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Isolation of environmental *Bacillus* spp. encoding bacteriocins with novel anti-*C. difficile* activity

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Declaration

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Unless otherwise stated, the work presented in this thesis is my own. No part has been submitted for another degree at The University of Nottingham or any other institute.

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Abstract

The emergence of antibiotic resistant, hypervirulent strains of the nosocomial pathogen *Clostridioides difficile*, coupled with increased concerns over the physiological impact of traditional broad-spectrum antibiotics on human health has led to an increased demand for alternative therapies. Bacteriocins are proteinaceous toxins produced by bacteria that kill other bacteria and their antimicrobial activity makes them an attractive potential alternative therapy to antibiotics. This study isolated two members of the *B. subtilis* species complex from porcine faeces that displayed potent anti-*C. difficile* activity against a range of *C. difficile* ribotypes: *B. velezensis* B18 and *B subtilis* 50B. Genome mining of these strains highlighted their incredible antimicrobial potential with them encoding nine and seven antimicrobial biosynthetic clusters respectively. Of these highlighted antimicrobials, multiple bacteriocins were thought to be responsible for the anti-*C. difficile* activity seen from the strains. Attempts to produce recombinant candidate bacteriocins highlighted a key issue in bacteriocin research: the lack of dedicated recombinant bacteriocin production workhorses. Part purification and characterisation of the anti-*C. difficile* compounds from the strains tentatively implies a novel activity against *C. difficile* from the lantibiotic amyloliquecidin, in addition to revealing a novel non-functional subunit that could be used to generate a semi-synthetic lantibiotic. Additionally, this study suggests a novel anti-*C. difficile* activity of the sactibiotic subtilosin A, which has never been described before. In addition to potentially highlighting the presence of other anti-*C. difficile* peptides produced by *B. subtilis* 50B overlooked by *in silico* mining methods.

Chapter 1 Introduction

1.1 Clostridioides difficile

1.1.1 *C. difficile* background and significance

Clostridioides difficile (formerly *Clostridium difficile*) is an anaerobic, spore forming, Gram-positive staining bacterium, which is associated naturally with the soil environment and also as a constituent of the normal human intestinal microflora for roughly 5% of the adult population (Lawson *et al*., 2016; Kachrimanidou and Malisiovas, 2011). However, since the 1970's *C. difficile* has become known as a prevalent cause of antibiotic associated disease and nosocomial infection (Bartlett, 2006). The economic burden of *C. difficile* infection (CDI) is high, with an estimated 3-billion-euro total cost accrued each year throughout Europe due to hospital acquired CDI (Reigadas-Ramírez and Bouza, 2018). Additionally, acquiring CDI during a hospital stay is associated with a higher risk of patient mortality (Enoch *et al*., 2020).

As stated, CDI is strongly promoted, particularly in the hospital environment, by the use of broad-spectrum antibiotics such as clindamycin, cephalosporins and fluoroquinolones (Bartlett, 2008). These antibiotics have a devastating effect on the intestinal microflora and result in gut dysbiosis, which leaves the host vulnerable to *C. difficile* colonisation and disease (Bartlett, 2006). The healthy microflora is thought to protect the host from pathogen invasion in a number of ways, including physical blocking of adherence sites, deprivation of nutrients and the production of antimicrobials (Chittim, Irwin and Balskus, 2018; Ferrer *et al*., 2017). Elimination of the protective microflora allows the opportunity for *C. difficile* and other opportunistic pathogens to establish. Once established *C. difficile* can then be carried asymptomatically or it causes symptomatic disease (Bagdasarian, Rao and Malani, 2015).

