriocin, while remaining immune to the active bacteriocin(s)[14]. Typically, bacteriocin production is controlled by a pheromone molecule that is secreted as part of a quorum sensing mechanism. Above a certain threshold, pheromones trigger a phosphorylation cascade, resulting in a response regulator activating the genes responsible for bacteriocin maturation and secretion[14; 15].

Whether located on a bacterial chromosome or on a plasmid, the genes associated with production of a particular bacteriocin are almost always found in close proximity to each other[14]. In the case of class II bacteriocins, a minimum of four different genes function to produce active bacteriocin. First, there is the gene required to express the aforementioned quorum-sensing system. In addition, there is the prepeptide gene, which encodes an inactive form of bacteriocin. This protein has an N-terminal region which acts as a leader peptide and is later cleaved in order to form the mature peptide[16]. The leader peptide serves a dual purpose. First, it prevents premature antimicrobial activity of the bacteriocin and therefore protects the producer strain from being attacked from the inside by its own weapon[17; 18]. It is thought that the leader peptide keeps the prebacteriocin in a more soluble state, preventing it from permeabilising the cell membrane. Second, it targets prepeptide to the appropriate transporter for cleavage and export from the cell. This was shown for class I lantibiotics, by

attaching leader peptides to non-lantibiotic proteins, and observing their export[19; 20]. In addition, for bacteriocins that undergo modification as part of their maturation process the leader sequence may be responsible for recognition and subsequent post-translational modification by specific enzymes[18; 21; 22]. This introduction is focused primarily on LAB bacteriocins.

Bacteriocin transporters are generally dedicated ATP-binding cassette (ABC) transporters[23]. While ABC transporters are functionally diverse, those involved with bacteriocins have two functions; (i) to cleave the leader peptide, creating an active bacteriocin, and (ii), to facilitate the export of the mature peptide into the extracellular environment. They are made up of 3 domains, typically part of a single polypeptide chain, consisting of cytoplasmic proteolytic N-terminal, and ATP – binding C-terminal domain, connected by a hydrophobic integral membrane domain[24]. The proteolytic domain is responsible for recognition of the prepeptide leader sequence, often containing a double glycine motif[24], and its cleavage to form a mature, active peptide. The C-terminal domain contains a ubiquitous and conserved ATP binding cassette, which assists protein translocation by utilising energy obtained from ATP binding and hydrolysis[25].

Finally, an immunity gene must be present to protect the producer strain from its own bacteriocin. In the case of nisin producing strains of *Lactococcus lactis*, full resistance is only attained by the expression of a dedicated immunity gene when the dedicated ABC transporters are functional[26]. Proteins involved in immunity may act by: actively exporting the bacteriocin[27], binding to and hence sequestering the bacteriocin[28], or by binding to the target receptor and blocking the effect of the bacteriocin[29]. Many immunity genes have been discovered, including *nisI* for nisin[30], *lcaB* for leucocin A[31] and *ltnI* and *ltnR* for lacticin 3147[32; 33]. Interestingly, the latter bacteriocin while usually active against *Staphylococcus aureus* strains, displays a rare case of cross-immunity with *S. aureus* strains producing staphyloccocin C55, a lantibiotic similar to lacticin 3147[34].

1.3 Ecological Significance

In nature, bacteria do not exist as pure cultures, but rather as diverse, interacting populations. In a nutrient rich environment neighbouring bacterial species may grow in a logarithmic fashion, until substrates are depleted. Towards the end of log phase and in stationary phase, competition for nutrients occurs with subsequent selection for species or strains that best suit

the environment creating a "bottle-neck" phase[35] and bacteriocins confer an advantage, increasing the likelihood of strain survival.

In a community, the ability to synthesise a substance that results in stasis, permeabilisation or even complete cell lysis of rival bacteria enables better access to pre-existing resources, and any released cellular nutrients such as proteins, carbohydrates[36] and nucleic acids[37]. At an ecological level, bacteriocin production leads to a shift in the species composition of the microbial community. Organisms sensitive to the antimicrobial peptides will rapidly decline. However, because of the narrow spectrum/range of most bacteriocins, many resistant strains or species may thrive. Bacterial populations are modified when a community is composed of several bacteriocin producers, leaving a hierarchy where tolerant/producer strains reign, ousting sensitive strains[38].

In a dynamic community of combined bacteriocin-producing, resistant and sensitive species, a "non-transitive interaction network" occurs[5]. This has been described in depth for the colicins, whereby the population of sensitive strain A is outcompeted by a bacteriocin-producer B. The population of B is itself reduced through competition with a strain C, a non-producer which is resistant to the bacteriocin. However, in the absence of bacteriocin the population of C does not grow as fast as strain A, which tends to take over[39]. These networks occur because bacteriocins prevent sensitive cells from thriving; however, bacteriocin biosynthesis, secretion and self-immunity incur a cost to the producer strain which has to devote resources to bacteriocin production that would otherwise be used in cell metabolism, effectively slowing producer growth and division relative to a non-producing strain. Resistant strains exploit this, and since the burden of maintaining immunity is less than that of bacteriocin production and immunity combined, they can grow faster than the producer. Since growth rates may be reduced in resistant strains compared to wild-type/sensitive strains[40], sensitive cells also have the potential to outgrow resistant strains in the absence of a bacteriocin producing organism.

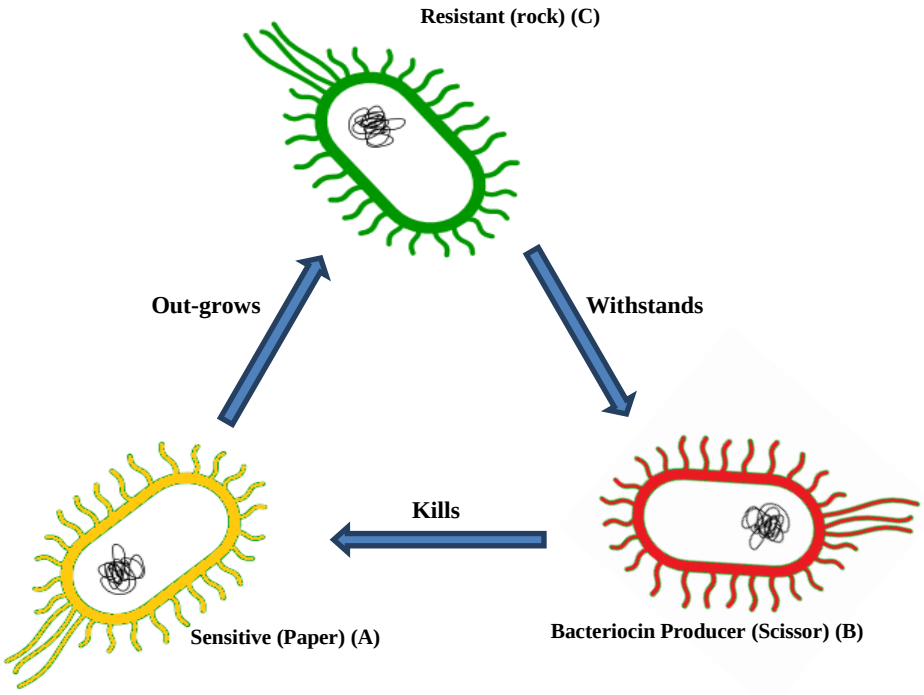


Figure 1.1 – A simplified diagram of a non-transitive competition network; a bacterial game of paper - scissors - rock in which strains vie for environmental supremacy[5]**.**

There are many examples of population effects on species dominance. *Bacillus subtilis* secretes a toxic peptide called SDP which acts against its own strain, targeting spores and cells that are genetically identical[41]. The toxin induces sibling autolysis via loss of proton motive force[42] resulting in nutrient release, which is then recycled back into the population in a process known as cannibalism. Competent pneumococcal cells express a two-peptide bacteriocin, CibAB, and deliver it to non-competent pneumococcal cells, in a process known as fratricide. This bacteriocin activates cell wall hydrolases resulting in "allolysis," a triggered release of intracellular virulence factors that aids invasive infection, while creating an isogenic population of competent cells[43]. A similar phenomenon has been seen in *Paenibacillus dentritiformus*. In order to prevent self-predation by colonies in close proximity, a protease is perpetually secreted into the environment, along with a prepeptide (DfsB, 20 kDa). When sibling colonies are close to each other, elevated concentrations of protease cause increased cleavage of the prepeptide to produce a mature bacteriocin (Slf, 12 kDa) that prevents its parent's growth. In this example, one colony does not cannibalise the other. Instead, both populations are permitted to thrive at the cost of each losing a small portion of cells[44].

Bacteriocins have been found in other species where antimicrobial activity does not appear to be the primary function of the peptide It has been proposed that bacteriocins also play a role in quorum-sensing, since many bacteriocins are not expressed until stationary phase where signalling molecules presumably surpass a concentration threshold[45; 46]. The filamentous bacterium, *Streptomyces coelicolor*, produces peptides SapB and SapT that act as biosurfactants, and are involved in the formation of aerial hyphae. These peptides contain lanthionine bridges, a post-translational modification (PTM) synonymous to lantibiotics, and have limited antimicrobial activity[47; 48; 49].

1.4 Classification

As more bacteriocins have been detected and characterised, researchers have developed classification schemes which continue to evolve as new bacteriocins are discovered. An early classification scheme introduced by Klaenhammer in 1993[50], divided LAB bacteriocins into four major classes. Class I consisted of the lantibiotics; small, heat stable peptides that are also active at low pH. Mature lantibiotics are characterised by an unusual post-translational modification in which internal peptide rings (lanthionines) are formed between specifically modified amino acid residues and cysteines. For example, nisin, a lantibiotic produced by *Lactococcus lactis*, has five serines and threonines dehydrated by specific enzymes encoded on the nisin operon. A cyclase then catalyses reactions between these newly formed dehydroamino acids and specific cysteine residues to form five (methyl) lanthionine rings, which are essential for nisin's antimicrobial function[18].

Class II bacteriocins are small (2 to 10 kDa) heat-stable unmodified peptides, some of which are characterised by a novel processing site on the prepeptide. Within this class are several subclasses: Class IIa is characterised by a unique, cationic N-terminal sequence motif, YGNGVXC[51]. This was first discovered in the bacteriocin pediocin, which is highly active against *Listeria*. This subclass is therefore referred to as pediocin-like, and/or anti-listerial[52; 53]. Class IIb bacteriocins require a pair of peptides for activity, and IIc bacteriocins are thiol activated.

Class III bacteriocins have large molecular masses, and are heat-labile[54; 55]. Finally, class IV bacteriocins are composed of protein plus one or more chemical moieties (lipid, carbohydrate) that are required for activity.

In 2005, Cotter *et al*. re-organised this classification system, largely due to lack of evidence for the class IV bacteriocins described by Klaenhammer[56]. They also changed class IIc, from bacteriocins that are thiol-activated peptides to those that are cyclic peptides, more specifically those that have linked C and N termini.

A mixed group (IId) was also introduced that contained bacteriocins that do not meet the requirements of the previous subclasses. In addition to these changes, Cotter *et al.* proposed that class III be reclassified as bacteriolysins, "non-bacteriocin lytic proteins."

Todorov made minor modifications to Cotter's classification system, replacing cyclic peptides (IIc) with *sec*-dependant bacteriocins. These are bacteriocins that do not possess their own transporter, but rather are exported via the *sec* pathway[23]. A combined general classification scheme for LAB bacteriocins is summarised in Table 1.1.

Class	Subclass	Name	Size	Features	Example(s)
I	a	Lantibiotics: elongated, cationic	2-4 kDa	Lanthionine rings, pore forming	Nisin, lacticin 3147
I	b	Lantibiotics: globular, non-charged	2-3 kDa	Lanthionine rings, interfere with enzymatic processes	Mersacidin
II	a	Pediocin-like / listerial-active	<10 kDa	Consensus N-terminal motif	Pediocin PA-1, sakacin A
II	b	Two-Peptide Complexes	<10 kDa	Activity requires both peptides	Lactacin F, lactococcin G
II	c	*sec* Dependent/Leaderless	<10 kDa	Lack their own ABC transporter	Enterocin P
II	d	Uncharacterised	<10 kDa	Do not meet criteria of IIa, b or c, but contain no PTMs	
III		Bacteriolysins	>30 kDa	Heat labile	Helveticin J, enterolysin
IV	a	Cyclic	<10 kDa	C-terminus fused to N-terminus	Garvicin ML, lactocyclin Q
IV	b, c, ...	Miscellaneous	?	Bacteriocins with other PTMs	Sublancin, Glycocin F

Table 1.1 – A proposed general bacteriocin classification scheme. This scheme is deduced from a combination of the aforementioned systems (excluding Zouhir's novel system, see next page), while being opening to accepting new classes of bacteriocins.

In 2010, Zouhir addressed the limitations and lack of stability within current classification schemes (including bacteriocins falling into more than one category) and proposed their own system based on amino acid sequence alignments, conserved motifs and phylogenetic relationships. Although certain bacteriocins did not fit their sequence-based model, it did allow the generation of new branches[57]. Current 'universal' bacteriocin classification schemes seem to be tending towards a simplified three-class system; Modified, Unmodified, and Large, with the first two classes being populated by small molecules < 10 kDa in size.

1.5 Modes of Action of Bacteriocins

Despite more than 100 bacteriocins having been discovered, sequenced and characterised, very few have had their modes of action elucidated. Lantibiotics were found early on in research to cause the leakage of low molecular weight compounds and dissipation of membrane potential. It was later found that nisin used lipid II, a cell wall precursor molecule, as a receptor in order to initiate its effect. While cell wall synthesis and hence cell growth is impaired, the bacteriocin-lipid II complex forms multimers, resulting in the formation of pores allowing the efflux of ATP, ions and amino acids (Figure 1.2A). The binding of nisin was also found to prevent concentration of lipid II, an important process in facilitating spore outgrowth[58].

The α subunit of the two-peptide lantibiotic lacticin 3147 first binds to lipid II in a manner similar to nisin, and subsequently recruits the β subunit to cause pore formation and bacteriolysis[59]. While the previous examples use a dual mechanism (i.e. (i) depleting available lipid II and (ii) forming membrane permeabilising pores) to bring about their antibiotic effect, some lantibiotics simply bind tightly to lipid II to inhibit target cell wall biosynthesis. Mersacidin, a 1.8 kDa lantibiotic, binds to the sugar-phosphate of lipid II, preventing transglycosylation, and halting cell wall synthesis, cell growth and division[60; 61].

Class IIa bacteriocins are highly active against *Listeria* species, but are also able to inhibit a broader spectrum of species. Initially they were found to dissipate both proton motive force and membrane potential[62], and to bind to the bacterial cell wall. Class IIa bacteriocins have been shown to exploit the mannose phosphotransferase system (Man-PTS) as a molecular target / receptor. This is an integral inner membrane protein that imports and phosphorylates

sugar molecules for intracellular metabolism. Diep *et al.* demonstrated that class IIa bacteriocins interact with the membrane associated IIC subunit of the man-PTS[29], compromising transport function, and causing ion efflux (Figure 1.2B), and subsequent cell death, although the precise mechanism remains unknown. Another effect is rapid ATP depletion, which is thought to be a result of the cells attempting to re-establish proton balance. It has also been shown that down-regulation of a man-PTS in *Listeria monocytogenes* leads to increased resistance to IIa bacteriocins, and re-introduction of the PTS components re-establishes sensitivity[63; 64]. Bioinformatics analysis of transporters from cells susceptible to class IIa bacteriocins showed there was a high sequence homology between their membrane domains (IIC and IID). Furthermore, sensitivity to class IIa bacteriocins is related to the expression level of these man-PTS transporters, and to their structural similarities[65], as class IIa bacteriocins have been shown to selectively target an extracellular loop on the IIC domain of the transporter[66].

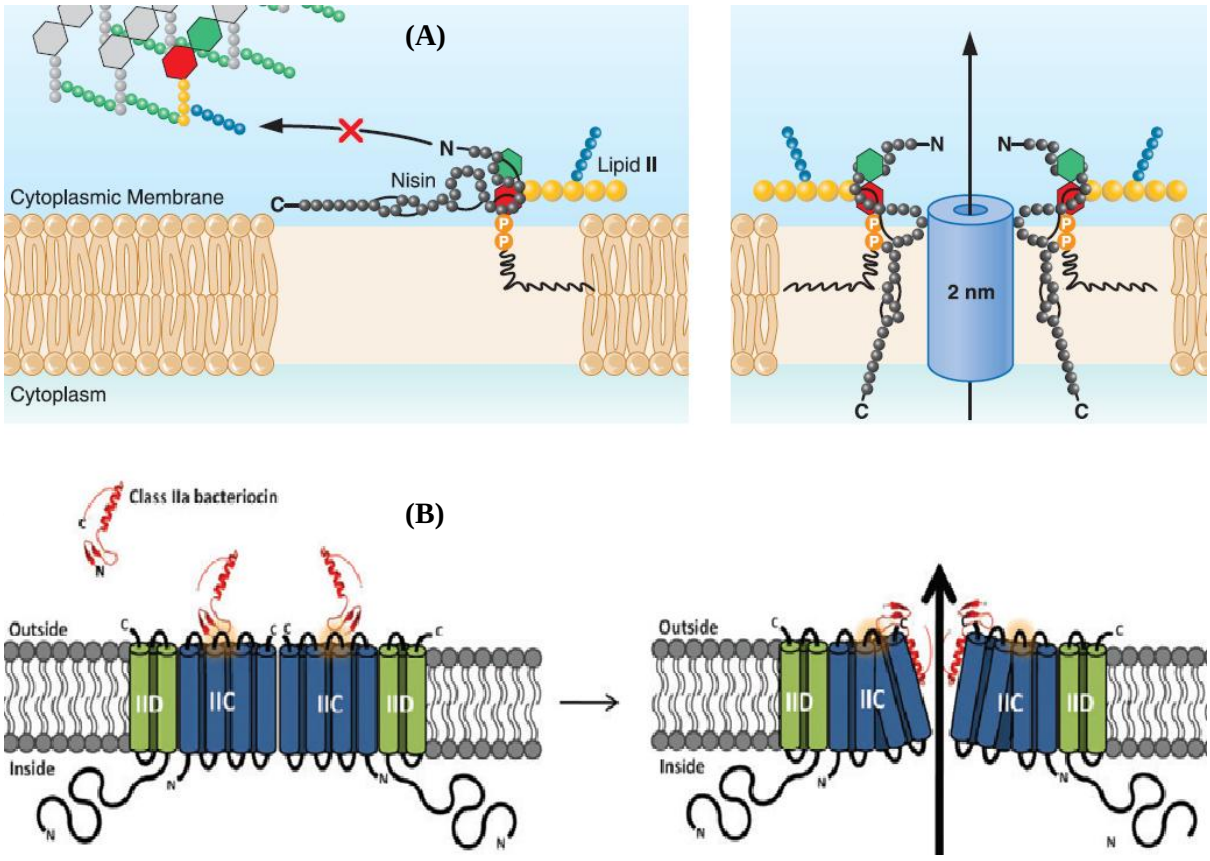


Figure 1.2 – Schematic diagrams of the proposed modes of action of bacteriocins. (A) The binding of nisin to Lipid II **(i)** prevents its use in peptidoglycan synthesis and **(ii)** leads to pore formation causing leakage of ATP and ions[2]. **(B)** A class IIa bacteriocin *e.g.* pediocin PA-1 interacting with the Man-PTS, inducing a conformational change to create a perpetually open pore, resulting in loss of proton and other ion gradients[3].

More recently, the mode of action of another class II bacteriocin, sakacin C2 has been elucidated in *E. coli*. Interest in the mechanism used by this bacteriocin to kill cells arose because of its ability to inhibit both Gram-positive and Gram-negative food spoilage organisms[67]. It was shown that sakacin C2 was able to depolarise *E. coli* transmembrane potential, indicating it operated by forming pores, allowing an efflux of intracellular ions[68]. There also appeared to be a substantial leakage of UV absorbing materials at 260 nm and 280 nm, indicating a possible leakage of nucleic acids and proteins. Flow cytometry confirmed there was an increase in cell membrane permeability due to pore formation upon exposure of *E. coli* cells to sakacin C2.

1.6 Applications

Because they have been assigned GRAS (generally regarded as safe) status and have the ability to enhance their environment (*e.g.* by lowering pH, and thereby deterring growth of pathogenic organisms) live lactobacilli have been exploited in the food and health industries. More recently, the potential for the production and use of bacteriocins in the food industry has been realised. Isolated in 1933 at the Dairy Research Institute in Palmerston North, New Zealand, first marketed in 1953 and added to the food additives list in 1983[56], nisin is the most commercially exploited bacteriocin today. Interestingly there are only a handful of others used commercially today, despite the intense interest in the properties and activities of known bacteriocins and the on-going search for new ones.

Both nisin and pediocin PA1 are currently utilised in the food industry to increase product shelf-life. They fulfil important criteria that include inhibiting a wide spectrum of bacterial species (including pathogens), a lack of toxicity to humans and stability to a variety of conditions[56]. Nisin A is commercially sold as Nisaplin™, and not only prevents bacterial growth, but can also prevent spores and vegetative cells from becoming viable, dividing cells[69]. Bacteriocins can be introduced into food products using a number of different methods that include adding a producer-strain, or adding a purified or semi purified additive solution. The use of nisin imbued in packaging materials has also been investigated in detail with largely positive results[70; 71]. The uses of bacteriocins in food preservation have expanded because they can be combined with agents such as organic acids, which increase net charge at varying pHs[72], enhancing bacteriocin activity; chelators, which diminish the barrier function

of Gram-negative outer membranes[13]; and other antimicrobial substances, which act synergistically with, for example, nisin to prevent pathogen growth[7].

Bacteriocins also confer benefits that may be of use in clinical medicine. Apb118 from *L. salivarius* UCC118 has a potent anti-listerial effect *in vivo*, preventing progressive liver infection in mice[73]. Mining of colonic commensal bacteria yielded *Bacillus thuringiensis* DPC 6431, a strain that secretes a two-peptide bacteriocin named thuricin CD. This bacteriocin kills several *Clostridium* species, in particular the causative agent of antibiotic associated diarrhoea, *C. difficile*, which colonises the host colon after antibiotic treatment has depleted the commensal gastrointestinal bacteria. Interestingly, when thuricin CD reduced the viability of *L. monocytogenes* cells, it did not impair the growth of commensal bacteria[74], whereas broad spectrum antibiotics, *e.g.* vancomycin, and the bacteriocin nisin, inhibited the growth of both *C. difficile* and gut commensals. Thuricin CD could therefore be useful in the treatment of antibiotic-induced *C. difficile* infections, precisely because of its narrow spectrum of activity.

Bacteriocins may also prove to be useful against bacteria resistant to conventional antibiotics. For example, lacticin 3147 has been shown to inhibit the *in vivo* growth of methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE) and an array of other Gram-positive pathogens[75].

The ability of non-pathogenic LAB and *Bacillus* species to produce bacteriocins may confer probiotic status. Recent mining of oral microflora revealed a strain of *Streptococcus salivarius* that is non-pathogenic, adheres to respiratory epithelial cells and also secretes bacteriocins or bacteriocin-like substances (BLISs), which prevent the growth of several respiratory pathogens[76]. These bacteriocins are now the cornerstone of a thriving biotechnology company, BLIS Technologies. In addition, two *Lactobacillus* species were recently found to down-regulate aflatoxin production by the fungus *Aspergillus*[77], a finding that may suggest an application for lactobacilli to control fungal contamination.

Potential applications for bacteriocins are rapidly expanding with recent advances in bioengineering. An example is the site-directed mutagenesis of nisin that has resulted in a peptide with greater stability[78]. Rink *et al.* used protein engineering to probe the lanthionine rings of nisin, discovering in the process that modifications of ring A lead not only to better activity but also to an altered activity spectrum[79]. Random mutagenesis has also been a powerful tool in yielding several novel nisin variants. For example, substitution of serine 29

of nisin A with specific amino acids increased activity against both Gram-positive, and Gram-negative pathogens[80].

1.7 Glycocin F of *Lactobacillus plantarum* KW30

In 1996 Kelly *et al.* discovered that *Lactobacillus plantarum* KW30 was able to prevent the growth of other *Lactobacillus* strains isolated from its environment, a corn fermentation. The compound responsible for antimicrobial activity was deduced to be a heat-and-pH-stable, protease-sensitive substance with a narrow range of activity that appeared bactericidal[81]. This substance was initially named plantaricin KW30 and appeared to be chromosomally encoded.

Plantaricin KW30 was subsequently purified and further characterised by Stepper *et al.* (2011), who renamed that bacteriocin 'glycocin F' (GccF) in recognition of its covalently- linked GlcNAc moieties[82]. The study determined the bacteriocin to be a 43 amino acid peptide with a "hair-pin architecture" formed by two internal disulfide bonds. In addition to this, the bacteriocin contains two post-translational modifications: an N-acetyl-D-glucosamine (GlcNAc), covalently *O*-linked to serine 18 and another GlcNAc, covalently *S*-linked to cysteine 43 (Figure 1.3), a novel form of glycosylation found only in this bacteriocin and sublancin. The presence of amino-sugar moieties in GccF and sublancin supports Klaenhammer's class IV category of complex bacteriocins[50], since these bacteriocins do not contain lanthionine modifications, are post-translationally modified and have a low molecular weights (~5200 Da for GccF).

The paper also showed the purified GccF was bacteriostatic and that the removal of the *O*-linked GlcNAc abolished activity against the susceptible *L. plantarum* indicator strain. The removal of the *S*-linked GlcNAc, however, severely reduced GccF potency, demonstrating the importance of both covalently linked glycans for GccF activity. The study also identified the *gcc* gene cluster (NCBI accession number GU552553; 6789 bp), encoding (pre)glycocin F, thioredoxin-domain protein genes (GccC and/or GccD) for disulfide bond formation, a dedicated proteolytic ABC transporter (GccB) and a glycosyltransferase (GccA) hypothesised to be responsible for GccF's glycosylation. A putative immunity gene (*gccI*) was annotated without experimental evidence of function.

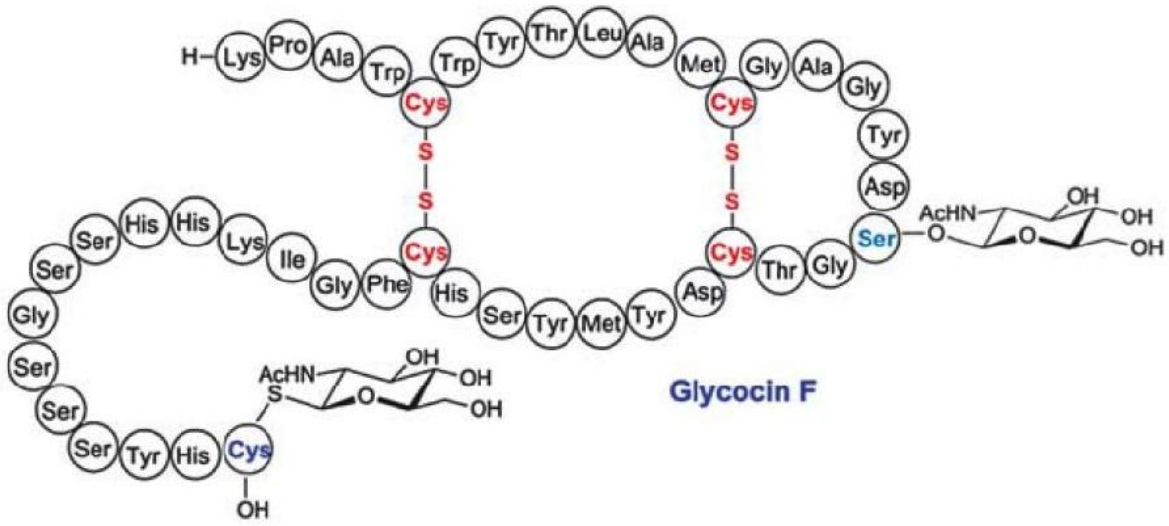


Figure 1.3 – A schematic diagram of the primary structure (*i.e.* the amino acid sequence) of GccF. Notable features are the disulfide bridges forming nested cystines, and N-acetyl-D-glucosamine moieties *O*-linked to serine and *S*-linked to the C terminal cysteine[1].

1.8 The Biological Roles of N-acetyl-D-glucosamine

The molecular receptor for several class IIa (unmodified) bacteriocins is the mannose PTS[29] which has previously been described as "an open door for macromolecular invasion"[83]. It is conceivable that susceptible bacteria could gain a measure of resistance to these bacteriocins by down-regulating the targeted phosphotransferase system and so lessen the impact of any disruption in PTS-mediated regulation of metabolic and other intracellular processes that might be caused by bacteriocin binding[84]. The possibility that the formation of bacteriocin-PTS complexes might affect intracellular sugar metabolism is a concept that may be particularly relevant to GccF.

The connection between GlcNAc and GccF, as previously mentioned, is two-fold: (i) the *O*- and *S*- linked amino-sugar moieties are crucial for GccF activity, and (ii) free GlcNAc both protects against GccF-mediated bacteriostasis, and reverses bacteriostasis after it has been established[82]. GlcNAc plays many roles throughout nature. In the human fungal pathogen, *Candida albicans*, GlcNAc acts via cell signalling pathways to instigate virulence[85] and GlcNAc's breakdown for energy[86]. In humans, GlcNAc can have a profound effect on cells involved in immunity[87] and is an important component of inter and intracellular communication[88]. For example, *O*-GlcNAcylation is a ubiquitous reversible regulatory PTM found on over 3,000 nuclear, cytoplasmic and mitochondrial proteins[89].

Like eukaryotes, bacteria are able to convert GlcNAc to fructose-6-phosphate which may serve as a source of energy via glycolysis or as a carbon source to form compounds required for cell growth[90], which is significant only if GlcNAc is either abundant, or the only sugar present. The chief role of GlcNAc in bacteria is its incorporation into peptidoglycan, the primary cell wall component[91]. In *Escherichia coli*, glucosamine (GlcN) -6-phosphate may be formed from fructose-6-phosphate (obtained from multiple metabolisable carbon sources) by GlmS (glutamine-fructose-6-phosphate transaminase), then isomerised to GlcN-1-P by GlmM (phosphoglucosamine mutase), and finally a dual-function enzyme, GlmU (bifunctional UDP-GlcNAc pyrophosphorylase / GlcN-1-phosphate N-acetyltransferase), both acetylates to GlcNAc-1-P and transfers a uridine-diphosphate (UDP) group to form UDP-GlcNAc[92; 93; 94]. In the event of an exogenous GlcNAc source, NagE, a GlcNAc phosphotransferase system, facilitates the transfer of GlcNAc across the cell membrane, and also its phosphorylation to GlcNAc-6-P. In *E. coli*, NagC acts as a transcriptional activator of the genes *glmU* and *glmS*, while repressing the *nag* operon that carries genes necessary for GlcNAc catabolism[95]. GlcNAc-6-P binds to NagC, altering the latter's DNA binding site and releasing it from the synthesis and catabolism genes[96]. This down-regulates the expression of GlcNAc synthesis machinery (*glm*), and up-regulates GlcNAc catabolism genes (*nag*), effectively inhibiting the conversion of a potential energy source (GlcNAc) into a precursor for cell wall synthesis (UDP-GlcNAc) and reducing resources dedicated to this conversion, optimising energy efficiency and substrate usage in a given environment.

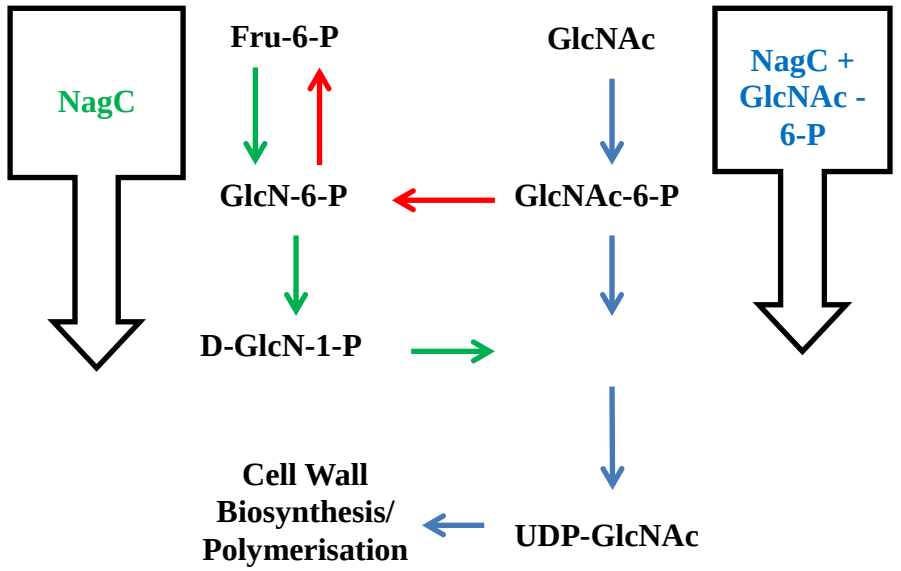


Figure 1.4 – Schematic diagram of GlcNAc's fate in bacterial cells. **Green** represents a GlcNAc-free environment where the transcriptional regulator NagC promotes expression of the *glm* genes for biosynthesis of GlcNAc from common sugars. **Blue** represents a GlcNAc-rich environment where GlcNAc-6-P stops *glm* expression and allows *nag* expression for GlcNAc catabolism. **Red** represents GlcNAc being used as a carbon / energy source by *nag*-encoded proteins.

1.9 Similar Bacteriocins (Other Glycocins?)

The mature form of the bacteriocin, sublancin 168 is composed of 37 residues with two nested disulphide bonds creating a constrained "hairpin-loop" (helix-loop-helix), and was initially thought to be a lantibiotic[97]. Sublancin 168 was reported to be bactericidal towards closely-related *Bacillus* strains. In the same study conducted on GccF, Stepper *et al.* reported that the producer strain of sublancin 168, *Bacillus subtilis* 168 did not possess the genes encoding the dehydratase and cyclise enzymes required to form lanthionine ring, undermining its classification as a lantibiotic. In addition, the team discovered an *S*-linked hexose on the non-disulfide bonded Cys22 of sublancin 168[82]. Other study published at the same time determined the *S*-linked hexose of sublancin to be a glucose moiety[98]. The latter group also suggested that glycosylation was required for sublancin's activity, although this interpretation may be incorrect[99].

Sublancin is not the only bacteriocin scaffold that shares the same fold as GccF. ASM1, produced by *Lactobacillus plantarum* A-1, inhibits growth of certain *Lactobacilli* species, as well as *Enterococcus faecalis* and *Leuconostoc mesenteroides*. Stepper *et al.* reported the very high (88.4%) sequence homology between GccF and ASM1[82]. A multiple sequence alignment of sublancin 168, GccF and ASM1 can be seen in **Figure 1.5**.

```
SunA: 18 -gsGLGKAQCAALWLQCASGGTIGCGGGAVACQNYRQFCR

GccF: 20 -gg--KPAWCWYTLAMCGAG--YDSG----TCDYMYSHCFGIKHHSSGSSSYHC

ASM1: 20 -gg--KPAWCWYTLAMCGAG--YDSG----TCDYMYSHCFGVKHSSGGGGSYHC
          *.    * *       *..*   ..*.   :*:   ..*
```

***** = Fully conserved residue
: = 'Strong' group fully conserved
. = 'Weak' group fully

Figure 1.5 – Multiple sequence alignment of bacteriocins similar to GccF. A ClustalW2 multiple sequence alignment of the mature sublancin 168 (SunA), GccF and ASM1 peptides (plus the known 'double glycine' motifs of the leader peptides, in lowercase italics) with manual adjustment. The C-X_6-C motifs in each peptide are underlined, and glycosylated residues are highlighted.

1.10 Research Aims

This study aims to test two hypotheses. First, that purified GccF can inhibit the growth of bacterial species not tested by Kelly *et al*.[101], including (but not limited to) the potentially pathogenic species *Enterococcus faecalis*. Second, that free GlcNAc may modify the susceptibility of target bacteria to GccF. This hypothesis is based on the observation that free GlcNAc antagonises GccF-mediated inhibition of susceptible *L. plantarum* strains.[102]

Experiments will also be directed towards obtaining more information about how GccF affects susceptible species or strains, and ideally elucidate a potential target/receptor by which GccF binds and is able to exert its inhibitory effect.

The significance of this research revolves around the exploitation of bacteriocins to enhance animal health, human health and food quality. Like nisin and pediocin PA-1, GccF may prove to be useful resource for the food and dairy industries, and in a clinical setting. As GccF is bacteriostatic, in contrast to the vast majority of bactericidal bacteriocins, and as it acts so quickly and at such low concentrations, understanding how it works may lead to the development of a new class of antimicrobials, one that does not lyse target cells and so avoids the release of toxins during the treatment of host infections. There has not been a new antibiotic developed since the late 1960s, yet pathogens are relentlessly mutating and adapting in their quest to overcome host immune systems and develop and spread antibiotic resistance genes.

2.0 MATERIAL AND METHODS

2.1 Disposable Materials

Materials are grouped by manufacturers/suppliers, listed in alphabetical order.

AMRESCO:

- EDTA disodium salt dehydrate.

Ansell Healthcare:

- Micro-Touch® DermaClean® Latex Gloves.

Axygen Scientific Inc:

- 10 µL "MAXYMum Recovery" Tips for P-2, Racked, Pre-Sterilized.
- 20 µL "MAXYMum Recovery" Filter Tips for P-20, Racked, Pre-Sterilized.
- 0.5-10 µL Clear Tips for P2/P10. T-300.
- 200 µL Yellow Tips. T-200-Y.
- 1000 µL Blue Tips. T-1000-B.
- 0.2 mL Thin Wall PCR Tubes, Flat Cap. Clear. PCR-02-C.
- 0.6 mL MaxyClear microtubes. Clear. MCT-060-C.
- 1.7 mL Microtubes. Clear, MCT-175-C.

BDH Laboratory Supplies:

- Sodium dodecyl sulfate.

Becton, Dickinson and Company:

- BD Bacto™ Agar.

Bio-Rad:

- 10 mg/mL Ethidium Bromide, 10 mL.

Carl Roth®:

- Glycerol.

Corning Incorporated:

- 100 – 1000 µL Universal Fit FilterTips. Non-Pyrogenic, RNase/DNase Free.
- 96 Well White Flat Bottom Polystyrene Not Treated Microplate.

Fisher Scientific

- Acetonitrile (Optima*) 99.9% min.
- Formic acid (Optima* LC/MS) ≥ 99.5%.
- Methanol ≥ 99.8%.
- Water (Optima* LC/MS).

GE Healthcare:

- illustra RNAspin Mini Kit.
- Iodoacetamide.

GoldBio:

- DL-Dithiothreitol (>99% pure) protease free.

Grace Davidson Discovery Sciences (Formerly Alltech Associates Inc.):

- Glass Beads, Regular, 60/80 mesh.

Greiner Bio-One:

- 5 mL Serological pipettes, 1/10 graduation, sterile.
- 15 mL Centrifuge tube, graduated, bagged, sterile.
- 50 mL Centrifuge tube, conical base, bagged, sterile.

LabServ:

- 90 mm x 15 mm plastic Petri dishes.

Life Technologies Corporation:

- Ambion® DNA-*free*™ DNase Treatment & Removal Reagents.
- LB Broth Base, Powder (Lennox L Broth Base)® (Invitrogen™).
- SuperScript® VILO™ cDNA Synthesis Kit.

LP Italiana:

- Special PS Micro Photometer Cuvette, 2 mL.
- Special PS Macro Photometer Cuvette, 4 mL.

Merck:

- Acrylamide:Bis 40% solution (19:1).

Neogen Corporation/Acumedia:

- Lactobacilli MRS Broth.

Neptune Scientific:

- BT200 Series - 200 µL Barrier Tip, Racked, Pre-Sterile.

New England Biolabs® Inc:

- Deoxynucleotide Solution Mix (10 mM of each nucleotide).

Oxoid Limited:

- Tryptone Soya Broth (TSB) (Casein soya bean digest medium).

Promega Corporation:

- Wizard® Genomic DNA Purification Kit.

Pure Science Limited:

- Glycine.
- Tris buffer Research grade/min.99.8%

Roche Applied Sciences, Roche Diagnostics:

- Complete, Mini Protease Inhibitor Cocktail
- Tablets. PCR Buffer with $MgCl_2$, 10 x
- concentrated.
- *Taq* DNA Polymerase, 5 U/µL.

SARSTEDT

- Micro tube, 2 mL, PP.

Sigma-Aldrich Co:

- Adenosine 5′-triphosphate (ATP) Bioluminescent Assay Kit.
- Ammonium bicarbonate.
- Ammonium persulfate (>98%).
- Coomassie Brilliant Blue G 250 (Fluka analytical).
- Lysozyme from chicken egg white, lyophilized powder, protein ≥90 %, ≥40,000 units/mg protein.
- *N*-Acetyl-D-glucosamine ≥ 99%.
- *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (~99%).
- Orange G.
- Trypsin from Porcine Pancreas, Proteomic Grade, BioReagent, hemimethylated.

ThermoFisher Scientific Inc.:

- 2 mL Short thread Vial. 32 x 11.6 mm, clear glass, wide opening.
- 0.1mL Micro-Insert, 29 x 5.7 mm, clear glass, with attached Plastic Spring.
- 9 mm Blue Cap TFE/Rubber.
- PageRuler Unstained Ladder.

United States Biochemical Corp.:

- Bromophenol Blue disodium salt.

VWR International:

- Ortho-boric acid, crystallized (Normapur®).

GccF was purified from culture supernatants of *L. plantarum* KW30 and quantitated spectrophotometrically as described by Stepper *et al*. (2011).[82]

2.2 Laboratory Equipment

Agilent Technologies:

- Cary 300 UV – Visible Spectrophotometer.
- 6220 Q-TOF LC/MS System.

Bio-Rad Laboratories:

- SmartSpecPlus™ Spectrophotometer.
- Molecular Imager® Gel Doc™ XR+ System with Image Lab™ Software.
- Mini-PROTEAN® 3 Cell. Electrophoresis System.
- PowerPac™ Basic Power Supply.

Biometra:

- T-Gradient Thermocycler.

BMG Labtech:

- FLUOstar Galaxy – Multidetection Microplate Reader.

Hitachi:

- U-1100 Spectrophotometer.

Hybaid:

- Ribolyser (model FP120HY-230).

Kendro®:

- Heraeus® Biofuge® Fresco.

Mettler:

- AE200 Analytical Balance.
- PM460 DeltaRange® Precision Balance.

Savant:

- Speed Vac DNA 110 Concentrator.

Sorvall:

- RT7 Benchtop Centrifuge.

ThermoFisher Scientific Inc.:

- NanoDrop™ 1000 Spectrophotometer.
- Barnstead NANOpure® Diamond Life Science (UV/UF) Ultrapure Water System.

2.3 Methods

2.3.1 Purified Water

A Barnstead NANOpure® Diamond Life Science (UV/UF) Ultrapure Water System supplied UV treated water (filtered to 0.2 μm), which in this section is referred to as "purified water".

2.3.2 Bacterial Growth Media

Media was prepared as per manufacturer's instructions (unless otherwise stated). All media were autoclaved at 121°C at 30 psi for 15 – 20 minutes. For solid media, BD Bacto™ Agar (1% or 1.2% agar) was added prior to autoclaving. *Lactobacillus* spp. were grown in/on deMan, Rogosa, and Sharpe (MRS) media, *Escherichia* and *Yersinia* spp. were grown in/on LB media and all other species were grown in/on TSB media.

Lactobacilli (MRS) Broth:

- 55 g MRS Broth Medium
- Make up to 1 L using purified water

Luria Broth (LB):

- 20 g LB Medium
- Make up to 1 L using purified water

Tryptone Soya Broth (TSB):

- 30 g TSB Medium
- (12 g glycine for TSB supplemented with 1.2% glycine, *i.e*. TSBgly)
- Make up to 1 L using purified water

2.3.3 Bacterial Stocks

Certain bacterial stocks were received from sources from Massey University as glycerol deeps stored at -80°C. Other cell cultures were imported from the New Zealand Reference Culture Collection (NZRCC) at the Institute of Environmental Science and Research (ESR, Wellington, New Zealand). Ampoules were received and

broken open to access freeze-dried bacteria. 0.5 mL of the appropriate liquid medium was added into the ampoule to rehydrate and resuspend the bacteria. Bacterial suspensions were then streaked using a flame sterilised loop onto a 1.2% agar plate of the appropriate media and incubated overnight at 30^{o}C. Single colonies were then picked from the plates and inoculated into liquid media and incubated again at 30°C with shaking if required.

The resulting pure cultures were then mixed in a 1:1 ratio with 40% (v/v) glycerol (40% glycerol, 60% purified water, autoclaved) and stored at -80°C until required.

A complete list of bacterial strains used in this research project can be found in the Appendix 6.1.

2.3.4 Susceptibility Plating

Bacterial strains were aseptically inoculated into media from -80°C glycerol stocks and incubated overnight at 37°C with shaking if required. 100 µL of this culture (typically in stationary phase) was added to 20 mL of molten media containing 1% agar and equilibrated to 40°C, poured into plastic Petri dishes and left to set. Solutions of various compounds (names and volumes are recorded in the Results and Discussion chapters) were applied to the surface of the agar plate and allowed to dry into the agar. Plates were then incubated overnight at 37°C and the effect of the compound on the growth of the embedded bacterial indicator strain was evaluated[100].

2.3.5 Measurement of Bacterial Cell Optical Density

A SmartSpecPlus™ Spectrophotometer (Bio-Rad) was first blanked at a wavelength of 600 nm (OD_{600}) with sterile medium. Overnight cultures were measured after ten-fold dilution with sterile medium. This was to keep within the instrument's accurate measurement range. Blanks and cultures were measured in 1 mL path-length cuvettes.

2.3.6 Colony Forming Unit (cfu) Plating

Cells scraped from the surface of frozen glycerol stocks were used to inoculate 5 mL of liquid medium which was incubated with shaking overnight at 37°C. Cells were then subcultured to an $O.D._{600nm}$ of ~0.2 (see Methods 2.3.5) and growth continued at 37°C.

Details of GccF and/or GlcNAc additions are alongside their data in the Results and Discussion section. Cultures were incubated at 37°C with shaking. Culture samples of 2 µL were serially diluted first in 998 µL media and then 5 µL in 995 µL media (total dilution factor (DF) of 10,000), 50 µL of which was pipetted onto 1.2% agar plates, spread and then incubated at 37°C.

The next day, colonies were counted, recorded and calculated back to cfu/mL. Final results were plotted using Microsoft® Excel 2010.

2.3.7 Spectrophotometric Assays

Initial experiments used a Hitachi U-1100 spectrophotometer at 30°C, using 4 mL cuvettes and manually recording readings $O.D._{600nm}$ values. Later experiments used a Cary 300 UV – Visible spectrophotometer under similar conditions and the temperature of each assay is shown in the results descriptions.

5 mL of appropriate medium was inoculated from single colony bacterial glycerol stocks. Cultures were then incubated O/N at 37°C, and subcultured the next day to an $O.D._{600nm}$ of 0.1 - 0.3 (see Methods 2.3.5). N.B. $O.D._{600nm}$ Y axes may be labelled as Abs units.

The timing, volumes and concentrations of various additives are recorded in the Results and Discussion chapter.

Results from the Hitachi spectrophotometer were entered manually into a Microsoft® Excel 2010 spreadsheet for plotting. Results from the Cary spectrophotometer were automatically plotted using the Cary WinUV software application, "Enzyme Kinetics."

2.3.8 ATP Efflux Assay for *Lactobacillus plantarum* ATCC 8014 treated with GccF.

L. plantarum ATCC 8014 was inoculated into 5 mL of sterile MRS in loosely capped 15 mL centrifuge tubes and incubated overnight at 37°C with shaking (rotary, 200rpm). The next morning $O.D._{600nm}$ was measured and the culture diluted to an $O.D._{600nm}$ of ~0.3 in 20 mL MRS and incubated at 37°C without shaking. 1 mL samples were taken at various time points prior to and after bacteriocin addition. Samples were centrifuged at 10,000 x g for 3 minutes. Supernatants were carefully transferred to new Eppendorf tubes and analysed immediately for ATP.

50 µL of each supernatant sample was pipetted into a 96 well plate, alongside dilutions of an ATP Standard (Sigma). To each sample or standard, an equal volume of undiluted ATP Assay Mix (Sigma) was added and mixed.

Bioluminescence was measured 5 times per sample using a FLUOstar Galaxy – Multidetection Microplate Reader (BMG Labtech). Measured values were then averaged and graphed using Microsoft® Excel 2010.

2.3.9 Protein Extraction of *Enterococcus faecalis* V583

TSBgly (5 mL) was inoculated with *E. faecalis* V583 from a glycerol stock and the culture incubated overnight at 37°C, shaking at 200 rpm. The next morning, OD_{600} was measured and the culture diluted to an $O.D._{600nm}$ of ~0.2 in 10 mL TSBgly and left for one hour to re-establish growth. Any additions to media were made at time 0, and cultures were incubated at 37°C for 2 hours with occasional inversion (approximately every 20 minutes). Cells were then centrifuged at 4,400 rpm for 10 minutes at 4°C and resuspended in 1.5 mL 1 x phosphate buffered saline (PBS) with protease inhibitor added.

1 mL of resuspended cells was added to 250 µL (packed volume) glass beads (0.2 mm diameter) and lysed using a Ribolyser (Hybaid) on a setting of 4, for three 40 second bursts at 4°C. After the glass beads had settled, the cell lysate was transferred to a clean tube and centrifuged at 14,000 x g for 20 minutes at 4°C to separate soluble and insoluble proteins. Supernatant (soluble fraction) was carefully aspirated, and the pellet (insoluble fraction) was resuspended in 1 x PBS with protease inhibitor to a volume equal to the aspirated supernatant.

An aliquot (nine volumes) of each sample was added to 10 x Protein Loading Dye solution (one volume), boiled for 3 minutes and either stored at -80°C, or loaded into the sample well of an SDS-PAGE gel.

10 x Phosphate Buffered Saline (PBS):

- 4 g NaCl
- 0.1 g KCl
- 0.12 g KH_2PO_4
- 0.72 g $Na_2HPO_4.7H_2O$
- 1 tablet Mini Protease Inhibitor Cocktail
- Make up to 100 mL using purified water, and adjust pH to 7.4

10 x Protein Loading Dye:

- 2 g Sodium dodecyl sulfate
- 2.5 mL 2 M Tris-HCl, pH 7.6
- 3 mL Glycerol
- 0.1 g Bromophenol Blue
- 0.31 mg Dithiothreitol
- Make up to 10 mL using purified water, and store at -20°C in 0.1 mL aliquots.

2.3.10 Protein Separation by Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Gels mixes for SDS-PAGE are described in **Table 2.1**. The separating gel (10%, 12% or 15% acrylamide) was poured and set first, followed by the stacking gel. Gels were 0.75 mm thick and the separating gel was three times the height of the stacking gel.

	4% Stacking Gel	**10% Separating Gel**	**12% Separating Gel**	**15% Separating Gel**
Purified Water	3.68 mL	4.85 mL	4.35 mL	3.6 mL
1 M Tris-HCl, pH 6.8	0.63 mL	-	-	-
1.5 M Tris-HCl, pH 8.8	-	2.5 mL	2.5 mL	2.5 mL
10% (w/v) Sodium Dodecyl Sulfate	100 µL	100 µL	100 µL	100 µL
40% (w/v) Acrylamide/Bis (19/1) Stock	0.5 mL	2.5 mL	3 mL	3.75 mL
VACUUM DEGAS FOR 15 MINUTES AT ROOM TEMPERATURE				
10% (w/v) Ammonium Persulfate	100 µL	100 µL	100 µL	100 µL
***N, N ,N', N'* – Tetramethyl ethylenediamine**	5 µL	5 µL	5 µL	5 µL

Table 2.1 – Reagents (in order of addition) and volumes for stacking and separating gel mixes for SDS-PAGE.

The cast gels were placed in tanks containing 1 x SDS-PAGE running buffer (diluted from a 5 x stock with dH_2O). 15 µL protein/dye samples were pipetted into stacking gel wells. Gels were run at 200 volts for 35-60 minutes.

5 x SDS-PAGE Running Buffer

- 72 g Glycine
- 15.1 g Tris base
- 5 g Sodium dodecyl sulfate
- Make up to 1 L using purified water.

2.3.11 Colloidal Coomassie Staining

After electrophoresis, SDS-PAGE gels were fixed in fixative solution with gentle agitation for 1 hour. Gels were then washed with gentle-agitation in purified water for 10 minutes three times. Gels were then stained overnight with working Colloidal Coomassie stain (4:1, dye stock:methanol) with agitation. The next morning gels were washed in ~50°C purified water to remove residual stain.

Fixative Solution:

- 100 mL Acetic acid
- 400 mL Methanol
- Make up to 1 L using purified water.

Dye Stock Solution:

- 50 g Ammonium sulfate
- 6 mL Phosphoric acid, 85% (w/v)
- 10 mL Coomassie Blue G250, 5% (w/v)
- Make up to 500 mL with purified water.

2.3.12 In-Gel Tryptic Digestion

After washing the SDS-PAGE gel, protein bands of interest (*e.g.* increased abundance shown by more intense colouration relative to other bands) were excised and the gel slices placed in separate clean 1.5 mL Eppendorf tubes and 200 µL of destain solution (D) was added. Gel slices were left overnight, incubated with a fresh 200 µL aliquot of D for another 3 hours. D was then discarded.

200 μL of acetonitrile (ACN) was added to dehydrate gel slices for ~5 minutes at room temperature. ACN was then removed, and the gel slices were dried in a vacuum centrifuge (Speed Vac) for 10-15 minutes at room temperature (RT).

30 μL of reducing agent (E) was added to hydrate gel slices, followed by incubation at RT for 30 minutes to reduce protein disulfide bonds to free cysteine residues. E was then replaced with 30 μL of alkylating reagent (F) at RT for 30 minutes. F was then replaced with 200 μL ACN to dehydrate gel slices for 5 minutes at RT. ACN was then removed and discarded.

Gel slices were next rehydrated in 200 μL of digest solution (C) for 10 minutes at RT. C was then replaced with 200 μL of ACN, and gel pieces were dehydrated for 5 minutes at RT. ACN was then removed and gel bands dried in a vacuum centrifuge for 10-15 minutes. 30 μL of trypsin solution (G) was added to the samples. Gel pieces were hydrated on ice for 10 minutes with occasional vortexing. Once gel pieces had fully hydrated, they were allowed to digest overnight at 37°C.

The next morning, 30 μL of Extraction Buffer (H) was added to the tubes containing the gel pieces and incubated for 10 minutes with occasional, gentle vortexing. Supernatants were collected and placed in fresh, washed 0.6 mL tubes. A second aliquot of 30 μL H was added to the gel pieces, and treated as before, and supernatant combined with the original. Samples were then centrifuged at 13,000 x g for 5 minutes to remove insoluble material, and supernatants transferred to HPLC sample vials.

Samples were then processed on an Agilent 6220 QTOF mass spectrophotometer. Mass data were subsequently processed with Mass Hunter Version B03.01 Rev B.02.01 SP1, and analysed using Mascot Daemon (© 2002-9 Matrix Science Ltd.) software, Version 2.3.2.

Solution A:

- 1 g Ammonium bicarbonate
- Make up to 25 mL with purified water

Solution B:

- 0.27 g $CaCl_2$
- Make up to 25 mL with purified water

Solution C:

- 0.5 mL Solution A
- 40 µL Solution B
- Make up to 4 mL with purified water

Solution D:

- 5 mL Methanol
- 0.5 mL Acetic acid
- Make up to 10 mL with purified water

Solution E:

- 1.5 mg Dithiothreitol
- 250 µL Solution A
- Make up to 1 mL with purified water

Solution F:

- 18 mg Iodoacetamide
- 250 µL Solution A
- Make up to 1 mL with purified water

Solution G:

- 1 mL Solution C
- 20 µg Trypsin, proteomic grade (Sigma)

Solution H:

- 5 mL Acetonitrile
- 0.5 mL Formic acid
- Make up to 10mL with purified water

2.3.13 Oligonucleotide Primers

Primers for the amplification of regions of three *Enterococcus faecalis* V583 genes were supplied by Integrated DNA Technologies. Primer sequences are listed in **Table 2.2**. Lyophilised primers were diluted to 100 μM stock solutions, and further diluted to 2 μM working solutions using purified water.

Gene to be Amplified	Gene Name	Forward Primer 5' – 3'	Reverse Primer 5' – 3'
GlcNAc PTS	*nagE*, EF1516 GI:29376082	GGCTTTGCCTTTAGTGCAGGTT	GCAACATCTGGCGTTTCTTCGC
Glyceraldehyde -3-phosphate dehydrogenase	*gap2* EF1964 GI:29376486	TCCACGCTTACACAGGTGACC	CGTTGAGCAGCGCCATCTAATT
DNA – directed RNA polymer-ase subunit α	*rpoA* EF0233 GI:29374877	GAGCTACTTTCCATGCTCGCT	TCATCACGACGACCAACACG

Table 2.2 – PCR Primer Pairs for three *E. faecalis* V583 genes. *nagE* is the gene encoding the GlcN Ac-specific PTS transporter hypothesised to be more abundant in *E. faecalis* grown in the presence of GlcNAc. *gap2* and *rpoA* are considered 'house-keeping genes', *i.e.* they are constitutively expressed and can serve as controls to help determine changes in the relative abundance of *nagE* transcript under different growth conditions.

2.3.14 Genomic DNA Extraction of *Enterococcus faecalis* using Promega – Wizard Genomic DNA Purification Kit

Enterococcus cells were inoculated into 5 mL of TSB supplemented with 1.2% w/v glycine (TSBgly) and grown overnight at 37°C with shaking at 200 rpm. Cells were then subcultured into 50 mL of TSBgly to a final $O.D._{600nm}$ of 0.2 and incubated at 37°C to an $O.D._{600nm}$ of ~1.0. Cell cultures were then centrifuged at 4°C, 2,700 x g for 10 minutes.

Cell pellets were then resuspended in 2 mL ice-cold 50 mM EDTA (pH 7.8) containing 10 mM Tris-HCl (pH 8). Suspensions were then split equally into 2 x 1.5 mL microcentrifuge tubes. 400 μL of 40 mg/mL lysozyme was added, then incubated at 37°C for 50 minutes, inverting every 10 minutes. Solutions were then centrifuged at

14,000 x g for 1 minute. Supernatant fractions were removed and cell pellets were resuspended in 800 µL of Nuclei Lysis Solution at room temperature. Solutions were then subjected to three freeze (-80°C) - thaw (80°C) cycles. Tubes were cooled to ~35°C and 5 µL of 4 mg/mL RNase solution was added. Tubes were incubated at 37°C for 45 minutes with inversion every 15 minutes. Tubes were put on ice and 270 µL Protein Precipitation Solution added. Tubes were inverted until the contents were well mixed, then cooled on ice for 5 minutes, and centrifuged at 14,000 x g for 5 minutes. Supernatant fractions were transferred to fresh 1.5 mL tubes and centrifuged for 3 minutes at 14,000 x g three times, transferring the supernatant after each spin. 620 µL isopropanol was layered on top of the resultant supernatant and mixed by gentle inversion. Tubes were then given physical shock to maximise DNA precipitation. The DNA was pelleted and washed twice in 70% ethanol. Washed DNA was resuspended in 50 µL purified H_2O and stored at -80°C.

2.3.15 Measuring Genomic DNA Concentration

1 µL of DNA solution was placed on the sample pedestal of a NanoDrop™ 1000 Spectrophotometer (ThermoFisher Scientific Inc.) and absorbance at 260 nm and 280 nm was measured. ND-1000 software V3.1.0 converted the absorbances into concentration and purity values.

2.3.16 Polymerase Chain Reaction (PCR) using Primers for Three *Enterococcus faecalis* V583 genes.

To amplify internal segments of the target genes, PCRs were done using a T-Gradient Thermocycler (Biometra). Reagents and solutions used per 20 µL reaction are shown in **Table 2.3**, while **Table 2.4** shows the thermocycling parameters for the PCR.

Reagent/Solution	Volume (µL)
100 ng/µL Genomic DNA	1
Forward Primer (2 µM)	1
Reverse Primer (2 µM)	1
10 X PCR Buffer with $MgCl_2$	2
Deoxynucleotide Solution Mix (10 mM)	1
Taq DNA Polymerase (5 U/µL)	0.2
Purified Water	13.8

Table 2.3 – Reagents/solutions and their respective volumes for a 20 µL PCR mix. Reagent suppliers are listed in the Materials section 2.1.

Step	Temperature (°C)	Duration (s)	Notes
Lid Preheating	98	N/A	Initial melting step commences when heated metal lid reaches 98°C
Initial Melting	92	60	
Melting	92	45	
Annealing	58	30	
Elongation	72	40	Returns to melting, annealing and elongation steps 30 x before proceeding to final elongation
Final Elongation	72	300	
Hold Phase	10	Indefinite	Amplified samples cooled until used or frozen at -80°C.

Table 2.4 – Steps involved in PCR amplification of regions of selected genes. As stated, melting, annealing and elongation steps are repeated 30 times (cycles) to give exponential product amplification.

2.3.17 DNA Gel Electrophoresis

Samples obtained from PCR were mixed with 6 x DNA gel loading dye to a final volume of 14 µL. Orange G was chosen as the gel dye since the bromophenol blue dye that is normally used migrates at the same rate as the expected product. A 0.8% agarose gel was cast, set, placed in a gel tank and submerged in 1 x TBE running buffer. 12 µL of the DNA/dye was loaded into wells alongside a ladder of DNA molecular size standards. Samples were run for ~45 minutes at 80 volts.

Gels were then stained in ethidium bromide (EtBr) solution for ~10 minutes, washed in purified water and visualised using a Molecular Imager® Gel Doc™ XR+ System with Image Lab™ Software (Bio-Rad Laboratories).

6 x DNA Loading Dye:

- 6 mL Glycerol
- 0.6 mL 1 M Tris HCl, pH 8.0

- 0.12 mL 0.5 M EDTA, pH 8.0
- 10 mg Orange G
- Make up to 10 mL using purified water.

10 x Tris-Borate-EDTA (TBE) Buffer, pH 8.0:

- 108 g Tris base
- 55 g Boric Acid
- 40 mL 0.5M EDTA, pH 8.0
- Make up to 1 L using purified water.

Ethidium Bromide (EtBr) Working Solution:

- 10 μL of 10 mg/mL EtBr
- Make up to 200 mL using purified water.

2.3.18 PCR Product Sequencing

PCR products were first purified, and then quantified using a NanoDrop™ 1000 Spectrophotometer (ThermoFisher Scientific). Samples containing 20 ng PCR product DNA and 3.2 pmol forward primer were made up to 15μL with purified water. Reactions were then sent to the Massey Genome Services to be sequenced using BigDye™ Terminator Version 1.1 Chemistry and a capillary ABI3730 Genetic Analyser instrument (ABI). Results were analysed using CLC Genomics Workbench 5 (Limited Mode).

2.3.19 RNA Isolation from *Enterococcus faecalis* V583

TSBgly (5 mL) was inoculated from a frozen glycerol stock and incubated overnight at 37°C with shaking. The following morning, part of the culture was diluted to an OD_{600} of 0.2 in 10 mL of TSBgly and incubated for one hour. A 1.5 mL sample of this culture was taken and labelled T0. GlcNAc (from a 0.905 M filter-sterilised stock) was then added to the remaining 8.5 mL culture to a final concentration of 10 mM. 1.5 mL samples were then taken every half hour for two hours. Each sample was immediately frozen at -80°C to minimise RNA degradation.

Once all samples had been taken, the illustra RNAspin Mini RNA Isolation Kit (GE Healthcare Life Sciences Ltd.) was used to isolate total RNA. "5.4. Protocol for total

RNA purification from up to 10^9 bacterial cells", can be retrieved from the following URL for the manufacturer's product booklet for this kit:

https://www.gelifesciences.com/gehcls_images/GELS/Related%20Content/Files/1314807262343/litdoc28982258AC_20110831205926.pdf

2.3.20 DNase Treatment of Extracted RNA

Once total RNA had been extracted from *E. faecalis* cells, residual DNA contaminants were removed by DNase digestion. This was done with a DNA-*free*™ Kit (Life Sciences). The manufacturer's protocol can be retrieved from the following URL:

http://tools.invitrogen.com/content/sfs/manuals/cms_055739.pdf

2.3.21 Reverse Transcription of Extracted RNA Molecules

After DNAse treatment of extracted RNA, cDNA formation was required prior to qPCR for determining the ratios of *nagE*, *gap2* and *rpoA* mRNA. For cDNA formation, extracted RNA was treated according to the manufacturer's instructions for the SuperScript® VILO™ cDNA Synthesis Kit (Life Sciences). The protocol can be retrieved from the following URL:

http://tools.invitrogen.com/content/sfs/manuals/vilo_cdna_synthesis_man.pdf

3.0 Determining the Antimicrobial Spectrum of Purified GccF

While Kelly *et al.* described a culture supernatant of *Lactobacillus plantarum* KW30 (containing GccF) as having an inhibitory effect on *L. plantarum* strains and one strain each of *Lactobacillus delbrueckii* supsp. *lactis* and *Lactobacillus brevis*, the range of bacteria tested was rather limited[101]. Since this early work, details of the structure of GccF have been reported, including the *S*- and *O*-linked N-acetyl-D-glucosamine moieties, attached to cysteine 43 and serine 28 respectively. The subsequent discovery that free GlcNAc specifically protects *L. plantarum* strains from GccF-mediated bacteriostasis[82] hinted at a mode of action that is novel, but has yet to be elucidated. Experiments carried out in this study were therefore designed to expand what is known about the phylogenetic spectrum of activity of GccF, and its mechanism, or mechanisms, of action.

This chapter presents the logic behind selecting the range of bacterial genera and species to be tested for their susceptibility to GccF, and the results of these tests.

3.1 Objectives

1) To identify other possible target organisms based on the presence of predicted GlcNAc PTS transporter genes in genomes.
2) To test the selected bacterial strains on solid media against a variety of antimicrobials, including GccF, with and without the addition of GlcNAc.
3) Use liquid culture assays to determine the nature and strength of GccF-mediated inhibition on selected bacteria, as well as the influence that GlcNAc has on GccF's activity.

3.2 Selecting Candidate Organisms for GccF Susceptibility Testing

As the GlcNAc moieties of GccF had been shown to be essential for its activity, the genome of a well characterised indicator strain, *Lactobacillus plantarum* WCFS1, was examined to see if a potential membrane receptor for GlcNAc could be identified.

Using the website Kyoto Encyclopedia of Genes and Genomes (www.kegg.jp/kegg/), the amino sugar metabolism pathway of GlcNAc for *L. plantarum* WCFS1 was examined.

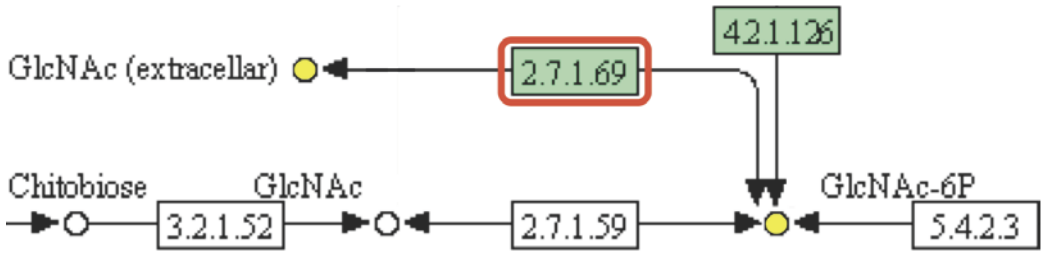


Figure 3.1 – KEGG amino sugar metabolism pathway for *L. plantarum* WCSF1. The enzyme bordered in red, a GlcNAc-specific PTS transporter (E.C. 2.7.1.69; pts18CBA and pts22CBA), represents the possible molecular receptor for GccF, as it selectively imports extracellular GlcNAc into cells, simultaneously phosphorylating it to GlcNAc-6-phosphate.

Two gene products were identified that are involved in the import of GlcNAc in WCFS1. These are pts18CBA and pts22CBA, both of which are phosphotransferase systems (PTSs), specific for importing and phosphorylating GlcNAc. In these particular transporters, the transmembrane EIIC protein and intracellular EIIB and EIIA proteins are fused into a single polypeptide. The transmembrane subunits, IIC and IID, of a mannose PTS transporter have recently been shown by Kjos *et al.* to be the receptor for the bacteriocin, pediocin PA-1[66]. It is therefore possible that a domain of the GlcNAc PTS transporter(s), the EIIC transmembrane domain, fulfils the same role acting as a target for GccF. This is an attractive hypothesis because (i) the GlcNAc PTSs are not promiscuous, but are GlcNAc specific, and GlcNAc is the only monosaccharide that protects *L. plantarum* from GccF[82] and (ii) PTSs are components of dynamic cell signalling pathways involved in metabolite system control and regulation. If GccF binds with one or both GlcNAc-PTS(s) it may: (a) force the transporter(s) open to allow efflux of intracellular material in a manner similar to type IIa bacteriocins, (b) block the transporter, preventing GlcNAc transport, (c) hijack the

intracellular phosphorelay signalling cascade associated with the PTS, leading to inefficient metabolic regulation, (d) be imported into the cell by the PTS and have a direct intracellular effect, or (e) a combination of effects (a) – (d). As GccF does not kill cells, but effectively inhibits growth, it is unlikely that (a) is the mechanism used, as this typically kills target cells.

On the basis of this hypothesis, the KEGG database was searched for orthologues of *L. plantarum* WCFS1 GlcNAc-specific transporter, pts18cba. This transporter was specifically chosen because it has been observed in populations of *L. plantarum* ATCC 8014, that two to five cells per million spontaneously become resistant to GccF. Within the ribosomal binding site of *pts18cba* gene for these resistant cells was found to be a single nucleotide polymorphism, which is likely to decrease the efficiency of its translation, resulting in fewer of these PTS transporters (pts18CBA) being present in the cell membrane[102]. Since the formation of a complex between GccF and a GlcNAc-specific PTS, such as pts18CBA, is hypothesised to be required for GccF-mediated bacteriostasis, fewer copies of pts18CBA in the cell membrane should decrease the susceptibility of target cells to GccF.

In order to be a suitable candidate for GccF testing, a bacterial species must not require containment facilities higher than a PC2 laboratory, which allows work with low level pathogens, and must be easily obtainable for testing. Some of the bacteria that were eventually tested are not listed in the orthologue tree in **Figure 3.2**, but were tested out of interest, or because they belong to the same genus as other susceptible species.