1.1.2 *C. difficile* pathogenesis

Once a strain of *C. difficile* has the opportunity to colonize the host, pathogenesis depends on the production of *C. difficile* toxins. The structure and mode of action of the *C. difficile* toxins were comprehensively reviewed by Papatheodorou *et al*., (2018). Toxin A (TcdA) and Toxin B (TcdB) are the primary virulence factors of toxigenic *C. difficile*. These toxins are large multi-domain protein toxins with a high degree of identity (50% amino acid sequence identity), reflecting their origin as a gene duplication event (Von Eichel Streiber *et al.,* 1992). Both toxins are secreted by *C. difficile,* following this they are internalised by the gut epithelial cells via receptor mediated endocytosis, wherein their enzymatic domains inactivate proteins essential in maintaining cellular cytoskeleton (Dingle *et al*.,2008). The intoxicated cells then die, and this results in loss of control of the intestinal epithelial barrier. Resulting presentation of CDI is outlined in Table 1.1, however, presentation is highly variable based on a myriad of factors and can vary from mild diarrhoea to severe complicated pseuodomembranous colitis, toxic megacolon and as mentioned previously, mortality (Martin, Monaghan and Wilcox, 2016).

CDI is further complicated by the persistence of *C. difficile* spores in the gut that can lead to recurrence of infection (Bagdasarian, Rao and Malani, 2015). Recurrence of CDI infection can occur in up to 20% - 30% of patients, with spore persistence being one of many factors that increase the risk of recurrence such as advanced age, gastric acid suppression and infection with hypervirulent strains of *C. difficile* (Song and Kim, 2019; Cole and Stahl, 2015). Throughout the early 2000's there was a sharp increase globally in the number of patients diagnosed with CDI, combined with an increase in infection recurrence and mortality rate, which increased the notoriety of *C. difficile* (Freeman *et al*., 2010). These increases were eventually attributed to the emergence of a hypervirulent strain of *C. difficile*, termed *C. difficile* B1/NAP1/027. The increased virulence of this ribotype was characterised by an increase in Toxin A and B production, the production of a third binary toxin and a marked increase in metronidazole resistance (Freeman *et al*., 2010). Since then, *C. difficile* B1/NAP1/027 strains have remained a significant health concern, in addition to the emergence of other clinically relevant ribotypes (Couturier *et al*., 2018).

Table 1.1: The categorization of CDI based on clinical presentation, the associated predisposing risk factors and the primary recommended treatment options. Recommended first line treatment is highlighted in bold. Based on Fitzpatrick *et al*., (2018) and Bagdasarian *et al*., (2015).

CDI Category	Associated risks	Clinical presentation	Treatment Options
Mild to Moderate	Antibiotic use, hospitalisation, immunosuppression, and chronic disease.	Diarrhoea without systemic infection	**500 mg Oral Metronidazole**
			125 mg oral vancomycin
			200 mg Fidaxomicin
Severe – Complicated	Advanced age, hypervirulent *C. difficile* ribotype and history of inflammatory bowel disease.	Diarrhoea with systemic infection including megacolon and pseudomembranous colitis.	**125 – 500 mg oral vancomycin**
			200 mg fidaxomicin
			500 mg intravenous metronidazole
			Surgery
Recurrent	Advanced age, antibiotic use, immunosuppression, chronic illness and severe initial infection	Presentation of symptoms following successful treatment of CDI	**200 mg Fidaxomicin**
			125 mg oral vancomycin
			500 mg oral Metronidazole
			Faecal Microbiota Transplantation (**FMT)**

1.1.3 Current therapies against *C. difficile*

Since the 1980's, the antibiotics metronidazole and vancomycin have been the treatments of choice against CDI (Bartlett, 2006). Due to its low cost, metronidazole is the recommended first line of treatment for mild to moderate CDI, with three, 500 mg doses taken orally for 10 – 14 days (Bagdasarian *et al*., 2015). For the treatment of severe/complicated CDI, vancomycin is the recommended antibiotic with 125 mg administered orally four times a day for 10 – 14 days, although increased dosages can be recommended (Fitzpatrick *et al*., 2018).

Also recommended for complicated CDI is surgical intervention, including complete colectomy, although less invasive surgical options are also available (Kelly *et al*, 2019). In 2011, the antibiotic fidaxomicin was approved as a treatment for CDI (Duggan, 2011), and it shows similar cure rates to vancomycin (Louie *et al*., 2011). However, despite its efficacy, the high cost of fidaxomicin means it is only recommended for recurrent CDI where other treatment options have failed or as a treatment for initial CDI where there is a high risk of severe complicated CDI (Fitzpatrick *et al*., 2018). Recurrent CDI remains a major hindrance in its successful treatment, data on recurrence rates is variable and it is difficult to predict recurrence due to a myriad of risk factors associated with the individual patients and *C. difficile* ribotypes themselves, however, an initial recurrence can increase the likelihood of subsequent recurrences to 45% (Fitzpatrick *et al*., 2018; Johnson, 2009). Additionally, recurrent CDI increases the risks for complicated CDI and is associated with increased mortality compared to non-recurrent CDI (Enoch *et al*., 2020).

1.1.4 Issues with current therapies

In addition to the risk of CDI recurrence, there are other issues that can arise in the use of traditional antibiotic therapies - primarily antibiotic resistance. As an antibiotic associated pathogen, resistance to clindamycin, chloramphenicol and fluoroquinolones are competitively advantageous to *C. difficile* and most isolates are not susceptible to these antibiotics (Spigaglia, Mastrantonio and Barbant, 2018). Of the antibiotics recommended for *C. difficile* treatment, metronidazole resistant strains of *C. difficile* have been reported since the 1980's, and more recently *C. difficile* B1/NAP1/027 strains have a marked increase in resistance to metronidazole, as have clinically associated ribotype 078 strains (Freeman *et al*., 2010). The most recent recommended treatment for *C. difficile*, fidaxomicin, has only one documented case of a resistant strain of *C. difficile* (Sholeh *et al*., 2020).

Most worrying is the emergence of strains of *C. difficile* in recent years that have a marked decrease in susceptibility to vancomycin, although these strains are not clinically resistant, the long-term health implications of these reduced susceptibilities is yet to be fully understood (Spigaglia, Mastrantonio and Barbant, 2018). An additional issue with the use of vancomycin, especially repetitive use treating recurrent infections, is its contribution to the emergence of vancomycin resistant *Enterococcus spp.* – another important nosocomial infection (Guzman Prieto *et al*., 2016).

In addition to this, there are concerns about the impact of use of antibiotics on human health. A study by Vrieze *et al*, (2014) showed that treatment with vancomycin for 1 week drastically reduced the diversity of the Gram-positive intestinal microbiota. These Gram-positive bacteria play a significant role in the transformation of primary bile acids to secondary bile acids, which have been shown to be essential in the maintenance of glucose homeostasis, particularly, the sensitivity of certain receptors for insulin (Vrieze *et al*., 2014). Therefore, showing even a short course of vancomycin can impact not only the intestinal microbiota, leading to increased susceptibility to pathogens, but also alter the composition of bile salts and reduce sensitivity to insulin. This evidence was corroborated in a population study by Mikkelsen *et al*., (2015), which identified those who take

multiple courses of antibiotics throughout their lives had a higher likelihood of developing type- 2 diabetes. The reduction in efficacy of the current arsenal of antibiotics and the impact antibiotic exposure has on human health means that future alternative therapies must always be considered.

1.1.5 Alternative therapies against CDI

As the predisposing factor for CDI is the depletion of the natural microbiota, faecal microbiota transplantation (FMT) is a current alternative to antibiotic treatment. FMT involves a homogenised faecal sample from a donor with a healthy microbiota being transplanted into the gastrointestinal tract of a patient with CDI; the rationale is to replace the depleted healthy microflora, which can then outcompete *C. difficile* (Rineh and Kelso, 2014). A lack of regulation of methods and often small sample sizes in FMT studies are a major drawback of the data collected. However, FMT has been proven to be efficacious against CDI and in some cases 90% of patients were successfully treated with only a small number experiencing recurrence of infection (Johnson, 2009). Although, more recent literature presents a highly variable outcome of CDI resolution following FMT, with success rates of 40-90% reported (Fitzpatrick *et al*., 2018).