Using the information collected from the databases, multiple species were selected, including those shown in **Figure 3.2**, to be challenged with GccF. Tests were conducted with and without GlcNAc, to see if species other than *L. plantarum* are similarly protected from the bacteriostatic effects of GccF.

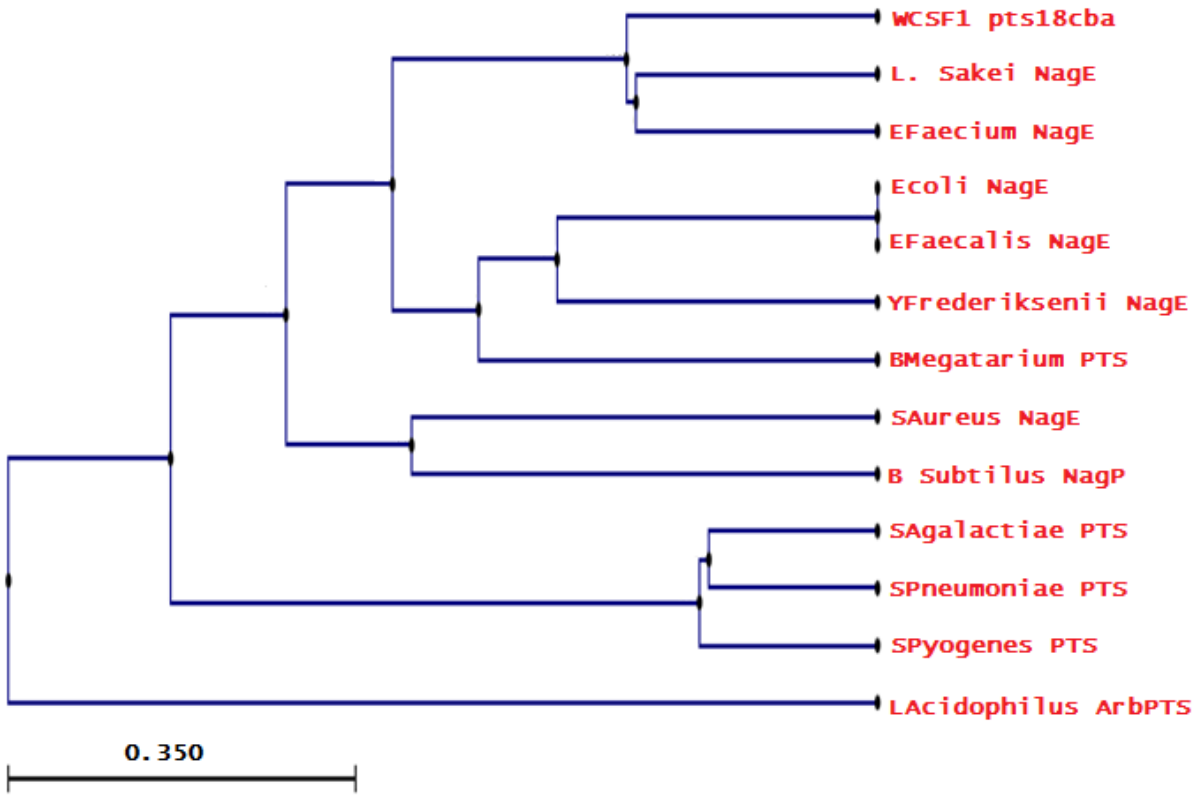


Figure 3.2 – Alignment Tree of pts18CBA Protein Orthologues. Amino acid sequences from NCBI and KEGG databases were loaded into CLC Genomics software, aligned and plotted as a tree. Not all phosphotransferase systems appear to be GlcNAc specific, but were selected as the most similar match per species.

3.2.1 In-plate Growth Assays

Refer to Methods: 2.3.3 and 2.3.4

Having obtained a variety of organisms from both the Institute of Environmental Science and Research (Wellington, New Zealand) and the culture collection at the Institute of Molecular Biosciences, Massey University (Palmerston North, New Zealand), indicator plates of each organism were poured, set and subsequently spotted with a number of antimicrobial solutions, including GccF, and GlcNAc.

Results were then visually scored on the basis of the size of the zone of bacterial growth inhibition caused by each antimicrobial.

Genus	Species	Strain	NZRM	ATCC	NCTC	GccF Inhibition	GlcNAc Effect
Bacillus	*megatarium*	DSM 319				++	+
	subtilis			6633		-	N/A
		BR151				-	N/A
Carnobacterium	*divergens*		3572			-	N/A
Enterococcus	*casseliflavus*		3293				
	faecalis	V583				+	+
		Stubbs IV	89		370	-	N/A
		Tissier	1106	19433	775	++	+
			1240	6055	5957	+++	-
			2244	29212		++	+
		PCl l325	2262	14506		++	+
		HH22	3178			+	+
		FC Tenover					
		NJ-3	3488	51299		+	+
			3601			+/-	0
		POW					
		1994	4061			++	+
		TX0104				-	+
	faecium	TYPE	1236	19434	7171	+++	0
			4037			+++	0
Lactobacillus	*acidophilus*	NCFM				++	+
		LMG1143					
	brevis	7				-	N/A
	plantarum	KW30				-	N/A
			1100	8014		+++	-
		Lp965				-	N/A
				8014 –		-	N/A

				Imm			
				14917		+++	-
		Lp39				+++	
		WCFS1				+++	-
		WRSF1				+++	-
		KW30 – 2111 – 4				+++	-
				14917		+++	-
		NC8				+++	-
	sakei	Lb790				+++	-
Leuconostoc	*mesenteroides*		3199			-	N/A
			3620			-	N/A
Listeria	*monocytogenes*			35152	7973	-	N/A
Staphylococcus	*aureus*	Newmans				-	N/A
Streptococcus	*agalactiae*	TYPE	2267	13813	8181	+++	0
			2721	27956		++	0
	mutans		987	25175	10449	+/-	0
	pneumoniae	TYPE	100	33400	7465	-	0
			2725	9163		-	N/A
			2762			++	0
			3399	49619		+	0
	pyogenes	TYPE	2264	12344	8198	-	N/A
			2724	21547		-	N/A
		Dochez					
		NY5	3604	12351		-	N/A
		T2/44/Rb 4			8322	+	0
	salivarius	TYPE	3489	7073	8618	-	N/A
	suis					-	N/A
Weissella	*viridescens*		3313			-	N/A
Yersinia	*frederiksenii*					+/-	+

Table 3.1 – Table of bacterial species and strains challenged with GccF in solid media. GccF inhibition has been scored on a scale of – (no inhibition) to +++ (strong inhibition). The effect of adjacent GlcNAc has also been assessed as either synergistic (+), protective (-), no effect (o) or not applicable (N/A).

The indicator plates served as a simple method of determining an approximate level of susceptibility to GccF. The presence of free GlcNAc next to GccF had a pronounced effect on

the inhibition of several bacterial species. The opposing effects that GlcNAc has on individual species must be due to differences in GlcNAc metabolism. For example, the protective effect of GlcNAc seen in *Lactobacillus plantarum* may be due to the target/receptor binding free GlcNAc and preventing the GlcNAc's attached to GccF from binding. In such a case, the receptor would have to have a higher affinity for free GlcNAc, compared to the *S*- or *O*- linked GlcNAcs found on GccF. For strains where GccF and GlcNAc act synergistically, the presence of GlcNAc in the environment may act as a signal to up-regulate GlcNAc-specific PTS transporter gene expression to facilitate GlcNAc uptake and optimise GlcNAc metabolism. However, in doing this cells may be "presensitising" themselves by increasing the number of receptors that may act as a target for GccF, thus promoting bacteriostasis.

These results demonstrate that the antimicrobial spectrum of activity of GccF extends far beyond *Lactobacillus plantarum*. Certain strains of pathogenic bacteria, including *Enterococcus faecalis* and *faecium*, *Streptococcus* spp. and to a lesser extent, *Yersinia frederiksenii* show suppressed growth when exposed to GccF. This supports the current hypothesis that GccF may act upon, at least as a primary target, the family of specific GlcNAc-specific PTS transporter, orthologous to those found in sequenced *L. plantarum* indicator strains.

3.2.2 Variation in Response to GccF and GlcNAc

Please refer to Methods: 2.3.4

Similar to the results found by Kelly *et al.* (1996)[101] certain strains of *L. plantarum*, were shown to develop resistance to GccF. For example, the indicator strain, ATCC 8014, became immune to GccF relatively easily through an unknown process that may involve mutation of key receptor residues or a change in regulation of receptor production[102].

Conversely, the producer strain, KW30 was able to undergo a modification which had the result of increasing its sensitivity to GccF. This could be due to the loss, or down-regulation of the entire *gcc* gene cluster, or of the immunity protein itself[102]. Unfortunately, none of these hypothesises has yet been proved. The natural variations within *L. plantarum* ATCC 8014, alongside novobiocin treated (2111-4) KW30 cells are shown in **Figure 3.3**.

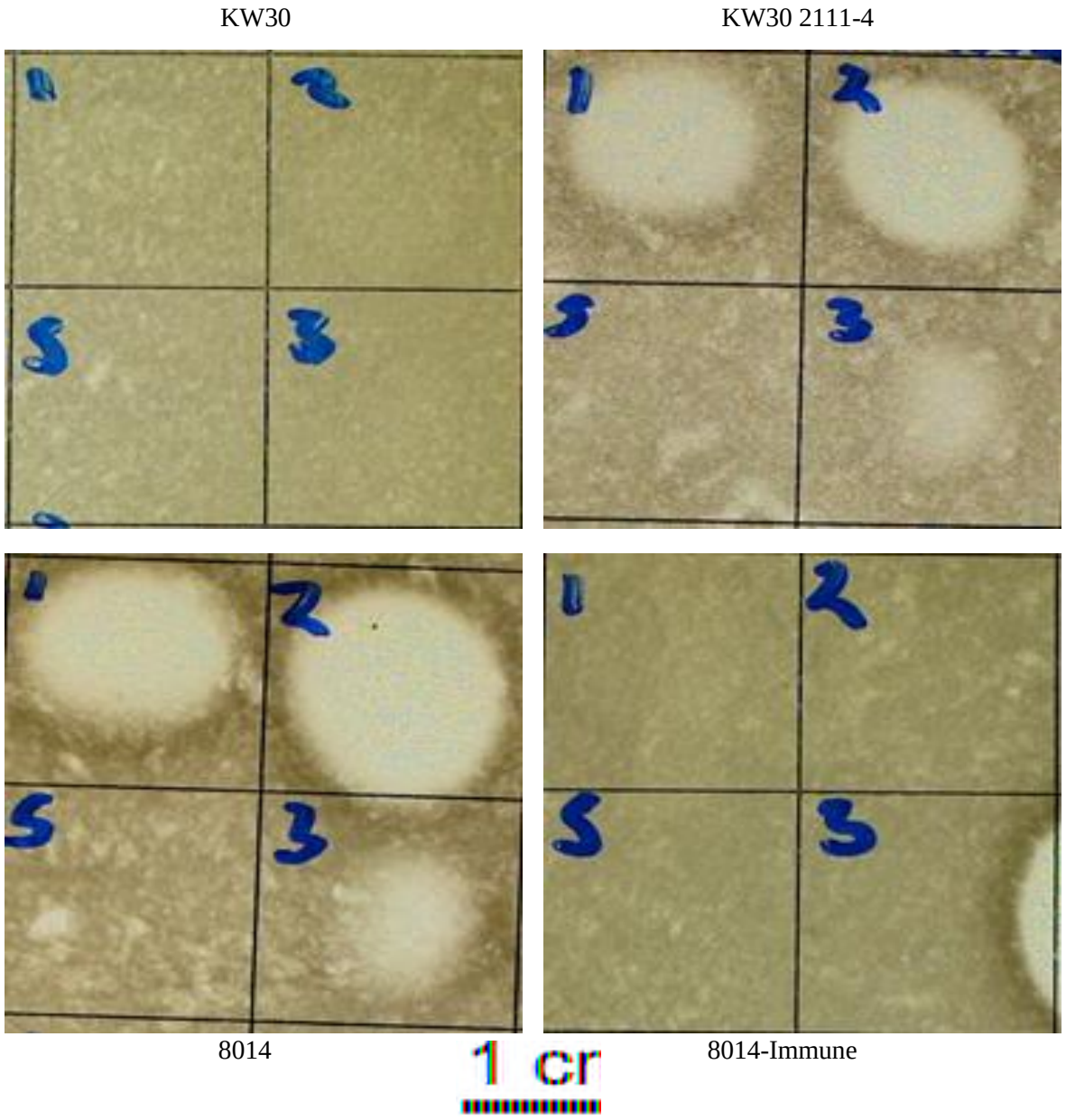


Figure 3.3 – Variation in susceptibility to GccF within *Lactobacillus plantarum*. Strains are given by the most adjacent caption. Inset squares starting at the top left, going clockwise: 1.5 µL 2.6 mg/mL GccF; 1.5 µL 0.52 mg/mL GccF; 1.5 µL 0.10 mg/mL GccF; 5 µL 20% (w/v) GlcNAc. White patches correspond to areas where the growth of indicator bacteria embedded in agar have been inhibited by pure GccF applied to the plate.

It should be noted that Kelly *et al.* (1996) found that strains of *L. plantarum* were the main targets for GccF.[101] However assays carried out in this work (**Table 3.1**), showed that a wide variety of bacteria are (a) susceptible to GccF to some degree and (b) that the addition of adjacent N-acetyl-D-glucosamine has varying effects on the activity of GccF, either attenuating, or enhancing its antimicrobial activity. The species that were most affected by free GlcNAc were *Lactobacillus plantarum* (attenuation) and *Enterococcus faecalis* (enhancement of GccF activity). Examples are seen in **Figure 3.4**.

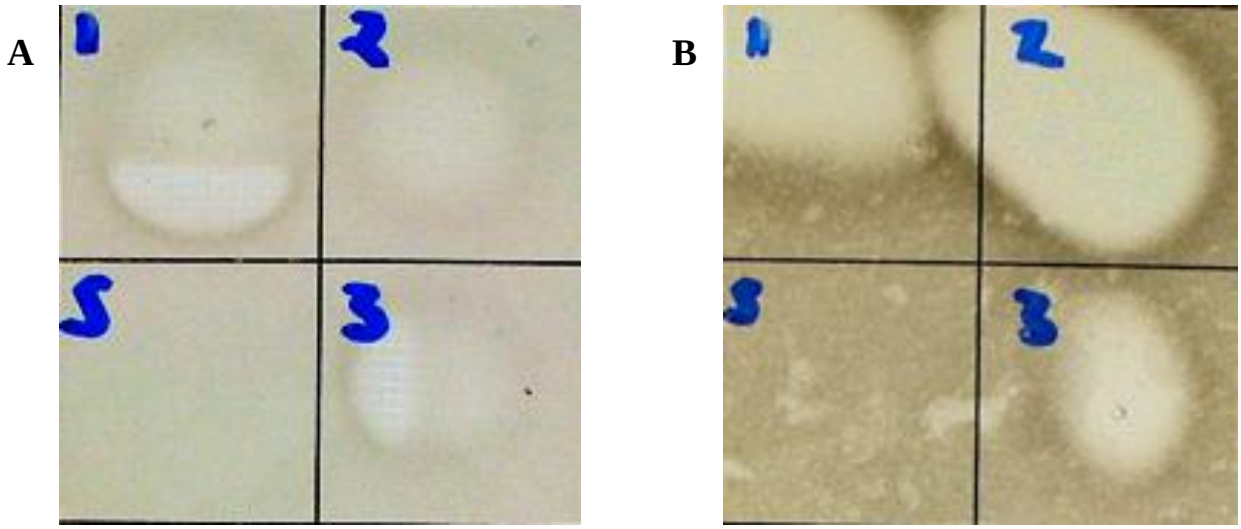
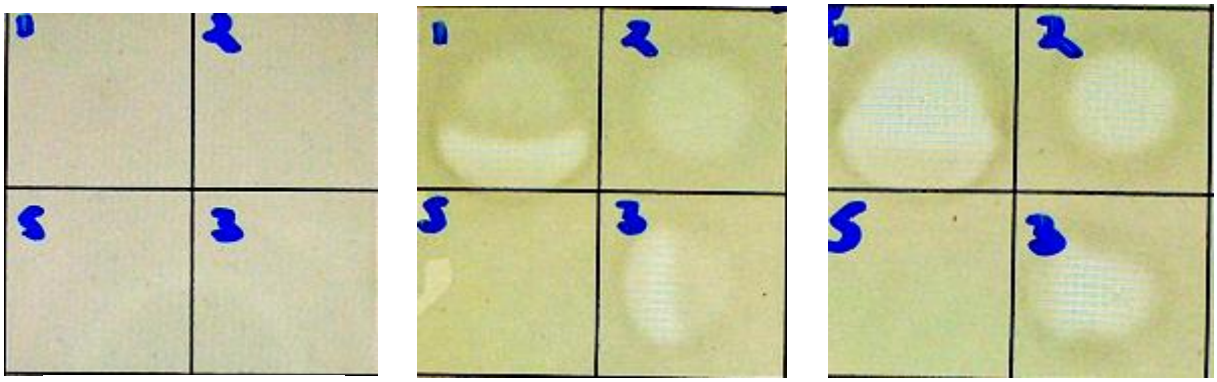


Figure 3.4 – Examples of differential GlcNAc effects between species. Samples of GccF or GlcNAc were pipetted into the centre of each square. Starting from the top left, going clockwise, each square was spotted with: 1.5 µL 2.6 mg/mL GccF; 1.5 µL 0.52 mg/mL GccF; 1.5 µL 0.10 mg/mL GccF; 5 µL 20% (w/v) GlcNAc. Plate (**A**) *E. faecalis* NZRM 4061 in TSB (1% agar); GlcNAc enhances the activity of GccF in the area where diffusion has resulted in the presence of both GccF and GlcNAc. Plate (**B**) *L. plantarum* KW30 2111-4 in MRS (1% agar); GlcNAc exhibits a protective effect where diffusion it has diffused into areas where growth would otherwise be inhibited by GccF.

Enterococcus faecalis, like *L. plantarum*, includes strains that are both resistant and sensitive to GccF. **Figure 3.5** shows three distinct levels of increasing susceptibility to GccF within the species, *Enterococcus faecalis*.



E. faecalis NZRM 89 | *E. faecalis* NZRM 1106 | *E. faecalis* NZRM 1240

Figure 3.5 – Variation in GccF susceptibility and GlcNAc response within *E. faecalis*. NZRM 89 appears completely resistant to the highest concentration of GccF, 1106 shows typical GlcNAc-dependent inhibition, and 1240 is very sensitive. Inset squares starting at the top left, going clockwise: 1.5 µL 2.6 mg/mL GccF; 1.5 µL 0.52 mg/mL GccF; 1.5 µL 0.10 mg/mL GccF; 5 µL 20% (w/v) GlcNAc.

NZRM 89 displays complete resistance to GccF, irrespective of the presence of GlcNAc. NZRM 1106 displays the typical response of the species, with mid-low level inhibition by GccF alone, but has enhanced activity in the presence of GlcNAc, shown on plates as a crescent-shaped zone of inhibited bacterial growth between the square containing GccF and that containing GlcNAc. NZRM 1240 is the most susceptible *E. faecalis* strain identified, and seemingly reverts to the typical *L. plantarum* response whereby GlcNAc becomes protective.

For 1240 in particular there is evidence of 3 overlapping regions of different response. For the top left panel, in the GccF-affected region, starting at lower diving line and working up, there is: (1) A zone where inhibition of growth has been partly prevented by a relatively high [GlcNAc]; (2) A region where inhibition is increased (note the wider diameter in this region of the 'circle') due to the synergistic action of GccF and a lower [GlcNAc]; and (3) A region where the [GlcNAc] is too low to affect GccF-mediated growth inhibition.

Although the concentration gradients of GccF and GlcNAc that formed in these indicator plates were clearly eliciting a complex range of behaviours from some susceptible bacteria embedded in agar, it was not feasible to develop methods for measuring these gradients, and/or the way in which local ratios of GccF and GlcNAc vary over time, in the time available for this study. Therefore an attempt was made to replicate GccF and GlcNAc's synergistic inhibition of *E. faecalis* strains in liquid cultures, where the concentration and timing of GlcNAc and GccF addition can be carefully controlled.

3.2.3 Real-Time Growth Determination in Liquid Media Using a Typical *Enterococcus faecalis* strain, V583

Please refer to Methods: 2.3.5, 2.3.7

As the identification of organisms susceptible to GccF had been done with agar diffusion assays (effectively end-point assays), the experiments were repeated in liquid culture growth assays using real time to optimise the GlcNAc/GccF synergy for inhibition and to visualise relative growth impairment by the bacteriocin.

Enterococcus faecalis V583 was used to investigate the synergy between GccF and GlcNAc because it is a sequenced strain and it exhibited a response typical reaction seen in most *E. faecalis* strains. Growth curve data was collected using a Cary 300 UV-Visible spectrophotometer (section 2.3.7).

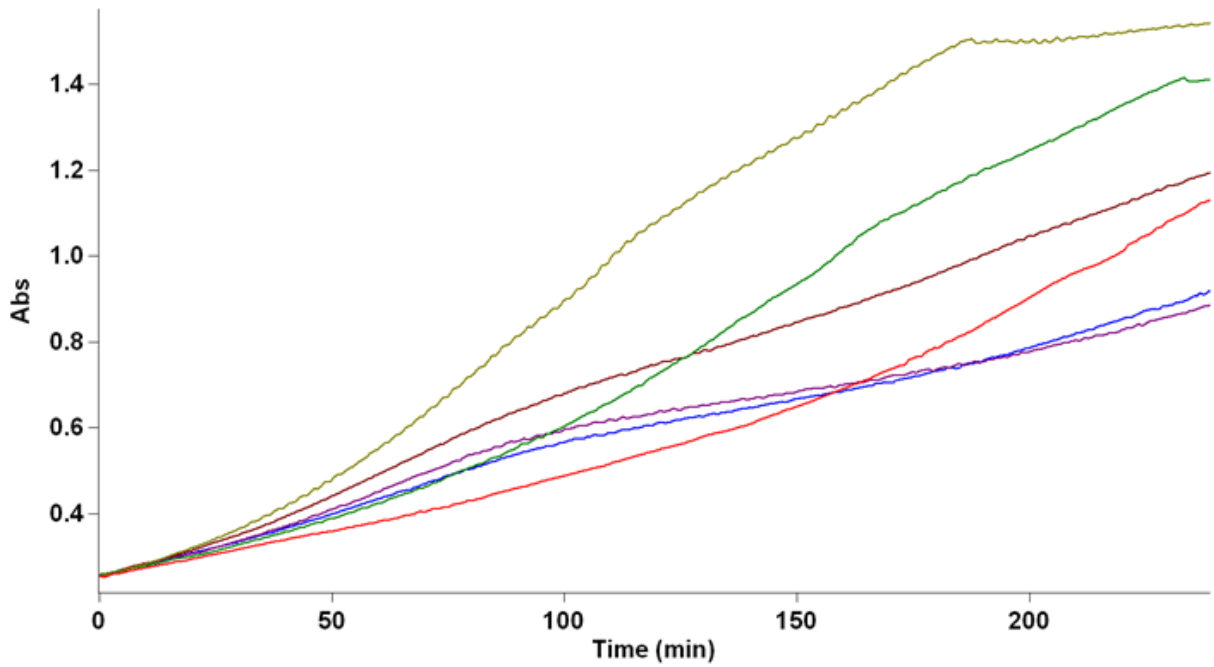


Figure 3.6 – OD_{600} growth curve of *E. faecalis* V583 with addition of 1 µM GccF at time 0. Positive Control/No GccF, GccF Only, GlcNAc added at T(0), GlcNAc added T(-20), GlcNAc added T(-40), GlcNAc added T(-60). GccF was not added to positive control. Final [GlcNAc] in cuvettes was 100 µM.

Figure 3.6 is a key finding, since it suggests that pre-exposure to GlcNAc is the key to sensitisation to GccF, a result that could not be elucidated from afar diffusion assays. The next step was to determine the effect of different concentrations of GlcNAc on GccF-induced bacteriostasis of *E. faecalis* V583. **Figures 3.7** and **3.8** show the effects of a range of low and high GlcNAc concentrations, respectively.

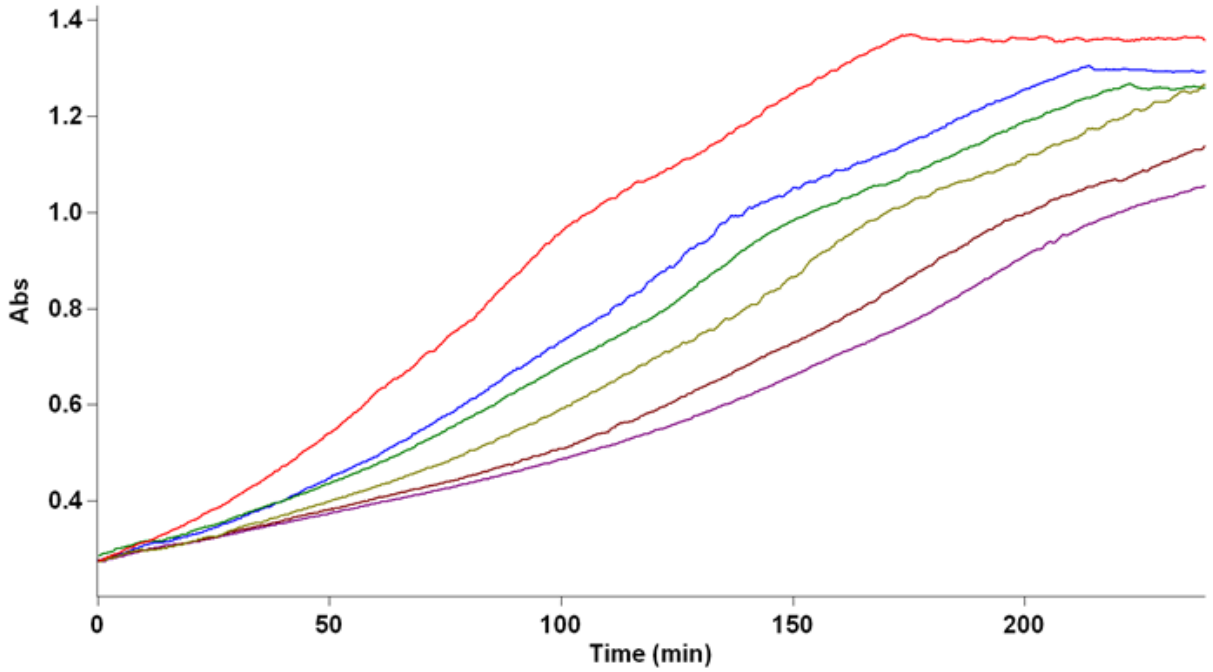


Figure 3.7 – OD_{600} growth curve of *E. faecalis* V583 with GlcNAc supplementation at T(-60) mins. Positive Control/No GccF, GccF Only, 1 µM GlcNAc, 10 µM GlcNAc, 25 µM GlcNAc, 50 µM GlcNAc. Final GccF concentration was 1 µM and was not added to positive control.

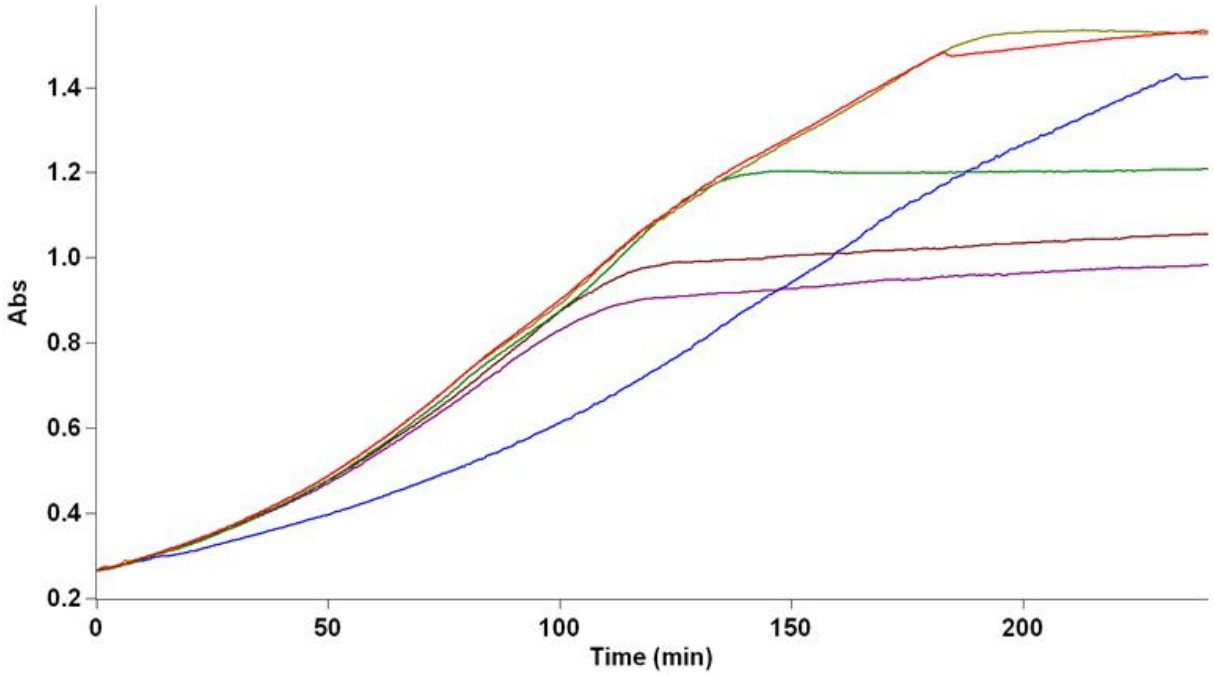


Figure 3.8 – OD_{600} growth curve of *E. faecalis* V583 with higher level GlcNAc supplementation at T(-60) mins. Positive Control/No GccF, GccF Only, 1 mM GlcNAc, 1.5 mM GlcNAc, 2.5 mM GlcNAc, 5 mM GlcNAc,. Final GccF concentration was 1 µM and was not added to positive control.

Figure 3.7 shows that GlcNAc acts synergistically with GccF, even at concentrations as low as 1 μM. Since TSB contains approximately 14 mM glucose the glucose:GlcNAc ratio is 14,000:1, indicating that *E. faecalis* V583 (and possibly other bacteria) has a very sensitive and specific GlcNAc-sensing mechanism. In addition, the result shows that the GlcNAc response at low concentrations is concentration dependent, and the extent of growth inhibition seems to correlate with the concentration of free GlcNAc that was added 60 minutes prior to GccF addition. A possible explanation could be that free GlcNAc has an extended effect that lasts long after GlcNAc has been taken up from the environment. This could potentially relate to the GlcNAc-PTS. If it were upregulated in response to free GlcNAc, the cells would be able to take it up from their immediate surroundings, which would be much more efficient than synthesising their own GlcNAc by a series of enzymatic reactions that utilise substrates for other key processes including ATP synthesis (see **Figure 1.4**). Subsequently, more copies of GlcNAc-PTS would result in more targets for GccF to act upon cells with perhaps a temporarily decreased capacity for GlcNAc synthesis. However, not enough is known about GlcNAc sensing and uptake, or regulation of GlcNAc metabolism in *E. faecalis* to draw any firm conclusions.

The higher GlcNAc concentrations in **Figure 3.8** initially provided protection against the effects of GccF, much like the effect seen for lower concentrations of GlcNAc with *L. plantarum* ATCC 8014 and GccF. The crossover point between protection and enhancement of growth inhibition occurred at ~2.5 mM GlcNAc, and was obvious at about 135 minutes after addition of GccF. The growth of the culture with 1 mM GlcNAc added at T(-60) minutes began to slow, relative to the control with no addition, ~90 minutes after addition of GccF, while for the culture containing 1.5 mM GlcNAc there was a longer delay of 105 minutes before the cells succumbed to strong inhibition by GccF Interestingly, 5 mM GlcNAc protected *E. faecalis* V583 cells from GccF inhibition for the entire duration of the 4 hour assay, closely mimicking the positive control. A logical interpretation is that extracellular free GlcNAc prevents GccF from inhibiting cell growth, possibly by competing with GccF for binding to its receptor and/or possibly by alleviating an intracellular shortage of GlcNAc (or UDP-GlcNAc) that might be caused by the GccF:GlcNAc PTS complex disrupting intracellular regulation of GlcNAc metabolism. Any temporary up-regulation of GlcNAc-PTS numbers in order to optimise GlcNAc uptake might also amplify the effect of GccF after the extracellular GlcNAc has been imported, as there would be more receptors for GccF to bind to, potentially amplifying its bacteriostatic effect. Experimental measurements

of GlcNAc concentrations in culture supernatants by capillary electrophoresis could confirm if the onset GccF-mediated growth inhibition coincided with the complete depletion of extracellular GlcNAc, but this was beyond the scope of this project.

Experiments were then carried out to determine whether *E. faecalis*, like *L. plantarum*, could be revived from GccF-induced bacteriostasis by GlcNAc.

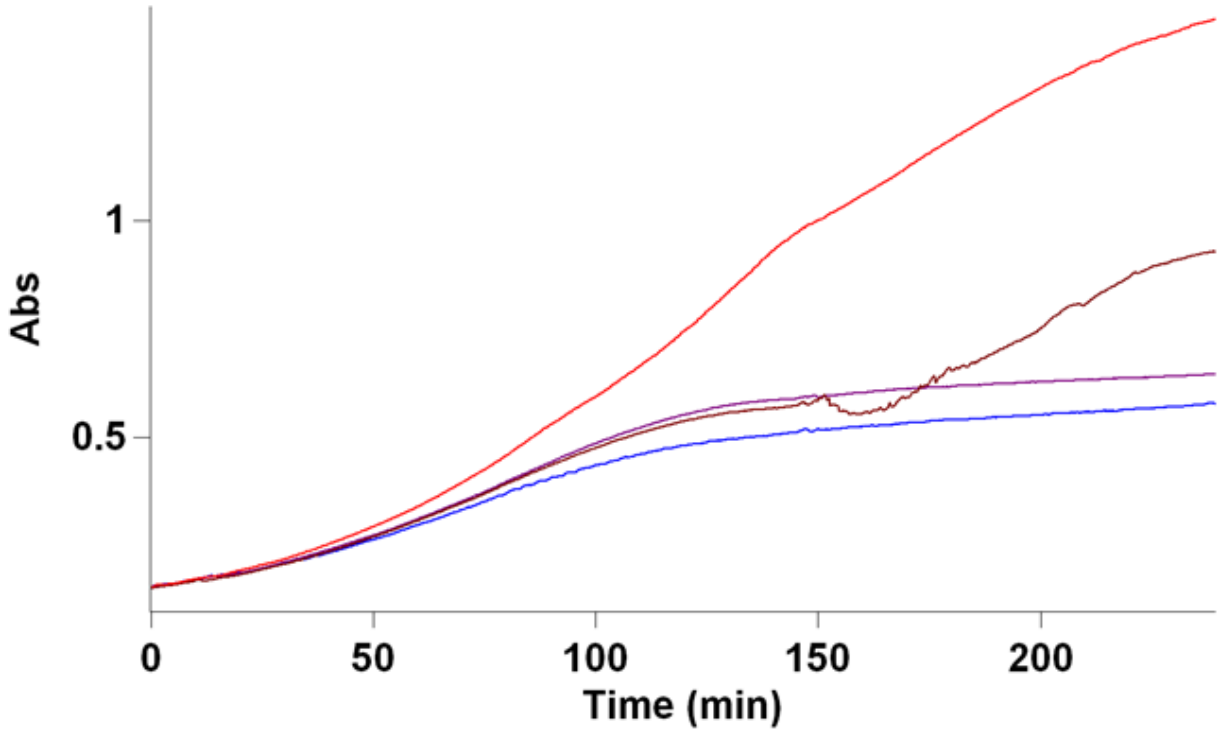


Figure 3.9 – OD_{600} growth curve of *E. faecalis* V583 showing GlcNAc-mediated recovery. Positive Control, GccF Only, 500 µM GlcNAc T(-60) + 1 mM GlcNAc T(150), 500 µM GlcNAc T(-60). Final GccF concentration was 1 µM and was not added to the positive control.