A major drawback of FMT is the composition of the donor microbiota itself, with not all constituents being beneficial and some can even have a directly negative effect on the recipient. Thus, before the faecal sample can be administered it must be screened for pathogens and despite rigorous screening processes, infective complications are still a risk, which is of particular concern for immunocompromised patients, who are most at risk of recurrent CDI (Di Bella, Gouliouris and Petrosillo, 2015). The healthy microbiota inhibits pathogenic microorganisms through physically occupying adherence sites, outcompeting the pathogens for nutrients and directly inhibiting pathogens through the production of antimicrobial molecules (Willing, Russell and Finlay, 2011).

An alternative to FMT whilst utilising the same principles is the use of probiotic therapy, in which a specific cocktail of beneficial bacteria are utilised to prevent *C. difficile* colonisation and persistence in the ileum, although there is little *in vivo* data

available for their efficacy (Valdés-Varela, Gueimonde, & Ruas-Madiedo, 2018). As with FMT a major drawback of probiotic therapy is the bacteria themselves, as many commensals can in turn be opportunistic pathogens to an immunocompromised host. Additionally, some "beneficial bacteria" that have been considered for use as anti-C. difficile probiotics can have unintentionally negative effects on the host such as *Clostridia scindens* that has the unintended negative effect of increasing the hosts risk of colorectal cancer (Wolf *et al*., 2020) Isolating inhibitory molecules often produced by probiotic bacteria and using them in a purified state as therapeutics would likely be safer than the introduction of potentially infective organisms into immunocompromised hosts.

Prophylactic vaccination is another alternative to traditional antibiotics against *C. difficile* that has been extensively studied. Vaccination functions by introducing components of the target bacteria (antigens) into the susceptible host to stimulate a host immune response with the aim of achieving long term antibody-based immunity (Bruxelle, Pechine and Collignon, 2018). As toxigenic *C. difficile's* primary virulence factor is the production of the toxins TcdA and TcdB, vaccine research and development has been particularly focussed on the *C. difficile* toxins as the target antigen (Bruxelle, Pechine and Collignon, 2018). Two toxin-based intramuscular vaccines against *C. difficile*, developed by Sanofi Pasteur and Pfizer, had proven promising at preventing CDI in early clinical trials. However, both vaccines recently failed Phase III of clinical trials when it was found that neither vaccine provided protection against primary CDI in the target group of individuals aged 50+ (de Bruyn *et al*., 2021; and Pfizer). As the intramuscular toxin-based vaccines against *C. difficile* have been shown to not be efficacious in the target demographic, other antigen targets from *C. difficile* and alternative vaccine delivery models are now being explored. A recent study by Hughes *et al*., (2022) highlighted successful immunization of hamster models against *C. difficile* adherence utilising oral delivery of a recombinant spore vaccine. This study, however, has not yet entered human clinical trials and the efficacy and effect of the vaccine in the target demographic is untested. As can be seen from the Sanofi Pasteur and Pfizer vaccines, promising early-stage

results do not equal an effective vaccine and so other alternative therapies still need to be explored in the treatment of *C. difficile*.

1.2 Bacteriocins: Potential alternatives to traditional antibiotics

One group of antimicrobial molecules that have the potential to be exploited as alternative antibiotics in the treatment of pathogens including *C. difficile* are the bacteriocins, particularly the bacteriocins of Gram-positive bacteria. Bacteriocins are a diverse group of ribosomally synthesised proteinaceous toxins produced by bacteria that are active against other closely related bacteria and against which the producer has a specific immunity to (Cotter, Ross and Hill, 2013). Bacteriocins have many properties that make them an attractive alternative to antibiotics. Primarily, bacteriocins are generally low toxicity and are safe for human consumption, for example the bacteriocins of Lactic Acid Bacteria (LAB) have an extensive history of use as preservatives in the meat and milk products against food spoilage organisms (Cleveland *et al.,* 2001). In addition to this an estimated 99% of bacteria, and even some archaea, produce at least one bacteriocin (Klaenhammer *et al.*, 1993). Given the extensive diversity in microbial communities this equates to a vast arsenal of undiscovered antimicrobial peptides that can be exploited as alternatives to antibiotics. Furthermore, many bacteriocins have a highly specific, narrow-spectrum of activity that is advantageous over traditional antibiotics when treating infection for two reasons. Firstly, traditional broad-spectrum antibiotics are indiscriminate in their killing capabilities, this has a devastating effect on the host's natural commensal bacteria with many negative health impacts (Jernberg *et al*., 2010). Secondly, broad-spectrum antibiotics exert a strong selection pressure on all organisms exposed to them and facilitates the emergence of resistant strains (Riley and Amherst, 2009). These resistant strains can then pass on the antibiotic resistance to other strains and species, including pathogens, through methods of horizontal genetic exchange, including conjugation, which involves the exchange of DNA through plasmids (Maiden, 1998). Finally, the proteinaceous nature of bacteriocins means they can be manipulated by bioengineering to a greater degree than traditional antibiotics, which in some cases leads to a greater killing capacity than the original peptide (Cotter, Ross and Hill, 2013).

1.3 Historical and current classification schemes of bacteriocins

The term bacteriocin was first used in 1953 to describe the colicins produced by the Gram-negative *Escherichia coli*, in the almost 70 years that have followed bacteriocins have been discovered in many species, including Gram-positives, and their diversity as a group has become more apparent. The bacteriocins of Gram-positive bacteria are of particular interest when searching for anti-*C. difficile* bacteriocins, as the bacteriocins of Gram-negative bacteria are generally strictly active against other members of the same species (Riley and Wertz, 2009). Klaenhammer proposed the initial classification scheme of Gram-positive bacteriocins, based on their sequence, biosynthesis, and size, which is outlined in Table 1.2.

Table 1.2: Original Klaenhammer classification scheme for Gram-positive bacteriocins, as summarised by Rea *et al*., (2011).

Class	Title	Features
I	Post translationally modified bacteriocins	Lanthionine/β-methyl lanthionine
II	Unmodified peptides	Small, heat stable peptides
III	Unmodified proteins	Large, heat labile enzymes
IV	Complex proteins	Lipid or carbohydrate moiety-containing proteins

Since Klaenhammer's initial proposed classification there have been many attempts to classify bacteriocins based on their biosynthesis or their mechanisms of action, retaining and expanding where need be. Modern bacteriocin classification schemes, however, omit the Class IV bacteriocins as no such bacteriocins have been described (Cotter, Hill and Ross 2005). Rea *et al*., reviewed the modern classification schemes of LAB in 2011 and proposed a classification scheme wherein, Class I bacteriocins were divided into the lantibiotics, the sactibiotics and the labyrinthopeptides; and Class II bacteriocins were subdivided into the pediocin-like bacteriocins, the two component bacteriocins, the head to tail cyclised bacteriocins and the linear peptides. They also proposed that the Class III bacteriocins be renamed the

bacteriolysins as a reflection of their enzymatic lytic activity (Rea *et al*., 2011). Further modifications to this scheme were proposed in 2013 to reflect increased understanding of the biosynthetic processes leading to mature bacteriocins and ribosomally synthesised, post translationally modified peptides (RiPPs) in general (Cotter *et al*., 2013; Arnison *et al*., 2013). The most recent classification scheme that is generally accepted is detailed below, with a summary shown in Table 1.3 (Alvarez-seirro *et al*., 2016). This scheme is based solely on the biosynthetic processes to achieve mature bacteriocins and is in adherence to Arnison's classification of RiPPs.