Figure 3.9 shows that when a culture of GccF-inhibited *E. faecalis* V583 cells was supplemented with an excess of GlcNAc, bacteriostasis was temporarily reversed and cells began to grow again within 15 minutes. This result is reminiscent of that found with *L. plantarum* and indicates that GlcNAc plays a dynamic role in GccF-induced inhibition in not just *L. plantarum*, but *E. faecalis* as well. The slight decrease in $O.D._{600nm}$ values in the 10 minute period after GlcNAc supplementation at 150 minutes is particularly interesting, since this was also observed for the GlcNAc-mediated revival of *L. plantarum* ATCC 8014[82].

The effect of "GccF Only" treatment on *E. faecalis* V583 growth in **Figure 3.9** was different from that seen in **Figures 3.6 - 3.8**. It appeared to cause enhanced growth inhibition

without the need for GlcNAc presensitisation. It is possible that the addition of GccF at a lower optical density (~0.1 rather than ~0.2 - 0.3) may have caused this effect.

3.2.4 Real-Time Growth Determination of Bacterial Cocci Strains, and Species Obtained from ESR

Refer to Methods: 2.3.5, 2.3.7

Given the diverse response of *Enterococcus* strains to GccF, it was important to verify that the results of liquid media assays would be consistent with the results of the agar diffusion assays.

E. faecalis was focused upon, not only due to its relative position near *L. plantarum* in terms of GlcNAc specific PTS orthology, but also due to its status as a pathogen. While part of normal gastrointestinal flora, *E. faecalis* has been known to cause endocarditis, meningitis and bacteraemia, especially in hospital environments. This species also makes up a large proportion of vancomycin-resistant-enterococci (VRE) and thus infections are becoming increasingly difficult to treat. The potential for a new antimicrobial product to slow or stop the growth of these pathogens, or even act synergistically with commonly used antimicrobials, would be of interest to the wider community.

Figure 3.10 presents the two extremes of GccF sensitivity found in *Enterococcus faecalis* strains shown in *E. faecalis* NZRM 89 (resistant) and NZRM 1240 (sensitive) under same conditions used for the strain V583.

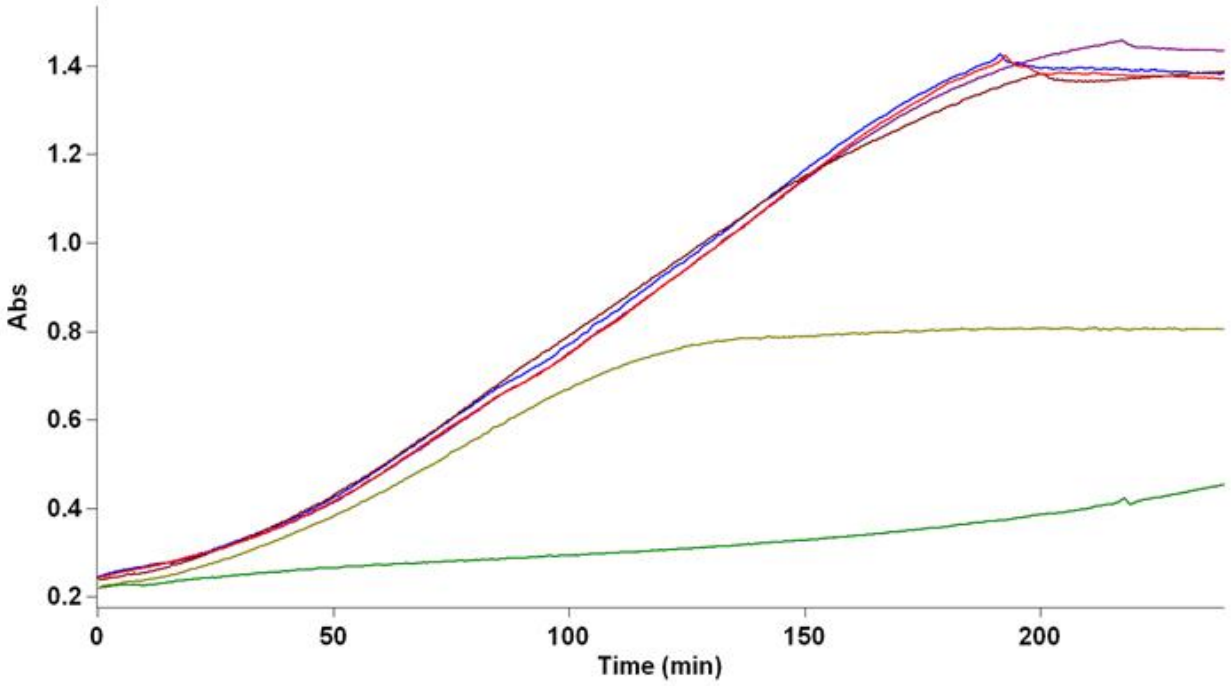


Figure 3.10 – OD_{600} growth curves of *E. faecalis* NZRM 89 and NZRM 1240. 89 Positive Control, 89 GccF Only, 89 GlcNAc T(-60) + GccF, 1240 GlcNAc T(-60) + GccF, 1240 GccF Only, 1240 Positive Control. Final GccF concentration was 1 µM, added at T(0) and was not added to positive controls. Final GlcNAc concentration at time of addition was 1 mM.

The results in **Figure 3.10** mirror those shown in **Figure 3.5**. *E. faecalis* 89 did not succumb to GccF outright, nor did GlcNAc presensitise cells to the bacteriocin since the growth curves were essentially the same as the positive control. *E. faecalis* NZRM 1240 curves were exactly as expected; GccF alone had a powerful inhibitory effect on bacterial growth, while GlcNAc allowed growth, albeit not as rapid as the positive control, before levelling off, presumably due to the possibility that all the free GlcNAc has been taken up by the cells, allowing GccF to assert its effect. This would explain the protective effect of GlcNAc seen on plates, where cells could grow for a period, before growth was slowed or halted. This 'partial' growth of bacteria was difficult to observe in agar diffusion assays, but is much clearer in this assay.

Figure 3.11 shows another two strains of *E. faecalis* responding to GccF alone or in combination with GlcNAc.

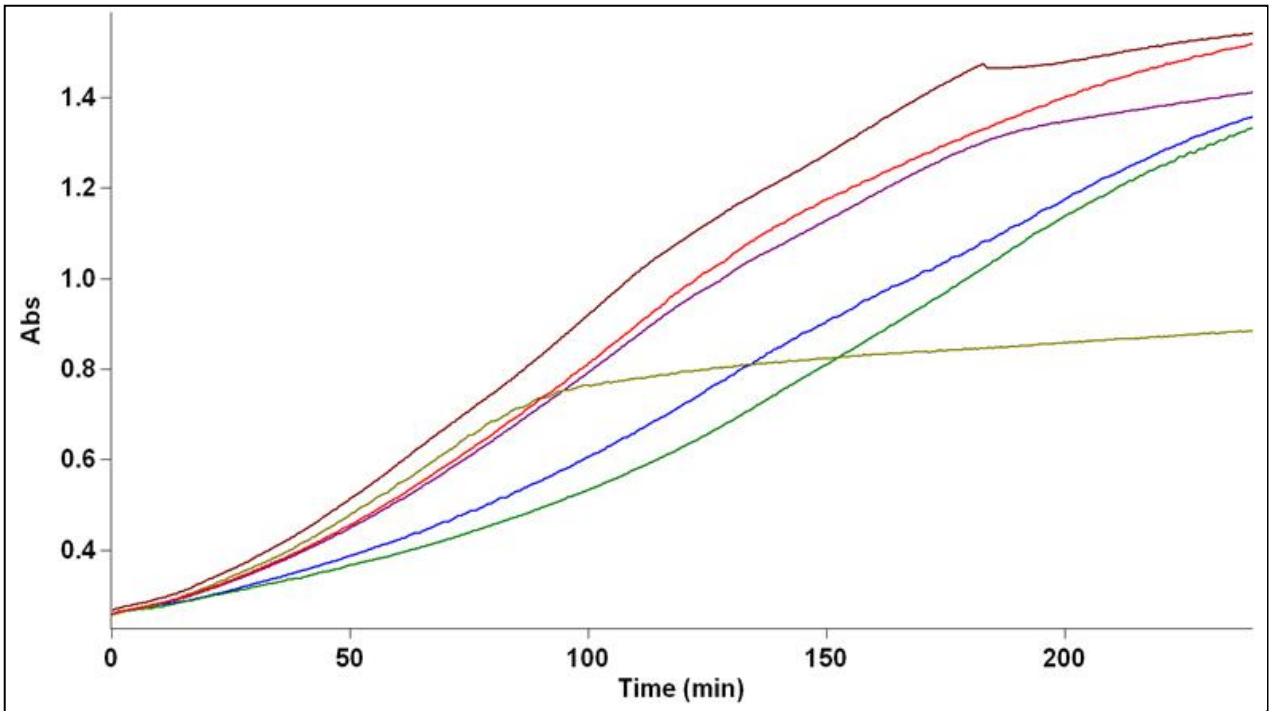


Figure 3.11 – OD_{600} growth curves of *E. faecalis* NZRM 3601 and NZRM 4061. 4061 Positive Control, 3601 Positive Control, 3601 GlcNAc T(-60) + GccF, 3601 GccF Only, 4061 GccF Only, 4061 GlcNAcT(-60) + GccF. Final GccF concentration was 1 µM, added at T(0) and was not added to positive controls. Final GlcNAc concentration was 1 mM.

In the case of NZRM 3601, pre-sensitisation with GlcNAc initially provided an extended protective effect, suggesting that its rate of GlcNAc uptake is slower than other *E. faecalis* strains, so that bacteriostasis by GccF does not occur during the initial growth phase. There was however some degree of susceptibility to GccF, as seen in the in-plate assay. The effect of GlcNAc on the efficacy of GccF inhibition of NZRM 4061 growth is more typical. In this case, pre-exposure of the cells to GlcNAc increase their sensitivity to GccF.

Potentially sensitive *Streptococcus* species from **Table 3.1** were tested for their response to GccF. *Streptococcus pneumoniae* is an important human pathogen which is a leading cause of pneumonia and may also cause an array of other invasive diseases including meningitis, sepsis and endocarditis. *Streptococcus pyogenes* is also a very invasive organism, causing necrotising fasciitis, toxic shock syndrome and sepsis. **Figure 3.12** shows their growth when exposed to GccF both alone and with pre-exposure to GlcNAc.

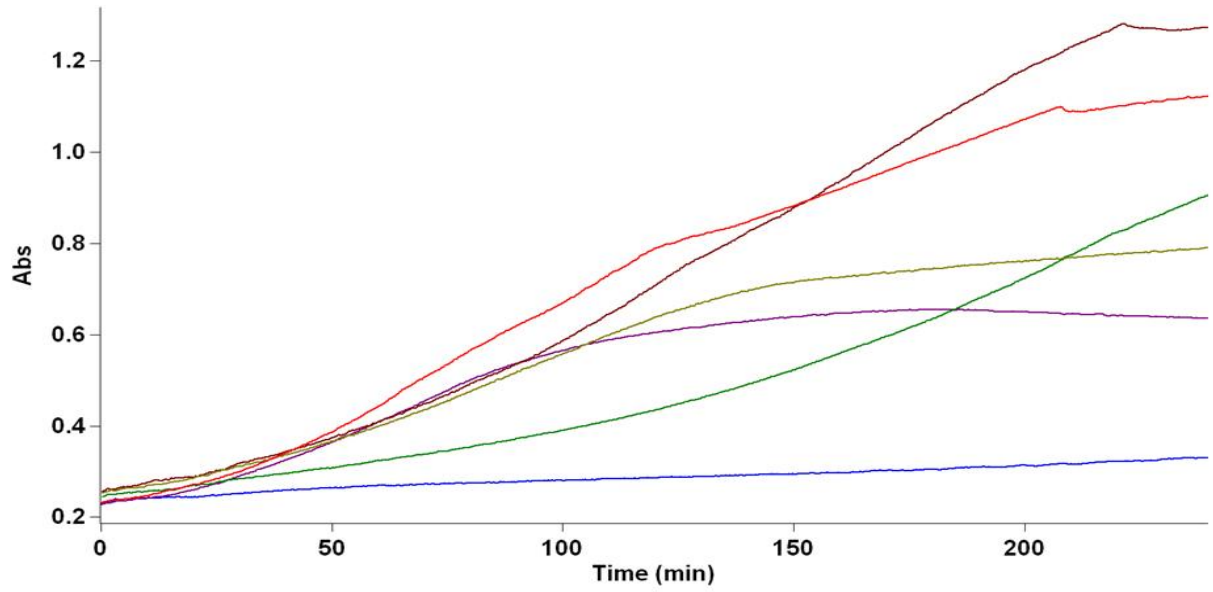


Figure 3.12 – OD_{600} growth curves of *Streptococcus* species: *pneumoniae* positive control, *pyogenes* positive control, *pyogenes* GccF only, *pneumoniae* GccF only, *pneumoniae* GlcNAc T(-60) + GccF, *pyogenes* GlcNAc T(-60) + GccF. Final GccF concentration was 1 µM, added at T(0) and was not added to positive controls. Final GlcNAc concentration was 1 mM.

The results shown are a contrast to those observed for in-plate assays. *S. pyogenes* showed slight inhibition on plates by GccF; however the liquid assay shows that GccF had a strong inhibitory effect on cell growth almost as soon as it was added. *S. pneumoniae* growth was also inhibited, although the inhibition was gradually overcome from 120 minutes onwards.

Both *Streptococcus* species were affected by the addition of 1 mM GlcNAc 60 minutes prior to GccF addition at the start of the assay. *S. pneumoniae* growth was inhibited more than it was with GccF alone, it is even possible that some cells might be lysing, as the OD_{600nm} actually decreases slightly from around 180 minutes onwards. However, this may be due to a combination of powerful bacteriostasis and cell settling, given that the cultures in the cuvettes were not agitated. *S. pyogenes* was initially protected from the effects of GccF by the addition of GlcNAc. However, after approximately 120 minutes it decreases to a rate similar to that seen when the cells are treated with GccF only, probably because of the depletion of the added GlcNAc. Despite GccF having the greater initial antimicrobial effect on *S. pneumoniae*, it recovers well after 150 minutes. Interestingly, while pre-exposure to GlcNAc seems protective in the initial stages of growth, it causes longer term enhancement of GccF activity seen by the lower growth rates at the end of the 4 hour assay.

This is an important result for the potential applications of GccF, since both *Streptococcus pneumoniae* and *pyogenes* are responsible for major invasive infections world-wide. The ability for GccF to slow the growth of these organisms in liquid culture indicates that the bacteriocin may be a useful therapeutic agent in infection treatment, overcoming resistance of these pathogens to conventional antibiotic treatments. It would be important to establish just how long GccF-mediated inhibition of growth can be sustained in cultures of those pathogens, as the (end-point) agar diffusion assay results would suggest that inhibition is eventually overcome.

3.2.5 Real-Time Growth Determination of Bacterial Rod Species in Liquid Media

Refer to Methods: 2.3.5 and 2.3.7

Species from the genus *Bacillus* (Gram-positive rods) were challenged with GccF. *Bacillus subtilis* strains tested on plates showed no sensitivity to GccF, while *Bacillus megaterium* exhibited GlcNAc-dependant inhibition by GccF. Following the growth of *B. megaterium* in liquid media proved difficult in the Cary 300 UV – Visible Spectrophotometer (Agilent Technologies), since unagitated growth led to rapid cell clumping, rendering results unusable. Instead, a U-1100 Spectrophotometer (Hitachi) was used in a 25^{o}C room, with manual reading and cuvette inversions to avoid cell clumping. Results are shown in **Figure 3.13**.

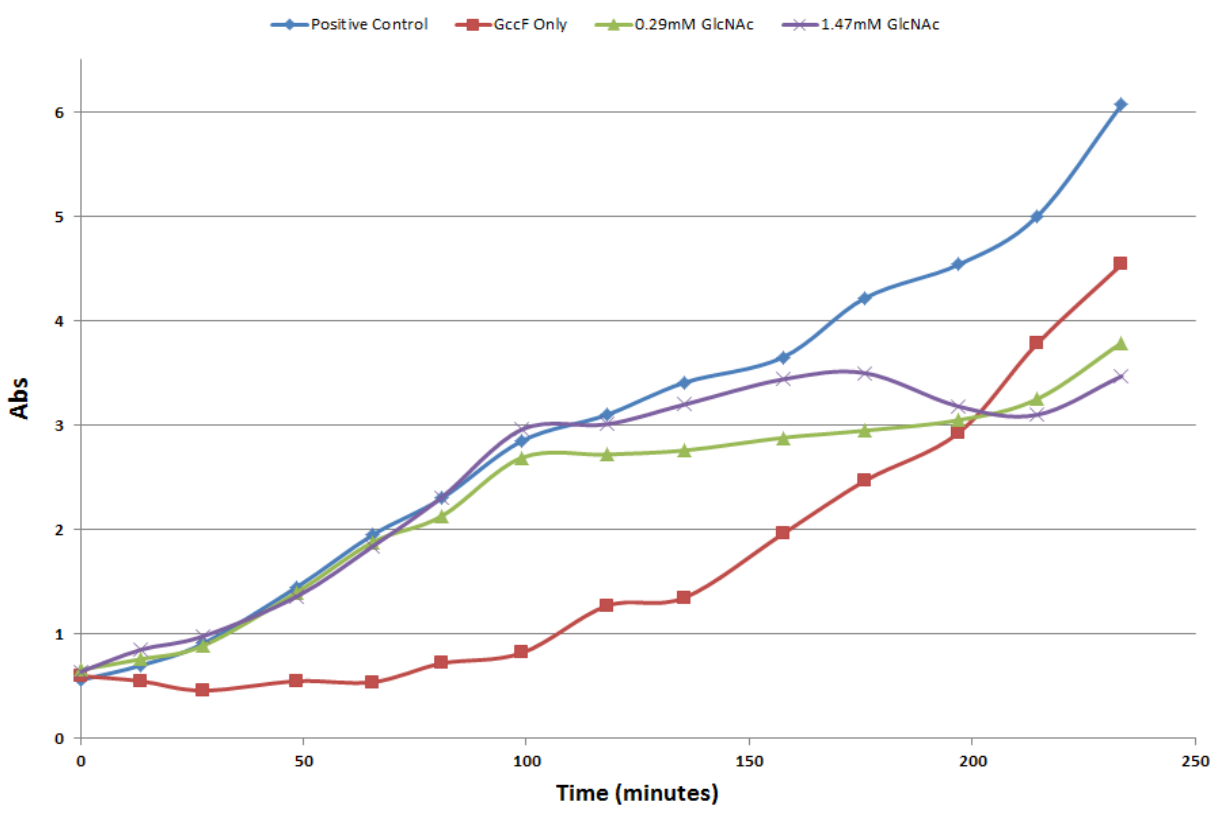


Figure 3.13 – OD_{600} growth curves of *Bacillus megaterium* DSM319. GlcNAc was added at T(-45). Final GccF concentration was 1 µM and was not added to the positive control. Cuvettes were inverted prior to each reading.

Bacillus megaterium is a very fast growing organism, reaching a maximum $O.D._{600nm}$ of more than 6 over the 4 hour assay. The rapid growth may have contributed to the cell clumping that made growth assays in the Cary Spectrophotometer problematic. The bacterium appeared to have strong bacteriostasis induced by GccF alone upon its addition at T(0), however, at approximately T(110) growth accelerated back to a rate similar to that of the positive control. The mechanism of overcoming GccF's toxicity is unknown. Perhaps GccF is metabolised by *B. megatarium*, since GccF is a conjugate of peptide and glycans that individually do not induce cell death. This means that temporarily inhibited cells may have the potential to exude enzymes able to degrade the bacteriocin. Another possibility is that *B. megaterium* possesses a resistance mechanism with the potential to re-establish any intracellular metabolic processes that GccF has disrupted.

Despite the atypical recovery from GccF inhibition by *B. megaterium*, the GlcNAc presensitisation was typical. Samples with pre-added GlcNAc resisted GccF inhibition, before succumbing to dramatically decelerated growth around T(100). It was unexpected that both sets of cultures exposed to 0.29 and 1.47 GlcNAc expressed inhibition at near identical times if we assume that GccF is able to take effect when GlcNAc levels have dropped sufficiently. This could be attributed to rapid cell growth. It may be speculated that the initial slowing of growth in the GccF only cuvette us due to the similarity between the *B. megaterium* GlcNAc-PTS and the *L. plantarum* PTS18CBA, which was the original basis for selecting this organism. However, the presence of a PTS18CBA-like PTS may not be the sole requirement for GccF susceptibility.

In addition to Gram-positive pathogens being potential target organisms, some Gram-negative organisms also possess GlcNAc specific PTSs that are orthologues of the *L. plantarum* transporters. One such genus is *Yersinia*, which encompasses *Yersinia pestis*, the infamous causative agent of the bubonic plague. *Yersinia* are Gram-negative rods, hence they have an outer cell membrane that serves as an extra barrier and level of selectivity for substances pass into, or out of the cell. GccF susceptibility was tested with and without polymyxin, an antibiotic that, allegedly, selectively targets lipopolysaccharide molecules in Gram-negative outer membranes (OM) causing damage via a "detergent-like" mode of action. *Yersinia frederiksenii*, a mild pathogen known to occasionally cause gastrointestinal infections in humans was used as a representative organism for this genus.

Figure 3.14 shows growth assays of *Y. frederiksenii* against polymyxin B as a basis to initially damage the OM, without killing the cells.

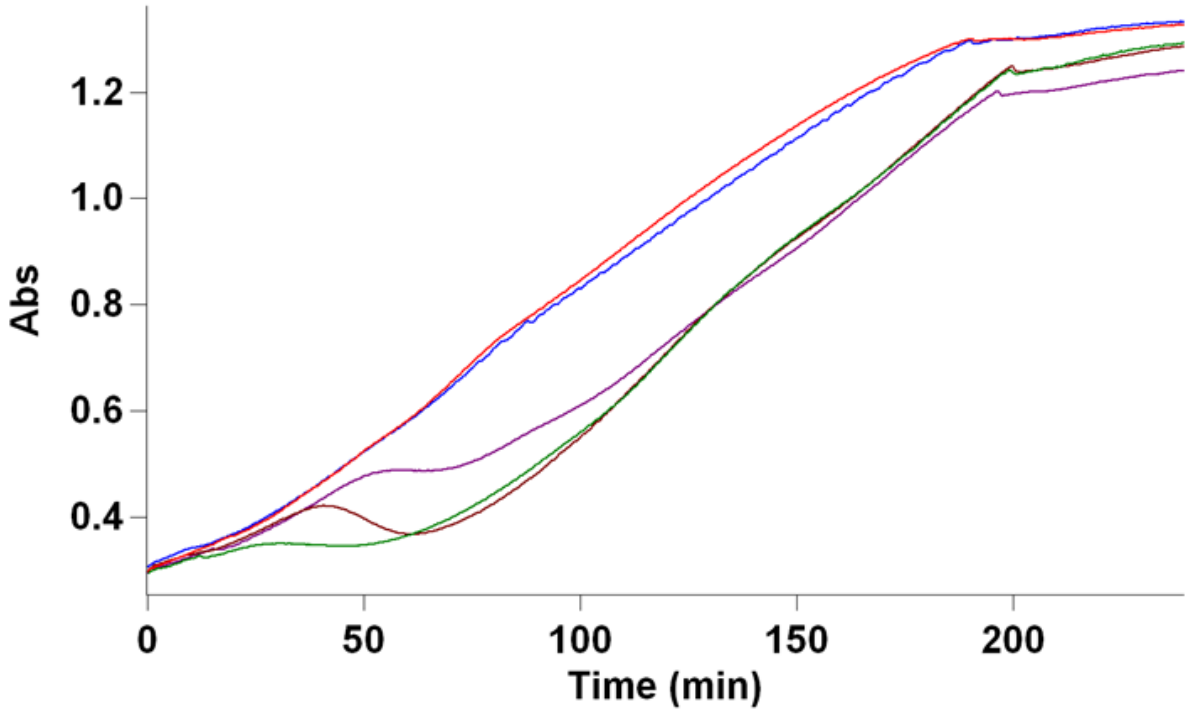


Figure 3.14 – OD_{600} growth curve of *Yersinia frederiksenii* in the presence of a range of concentrations of polymyxin B. **Positive Control, 0.5 µM polymyxin, 1 µM polymyxin. 2 µm polymyxin, 5 µM polymyxin,** Polymyxin B was added at T(0).

Y. frederiksenii displayed moderate resistance to polymyxin, which was unexpected. Upon investigation, *Y. frederiksenii* strain ATCC 33641 was found to carry a gene that encodes for a polymyxin resistance protein[103]. Perhaps the unexpected resistance could be attributed to this protein. Nonetheless, all concentrations of polymyxin, except 0.5 µM caused a minor concentration-dependent inhibition of growth shortly after addition of polymyxin.

From these results, a concentration of 0.5 µM was chosen for the next set of assays to assess the effect of GccF on *Y. frederiksenii*. It was hoped that 0.5 µM polymyxin would permeabilise the OM sufficiently to allow GccF to access targets in the inner membrane, *e.g.* PTS18CBA.

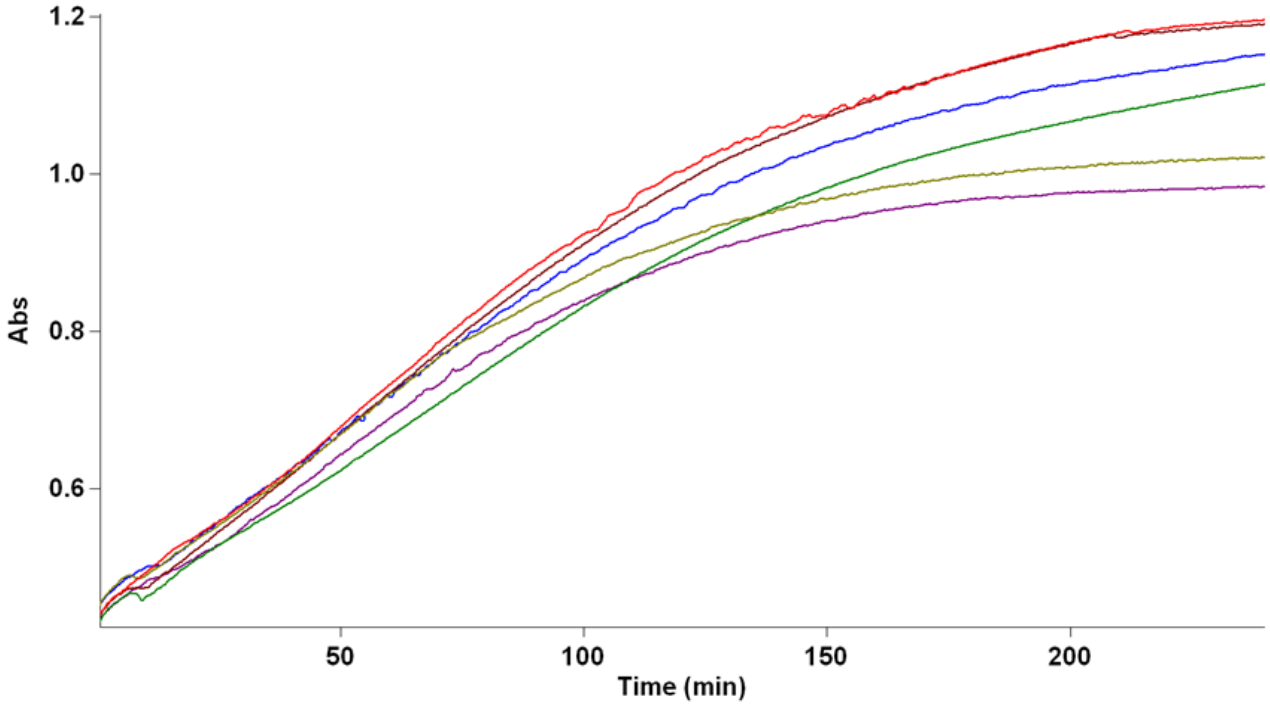


Figure 3.15 – OD_{600} growth curves of *Yersinia frederiksenii* in the presence of GccF with and without the addition of polymyxin B. **Positive Control, Positive Control (PM), GccF Only, GccF Only (PM), GlcNAc T(-60) (PM), GlcNAc T(-60).** GccF was not added to positive control. Final concentrations were 1mM GlcNAc, 1 µM GccF and 0.5 µM polymyxin. PM indicates the addition of polymyxin at T(0).

Figure 3.15 demonstrates that GccF does slightly inhibit the growth of *Y. frederiksenii*. This effect, typically seen for most previous results, was exacerbated by exposure to GlcNAc an hour prior to commencing the assay, but the sensitisation is not quite as dramatic as seen for other genera. In addition, the presence of polymyxin appeared to slightly enhance GccF's ability to inhibit cell growth. It was hypothesised that the disruption of the Gram-negative OM, GccF would have easier access to its theoretical target (a GlcNAc-PTS), which is present in the inner membrane. This hypothesis is investigated further in Chapter 4.

4.0 Investigating the Mode of Action of GccF

Kelly *et al.* (1996)[101] and Stepper *et al.* (2011)[82] obtained conflicting results regarding the effect of GccF on the indicator strain, *L. plantarum* ATCC 8014. Kelly *et al.* observed that the culture supernatant of *L. plantarum* KW30, containing GccF, was bactericidal[101], while Stepper *et al.* showed that purified GccF was bacteriostatic[82]. It remains unclear how GccF exerts its antimicrobial effect, and given its distinct structural features, including novel post-translational modifications (PTMs), and its bacteriostatic activity, it is likely that it employs a novel inhibitory mechanism.

While circumstantial evidence suggests that the potential target of GccF is a GlcNAc-specific pts18CBA/22CBA/NagE, this is by no means certain. It was therefore necessary to rule out other common antimicrobial mechanisms of bacteriocins. Due to Gram-negative bacteria possessing an addition barrier (the outer membrane) that may enable cells to resist GccF, *E. coli* outer membranes were made 'leaky' to test if this increased inhibition by GccF. Because of the different effects of free GlcNAc on different bacterial strains, the effect of this amino-sugar on the proteome and transcriptome of various susceptible strains was also investigated.

4.1 Objectives

1) To determine whether GccF is bactericidal, or bacteriostatic towards both *L. plantarum* and GlcNAc-presensitised *E. faecalis*.
2) To determine if GccF, like nisin, causes an efflux of ATP through pores formed in the bacterial membrane, using a sensitive bioluminescent assay for ATP.
3) Use the Gram-negative bacterium *E. coli* expressing "leaky" secretins in the outer membrane to deduce the location of GccF's receptor in *E. coli*.
4) Identify differentially expressed proteins in *E. faecalis* after GlcNAc addition using proteomics techniques.
5) Use qPCR to investigate the effect of GlcNAc addition on the expression of *nagE* in *E. faecalis*.

4.2 Is GccF bactericidal, or bacteriostatic?

Refer to methods: 2.3.5- 2.3.7

The first article documenting plantaricin KW30 by Kelly *et al.* concluded that the bacteriocin was bactericidal[101]. Their bacteriocins activity assays were, however, carried out using *L. plantarum* KW30 supernatant containing GccF, rather than purified GccF. It is possible that the culture supernatant contained additional antibacterial substances, for example an organic acid and/or another bacteriocin, and that the death of the indicator *L. plantarum* ATCC 8014 cells occurs only when these other substances are present in addition to GccF. In the 17 years since this paper was published there has been steady progress in peptide purification techniques for the isolation bacteriocin molecules. Stepper *et al*. studied the effect of purified GccF on *L. plantarum* ATCC 8014 using a Live/Dead assay to determine cell death[82]. They found that after three hours exposure to a high concentration of GccF (200 nM, *i.e.* approximately 100-times the IC_{50}), only 5% of the indicator cells were dead. This, in conjunction with the fact that prolonged, complete bacteriostasis induced by purified GccF can be reversed by adding free GlcNAc (Stepper *et al.* (2011) and this study), strongly suggests that, when acting alone, GccF is primarily a bacteriostatic agent.

Both GlcNAc moieties on GccF contributed to antibacterial activity, indeed the *O*-linked GlcNAc is essential for activity in agar diffusion assay[82]. Recombinant *N*-acetyl-β-D-glucosaminidase (GcnA) from *Streptococcus gordonii* was used to specifically cleave the *O*-GlcNAc residues from GccF, leaving the *S*-linked GlcNAc attached to the peptide. In this thesis the agar diffusion assays were repeated in liquid culture, where *L. plantarum* cells were treated with various concentrations of *O*-deglycosylated GccF, and GccF. Untreated cells grown under identical conditions were used as a positive control. Cell viability was determined by counting colonies. Results are expressed as colony forming units (cfu) per millilitre of culture and are shown in **Figure 4.1**.

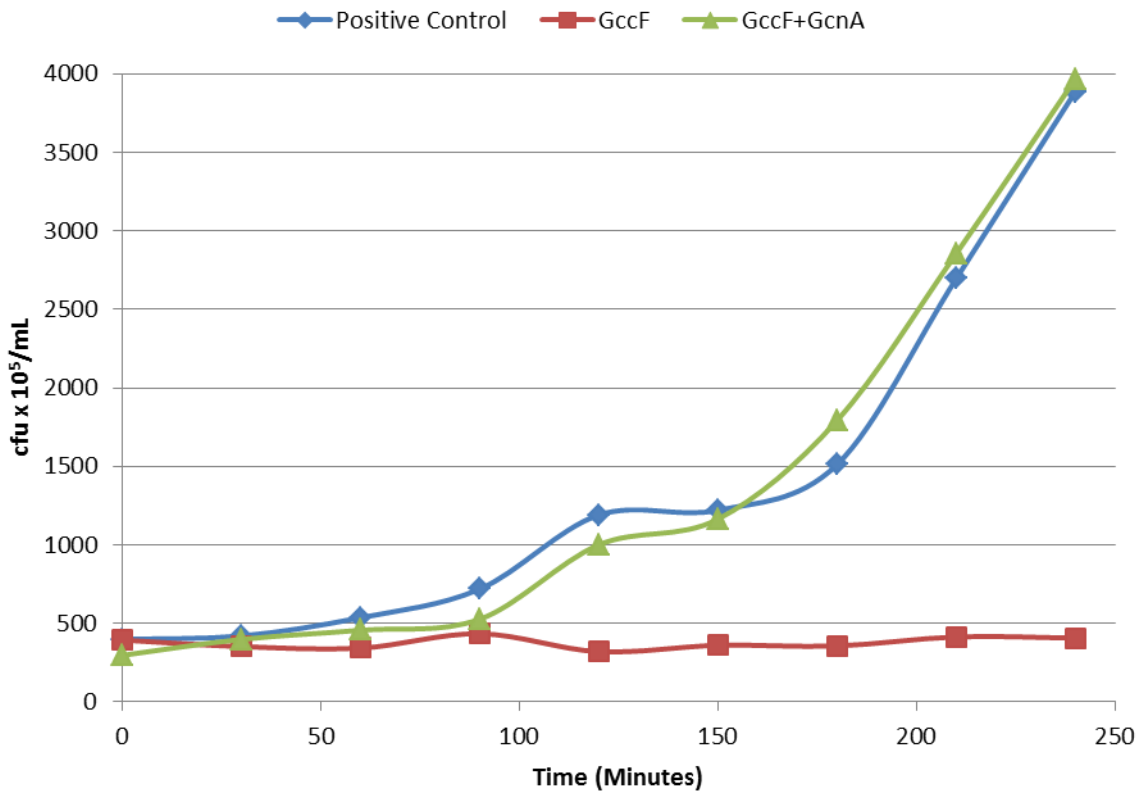


Figure 4.1 – Calculated cfu/mL for *L. plantarum* ATCC 8014 cultures, at 30 minute intervals after addition of GccF or *O*-deglycosylated GccF. The positive control (no addition) and GcnA+GccF (~200 nM *O*-deglycosylated GccF) have similar trends in both lag and log phase. GccF (200 nM) treated culture shows no growth but also no decrease in numbers of viable cells. All additions were made at T(0).

Contrary to the finding by Kelly *et al.* (1996)[101] of bactericidal activity in *L. plantarum* KW30 supernatants, *L. plantarum* ATCC 8014 cfu/mL did not decrease at all as a result of treatment with purified GccF. Bacterial growth was completely inhibited for the duration of the assay, and colonies grown from these GccF-inhibited cells were smaller than those seen on positive control plates with similar cfu numbers. This may be a result of residual amounts of GccF being transferred to agar plates when culture samples were taken for spread plates. Another explanation may be that GccF-induced bacteriostasis is slowly reversed over time once the GccF concentration has decreased through culture dilution prior to spreading on agar plates, where further dilution of GccF occurs.

Figure 4.1 supports the results obtained in the Live/Dead cell assay conducted by Stepper *et al.* (2011)[82]. In both cases *L. plantarum* ATCC 8014 responded rapidly to purified GccF. In the above experiment, intact GccF caused cfu/mL values to stabilise; they did not increase (whereas the untreated cultures did), but nor did they decrease, whereas *L. plantarum* KW30 culture supernatants caused a 100-fold decrease in cfu/mL values of ATCC 8014 log-phase cultures in 30 minutes, and a 1000-fold decrease after 4 hours [101].

A similar viable cell count experiment was done with *E. faecalis* V583 cultured in TSB medium. In one culture, cells were pre-treated with GlcNAc to maximise the GccF-mediated inhibition of *E. faecalis* growth. The aim was to determine whether cells sampled after the point where the culture OD_{600nm} values level off, in a seemingly premature stationary phase, remained viable, as was shown for *L. plantarum* in **Figure 1.4**.

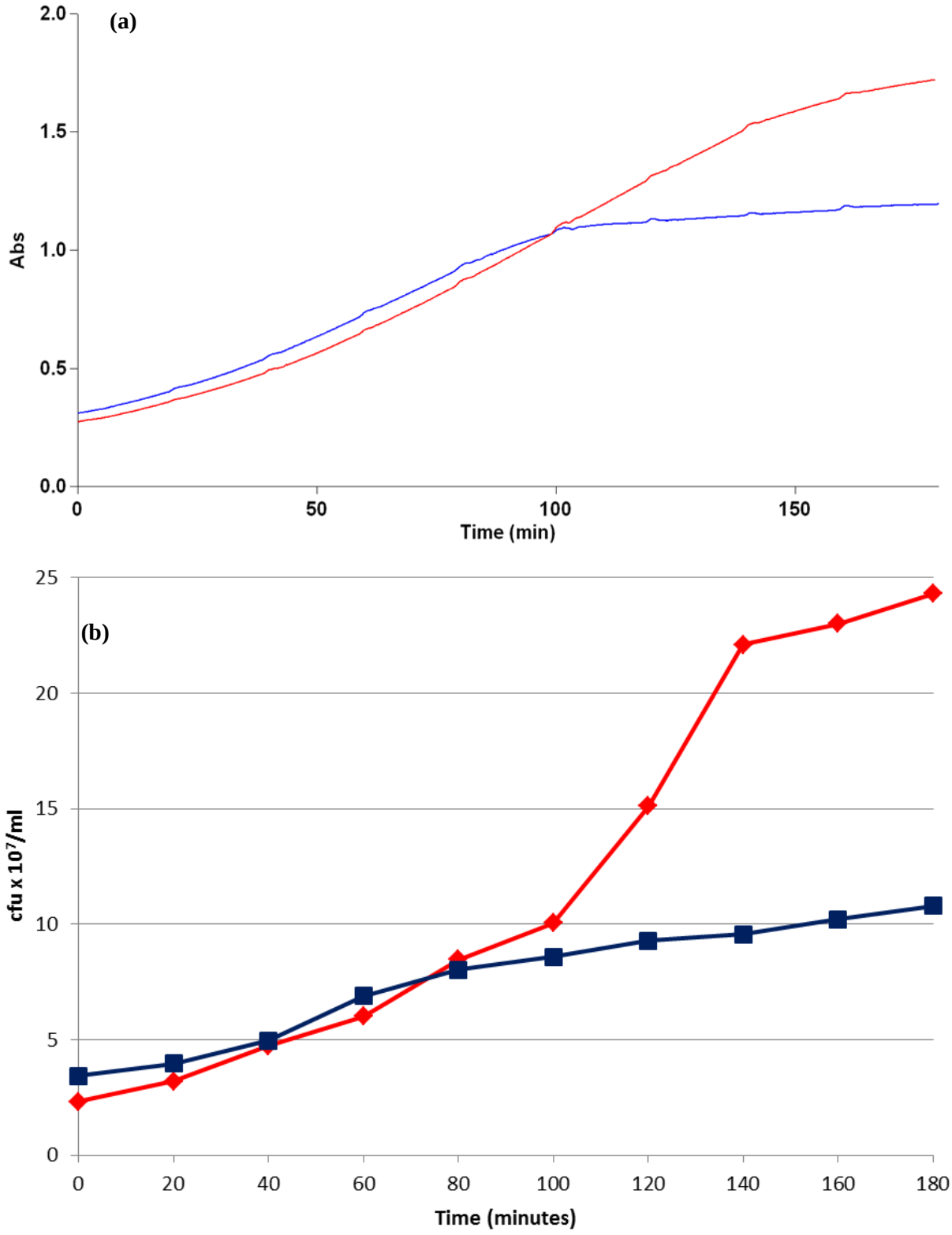


Figure 4.2 – Growth curves of *E. faecalis* V583 with and without 1 mM GlcNAc added at T(-60) and 1 µM GccF added at T(0). (a) OD_{600} values **(b)** cfu/mL. **Positive Control** (no addition), **GlcNAc and GccF treatments**.

For *E. faecalis* V583, both the positive control and cells treated with GlcNAc (T(-60)) and GccF (T(0)), continued to multiply. Although the GccF treatment did decrease in growth rate after 100 minutes, as shown by both $O.D._{600nm}$ and cfu/mL values, there was no indication of cell death.

Figures 4.2a and **b** together show a fair correlation between $O.D._{600nm}$ and the number of cells that can form colonies (cfu/mL). $O.D._{600nm}$ is roughly proportional to the number of cells per mL of culture, regardless of whether the cells are viable or dead, while cfu values measure only live cells. Thus a correlation between the two values would indicate that GccF-induced inhibition of *E. faecalis* growth is due to bacteriostasis, and that few, if any, cells are actually killed by GccF treatment. It is notable, however, that for untreated cells, the ratio of cfu/mL : OD_{600} values roughly doubles over the 180 minutes of culture growth (from 7.5 to 14.5), while the ratio for the treated cells is relatively uniform (10 ± 1 across all time points), so the correlation may depend on additional factors such as the growth phase of the culture, and will also be confounded by errors in the individual measurements.

Overall, the results obtained for experiments with *L. plantarum* and *E. faecalis* suggest that GccF is indeed bacteriostatic. It may be possible that the supernatant of *L. plantarum* KW30 cultures contain some substance(s) that work synergistically with GccF to induce cell killing and the subsequent 1-3 log drops in cfu/mL seen by Kelly *et al.*[101], however purified GccF is not significantly bactericidal in the short term, *i.e.* up to 240 minutes exposure (**Figure 4.1**).

4.3 Monitoring Bacteriocin-Induced Efflux of Cellular ATP via Bioluminescence Assay

Refer to methods: 2.3.8

Several bacteriocins, including nisin and some characterised type IIa bacteriocins, form pores in bacterial cell membranes, and in many cases, these pores result in the leakage of ATP and other intracellular metabolites or ions. Indeed, the loss of cell membrane integrity is considered to be a common mechanism of action for many bactericidal bacteriocins[104]. To determine whether GccF treatment compromises the integrity of target cell membranes, ATP efflux from treated cells was measured using a sensitive bioluminescence assay for ATP. Nisin, a pore-forming bacteriocin known to cause ATP efflux[105; 106] was used as a positive control.

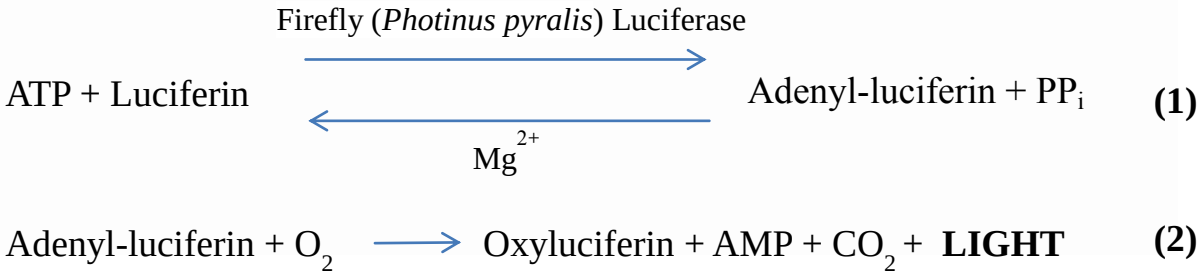


Figure 4.3 – **Outline of reactions taking place in the ATP bioluminescence assay** - "Reaction (1) is reversible and the equilibrium lies far to the right. Reaction (2) is essentially irreversible. When ATP is the limiting reactant, the light emitted is proportional to the ATP present" – Excerpt taken from Sigma® ATP Bioluminescent Assay Kit outlining the principle on how the kit detects ATP and yields a result.

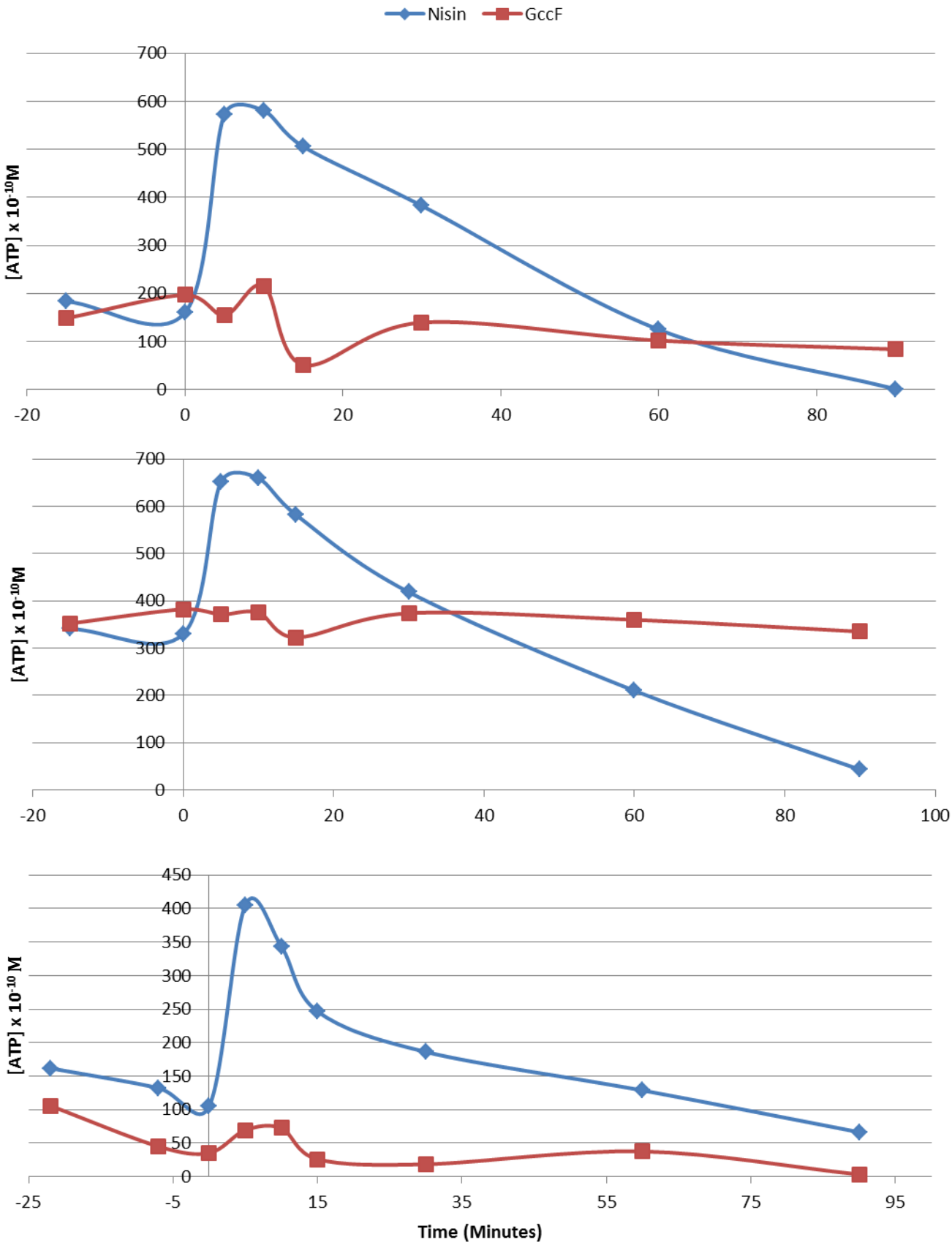


Figure 4.4 – Change in ATP levels in cell-free supernatants over time. Bacteriocins were added to *L. plantarum* ATCC 8014 cultures at T(0); final concentrations for nisin were all 1 µM (estimated) and for GccF 1.56 µM. Fluorescence was converted to ATP concentration using a standard (see appendices). The experiment for the top graph was conducted on 24/07/12, the middle on the 27/07/12, and the bottom on 16/08/12.

ATP assay results show that GccF treatment did not cause any substantial efflux of ATP upon its addition to *L. plantarum* ATCC 8014 cultures. It is interesting however, that in GccF-treated cultures there is a slight rise followed by a drop in ATP levels, in all cases, 15 minutes after addition of GccF. This might be due to a modification in cellular messaging, since ATP plays more roles than just as a cell's energy currency. This result is evidence that GccF does not trigger the formation of ATP-permeable pores in the cell membrane, unlike the positive control bacteriocin, nisin. This result is consistent with a bacteriostatic mode of action, and the hypothesis that the ultimate target of GccF may be intracellular regulatory mechanisms. Therefore, GccF-induced bacteriostasis of *L. plantarum* ATCC 8014 is not due to ATP efflux, and this is consistent with an inhibitory mechanism that does not compromise membrane integrity.

The positive control, treatment with nisin, shows efflux of cellular ATP, consistent with nisin's well documented bactericidal effect due to membrane pore formation[107; 108]. The rapid decrease in ATP post-efflux may be due to the hydrolysis of ATP, catalysed by enzymes present in the incubation mixture. Apparently this phenomenon has not previously been reported in assays of ATP released from nisin treated cells. Many articles focus on the immediate ATP efflux at a single time point, for example, intracellular/extracellular fluctuations of ATP due to nisin in *Listeria monocytogenes* by Winkowski *et al.*[109] rather than a time course of ATP measurements after addition of the bacteriocin. In one study where [ATP] was recorded for periods up to 15 minutes after nisin addition to *L. monocytogenes* cells, no ATP depletion was detected[110]. It is possible that the phenomenon seen in **Figure 4.4** is restricted to *L. plantarum*, or that *L. monocytogenes* does not release ATP-metabolising proteins.

It should be noted that the differences in [ATP] values between each graph may be due to a decrease in the activity of the luciferase in the assay kit over time. The manufacturer states "This solution is stable for at least two weeks when stored at 0-5^{o}C and protected from light. A slight decrease in light production and sensitivity may occur during this time." Because one of the 'reagents' in the ATP assays is bacterial cells, it is also possible that differences in the growth media and other growth conditions may have contributed to the poor reproducibility of this assay, so all three results have been included for information. It is also possible that other components in the MRS growth medium, *e.g.* Mn^{2+}, may be responsible

for catalysing the hydrolysis of released ATP. Most assays for bacteriocin-induced release of ATP are done with washed bacterial cells resuspended in a relatively simple buffer.

4.4 Compromising Outer Membrane Integrity Sensitises Gram -ve Bacteria to GccF

Bacteriocins from Gram-positive (G +ve) bacteria invariably inhibit G +ve bacteria more effectively than Gram-negative (G –ve) bacteria, and this difference in sensitivity is usually attributed to the protective effect of the outer membrane of G -ve bacteria. This acts as a physical barrier, preventing bacteriocins from binding to their molecular receptors/targets[111; 112]. At 5200 Da, GccF is too large to pass through the outer membrane. The finding that GccF is slightly inhibitory towards the G -ve bacterium, *Yersinia frederiksenii* (see **Table 3.1**) was therefore an unexpected result. Inhibition of *Y. frederiksenii* growth inhibition by GccF was enhanced in the presence of polymyxin, an antibiotic produced by *Bacillus polymyxa*, which acts in part by damaging the outer membrane[113].

Secretins are a family of homologous selective outer membrane channels that facilitate the export of specific substrates and sometimes aid in the assembly of products/complexes, including virulence factors. Secretins are dynamic, in that they must undergo extensive structural changes in order to permit export, without compromising the barrier function of the outer membrane.

pIV is a unique secretin which spans both the outer and inner membranes. It is involved in the assembly and export of filamentous bacteriophage f1. The pIV secretin was found to constrict to an internal diameter of 6.0 nm, in addition to having an internal "plug," yet is able to export phage particles 6.5 nm in diameter, indicating that pIV undergoes dramatic conformational changes in order to permit substrate passage[114]. It was found in 2010 that amino acid substitutions within certain "gating" regions of the secretin pIV, makes the channel 'leaky' without disrupting its overall structural integrity. The substitutions allowed an influx of relatively large molecules, *e.g.* maltopentose (829 Da), vancomycin (1486 Da) and deoxycholate (393 Da), that would otherwise be excluded by the outer membrane[115] and therefore be unable to support (maltopentose) or inhibit (vancomycin, deoxycholate) growth.

PulD is the secretin responsible for the secretion of pullulanase (PulA), a starch de-branching lipoprotein, in *Klebsiella oxytoca*. PulD undergoes chaperone (PulS) dependent localisation[116; 117] to the outer membrane (OM), spans only the OM and, like pIV, has been found to have similar regions within its sequence that once modified, compromise gating function. The studies on pIV[115] and PulD[118] involved introducing the relevant mutated

secretin genes into *E. coli*, expressing them, and scoring appropriate phenotypes, *e.g.* sensitivity to vancomycin.

The hypothesis that secretins with compromised gating functions might allow GccF direct access to either the periplasm/inner membrane in the case of *pulD* mutants, or cytoplasm in the case of *pIV* mutants (**Figure 4.5**), was tested by comparing the GccF-induced growth inhibition seen in wild-type *E. coli* strains, to strains with mutated secretin genes expressing 'leaky' secretins.

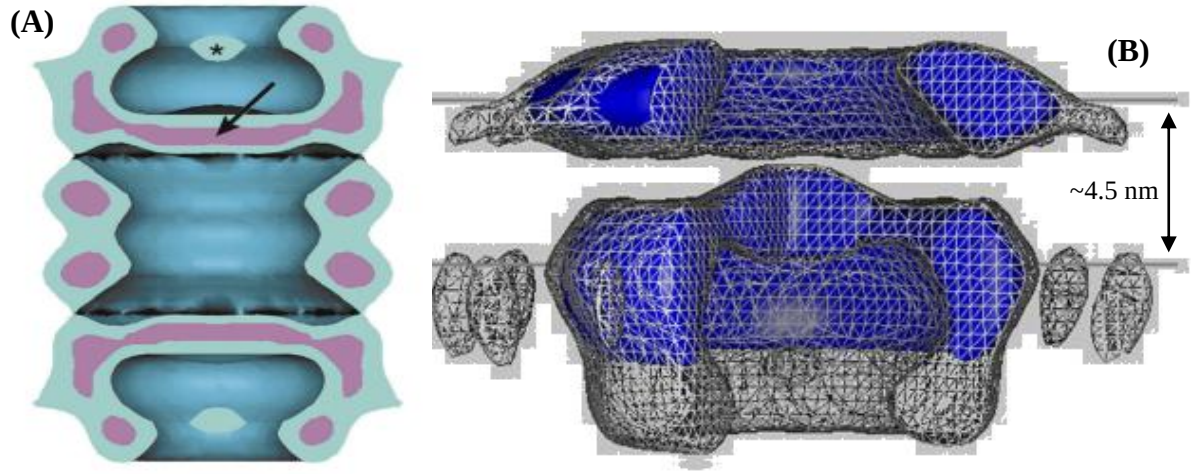


Figure 4.5 – A computer generated reconstruction of secretin multimers. (A) 3D cross-sectional density map of pIV showing the plug (*) and inner constriction (arrow). (B) Constructed by Chami *et al.*,[4] this 3D projection shows PulD spanning the outer membrane (~4.5 nm wide), with a minimum internal diameter of 5.4 nm at the entrance to the periplasm.

4.4.1 Real-Time Growth in Liquid Media of *Escherichia coli* Secretin Mutants

Refer to Methods: 2.3.7

Figure 4.6 shows a simple schematic diagram of the hypothesised mechanism of improved GccF access to putative targets in the cytosol or inner membrane via 'leaky' pores. Using secretin mutant strains, there is no need to treat the cell with compounds that destabilise the OM, for example polymyxin or EDTA, which has the advantage of avoiding any potential for additive or synergistic inhibition of growth from exposure to multiple inhibitory substances.

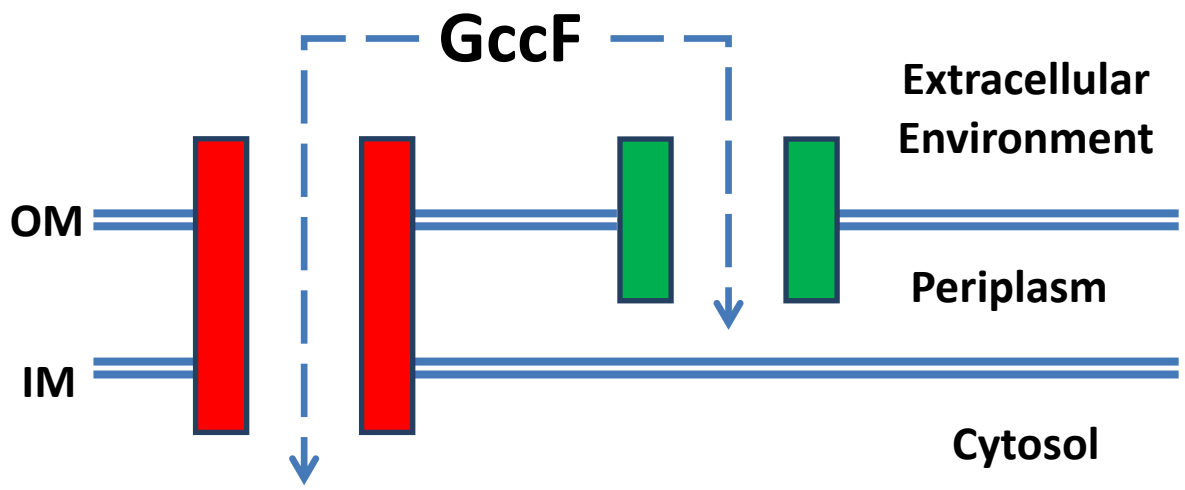


Figure 4.6 – Schematic diagram of GccF's hypothetical entry through 'leaky' pIV or PulD. If GccF can pass through 'leaky' pIV channels, then it can interact directly with cytosolic molecules, while access through leaky PulD would allow GccF to interact with periplasmic and inner membrane proteins.

Figure 4.7 shows the results of initial experiments testing the effect of GccF, with and without GlcNAc, on the growth of *ΔpulD+pulS, ΔpIV*, and the parental K1508 *E. coli* strains. Note that *E. coli* was a faster growing organism than others used in this study, so typically growth was monitored for a shorter period of time. As with prior experiments, GlcNAc (1 mM final concentration) was added to selected cuvettes at T(-60). At T(0), GccF only and GlcNAc+GccF treatments received GccF to a final concentration of 1 μM.

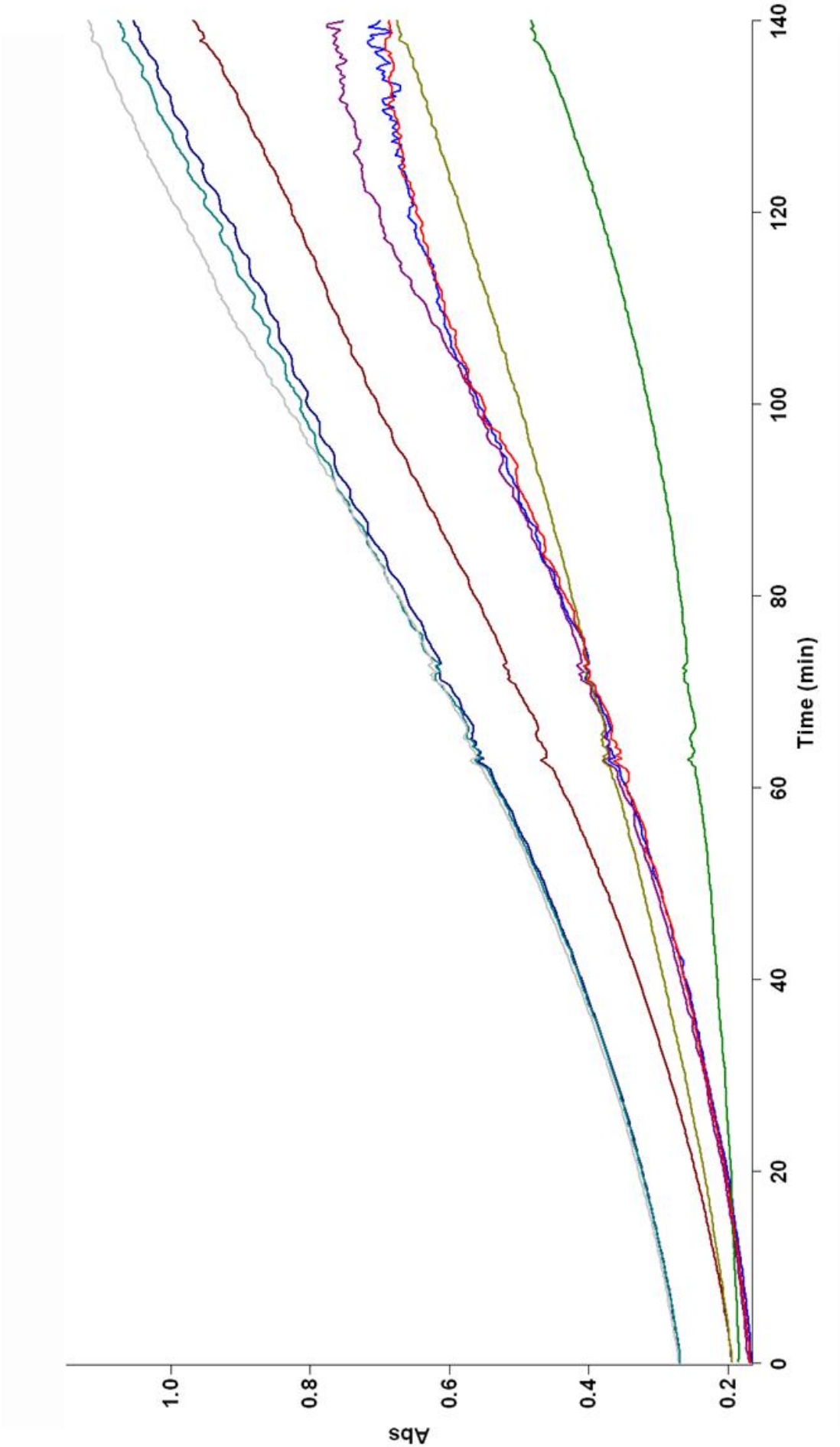


Figure 4.7 – Growth curves of *E. coli* parental strain and secretin mutants. Parent Positive, Parent GccF, Parent GlcNAc+GccF, *ΔpulD+pulS* Positive, *ΔpulD+pulS* GccF, *ΔpulD+pulS* GlcNAc+GccF, *ΔpIV* Positive, *ΔpIV* GccF, *ΔpIV* GlcNAc+GccF

Firstly, it should be noted that the parental *E. coli* K1508 strain grew more slowly than the mutant secretin strains, possibly because the 'leaky' secretin mutants allow a more efficient uptake of metabolites from the rich LB growth medium. In addition, GccF and GlcNAc+GccF treated parent (K1508) samples did not deviate significantly from the strain's positive control. This indicates that GccF has little to no effect on wild type *E. coli* cells, with or without 60 minutes prior sensitisation to 1 mM GlcNAc.

Secondly, the growth of *ΔpulD+pulS* is significantly inhibited by GccF addition, compared to its positive control (no GccF). This inhibition is also apparent when 1 mM GlcNAc is added before GccF, but to a lesser extent, suggesting a protective role for GlcNAc.

Lastly, the growth of *ΔpIV* mutants was not significantly inhibited by GccF. The *ΔpIV* cultures grew the fastest in this experiment, suggesting that, if GccF has been permitted access to the cytosol through the 'leaky' pIV secretin, it has not targeted cytosolic proteins, at least as its primary target. If 'leaky' pIV secretins allow GccF to access the *E. coli* cytosol, then there is no indication that cytosolic GccF is inhibitory to growth, but this is hypothetical because there is no test to determine whether GccF enters the cytosol of these cells

The results suggest that it is unlikely that GccF exerts its effect by interacting directly with cytosolic proteins, since ΔpIV could hypothetically allow free passage of GccF into the cell. In addition to this, the set of ΔPulD(+PulS)-carrying cells were affected considerably by GccF. The introduction of this "leaky" secretin hypothetically permits the passage of GccF through the outer membrane by effectively increasing its permeability without the need for treatment with extra compounds that would also increase the permeability of the OM.

The fact that in this instance GccF inhibited cell growth, suggests that the initial target/ receptor for GccF is a molecule, or a region of a molecule, that is exposed to the periplasm. The sole GlcNAc-specific PTS in *E. coli*, NagE, is an inner membrane protein (as are all active sugar transporters), which binds periplasmic GlcNAc and actively translocates it into the cytosol. Thus the increased GccF-mediated inhibition seen for ΔPulD(+PulS)-carrying cells is consistent with the hypothesis that GlcNAc PTS transporters are receptors for GccF.

Figure 4.8 shows that sugars, and possibly molecules that contain sugar moieties, such as GccF, can be bound by periplasmic-facing sugar-specific binding sites of PTSs (GlcNAc-specfic NagE in the case of *E. coli* and GccF), and that this binding can influence the phosphorylation status of intracellular domains and proteins in the phosphorelay system. The ratio of phosphorylated and non-phosphorylated forms or various intracellular domains and proteins can in turn act as signals to control a variety of processes, including gene expression and/or intracellular metabolism.

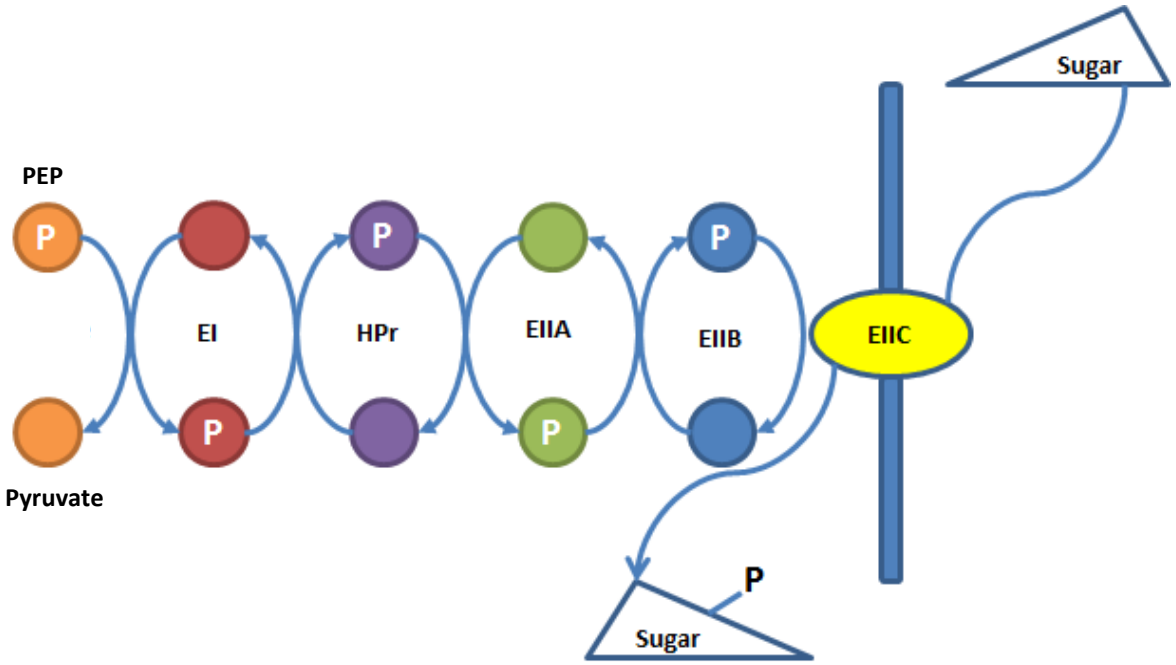


Figure 4.8 – A generalised schematic of a sugar phosphotransferase system. The EIIC protein (or transporter domain) is responsible for coordinating the import and phosphorylation (by EIIB) of extracellular sugars, trapping them inside the cell. EIIB is then re-phosphorylated by a phosphorelay system, with phospho-enol-pyruvate (PEP, the orange circle with a 'P' in the middle) as the phosphate donor. In the case of N-acetylglucosamine PTSs, EIIC, EIIB and EIIA are fused into one multidomain protein, with the EIIC embedded in the inner membrane.

4.4.2 Testing Experimental Controls for a 'Leaky' PulD Secretin Mutant

Refer to Methods: 2.3.5 and 2.3.7

The presence of two plasmids (pCHAP362 Δ477-481, and pAH181, refer to **Table 6.2**) in the *E. coli* K1508 expressing a leaky PulD secretin meant that additional controls were needed. It is important to note that wild type *E. coli* lack a *pulD* gene. Specific questions included:

- Does expression of the chaperone PulS alone increase GccF-induced inhibition?
- If the 'leaky' PulD was mislocalised due to absence of its chaperone, would the strain still exhibit increased sensitivity to GccF?
- Does expression of wild-type PulD also increase sensitivity to GccF?

Figure 4.9 shows the growth curves for these additional controls (±1 µM GccF). The original result of GccF-induced growth inhibition for the 'leaky' *pulD* mutant, compared to wild-type *pulD* which was not inhibited, was confirmed.

The presence of PulS alone did not increase GccF susceptibility, which was an expected result, since PulS is a chaperone protein and is not present in the other susceptible organisms. Finally, the presence of 'leaky' PulD by itself had no impact on GccF-induced bacteriostasis. This was also an expected result, since without PulS, functional PulD secretin channels localise to the inner, rather than outer membrane[119].

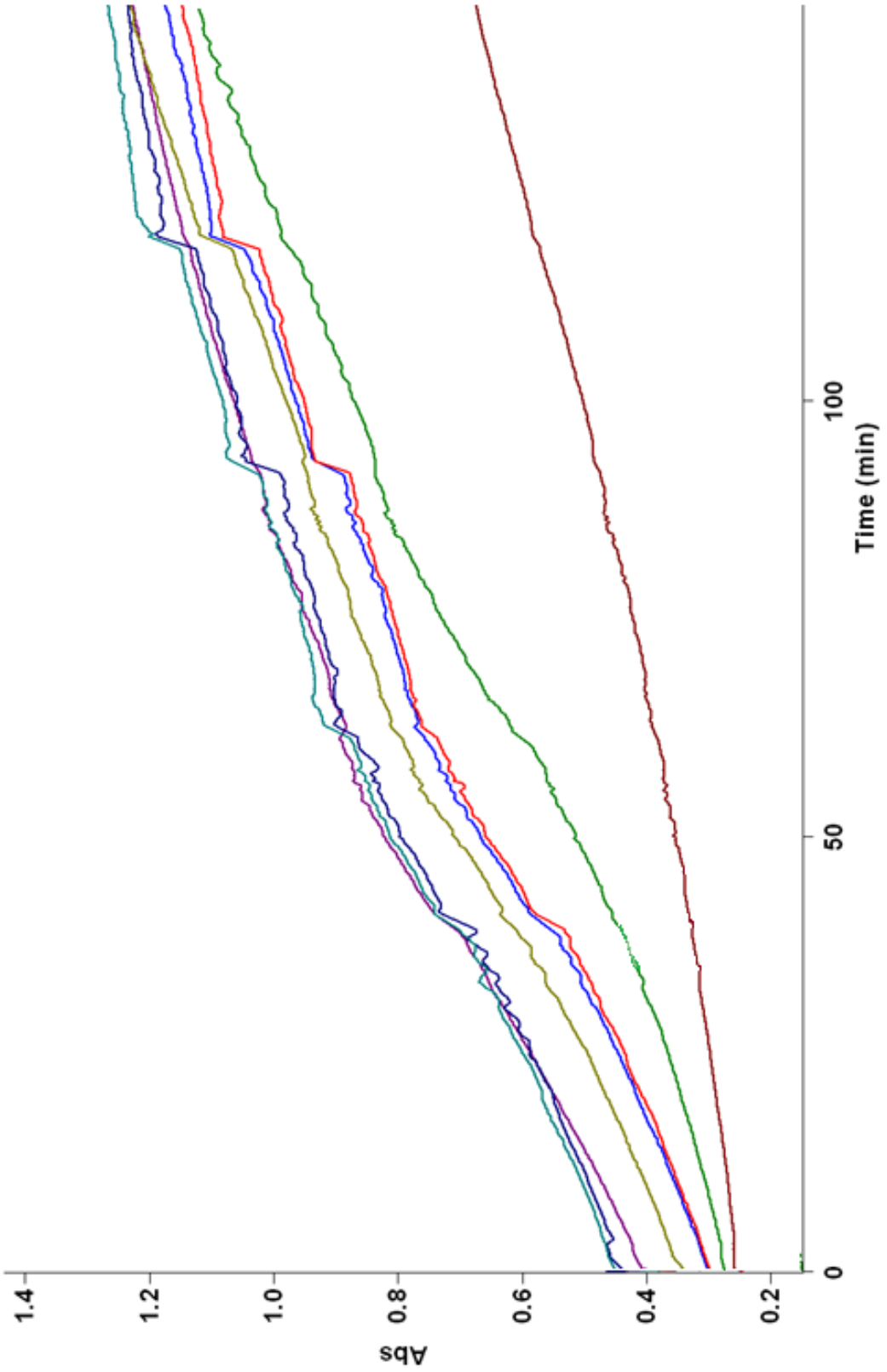


Figure 4.9 – Growth curves of *E. coli* with PulD-related plasmids. *ΔpulD* Positive, *ΔpulD* GccF, *ΔpulD+pulS* Positive, *ΔpulD+pulS* GccF, *WTpulD+pulS* Positive, *WTpulD+pulS* GccF, *pulS* Positive, *pulS* GccF.

4.4.3 GlcNAc-Mediated Revival in *ΔpulD+pulS E. coli* from GccF-Induced Bacteriostasis

Refer to Methods: 2.3.5 and 2.3.7

The addition of free GlcNAc to cultures of *Lactobacillus plantarum* and *Enterococcus faecalis* in GccF-induced complete or partial bacteriostasis (respectively), restored near-normal growth in these cultures. If free GlcNAc can also reverse the GccF-induced partial bacteriostasis of *E. coli* cultures, then this would suggest that a common mechanism of GccF-induced growth inhibition is operating in both Gram positive, and Gram negative species.

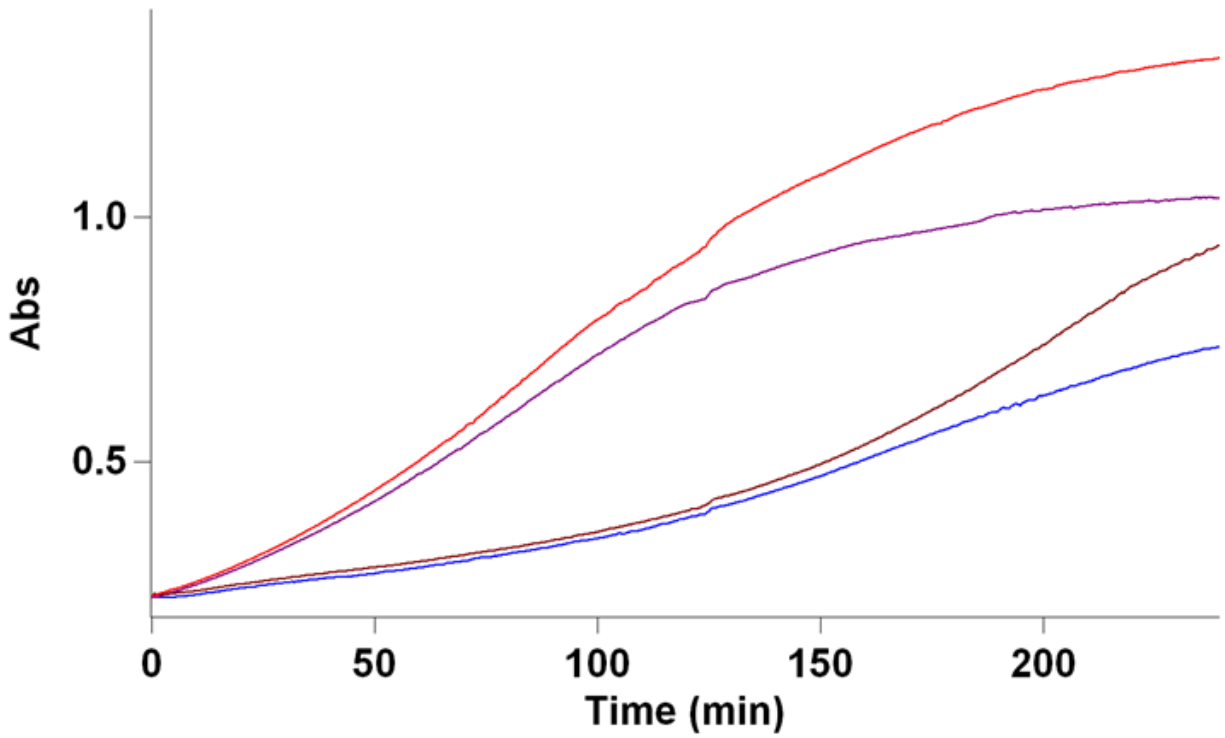


Figure 4.10 – Restoring growth of GccF-inhibited *ΔpulD+pulS* GccF with GlcNAc. **Positive Control, GlcNAc T(-60) + GccF T(0), GccF T(0) + GlcNAc T(120), GccF T(0).** Final concentration for GccF was 1 µM and GlcNAc was 1 mM. At T(120) all cultures were mixed by inverting the cuvettes.

Figure 4.10 shows similar responses to those seen in **Figure 3.4**, *i.e.* addition of GlcNAc to cultures in the GccF-induced partial bacteriostasis acts to stimulate growth (compare the blue (no GlcNAc) and brown (GlcNAc added at T(120)) lines in **Figure 4.10**). This reversal of bacteriostasis in *E. coli* is not as dramatic as seen in *L. plantarum*, perhaps because GccF

induced only partial bacteriostasis in *E. coli* under these culture conditions. Towards the end of the assay (220-240 minutes), it appears that the "GccF T(0) + GlcNAc T(120)" curve begins to level off again. It may be that all exogenous GlcNAc has been taken up and metabolised by the cells, and hence the protective effect of GlcNAc is lost and GccF-induced bacteriostasis is being re-established. This hypothesis could be tested by repeating the experiment and varying the concentration of GlcNAc added at T(120), and/or by measuring the concentration of GlcNAc in the growth medium, although the latter is not trivial for a rich growth medium such as the LB medium used here for *E. coli* cultures.

Another point of interest is that, as for *E. faecalis* V583 and *B. megaterium*, growth of the GccF T(0) culture appeared to recover towards the end of the assay. While careful measurements of specific growth rates would be needed to compare these responses across species, it is possible that these organisms employ similar mechanisms to overcome partial bacteriostasis *e.g.* the adjustment of metabolic regulation, or perhaps proteolytic inactivation of GccF. Lastly, while the addition of 1 mM GlcNAc at T(-60) initially strongly protects against GccF added at T(0), this protection also only lasts about 120 minutes, after which the red (control) and purple growth curves begin to diverge. One could speculate that the protective effect of GlcNAc has been lost due to uptake and metabolism, although the only way to be sure of this would be to measure free GlcNAc in the culture medium. While analytical methods exist to measure GlcNAc (*e.g.* high performance liquid chromatography, or colorimetric[120]), it is not trivial to apply these methods to a complex matrix such as rich growth media, and these measurements were deemed beyond the scope of this project.

4.5 Using Proteomics Methods to Investigate Proteins Involved in GccF Susceptibility

Refer to Methods: 2.3.9 – 2.3.12

The discovery that pre-incubation of *E. faecalis* with free GlcNAc sensitises some strains to GccF-induced growth inhibition led to a hypothesis that exogenous GlcNAc changes the abundance of the GccF receptor and/or the regulation of intracellular processes in a way that enhances GccF activity. Intracellular regulation to change, for example, enzyme activity, might be due to change either in the abundance or post-translational modifications (*e.g.* phosphorylation) of intracellular proteins.

The aim of this part of the research was to use proteomics methods to investigate the effect of supplementing TSB growth medium with 5 mM GlcNAc on the abundance of soluble and insoluble proteins extracted from *E. faecalis* V583. The 5 mM GlcNAc concentration was higher than the 1 mM used in culture growth assays to ensure that GlcNAc would be present in the culture throughout the 120 minute period the cells had to adapt to the presence of GlcNAc. If the free GlcNAc were to be depleted before the T(120) sample, then proteolysis might reverse any changes in protein abundance.

The proteins in the soluble and insoluble fractions extracted from cells were separated by one-dimensional SDS-PAGE, the selected bands were subjected to in-gel digest with trypsin. An apparent increase in bands intensity during the 120 minute incubation was the main criterion for band selection. The tryptic peptides were eluted from gel slices and analysed using mass spectrometry at The Riddet Institute on Massey University's Manawatu Campus in Palmerston North. **Figure 4.11** shows the gel with locations of the eight bands of interest indicated, before their excision, subsequent digestion with trypsin, and mass spectrometry analysis.

Table 4.2 shows the top two relevant proteins from each band in terms of organism (only *E. faecalis* proteins were considered), size and protein score. A high protein score indicates a low probability that the protein observed was due to a random event, *i.e.* the higher the protein score, the more significant the result.

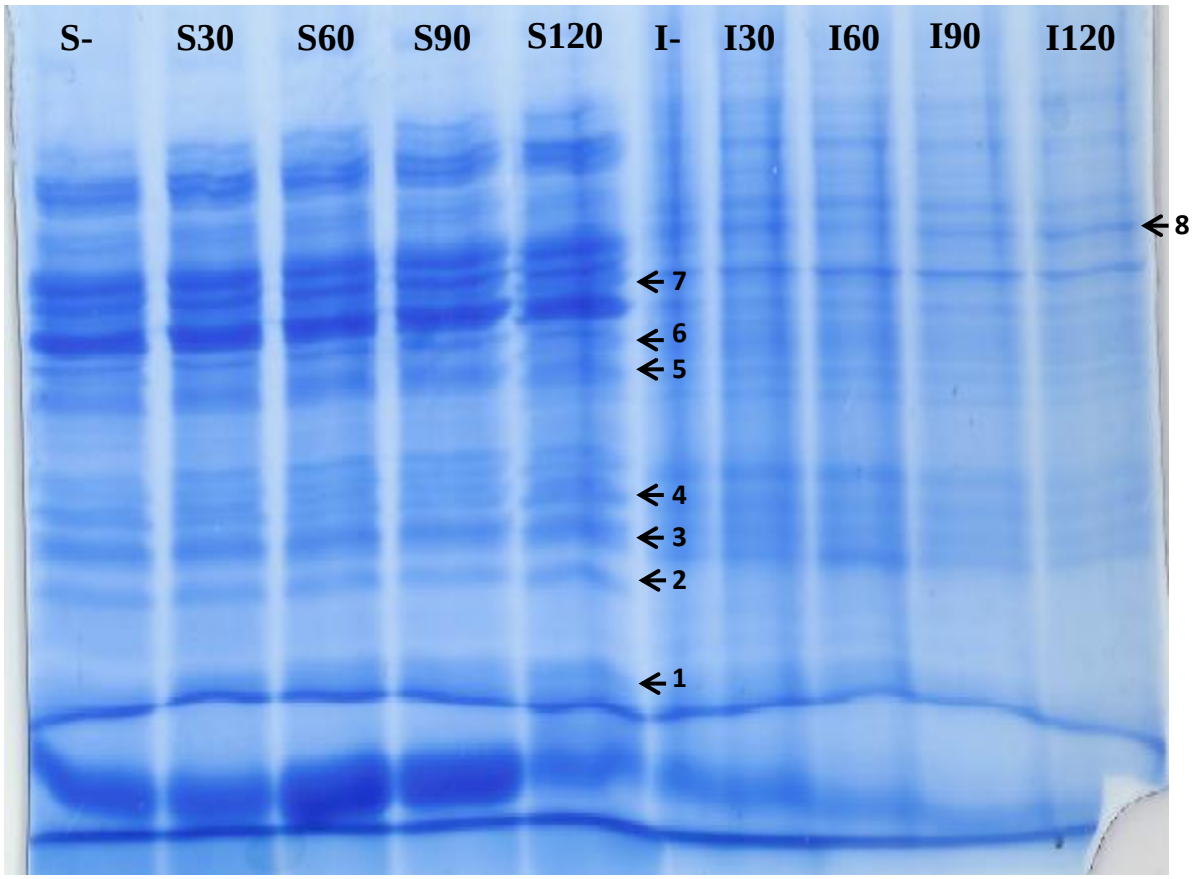


Figure 4.11 – Coomassie-stained SDS-PAGE of proteins extracted from lysed *E. faecalis* V583 cells grown in the presence of GlcNAc. S = Soluble fraction, I = Insoluble fraction. The suffixes indicate the time (in minutes) after GlcNAc addition to cultures, and the '-' samples were those taken immediately before GlcNAc addition. Numbered arrows show the excised bands. Sample volume loaded per lane: 20 µL. N.B. Molecular Marker absent.

Band Number	Protein Name	Protein Size (Da)	ID Number	Score
1	Acyl carrier protein	8610	525278	166
	Peptidase propeptide and YPEB domain protein	19488	749518	156
2	Superoxide dismutase	22683	226185	433
	Xanthine phosphoribosyltransferase	20912	749497	103
3	Triosephosphate isomerase	27518	565646	335
	General stress protein Gls20	20540	491075	220
4	Triosephosphate isomerase	27112	699185	46
	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	26047	525271	40
5	Putative uncharacterized protein	26579	749518	82
	Carbamate kinase	36034	749511	73
6	Probable manganese-dependent inorganic pyrophosphatase	33604	749494	137
	Putative PTS system mannose-specific EIIAB component	41571	749506	128
7	Phosphoglycerate kinase	42428	657310	327
	Glyceraldehyde-3-phosphate dehydrogenase	35920	525278	303
8	Elongation factor	43418	657310	386

Table 4.1 – Top two protein hits for each band from Figure 4.11. All proteins appear to show an increase in relative intensity upon GlcNAc addition and hence may have a role in increasing cell susceptibility to GccF. The "Score" number is related to the 'Expect values' in Blast sequence similarity search, and is a measure of the significance of the protein identification. Higher scores indicate a better identification.

Band 1's best 'hit' was acyl carrier protein which is involved in fatty acid and polyketide synthesis, while the second hit, a peptidase, is involved in protein turnover and degradation. Band 2's hits include superoxide dismutase, which eliminates free radicals resulting from oxidative phosphorylation, and xanthine phosphoribosyltransferase, an enzyme involved in purine metabolism. Band 3's and also 4's top hit, triosephosphate isomerase, is essential to glycolysis and hence energy production, while the general stress protein, Gls20, is a response to different environmental pressures. Band 4's 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase is an enzyme that catalyses the conversion of 2,3-bisphosphoglycerate to 3-phosphoglycerate in glycolysis. Band 5's top hit is of unknown function while the second, carbamate kinase, catalyses the synthesis of carbamoyl phosphate, an anion used for nitrogen disposal and pyrimidine synthesis. Band 6's top hit is responsible for splitting linked phosphate groups via hydrolysis. The second, however, is a PTS component which allows the "specific" cellular import of mannose; however these

transporters are often promiscuous and may facilitate the influx of other monosaccharides, *e.g.* glucose. Band 7's top protein of interest catalyses the ATP producing step in glycolysis, and the second is also involved in glycolysis. Finally, the only protein from Band 8 which had an *E. faecalis* origin was elongation factor, a protein that facilitates translational elongation.

From the results of the mass spectrometry, no proteins were found which would conclusively explain the increased susceptibility of GlcNAc-treated *E. faecalis* cells to GccF. Five of the fifteen proteins were involved with glycolysis, which may have been up-regulated due to the ability for GlcNAc to be channelled into the energy yielding glycolytic pathway. A possible candidate for a GccF susceptibility protein is the mannose PTS EIIAB component. This is because some PTSs can translocate more than one substrate, and therefore may be related to GccF susceptibility should the hypothesis that an increase in the abundance of GlcNAc PTSs would provide more targets for GccF prove correct.

From the point of view confirming this hypothesis, the ideal protein(s) to identify would have been directly related to N-acetylglucosamine metabolism, including either NagE itself, or perhaps proteins encoded by the Nag and/or Glm pathways of *E. faecalis* (see **Figure 1.4**).

A second round of mass spectrometry was undertaken to investigate bands that appeared to decrease in intensity upon GlcNAc supplementation, using gels of varying percentages in order to increase the resolving power of the separation. Unfortunately, the results did not yield any substantial data, and the experiment could not be repeated within the time constraints of the project. This experiment should be repeated in the future in order to identify which specific proteins increase or decrease in response to either GlcNAc and/or GccF treatment.

4.6 Designing a qPCR Experiment for *nagE* of *E. faecalis* V583

The discovery that pre-incubation of *E. faecalis* with free GlcNAc sensitises some strains to GccF-induced inhibition led to a hypothesis *nagE*, encoding the predicted GccF receptor, the GlcNAc-specific NagE, is up-regulated in response to the addition of GlcNAc to the growth medium. The sugar specific induction of genes involved in sugar uptake and subsequent metabolism is one of the most well characterised examples of prokaryotic gene regulation, *e.g.* the lac operon in *E. coli*[121]. An increase in *nagE* transcription, and subsequent increase in NagE abundance, would present more receptor molecules for GccF to bind to and therefore strengthen its effect. This hypothesis is described visually in **Figure 4.12**.

This section of research aimed to prepare and test materials needed to measure *nagE* mRNA via quantitative polymerase chain reaction (qPCR) before and after GlcNAc addition, to confirm that there is an increase.

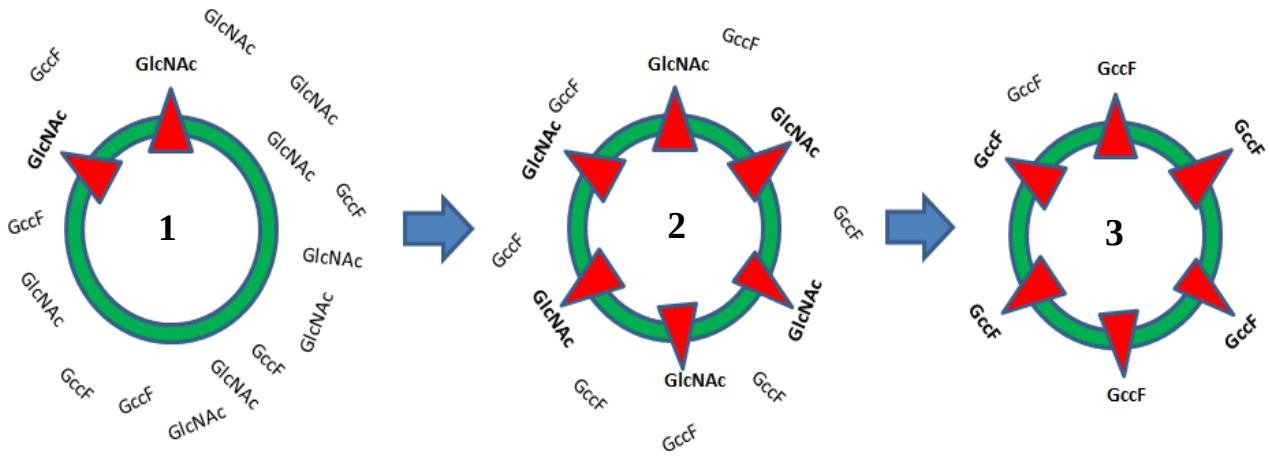


Figure 4.12 – Schematic diagram of the hypothetical mechanism of GlcNAc's influence on susceptibility to GccF **Green** represents the *E. faecalis* cell membrane, **Red** is NagE/GlcNAc-PTS, **Bold** substrates are bound to NagE, and the rest are free. **(1)** Low abundance NagE; GlcNAc and GccF have just been added to the culture. **(2)** Increased GlcNAc has led to increased NagE, efficiently taking up GlcNAc, N.B. Relative affinities of free GlcNAc and GccF for NagE are unknown, but while free GlcNAc is in large excess to GccF, the inhibitory action of GccF is minimal. This may be because free GlcNAc competes with GccF for binding to NagE, or it may be that free GlcNAc protects the cell against the downstream effects of a GccF-NagE complex. **(3)** Upon GlcNAc depletion, GccF is free to bind to the (more abundant) NagE and exert its bacteriostatic effect more strongly compared to cells not exposed to GlcNAc.

4.6.1 Verification of PCR Primer Specificity

Refer to Methods: 2.3.13 - 2.3.18

To check that the oligonucleotide primers (listed in the Materials and Methods section, **Table 2.2**) specifically amplify an internal segment of *E. faecalis* target gene, *nagE,* and the control genes *gap2* and rpoA, PCR was done using these primers and *E. faecalis* V583 genomic DNA as template. *E. faecalis* V583 is a mild human pathogen, and its genome was sequenced in 2003[122]. Using Integrated DNA Technology's data and manually checking the primer sequence vs. the gene sequences, the predicted lengths of PCR products would be the following:

- *nagE*: 200 bp
- *gap2*: 172 bp
- *rpoA*: 172 bp

PCR products were analysed on an agarose gel, stained and visualised.

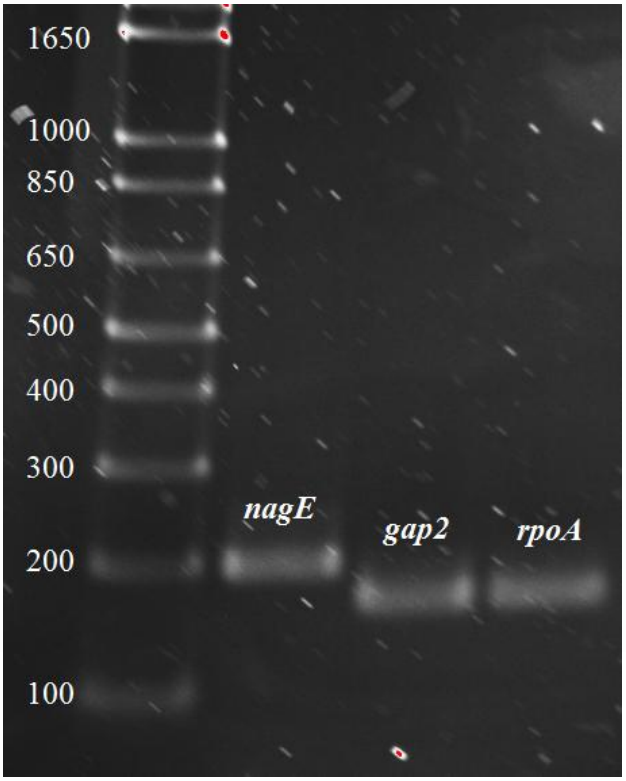


Figure 4.13 - 1% Agarose Gel Electrophoresis of PCR Products. The gel was stained with ethidium bromide. The 1kb+ ladder bands are labelled in base pairs (bp). The PCR product lanes each contain a single band of the predicted size. Each lane was loaded with 15 μL of dye/product.

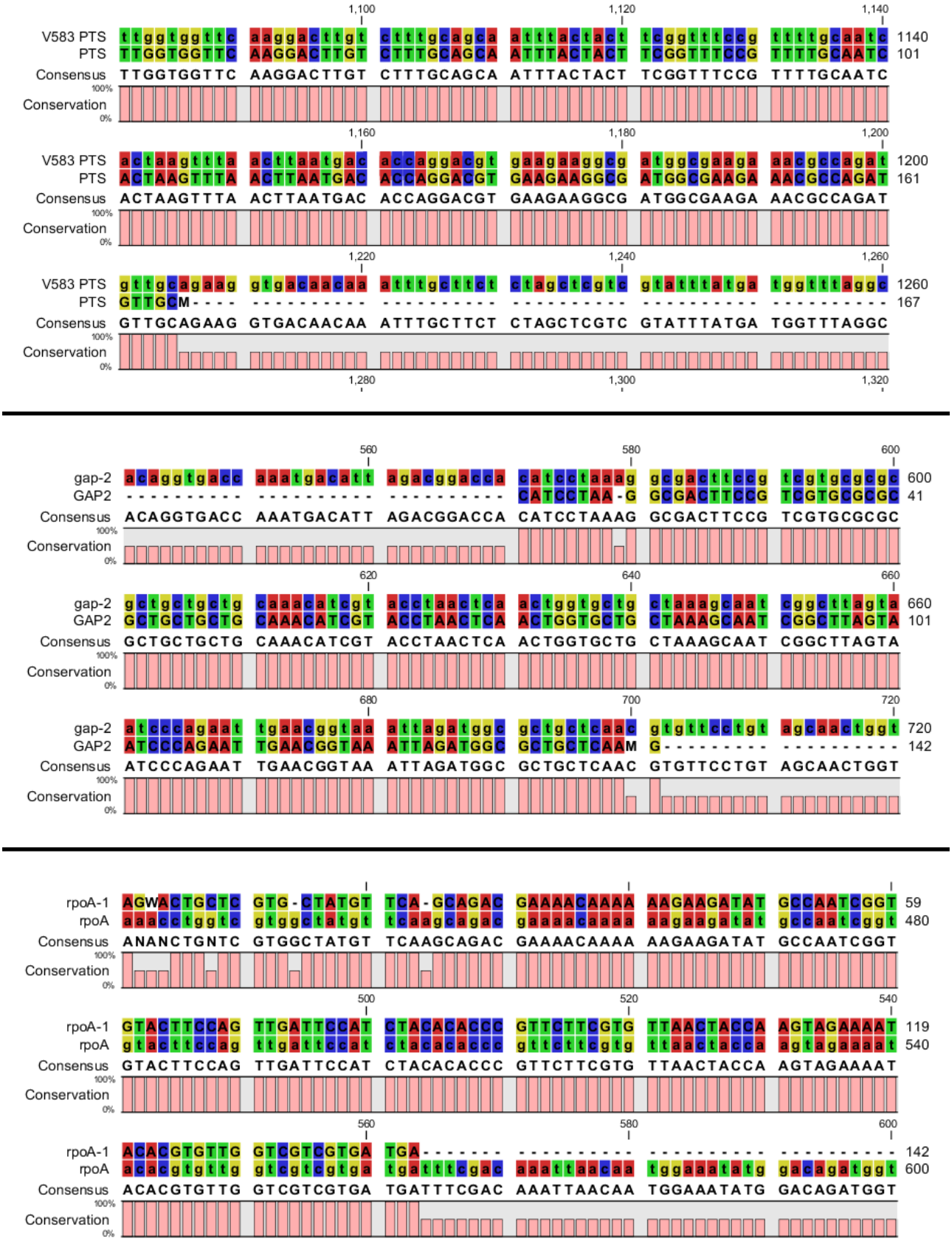


Figure 4.14 – CLC Genomics Alignments of Sequenced PCR Products. *nagE* (top), *gap2* (middle) and *rpoA* (bottom) sequences from Massey Genome Services (MGS) aligned against those from the NCBI database. Top row sequences are the MGS results, bottom rows are database sequences.

Figure 4.13 shows that only one product of the expected size was detected in each of the three reactions. To confirm the identity of the PCR products, and the suitability of the primers for qPCR experiments, each product was sequenced by Massey Genome Services (MGS). **Figure 4.14** shows the alignments of MGS sequencing results and the corresponding genes of interest, downloaded from the NCBI database. These alignments confirmed the identity of each PCR product.

Having run this control, further preparation for the quantification of corresponding mRNA in GlcNAc induced and non-induced cells could proceed.

4.6.2 RT-PCR of *nagE, gap2* and *rpoA*

Refer to Methods: 2.3.16, 2.3.17, 2.3.20 and 2.3.21

After isolating total RNA from GlcNAc-induced and non-induced cells, the next step was an incubation with DNase to remove residual genomic DNA contamination that would otherwise confound the interpretation of downstream experiments. After this, a reverse transcription (RT) step was used to form cDNA from the RNA, using the primers validated in the previous conventional PCR experiment (Section 4.6.1). The resultant cDNA was then amplified by conventional PCR and run on an agarose gel to check that cDNA had been synthesised. The best agarose gel for this analysis is shown in **Figure 4.15**.

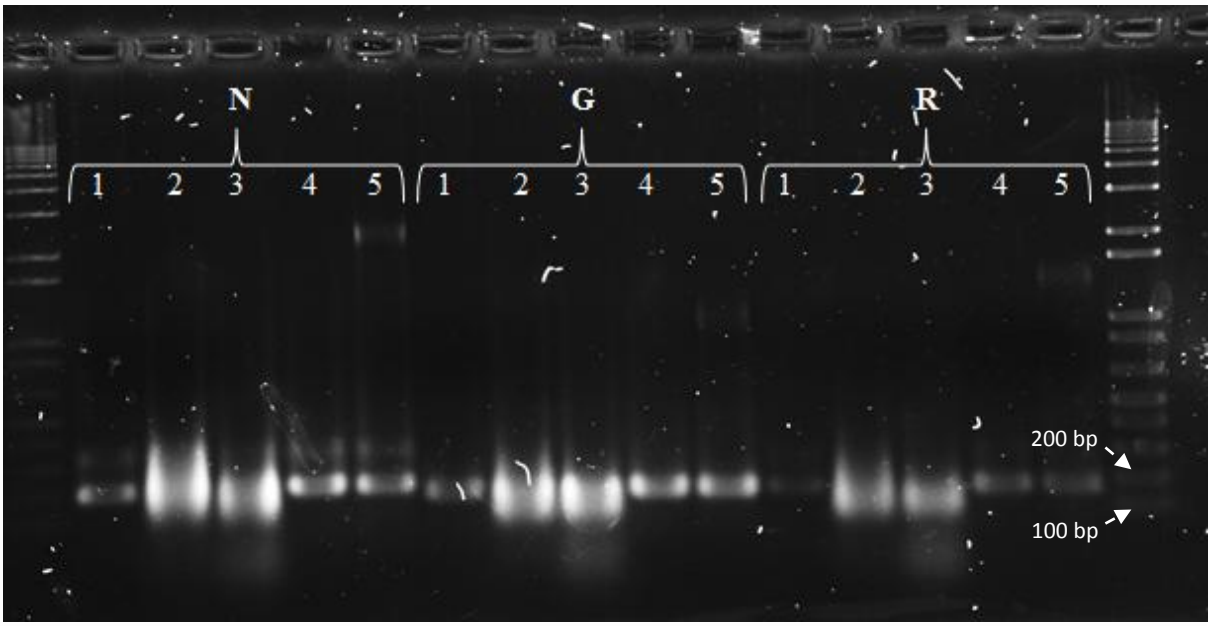


Figure 4.15 – 1% Agarose Gel of RT-PCR Products. (N) *nagE,* (G) *gap2* and (R) *rpoA* genes. (1) No GlcNAc, (2) 30 min GlcNAc, (3) 60 min GlcNAc, (4) 90 min GlcNAc (5) 120 min GlcNAc. Each lane was loaded with 10 µL of dye/product. 1 kb+ ladders flank each side of the gel.

Figure 4.15 shows PCR products of the expected size in every lane. Despite this, many of the results are far from ideal. For example, lanes N1, N4, N5, G5 and R5 contain a second band (120 min), and some bands appear smeared (primarily at 30 min and 60 min), although this could be caused by too much DNA in the sample. This result brought the purity of reagents, primers and other PCR components into question.

The *gap2* primers were used to test if the total RNA samples were contaminated with genomic DNA. *gap2* was selected because *rpoA* had low product yields, and *nagE* showed multiple PCR products.

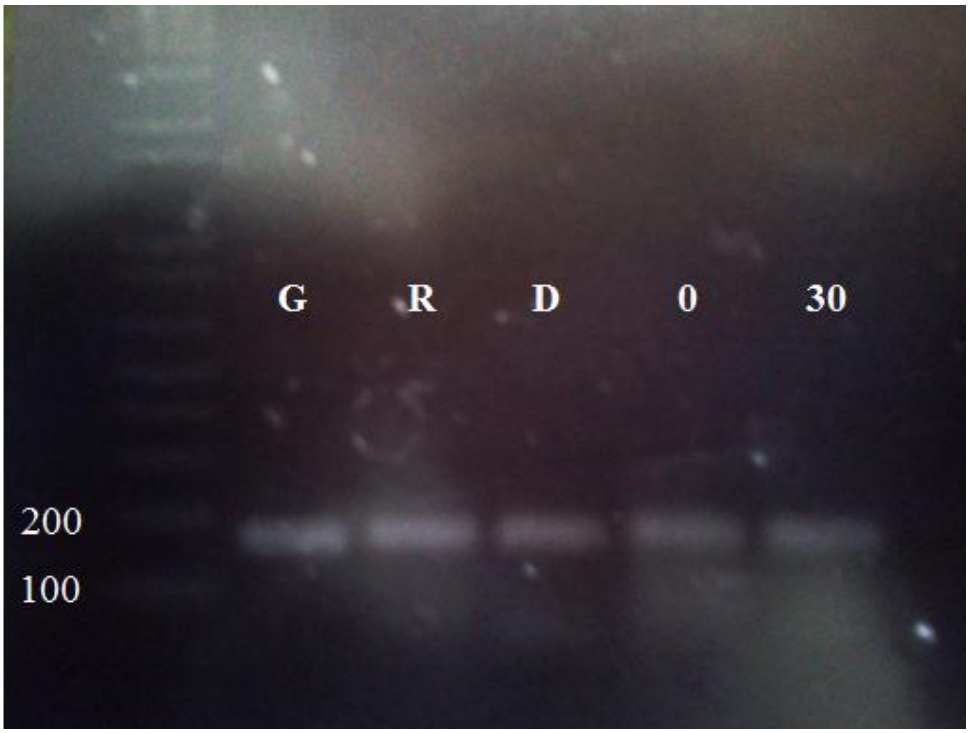


Figure 4.16 – 1% Agarose Gel of RT-PCR Step Products. (G) Genomic DNA, (R) Extracted total RNA (D) DNase treated RNA. (0) No GlcNAc cDNA, (30) 30min GlcNAc cDNA. Each lane was loaded with 15 μL of dye/product.

Figure 4.16 shows a major problem with sample workup in the RT-PCR process. The formation of a PCR product from the DNase treated RNA sample indicates that despite the DNase treatment, this sample still contains genomic DNA, as *Taq* DNA polymerase is unable to amplify mRNA. This indicates contamination, but not how the contamination occurred. Further attempts to eliminate the DNA contamination were unsuccessful, and due to time restrictions, the qPCR of the desired genes was postponed until the DNase treatment is optimised to decrease the DNA contamination to acceptable levels. Despite this problem, the primers can be used for a future qPCR experiment.

5.0 Conclusions and Future Directions

The experimental results presented in this thesis have demonstrated many formerly unknown attributes of the diglycosylated bacteriocin, glycocin F (GccF). GccF was found to have a much wider spectrum of activity than previously realised, and even the Gram-negative bacterium *Yersinia frederiksenii* was slightly susceptible. Appendix 2 contains a photographic record of most agar diffusion assays of GccF activity performed in this thesis. It was also found that if the integrity of the outer membrane (OM) was compromised by polymyxin treatment or by the presence of a 'leaky' mutant of the OM secretin, PulD, then the susceptibility of Gram-negative bacteria to GccF was increased, suggesting that in these bacteria the GccF receptor is located in, and binds GccF at the periplasmic surface of, the inner membrane.

In addition, new information about how this bacteriocin's activity is modulated either up or down by GlcNAc has been revealed. Apart from GlcNAc's established role as an antagonist of GccF-mediated inhibition of *L. plantarum* strains, at lower concentrations GlcNAc can enhance activity of GccF against *E. faecalis* and other species, which was a most unexpected result. An attempt to unravel this apparently synergistic effect of GccF and GlcNAc led to the conclusion that exposing *E. faecalis* cells to GlcNAc prior to treatment with GccF results in a stronger inhibitory effect. A possible explanation of this startling behaviour is that free GlcNAc increases the number of GccF receptors, and/or changes an intracellular process, for example regulation of GlcNAc metabolism, in a way that increases the inhibitory activity of GccF added at a later time (see **Figures 5.1** and **5.2**).

It was also demonstrated that GccF does not induce significant ATP efflux in sensitive *L. plantarum* cells, indicating that GccF does not form large pores through which ATP can leak from the cell. Thus GccF's mode of action is quite different to that of the bactericidal pore-forming lantibiotic, nisin. Despite not having obtained convincing results from either mass spectrometry or qPCR, the methodological groundwork has been laid for future experiments to use these methods to detect changes in the putative GccF receptor, a GlcNAc-specific PTS transporter (pts18/22CBA, NagE).

From these results of these and other experiments, the hypothetical mechanism of action for GccF-induced bacteriostasis was developed and is presented in **Figures 5.1** and **5.2**. GccF

may ‘hijack’ the regulatory signalling pathway associated with a GlcNAc-specific PTS, with its *O*-linked GlcNAc tricking the cell into a response that occurs when an exogenous GlcNAc source is discovered (as per **Figure 5.1**). The cells decrease internal GlcNAc-1P synthesis, but are unable to take up the *O*-linked GlcNAc (which may be phosphorylated by the PTS). Thus, cells are manipulated into creating a GlcNAc(-1P)-depleted cytosol. The absence of this peptidoglycan precursor would halt cell wall synthesis, slowing or even stopping cell growth without killing cells (**Figure 5.2**). In the event that cells have been pre-exposed to GlcNAc, the GlcNAc-specific PTS may have already be up-regulated, and GlcNAc synthesis machinery already been down-regulated, resulting in a stronger inhibitory response once all free GlcNAc is imported and only GccF remains to bind to the GlcNAc-PTS transporter,

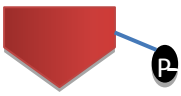
Symbol in Diagrams	Designation
	GlcNAc
	GlcNAc precursors
	GlcNAc-1/6-phosphate
	GccF, emphasising *O*-linked GlcNAc
	Phosphate
	GlcNAc Phosphotransferase system
	Glm (GlcNAc synthesis) Pathway
	(Inner) Cell Membrane

Table 5.1 - Key to Figures 5.1 and 5.2

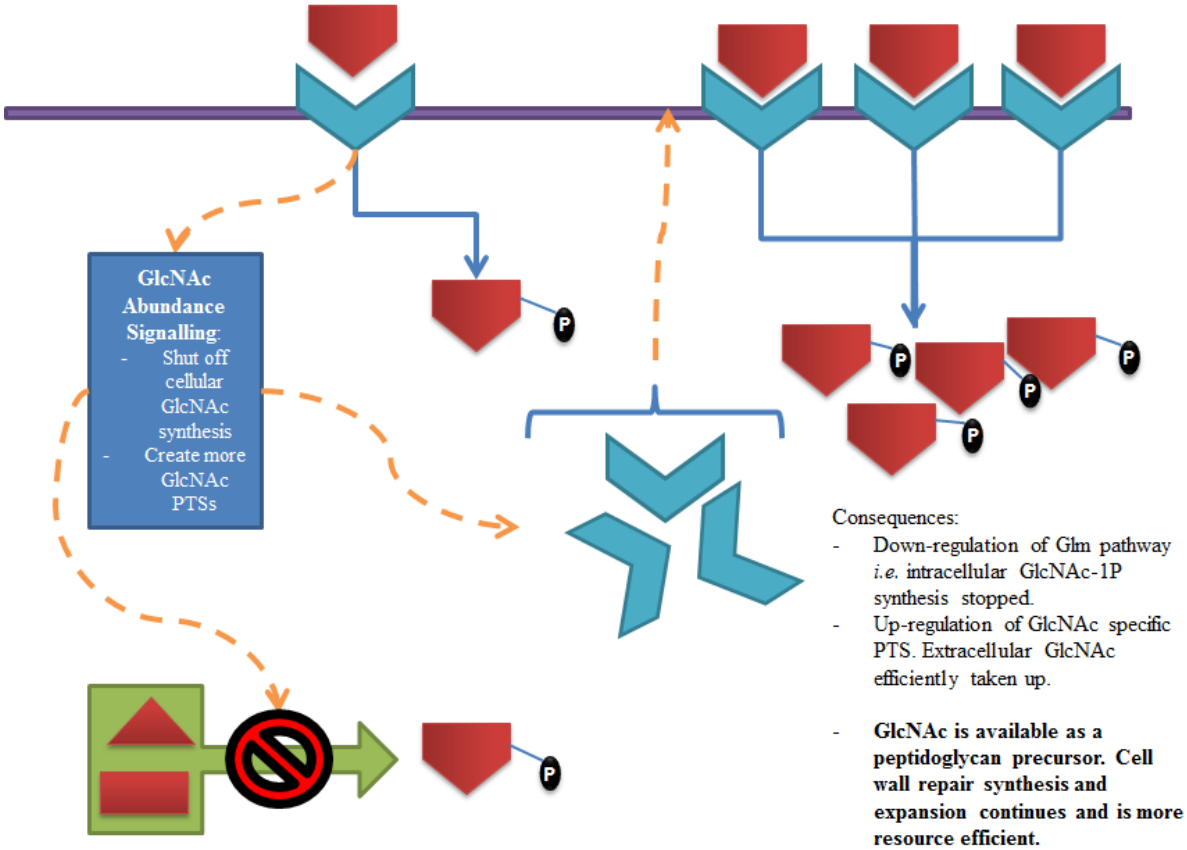


Figure 5.1 - Schematic Diagram of GlcNAc-Induced Metabolic Regulation. Upon sensing abundant exogenous GlcNAc, cells use the readily available GlcNAc, rather than catabolising potentially high energy yielding intermediates, *e.g.* fructose-1-phosphate.

Figure 5.2 illustrates a hypothetical model for the mechanism of GccF-induced growth inhibition. Some aspects of this model could be tested using established methods. For example. the model requires that a GlcNAc moiety of GccF binds to a GlcNAc-specific PTS transporter. If the *pts18CBA* and/or *pts22CBA* genes that encode these transporters in susceptible *L. plantarum* strains could be knocked out, then knockout strains could be assessed for susceptibility to GccF. According to the model proposed in Figure 5.2, the prediction would be that at least some of the knockout strains would show greatly reduced susceptibility to GccF. Similar (*nagE*) knockouts could be generated in Gram-negative bacteria such as *E. coli*, although it would be necessary to optimise the assay conditions for GccF activity against *E. coli* before testing whether knocking out the *nagE* gene decreases susceptibility to GccF.

Whether *L. plantarum* strains would be viable after the *pts18CBA* and/or *pts22CBA* have been knocked out is unknown, although the nagE deletion mutant *E. coli* is viable.[123]

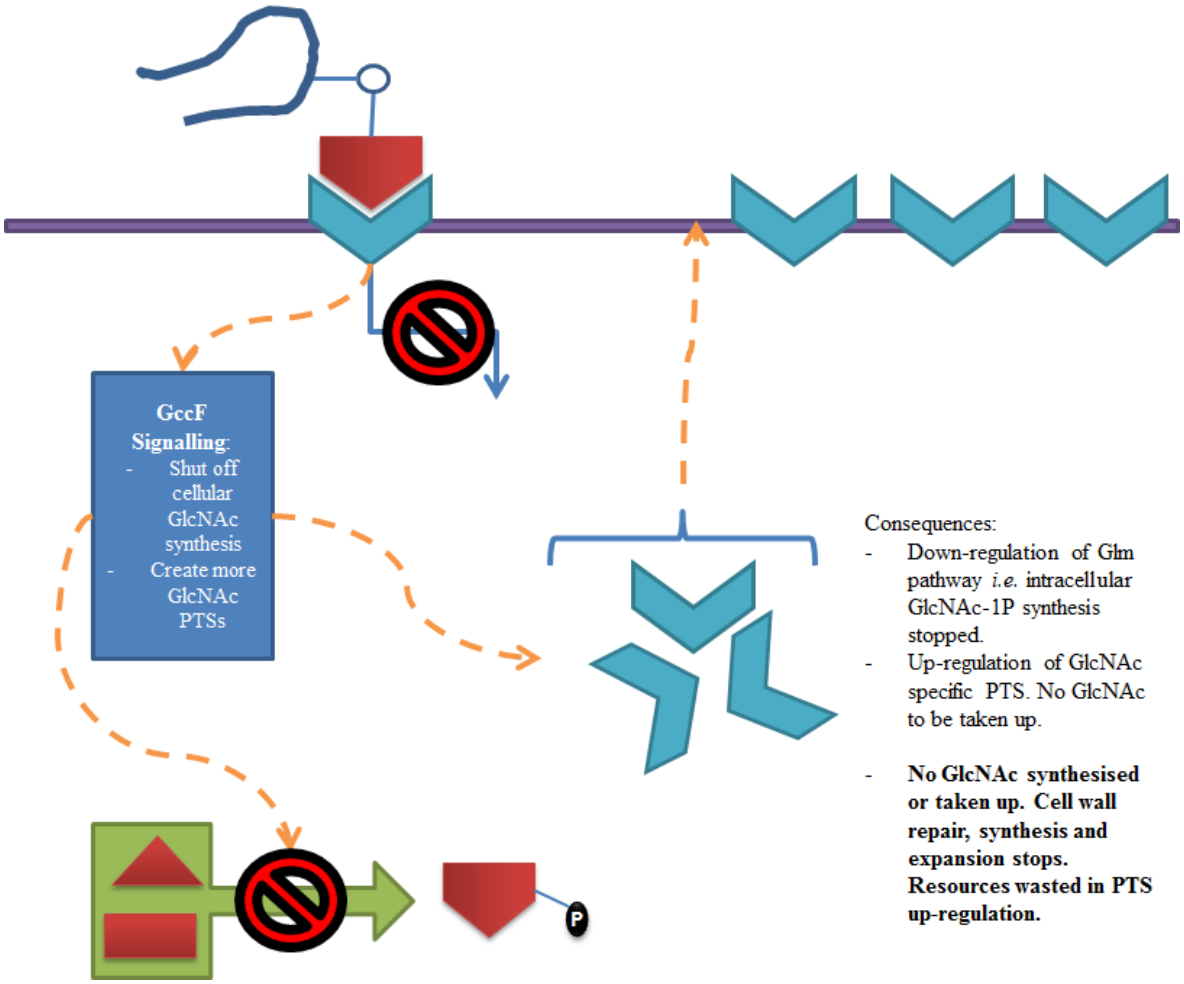


Figure 5.2 - Schematic Diagram of Hypothesised GccF Mechanism of Action. GccF acts as GlcNAc, and cells change metabolic regulation according to GlcNAc abundance. However, there is no GlcNAc readily available to be taken up into cells.

Further testing of the effects of GccF inside susceptible cells might require the use of quantitative proteomic, transcriptomic and/or metabolomic methods. An example of the latter would be to measure the concentration of UDP-GlcNAc, an essential GlcNAc-derived precursor for peptidoglycan biosynthesis, with and without GccF treatment. The activities of enzymes of the Glm pathway for UDP-GlcNAc synthesis (see Figure 1.4) could also be measured before and after GccF treatment. These tests could be carried out on susceptible strains of *L. plantarum* or *E. faecalis*.

Only when the molecular mechanism of GccF is understood will it be possible to leverage nature's design for the development of new antibiotics that exploit the novel inhibitory principles illustrated in Figure 5.2.

6.0 Appendices

6.1 - Bacterial strains and plasmids used in this study

Genus	Species	Strain	NZRM	ATCC	NCTC
Bacillus	*megaterium*	DSM 319			
	subtilis			6633	
		BR151			
Carnobacterium	*divergens*		3572		
Enterococcus	*casseliflavus*		3293		
	faecalis	V583			
		Stubbs IV	89		370
		Tissier	1106	19433	775
			1240	6055	5957
			2244	29212	
		PCl l325	2262	14506	
		HH22	3178		
		FC Tenover NJ-3	3488	51299	
			3601		
		POW 1994	4061		
		TX0104			
	faecium	TYPE	1236	19434	7171
			4037		
Lactobacillus	*acidophilus*	NCFM			
	brevis	LMG11437			
	plantarum	KW30			
			1100	8014	
				8014 - Imm	
				14917	
		Lp39			
		WCFS1			
		WRSF1			
		KW30 - 2111 - 4			
		KW30 - 2111 - 4 Imm			
				14917	
		NC8			
	sakei	Lb790			
Leuconostoc	*mesenteroides*		3199		
			3620		
Listeria	*monocytogenes*			35152	7973
Staphylococcus	*aureus*	Newmans			
Streptococcus	*agalactiae*	TYPE	2267	13813	8181
			2721	27956	
	mutans		987	25175	10449

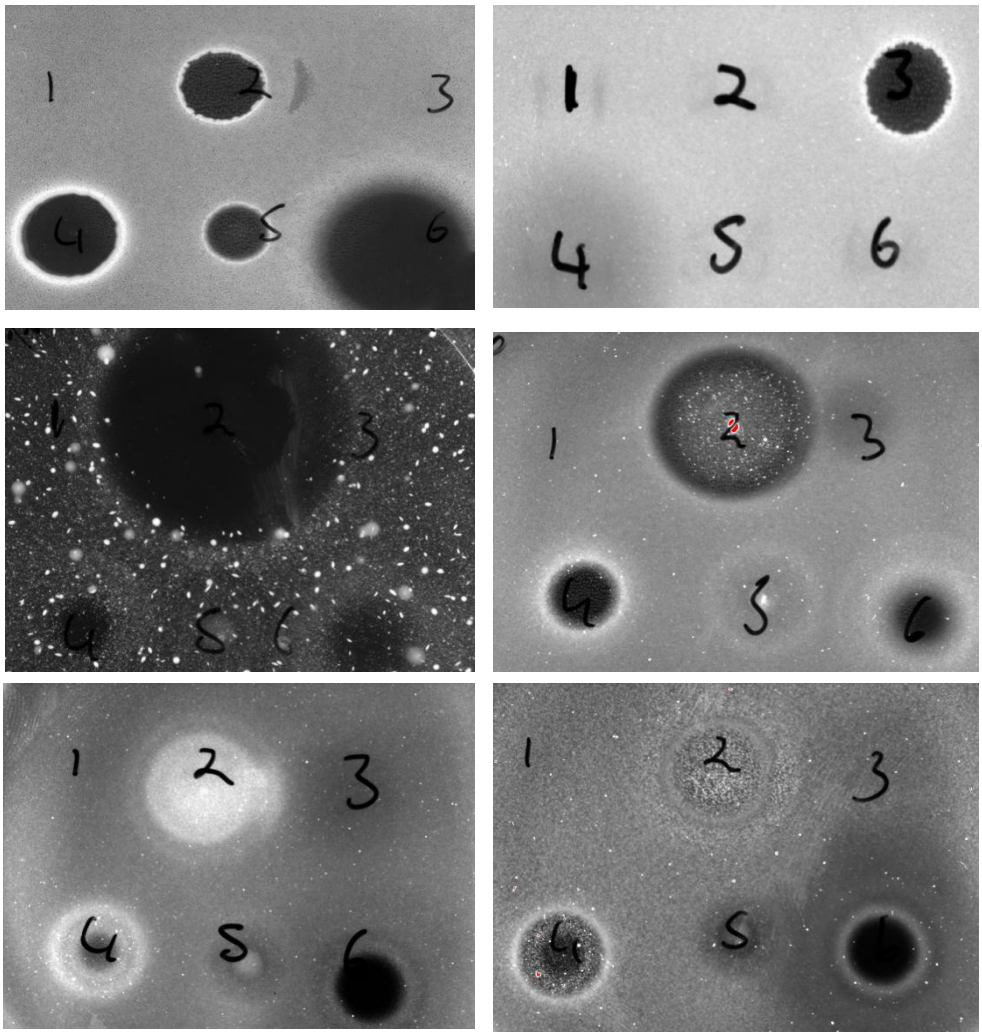
	pneumoniae	TYPE	100	33400	7465
			2725	9163	
			2762		
			3399	49619	
	pyogenes	TYPE	2264	12344	8198
			2724	21547	
		Dochez NY5	3604	12351	
		T2/44/Rb4			8322
	salivarius	TYPE	3489	7073	8618
	suis				
Weissella	*viridescens*		3313		
Yersinia	*frederiksenii*				

Table 6.1 - Comprehensive list of non-genetically modified bacteria used in this study.

Strain	**Parental Strain**	**Plasmids**	**Notes**	**Reference(s)**
K1508	MC4100	N/A	*ΔlamB106*, faulty maltopentose uptake	
K2162	K1508	pPMR132 T327A	*pIV* his tagged (as in pPMR132) has leaky T327A mutation	[115; 124]
	K1508	pCHAP362 Δ477-481	*pulD* (as in pCHAP362) contains Δ477-481 mutation	[118]
	K1508	pAH181	*pulS* (as in pAH181)	[116; 118; 125]
K2234	K1508	pCHAP362 Δ477-481 + pAH181	*pulD* (as in pCHAP362) contains Δ477-481 mutation, *pulS* (as in pAH181)	[116; 118; 125]
K2235	K1508	pCHAP362 + pAH181	*pulD* (as in pCHAP362) is wild-type, *pulS* (as in pAH181)	[116; 125]

Table 6.2 - *Escherichia coli* strains and plasmids used in secretin mutant analysis. K1508 strains were transformed and obtained courtesy of Helipad Lab, Massey University, Palmerston North.

6.2 – Antimicrobial Spotting on Indicator Bacterial Lawns



Left: *Bacillus megaterium* DSM 319

Right: *Bacillus subtilis* BR151

Left: *Enterococcus faecium* NZRM 1236

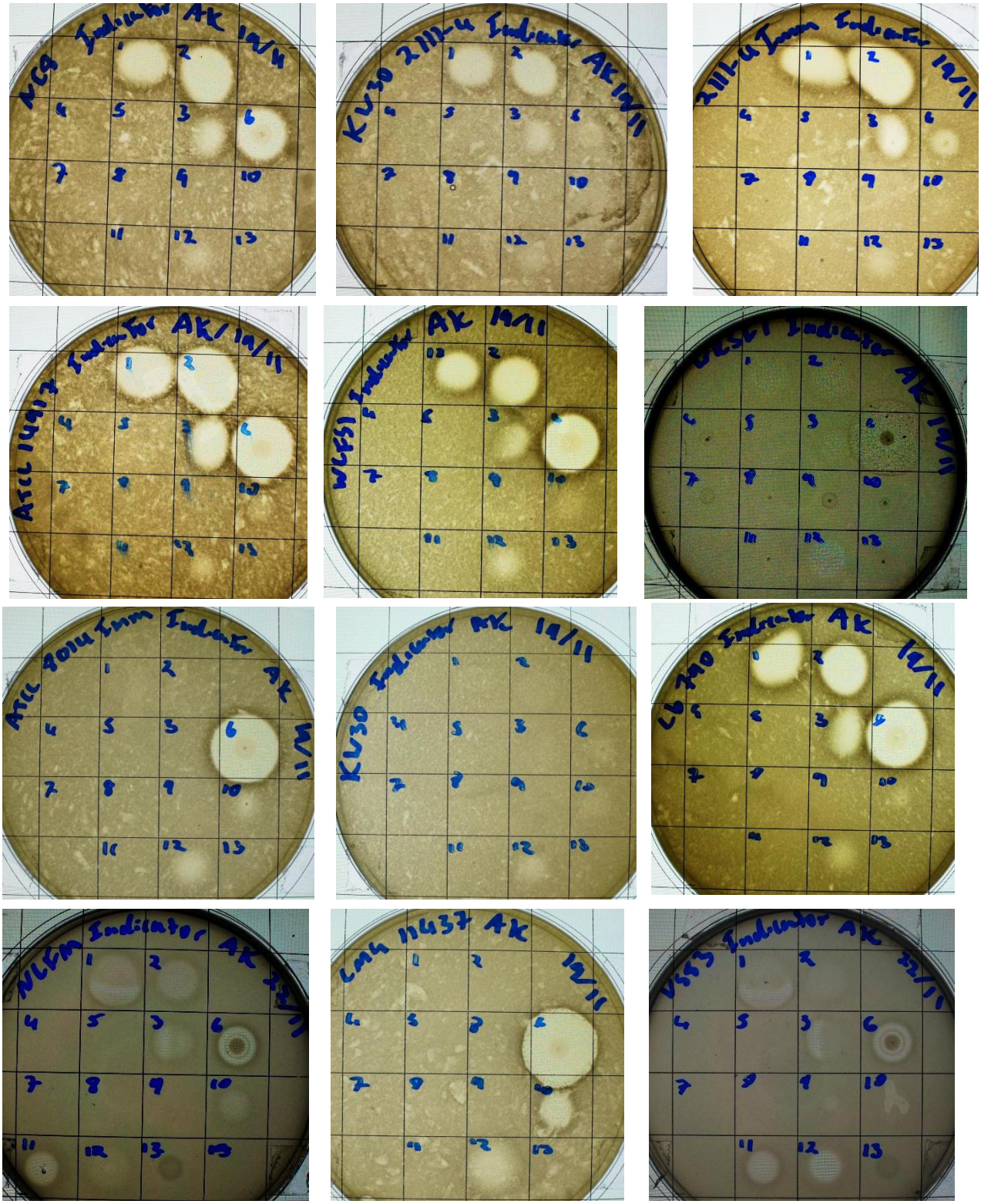
Right: *Streptococcus agalactiae* NZRM 2267

Left: *Streptococcus pneumoniae* ATCC 2762

Right: *Streptococcus pyogenes* NCTC 8322

Figure 6.1 – Indicator Plates for Antimicrobial Agar Diffusion Assays. Key: **1** – No spot/Positive control, **2** – 2.8 mg/mL GccF, **3** – 20% w/v GlcNAc, **4** – Nisaplin (10% w/v), **5** - PlnA (5 mg/mL), **6** – Polymyxin B (100mM). N.B. All samples were 2 μL drops.

Figure 6.2 (below) – More Indicator Plates for Antimicrobial Agar Diffusion Assays. Key: **1** - 2.6 mg/mL GccF, **2** - 5-Fold dilution of 1, **3** - 5-Folid dilution of 2, **4** - V583/TX0104 mixed culture + 9mM GlcNAc supernatant, **5** - 20% w/v GlcNAc, **6** - 10% w/v Nisaplin (nisin), **7** - V583/TX0104 mixed culture supernatant, **8** - V583 supernatant (TSB), **9** - TX0104 supernatant (TSB), **10** - 5 mg/mL plnA, **11** - Vancomycin.HCl (1 mg/mL in H_2O), **12** - Bacitracin (1.8 mM), **13** - plnEF/JK (DSM 13273 plnA supernatant).



Top - Down:

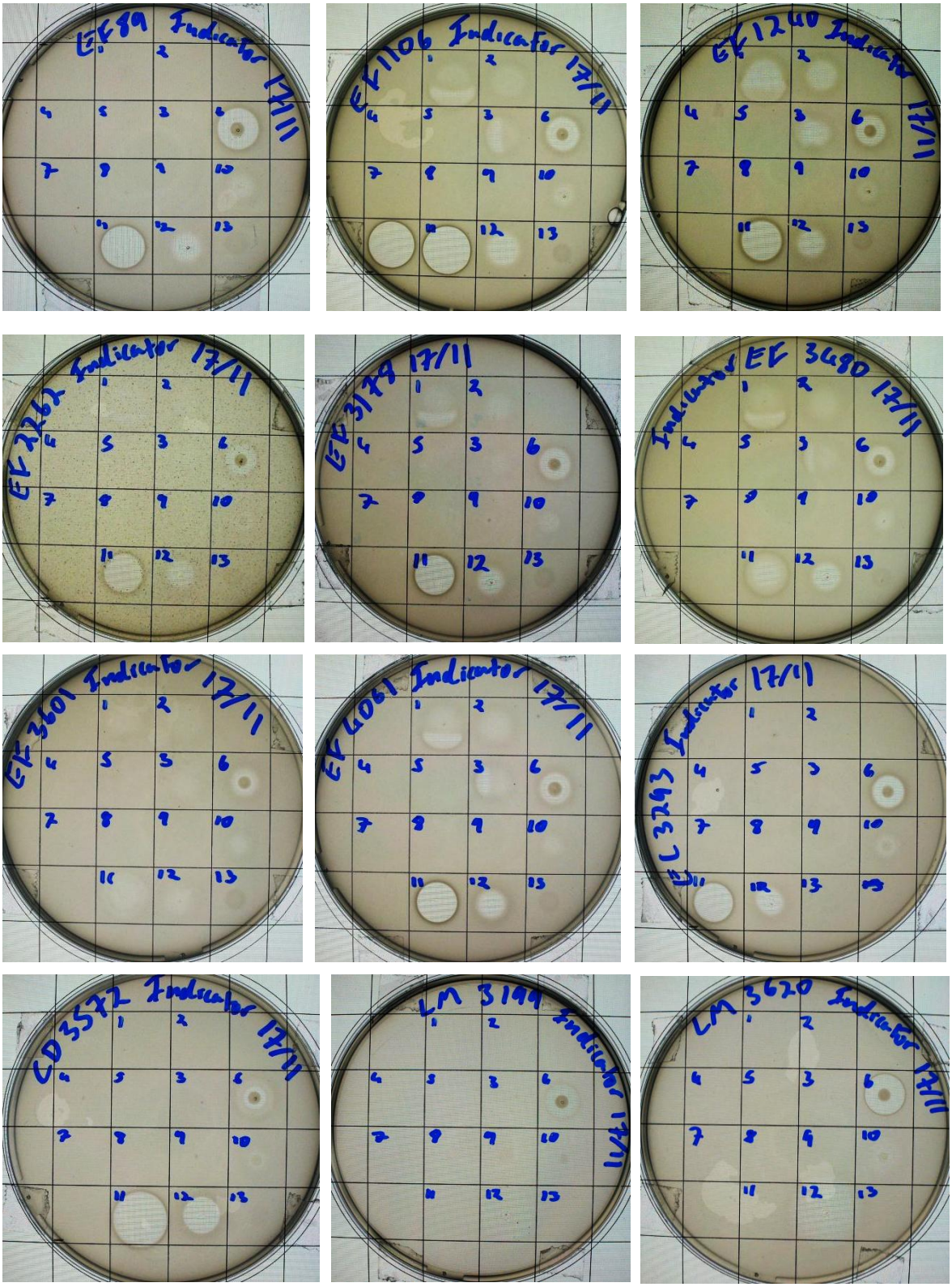
- *Lactobacillus plantarum* NCFM
- *Lactobacillus plantarum* ATCC 14917
- *Lactobacillus plantarum* ATCC 8014
- *Lactobacillus acidophilus* NCFM

Top - Down:

- *Lactobacillus plantarum* KW30 2111-4
- *Lactobacillus plantarum* WCSF1
- *Lactobacillus plantarum* KW30
- *Lactobacillus brevis* LMG11437

Top - Down:

- *Lactobacillus plantarum* NCFM
- *Lactobacillus plantarum* WRSF1
- *Lactobacillus sakei* Lb790
- *Enterococcus faecalis* V583



Top - Down:

- *Enterococcus faecalis* NZRM 89
- *Enterococcus faecalis* NZRM 2262
- *Enterococcus faecalis* NZRM 3601
- *Carnobacteria divergens* NZRM 3572

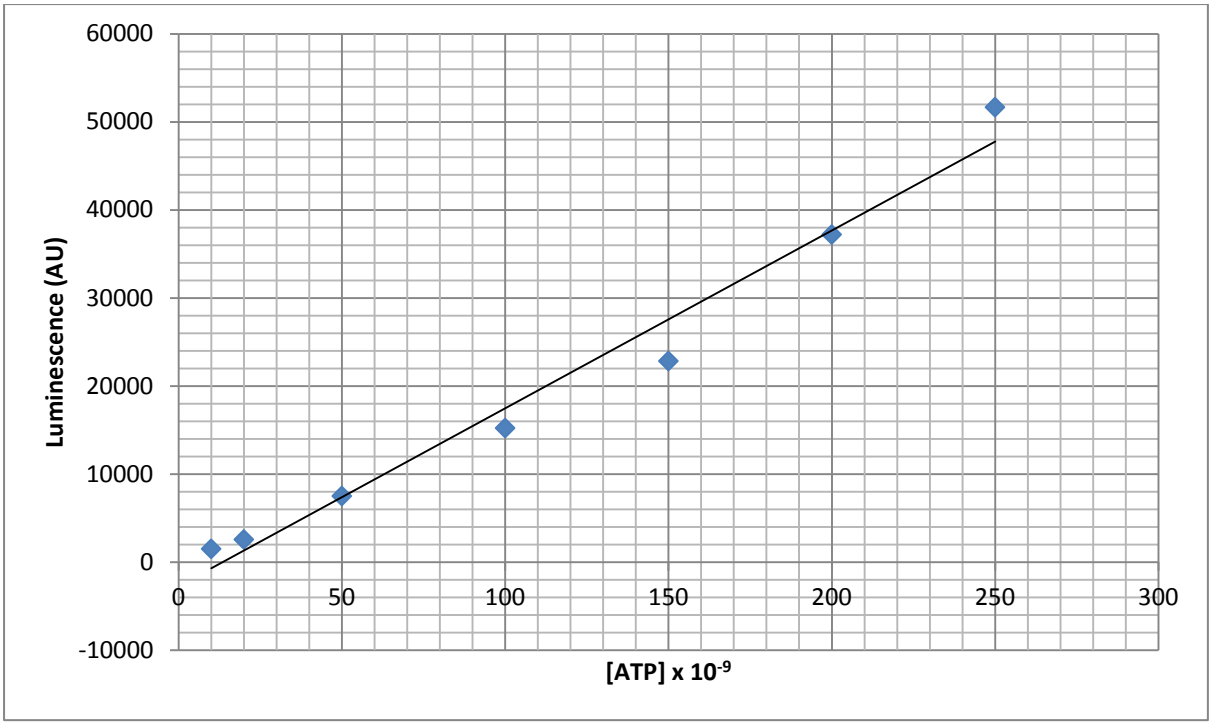
Top - Down:

- *Enterococcus faecalis* NZRM 1106
- *Enterococcus faecalis* NZRM 3178
- *Enterococcus faecalis* NZRM 4061
- *Leuconostoc mesenteroides* NZRM 3572

Top - Down:

- *Enterococcus faecalis* NZRM 1240
- *Enterococcus faecalis* NZRM 3480
- *Enterococcus cassiflavus* NZRM 3293
- *Leuconostoc mesenteroides* NZRM 3572

6.3 – ATP Assay Standard Curves, [ATP] vs. Luminescence



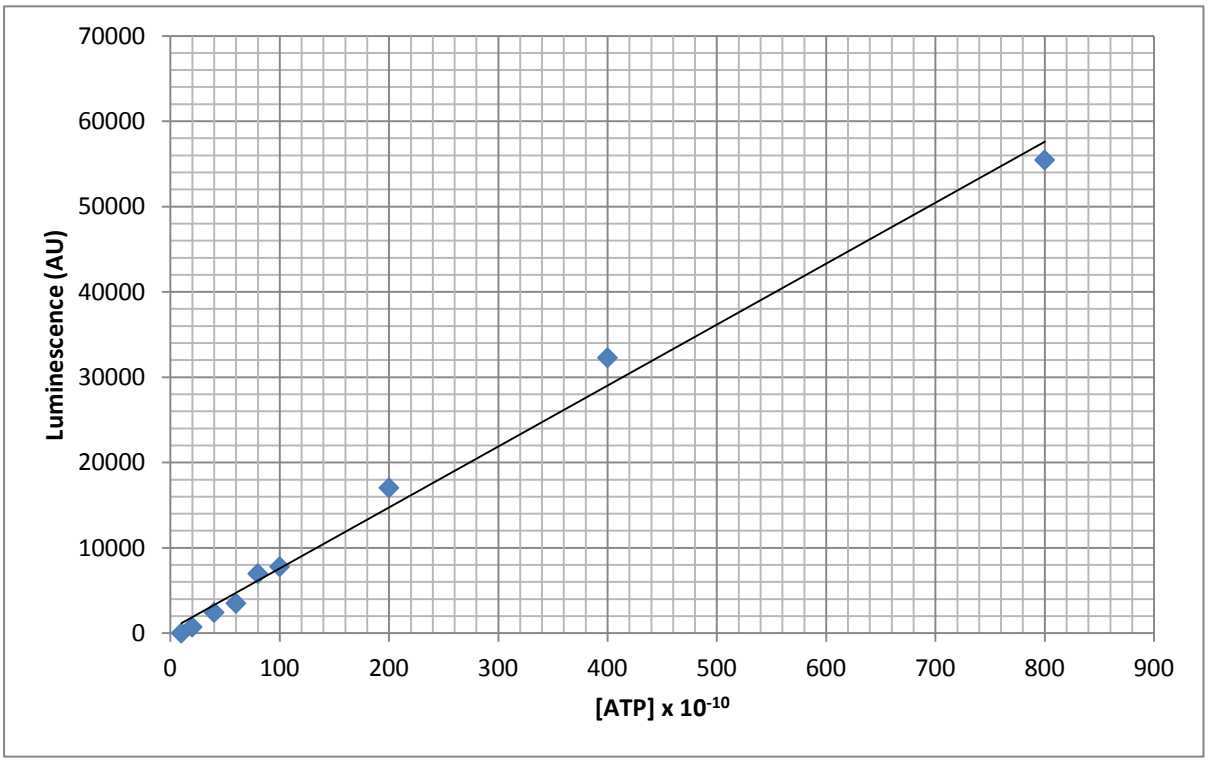


Figure 6.3 – Standard Curves for ATP bioluminescence assay taken for Figure 4.4. Top was taken on 24/07/2012 for data taken on 24/07/2012 and 27/07/2012. **Bottom** was taken on 16/08/2012 for the data taken on the same day.

7.0 References

1. Arnison, P. G., Bibb, M. J., Bierbaum, G., Bowers, A. A., Bugni, T. S., Bulaj, G., Camarero, J. A., Campopiano, D. J., Challis, G. L., Clardy, J., Cotter, P. D., Craik, D. J., Dawson, M., Dittmann, E., Donadio, S., Dorrestein, P. C., Entian, K.-D., Fischbach, M. A., Garavelli, J. S., Goransson, U., Gruber, C. W., Haft, D. H., Hemscheidt, T. K., Hertweck, C., Hill, C., Horswill, A. R., Jaspars, M., Kelly, W. L., Klinman, J. P., Kuipers, O. P., Link, A. J., Liu, W., Marahiel, M. A., Mitchell, D. A., Moll, G. N., Moore, B. S., Muller, R., Nair, S. K., Nes, I. F., Norris, G. E., Olivera, B. M., Onaka, H., Patchett, M. L., Piel, J., Reaney, M. J. T., Rebuffat, S., Ross, R. P., Sahl, H.-G., Schmidt, E. W., Selsted, M. E., Severinov, K., Shen, B., Sivonen, K., Smith, L., Stein, T., Sussmuth, R. D., Tagg, J. R., Tang, G.-L., Truman, A. W., Vederas, J. C., Walsh, C. T., Walton, J. D., Wenzel, S. C., Willey, J. M. & van der Donk, W. A. (2013). Ribosomally synthesized and post-translationally modified peptide natural products: overview and recommendations for a universal nomenclature. *Natural Product Reports* **30**, 108-160.
2. Sahl, H. G. & Bierbaum, G. (2008). Multiple Activities in Natural Antimicrobials-Multiple activities of natural agents such as defensins and bacteriocins suggest a change in strategy when developing new antimicrobials. *Microbe* **3**, 467.
3. Kjos, M., Borrero, J., Opsata, M., Birri, D. J., Holo, H., Cintas, L. M., Snipen, L., Hernandez, P. E., Nes, I. F. & Diep, D. B. (2011). Target recognition, resistance, immunity and genome mining of class II bacteriocins from Gram-positive bacteria. *Microbiology-SGM* **157**, 3256-3267.
4. Chami, M., Guilvout, I., Gregorini, M., Remigy, H. W., Muller, S. A., Valerio, M., Engel, A., Pugsley, A. P. & Bayan, N. (2005). Structural insights into the secretin PulD and its trypsin-resistant core. *The Journal of Biological Chemistry* **280**, 37732- 37741.
5. Hibbing, M. E., Fuqua, C., Parsek, M. R. & Peterson, S. B. (2010). Bacterial competition: surviving and thriving in the microbial jungle. *Nature Reviews Microbiology* **8**, 15-25.
6. Peschel, A. & Sahl, H. G. (2006). The co-evolution of host cationic antimicrobial peptides and microbial resistance. *Nature Reviews Microbiology* **4**, 529-536.
7. Luders, T., Birkemo, G. A., Fimland, G., Nissen-Meyer, J. & Nes, I. F. (2003). Strong synergy between a eukaryotic antimicrobial peptide and bacteriocins from lactic acid bacteria. *Applied and Environmental Microbiology* **69**, 1797-1799.
8. Riley, M. A. & Wertz, J. E. (2002). Bacteriocin diversity: ecological and evolutionary perspectives. *Biochimie* **84**, 357-364.
9. Klaenhammer, T. R. (1988). Bacteriocins of Lactic-Acid Bacteria. *Biochimie* **70**, 337-349.
10. Riley, M. A. (1998). Molecular mechanisms of bacteriocin evolution. *Annual Review of Genetics* **32**, 255-278.
11. Nes, I. F., Yoon, S. S. & Diep, D. B. (2007). Ribosomally synthesiszed antimicrobial peptides (Bacteriocins) in lactic acid bacteria: A review. *Food Science and Biotechnology* **16**, 675-690.
12. Chung, W. & Hancock, R. E. W. (2000). Action of lysozyme and nisin mixtures against lactic acid bacteria. *International Journal of Food Microbiology* **60**, 25-32.
13. Martin-Visscher, L. A., Yoganathan, S., Sit, C. S., Lohans, C. T. & Vederas, J. C. (2011). The activity of bacteriocins from *Carnobacterium maltaromaticum* UAL307 against Gram-negative bacteria in combination with EDTA treatment. *FEMS Microbiology Letters* **317**, 152-159.

14. Nes, I. F., Diep, D. B., Havarstein, L. S., Brurberg, M. B., Eijsink, V. & Holo, H. (1996). Biosynthesis of bacteriocins in lactic acid bacteria. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **70**, 113-128.
15. Kleerebezem, M., Beerthuyzen, M. M., Vaughan, E. E., deVos, W. M. & Kuipers, O. P. (1997). Controlled gene expression systems for lactic acid bacteria: Transferable nisin-inducible expression cassettes for *Lactococcus*, *Leuconostoc*, and *Lactobacillus* spp. *Applied and Environmental Microbiology* **63**, 4581-4584.
16. Jack, R. W., Tagg, J. R. & Ray, B. (1995). Bacteriocins of Gram-Positive Bacteria. *Microbiological Reviews* **59**, 171-200.
17. van der Meer, J. R., Rollema, H. S., Siezen, R. J., Beerthuyzen, M. M., Kuipers, O. P. & Devos, W. M. (1994). Influence of Amino-Acid Substitutions in the Nisin Leader Peptide on Biosynthesis and Secretion of Nisin by *Lactococcus lactis*. *Journal of Biological Chemistry* **269**, 3555-3562.
18. Li, B., Yu, J. P. J., Brunzelle, J. S., Moll, G. N., van der Donk, W. A. & Nair, S. K. (2006). Structure and mechanism of the lantibiotic cyclase involved in nisin biosynthesis. *Science* **311**, 1464-1467.
19. Kuipers, A., de Boef, E., Rink, R., Fekken, S., Kluskens, L. D., Driessen, A. J. M., Leenhouts, K., Kuipers, O. P. & Moll, G. N. (2004). NisT, the transporter of the lantibiotic nisin, can transport fully modified, dehydrated, and unmodified prenisin and fusions of the leader peptide with non-lantibiotic peptides. *Journal of Biological Chemistry* **279**, 22176-22182.
20. Izaguirre, G. & Hansen, J. N. (1997). Use of alkaline phosphatase as a reporter polypeptide to study the role of the subtilin leader segment and the SpaT transporter in the posttranslational modifications and secretion of subtilin in *Bacillus subtilis* 168. *Applied and Environmental Microbiology* **63**, 3965-3971.
21. Rink, R., Wierenga, J., Kuipers, A., Muskens, L. D., Driessen, A. J. M., Kuipers, O. P. & Moll, G. N. (2007). Production of dehydroamino acid-containing peptides by *Lactococcus lactis*. *Applied and Environmental Microbiology* **73**, 1792-1796.
22. Kluskens, L. D., Kuipers, A., Rink, R., de Boef, E., Fekken, S., Driessen, A. J. M., Kuipers, O. P. & Moll, G. N. (2005). Post-translational modification of therapeutic peptides by NisB, the dehydratase of the lantibiotic nisin. *Biochemistry* **44**, 12827-12834.
23. Todorov, S. D. (2009). Bacteriocins from *Lactobacillus Plantarum* - Production, Genetic Organization and Mode of Action. *Brazilian Journal of Microbiology* **40**, 209-221.
24. Havarstein, L. S., Diep, D. B. & Nes, I. F. (1995). A Family of Bacteriocin ABC Transporters Carry out Proteolytic Processing of Their Substrates Concomitant with Export. *Molecular Microbiology* **16**, 229-240.
25. Fath, M. J. & Kolter, R. (1993). ABC Transporters - Bacterial Exporters. *Microbiological Reviews* **57**, 995-1017.
26. Ra, R., Beerthuyzen, M. M., de Vos, W. M., Saris, P. E. & Kuipers, O. P. (1999). Effects of gene disruptions in the nisin gene cluster of *Lactococcus lactis* on nisin production and producer immunity. *Microbiology* **145**, 1227-1233.
27. Lubelski, J., Rink, R., Khusainov, R., Moll, G. N. & Kuipers, O. P. (2008). Biosynthesis, immunity, regulation, mode of action and engineering of the model lantibiotic nisin. *Cellular and Molecular Life Sciences* **65**, 455-476.
28. Nagao, J., Asaduzzaman, S. M., Aso, Y., Okuda, K., Nakayama, J. & Sonomoto, K. (2006). Lantibiotics: insight and foresight for new paradigm. *Journal of Bioscience and Bioengineering* **102**, 139-49.

29. Diep, D. B., Skaugen, M., Salehian, Z., Holo, H. & Nes, I. F. (2007). Common mechanisms of target cell recognition and immunity for class II bacteriocins. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 2384-2389.
30. Kuipers, O. P., Beerthuyzen, M. M., Siezen, R. J. & De Vos, W. M. (1993). Characterization of the nisin gene cluster *nisABTCIPR* of *Lactococcus lactis*. Requirement of expression of the *nisA* and *nisI* genes for development of immunity. *European Journal of Biochemistry* **216**, 281-291.
31. van Belkum, M. J. & Stiles, M. E. (1995). Molecular characterization of genes involved in the production of the bacteriocin leucocin A from *Leuconostoc gelidum*. *Applied and Environmental Microbiology* **61**, 3573-3579.
32. McAuliffe, O., O'Keeffe, T., Hill, C. & Ross, R. P. (2001). Regulation of immunity to the two-component lantibiotic, lacticin 3147, by the transcriptional repressor LtnR. *Molecular Microbiology* **39**, 982-993.
33. McAuliffe, O., Hill, C. & Ross, R. P. (2000). Identification and overexpression of ltnI, a novel gene which confers immunity to the two-component lantibiotic lacticin 3147. *Microbiology* **146**, 129-138.
34. Draper, L. A., Grainger, K., Deegan, L. H., Cotter, P. D., Hill, C. & Ross, R. P. (2009). Cross-immunity and immune mimicry as mechanisms of resistance to the lantibiotic lacticin 3147. *Molecular Microbiology* **71**, 1043-1054.
35. Leisner, J. J. & Haaber, J. (2012). Intraguild predation provides a selection mechanism for bacterial antagonistic compounds. *Proceedings of the Royal Society B-Biological Sciences* **279**, 4513-4521.
36. Adamberg, K., Antonsson, M., Vogensen, F. K., Nielsen, E. W., Kask, S., Moller, P. L. & Ardo, Y. (2005). Fermentation of carbohydrates from cheese sources by non-starter lactic acid bacteria isolated from semi-hard Danish cheese. *International Dairy Journal* **15**, 873-882.
37. Finkel, S. E. & Kolter, R. (2001). DNA as a nutrient novel role for bacterial competence gene homologs. *Journal of Bacteriology* **183**, 6288-6293.
38. West, S. A. & Gardner, A. (2010). Altruism, Spite, and Greenbeards. *Science* **327**, 1341-1344.
39. Kerr, B., Riley, M. A., Feldman, M. W. & Bohannan, B. J. M. (2002). Local dispersal promotes biodiversity in a real-life game of rock-paper-scissors. *Nature* **418**, 171-174.
40. Gravesen, A., Axelsen, A. M. J., da Silva, J. M., Hansen, T. B. & Knochel, S. (2002). Frequency of bacteriocin resistance development and associated fitness costs in *Listeria monocytogenes*. *Applied and Environmental Microbiology* **68**, 756-764.
41. Gonzalez-Pastor, J. E., Hobbs, E. C. & Losick, R. (2003). Cannibalism by sporulating bacteria. *Science* **301**, 510-513.
42. Lamsa, A., Liu, W. T., Dorrestein, P. C. & Pogliano, K. (2012). The *Bacillus subtilis* cannibalism toxin SDP collapses the proton motive force and induces autolysis. *Molecular Microbiology* **84**, 486-500.
43. Guiral, S., Mitchell, T. J., Martin, B. & Claverys, J. P. (2005). Competence-programmed predation of noncompletent cells in the human pathogen *Streptococcus pneumoniae*: Genetic requirements. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 8710-8715.
44. Be'er, A., Ariel, G., Kalisman, O., Helman, Y., Sirota-Madi, A., Zhang, H. P., Florin, E. L., Payne, S. M., Ben-Jacob, E. & Swinney, H. L. (2010). Lethal protein produced in response to competition between sibling bacterial colonies. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 6258-6263.

45. Eijsink, V. G. H., Axelsson, L., Diep, D. B., Havarstein, L. S., Holo, H. & Nes, I. F. (2002). Production of class II bacteriocins by lactic acid bacteria; an example of biological warfare and communication. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **81**, 639-654.
46. Quadri, L. E. N. (2002). Regulation of antimicrobial peptide production by autoinducer-mediated quorum sensing in lactic acid bacteria. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **82**, 133-145.
47. Kodani, S., Lodato, M. A., Durrant, M. C., Picart, F. & Willey, J. M. (2005). SapT, a lanthionine-containing peptide involved in aerial hyphae formation in the streptomycetes. *Molecular Microbiology* **58**, 1368-1380.
48. Kodani, S., Hudson, M. E., Durrant, M. C., Buttner, M. J., Nodwell, J. R. & Willey, J. M. (2004). The SapB morphogen is a lantibiotic-like peptide derived from the product of the developmental gene *ramS* in *Streptomyces coelicolor*. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 11448-11453.
49. Kreth, J., Merritt, J., Shi, W. & Qi, F. (2005). Co-ordinated bacteriocin production and competence development: a possible mechanism for taking up DNA from neighbouring species. *Molecular Microbiology* **57**, 392-404.
50. Klaenhammer, T. R. (1993). Genetics of Bacteriocins Produced by Lactic-Acid Bacteria. *FEMS Microbiology Reviews* **12**, 39-86.
51. Moll, G. N., Konings, W. N. & Driessen, A. J. M. (1999). Bacteriocins: mechanism of membrane insertion and pore formation. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **76**, 185-198.
52. Lozano, J. C. N., Meyer, J. N., Sletten, K., Pelaz, C. & Nes, I. F. (1992). Purification and amino-acid-sequence of a bacteriocin produced by *Pediococcus acidilactici*. *Journal of General Microbiology* **138**, 1985-1990.
53. Yousef, A. E., Luchansky, J. B., Degnan, A. J. & Doyle, M. P. (1991). Behavious of *Listeria monocytogenes* in wiener exudates in the presence of *Pediococcus acidilactici* H or pediocin-ACH during storage at 4 degrees-C or 25 degrees-C. *Applied and Environmental Microbiology* **57**, 1461-1467.
54. Vaughan, E. E., Daly, C. & Fitzgerald, G. F. (1992). Identification and characterization of Helveticin V-1829, a bacteriocin produced by *Lactobacillus helveticus* 1829. *Journal of Applied Bacteriology* **73**, 299-308.
55. Joerger, M. C. & Klaenhammer, T. R. (1986). Characterization and purification of Helvetican-J and evidence for a chromosomoally determined bacteriocin produced by *Lactobacillus helveticus* 481. *Journal of Bacteriology* **167**, 439-446.
56. Cotter, P. D., Hill, C. & Ross, R. P. (2005). Bacteriocins: Developing innate immunity for food. *Nature Reviews Microbiology* **3**, 777-788.
57. Zouhir, A., Hammami, R., Fliss, I. & Ben Hamida, J. (2010). A New Structure-based Classification of Gram-positive Bacteriocins. *Protein Journal* **29**, 432-439.
58. Hasper, H. E., Kramer, N. E., Smith, J. L., Hillman, J. D., Zachariah, C., Kuipers, O. P., de Kruijff, B. & Breukink, E. (2006). An alternative bactericidal mechanism of action for lantibiotic peptides that target lipid II. *Science* **313**, 1636-7.
59. McAuliffe, O., Ryan, M. P., Ross, R. P., Hill, C., Breeuwer, P. & Abee, T. (1998). Lacticin 3147, a broad-spectrum bacteriocin which selectively dissipates the membrane potential. *Applied and Environmental Microbiology* **64**, 439-445.
60. Brotz, H., Bierbaum, G., Reynolds, P. E. & Sahl, H. G. (1997). The lantibiotic mersacidin inhibits peptidoglycan biosynthesis at the level of transglycosylation. *European Journal of Biochemistry* **246**, 193-199.

61. Brotz, H., Bierbaum, G., Leopold, K., Reynolds, P. E. & Sahl, H. G. (1998). The lantibiotic mersacidin inhibits peptidoglycan synthesis by targeting lipid II. *Antimicrobial Agents and Chemotherapy* **42**, 154-160.
62. Bruno, M. E. C. & Montville, T. J. (1993). Common Mechanistic Action of Bacteriocins from Lactic-Acid Bacteria. *Applied and Environmental Microbiology* **59**, 3003-3010.
63. Ramnath, M., Arous, S., Gravesen, A., Hastings, J. W. & Hechard, Y. (2004). Expression of *mptC* of *Listeria monocytogenes* induces sensitivity to class IIa bacteriocins in *Lactococcus lactis*. *Microbiology* **150**, 2663-2668.
64. Gravesen, A., Ramnath, M., Rechinger, K. B., Andersen, N., Jansch, L., Hechard, Y., Hastings, J. W. & Knochel, S. (2002). High-level resistance to class IIa bacteriocins is associated with one general mechanism in *Listeria monocytogenes*. *Microbiology* **148**, 2361-2369.
65. Kjos, M., Nes, I. F. & Diep, D. B. (2009). Class II one-peptide bacteriocins target a phylogenetically defined subgroup of mannose phosphotransferase systems on sensitive cells. *Microbiology-SGM* **155**, 2949-2961.
66. Kjos, M., Salehian, Z., Nes, I. F. & Diep, D. B. (2010). An Extracellular Loop of the Mannose Phosphotransferase System Component IIC Is Responsible for Specific Targeting by Class IIa Bacteriocins. *Journal of Bacteriology* **192**, 5906-5913.
67. Gao, Y. R., Jia, S. R., Gao, Q. & Tan, Z. L. (2010). A novel bacteriocin with a broad inhibitory spectrum produced by *Lactobacillus sake* C2, isolated from traditional Chinese fermented cabbage. *Food Control* **21**, 76-81.
68. Gao, Y. R., Li, D. P., Sheng, Y. & Liu, X. Y. (2011). Mode of action of sakacin C2 against *Escherichia coli*. *Food Control* **22**, 657-661.
69. Zendo, T., Yoneyama, F. & Sonomoto, K. (2010). Lactococcal membrane-permeabilizing antimicrobial peptides. *Applied Microbiology and Biotechnology* **88**, 1-9.
70. Kim, Y. M., An, D. S., Park, H. J., Park, J. M. & Lee, D. S. (2002). Properties of nisin-incorporated polymer coatings as antimicrobial packaging materials. *Packaging Technology and Science* **15**, 247-254.
71. Cha, D. S., Chen, J. R., Park, H. J. & Chinnan, M. S. (2003). Inhibition of *Listeria monocytogenes* in tofu by use of a polyethylene film coated with a cellulosic solution containing nisin. *International Journal of Food Science and Technology* **38**, 499-503.
72. Galvez, A., Abriouel, H., Lopez, R. L. & Ben Omar, N. (2007). Bacteriocin-based strategies for food biopreservation. *International Journal of Food Microbiology* **120**, 51-70.
73. Corr, S. C., Li, Y., Riedel, C. U., O'Toole, P. W., Hill, C. & Gahan, C. G. M. (2007). Bacteriocin production as a mechanism for the antfinfective activity of *Lactobacillus salivarius* UCC118. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 7617-7621.
74. Rea, M. C., Dobson, A., O'Sullivan, O., Crispie, F., Fouhy, F., Cotter, P. D., Shanahan, F., Kiely, B., Hill, C. & Ross, R. P. (2011). Effect of broad- and narrow-spectrum antimicrobials on *Clostridium difficile* and microbial diversity in a model of the distal colon. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 4639-4644.
75. Galvin, M., Hill, C. & Ross, R. P. (1999). Lacticin 3147 displays activity in buffer against Gram-positive bacterial pathogens which appear insensitive in standard plate assays. *Letters in Applied Microbiology* **28**, 355-358.

76. Santagati, M., Scillato, M., Patane, F., Aiello, C. & Stefani, S. (2012). Bacteriocin-producing oral streptococci and inhibition of respiratory pathogens. *FEMS Immunology and Medical Microbiology* **65**, 23-31.
77. Gerbaldo, G. A., Barberis, C., Pascual, L., Dalcero, A. & Barberis, L. (2012). Antifungal activity of two *Lactobacillus* strains with potential probiotic properties. *FEMS Microbiology Letters* **332**, 27-33.
78. Rollema, H. S., Kuipers, O. P., Both, O., Devos, W. M. & Siezen, R. J. (1995). Improvement of solubility and stability of the antimicrobial peptide Nisin by protein engineering. *Applied and Environmental Microbiology* **61**, 2873-2878.
79. Rink, R., Wierenga, J., Kuipers, A., Kluskens, L. D., Driessen, A. J. M., Kuipers, O. P. & Moll, G. N. (2007). Dissection and modulation of the four distinct activities of nisin by mutagenesis of rings A and B and by C-terminal truncation. *Applied and Environmental Microbiology* **73**, 5809-5816.
80. Field, D., Begley, M., O'Connor, P. M., Daly, K. M., Hugenholtz, F., Cotter, P. D., Hill, C. & Ross, R. P. (2012). Bioengineered Nisin A Derivatives with Enhanced Activity against Both Gram Positive and Gram Negative Pathogens. *PloS One* **7**.
81. Kelly, W. J., Asmundson, R. V. & Huang, C. M. (1996). Isolation and characterization of bacteriocin-producing lactic acid bacteria from ready-to-eat food products. *International Journal of Food Microbiology* **33**, 209-218.
82. Stepper, J., Shastri, S., Loo, T. S., Preston, J. C., Novak, P., Man, P., Moore, C. H., Havlicek, V., Patchett, M. L. & Norris, G. E. (2011). Cysteine *S*-glycosylation, a new post-translational modification found in glycopeptide bacteriocins. *FEBS Letters* **585**, 645-650.
83. Erni, B. (2006). The mannose transporter complex: an open door for the macromolecular invasion of bacteria. *Journal of Bacteriology* **188**, 7036-7038.
84. Kjos, M., Nes, I. F. & Diep, D. B. (2011). Mechanisms of Resistance to Bacteriocins Targeting the Mannose Phosphotransferase System. *Applied and Environmental Microbiology* **77**, 3335-3342.
85. Castilla, R., Passeron, S. & Cantore, M. L. (1998). *N*-acetyl-D-glucosamine induces germination in *Candida albicans* through a mechanism sensitive to inhibitors of cAMP-dependent protein kinase. *Cellular signalling* **10**, 713-719.
86. Kumar, M. J., Jamaluddin, M. S., Natarajan, K., Kaur, D. & Datta, A. (2000). The inducible *N*-acetylglucosamine catabolic pathway gene cluster in *Candida albicans*: discrete *N*-acetylglucosamine-inducible factors interact at the promoter of *NAG1*. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 14218-14223.
87. Grigorian, A., Araujo, L., Naidu, N. N., Place, D. J., Choudhury, B. & Demetriou, M. (2011). *N*-acetylglucosamine inhibits T-helper 1 (Th1)/T-helper 17 (Th17) cell responses and treats experimental autoimmune encephalomyelitis. *The Journal of Biological Chemistry* **286**, 40133-40141.
88. Hart, G. W., Slawson, C., Ramirez-Correa, G. & Lagerlof, O. (2011). Cross talk between *O*-GlcNAcylation and phosphorylation: roles in signaling, transcription, and chronic disease. *Annual Review of Biochemistry* **80**, 825-858.
89. Rexach, J. E., Rogers, C. J., Yu, S. H., Tao, J., Sun, Y. E. & Hsieh-Wilson, L. C. (2010). Quantification of *O*-glycosylation stoichiometry and dynamics using resolvable mass tags. *Nature Chemical Biology* **6**, 645-651.
90. Calcagno, M., Campos, P. J., Mulliert, G. & Suastegui, J. (1984). Purification, molecular and kinetic properties of glucosamine-6-phosphate isomerase (deaminase) from *Escherichia coli*. *Biochimica et Biophysica Acta* **787**, 165-173.

91. Anderson, J. S., Matsuhashi, M., Haskin, M. A. & Strominger, J. L. (1967). Biosynthesis of the Peptidoglycan of Bacterial Cell Walls: II. PHOSPHOLIPID CARRIERS IN THE REACTION SEQUENCE. *Journal of Biological Chemistry* **242**, 3180-3190.
92. Dutka-Malen, S., Mazodier, P. & Badet, B. (1988). Molecular cloning and overexpression of the glucosamine synthetase gene from *Escherichia coli*. *Biochimie* **70**, 287-290.
93. Mengin-Lecreulx, D. & van Heijenoort, J. (1996). Characterization of the essential gene *glmM* encoding phosphoglucosamine mutase in *Escherichia coli*. *The Journal of Biological Chemistry* **271**, 32-39.
94. Mengin-Lecreulx, D. & van Heijenoort, J. (1994). Copurification of glucosamine-1-phosphate acetyltransferase and *N*-acetylglucosamine-1-phosphate uridyltransferase activities of *Escherichia coli*: characterization of the *glmU* gene product as a bifunctional enzyme catalyzing two subsequent steps in the pathway for UDP-*N*-acetylglucosamine synthesis. *Journal of Bacteriology* **176**, 5788-5795.
95. Plumbridge, J. (1995). Coordinated Regulation of Amino Sugar Biosynthesis and Degradation - the Nagc Repressor Acts as Both an Activator and a Repressor for the Transcription of the *Glmus* Operon and Requires 2 Separated Nagc Binding-Sites. *The EMBO Journal* **14**, 3958-3965.
96. Konopka, J. B. (2012). *N*-Acetylglucosamine Functions in Cell Signaling. *Scientifica* **2012**, 15.
97. Paik, S. H., Chakicherla, A. & Hansen, J. N. (1998). Identification and characterization of the structural and transporter genes for, and the chemical and biological properties of, sublancin 168, a novel lantibiotic produced by *Bacillus subtilis* 168. *Journal of Biological Chemistry* **273**, 23134-23142.
98. Oman, T. J., Boettcher, J. M., Wang, H. A., Okalibe, X. N. & van der Donk, W. A. (2011). Sublancin is not a lantibiotic but an *S*-linked glycopeptide. *Nature Chemical Biology* **7**, 78-80.
99. Wang, H. & van der Donk, W. A. (2011). Substrate selectivity of the sublancin S-glycosyltransferase. *Journal of the American Chemical Society* **133**, 16394-7.
100. Tagg, J. R. & McGiven, A. R. (1971). Assay system for bacteriocins. *Applied Microbiology* **21**, 943.
101. Kelly, W. J., Asmundson, R. V. & Huang, C. M. (1996). Characterization of plantaricin KW30, a bacteriocin produced by *Lactobacillus plantarum*. *Journal of Applied Bacteriology* **81**, 657-662.
102. Patchett, M. L. (2013). Conversation on logic behind PTS choices, GccF and GlcNAc. (Kerr, A., ed.), Palmerston North.
103. Felek, S., Muszynski, A., Carlson, R. W., Tsang, T. M., Hinnebusch, B. J. & Krukonis, E. S. (2010). Phosphoglucomutase of Yersinia pestis is required for autoaggregation and polymyxin B resistance. *Infection and mmunity* **78**, 1163-1175.
104. Brotz, H. & Sahl, H. G. (2000). New insights into the mechanism of action of lantibiotics--diverse biological effects by binding to the same molecular target. *The Journal of Antimicrobial Chemotherapy* **46**, 1-6.
105. Chung, H. J., Montville, T. J. & Chikindas, M. L. (2000). Nisin depletes ATP and proton motive force in mycobacteria. *Letters in Applied Microbiology* **31**, 416-420.
106. Breukink, E., Wiedemann, I., van Kraaij, C., Kuipers, O. P., Sahl, H. G. & de Kruijff, B. (1999). Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic. *Science* **286**, 2361-2364.

107. Brotz, H., Josten, M., Wiedemann, I., Schneider, U., Gotz, F., Bierbaum, G. & Sahl, H. G. (1998). Role of lipid-bound peptidoglycan precursors in the formation of pores by nisin, epidermin and other lantibiotics. *Molecular Microbiology* **30**, 317-327.
108. Breukink, E., van Heusden, H. E., Vollmerhaus, P. J., Swiezewska, E., Brunner, L., Walker, S., Heck, A. J. & de Kruijff, B. (2003). Lipid II is an intrinsic component of the pore induced by nisin in bacterial membranes. *The Journal of Biological Chemistry* **278**, 19898-19903.
109. Winkowski, K., Bruno, M. E. & Montville, T. J. (1994). Correlation of bioenergetic parameters with cell death in *Listeria monocytogenes* cells exposed to nisin. *Applied and Environmental Microbiology* **60**, 4186-4188.
110. Waite, B. L., Siragusa, G. R. & Hutkins, R. W. (1998). Bacteriocin inhibition of two glucose transport systems in *Listeria monocytogenes*. *Journal of Applied Microbiology* **84**, 715-721.
111. Gao, Y., van Belkum, M. J. & Stiles, M. E. (1999). The outer membrane of Gram-negative bacteria inhibits antibacterial activity of brochocin-C. *Applied and Environmental Microbiology* **65**, 4329-4333.
112. Ananou, S., Galvez, A., Martinez-Bueno, M., Maqueda, M. & Valdivia, E. (2005). Synergistic effect of enterocin AS-48 in combination with outer membrane permeabilizing treatments against *Escherichia coli* O157:H7. *Journal of Applied Microbiology* **99**, 1364-1372.
113. Pamp, S. J., Gjermansen, M., Johansen, H. K. & Tolker-Nielsen, T. (2008). Tolerance to the antimicrobial peptide colistin in *Pseudomonas aeruginosa* biofilms is linked to metabolically active cells, and depends on the *pmr* and *mexAB-oprM* genes. *Molecular Microbiology* **68**, 223-240.
114. Opalka, N., Beckmann, R., Boisset, N., Simon, M. N., Russel, M. & Darst, S. A. (2003). Structure of the filamentous phage pIV multimer by cryo-electron microscopy. *Journal of Molecular Biology* **325**, 461-470.
115. Spagnuolo, J., Opalka, N., Wen, W. X., Gagic, D., Chabaud, E., Bellini, P., Bennett, M. D., Norris, G. E., Darst, S. A., Russel, M. & Rakonjac, J. (2010). Identification of the gate regions in the primary structure of the secretin pIV. *Molecular Microbiology* **76**, 133-150.
116. Daefler, S., Guilvout, I., Hardie, K. R., Pugsley, A. P. & Russel, M. (1997). The C-terminal domain of the secretin PulD contains the binding site for its cognate chaperone, PulS, and confers PulS dependence on pIVf1 function. *Molecular Microbiology* **24**, 465-475.
117. Nouwen, N., Ranson, N., Saibil, H., Wolpensinger, B., Engel, A., Ghazi, A. & Pugsley, A. P. (1999). Secretin PulD: association with pilot PulS, structure, and ion-conducting channel formation. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 8173-8177.
118. Whitaker, R. (2012). Analysis of gate residues in the type 2 secretin, PulD, Massey University.
119. Guilvout, I., Chami, M., Engel, A., Pugsley, A. P. & Bayan, N. (2006). Bacterial outer membrane secretin PulD assembles and inserts into the inner membrane in the absence of its pilotin. *The EMBO Journal* **25**, 5241-52419.
120. Morgan, W. T. & Elson, L. A. (1934). A colorimetric method for the determination of *N*-acetylglucosamine and *N*-acetylchrondrosamine. *The Biochemical Journal* **28**, 988-895.
121. Eron, L. & Block, R. (1971). Mechanism of initiation and repression of in vitro transcription of the *lac* operon of *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* **68**, 1828-1832.

122. Paulsen, I. T., Banerjei, L., Myers, G. S., Nelson, K. E., Seshadri, R., Read, T. D., Fouts, D. E., Eisen, J. A., Gill, S. R., Heidelberg, J. F., Tettelin, H., Dodson, R. J., Umayam, L., Brinkac, L., Beanan, M., Daugherty, S., DeBoy, R. T., Durkin, S., Kolonay, J., Madupu, R., Nelson, W., Vamathevan, J., Tran, B., Upton, J., Hansen, T., Shetty, J., Khouri, H., Utterback, T., Radune, D., Ketchum, K. A., Dougherty, B. A. & Fraser, C. M. (2003). Role of mobile DNA in the evolution of vancomycin-resistant *Enterococcus faecalis*. *Science* **299**, 2071-4.

123. Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K. A., Tomita, M., Wanner, B. L., and Mori, H. (2006) Construction of *Escherichia coli* K-12 inframe, single-gene knockout mutants: the Keio collection. *Molecular Systems Biology* **2**, 2006.0008.

124. Linderoth, N. A., Simon, M. N. & Russel, M. (1997). The filamentous phage pIV multimer visualized by scanning transmission electron microscopy. *Science* **278**, 1635-1638.

125. Daefler, S., Russel, M. & Model, P. (1997). Module swaps between related translocator proteins pIV(f1), pIV(IKe) and PulD: identification of a specificity domain. *Journal of Molecular Biology* **266**, 978-992.