1.4 Class 1: Small, post-translationally modified peptides

1.4.1 Class Ia: The Lantibiotics

The subclass Ia bacteriocins – the lantibiotics – are bacteriocins belonging to the wider class of RiPPs known as the lanthipeptides. Lanthipeptides are characterised by the incorporation of the unusual amino acids lanthionine (Lan) and β-methyllanthionine (MeLan) in their structures (Alvarez-seirro *et al*., 2016). These unusual amino acids are formed from the dehydration of serine and threonine residues to form dehydroalanine (Dha) or dehydrobutyrine (Dhb) respectively, a subsequent addition of cysteine thiols results in the formation of the Lan/ MeLan and a cyclic shape (Montalbon-Lopez *et al.,* 2020). The enzymes involved in the dehydration and cyclization reaction form the basis of classification schemes for the lanthipeptides, however, based on these classifications only type I and type II can be classed as lantibiotic bacteriocins due to their antimicrobial activity (Arnison *et al*., 2013).

The subclass I lantibiotics are produced as an inactive prepeptide that is subsequently dehydrated in an initial reaction by LanB dehydratase enzymes, following this a cyclisation reaction catalysed by LanC occurs (Montalbon-Lopez *et al.,* 2020). The genes for these dedicated enzymes are present in the same operon as the structural genes (Figure 1.1), in addition to genes encoding for a serine protease required for leader cleavage (LanP), ABC-transport related proteins (LanT) and immunity related proteins (Cheigh and Pyun, 2005). The type I lantibiotic nisin, produced by many strains of *Lactococcus*, has a long history as a commercial anti-spoilage agent and is

thus the best characterised of the lantibiotics (Younes *et al*., 2017). Whilst four distinct variants of nisin have been described, their similarity in sequence and activity generally means that nisin is used generically (Piper *et al*., 2009). Whilst nisin has historically been used in the past as anti-spoilage agent, it has garnered significant attention due to its inhibitory activity against nosocomial pathogens including *C. difficile (*Egan *et al.,* 2016; Shin *et al*., 2016). Nisin is relatively broad spectrum with activity against many Gram-positive bacteria although activity against Gram-negative bacteria has been reported (Stevens *et al*., 1991). As the best characterised of the Type I lantibiotics, the mode of action of nisin is relatively well understood. The mature nisin peptide binds to and sequesters the cell wall precursor, Lipid II, in target bacteria, inhibiting cell-wall synthesis. Binding to lipid II also facilitates pore formation in the target wall by nisin, allowing leakage of key intracellular components and cell death (Piper *et al*., 2009). Lipid II is also the target for another type I lantibiotic, epidermin – originally produced by *Staphylococcus epidermidis* (Götz *et al.,* 2014). However, unlike nisin, epidermin has a relatively narrow spectrum of activity with only the lipid II of certain species targeted (Brötz *et al*., 1998). Production of mature nisin also stimulates the producing bacteria to produce proteins associated with host immunity to nisin. These proteins (NisIEFG) are thought to act synergistically to protect the host from nisin, although the exact mechanism remains unclear (Draper *et al.,* 2008).

Type II lantibiotics are classified in accordance with their dedicated LanM synthetase enzyme, which carries out the dual functions of dehydration and cyclisation to produce the mature lantibiotic (Arnison *et al*., 2013). The structural protein and the LanM synthetase are encoded on the same operon, in addition to immunity associated proteins and ABC-transport related proteins required for secretion (Figure 1.1; Cotter, Hill and Ross 2005). Interestingly, many of the Type II lantibiotic bacteriocins encode two structural prepeptides, which are each processed to maturity by a dedicated LanM enzyme and subsequently act in a synergistic manner against their target cells (Mcauliffe *et al.,* 1998; Begley *et al.*, 2009; Lawton *et al.*, 2007). The mode of action of the Type II lantibiotic, lacticin 3147 that was originally isolated from *L. lactis* DPC3147 has been studied in detail (Mcauliffe *et al.,* 1998).

Lacticin 3157 is comprised of two subunit peptides, Ltnα and Ltnβ, which act in concert to depolarise the cytoplasmic membrane of a sensitive cell through pore formation and an efflux of potassium ions and phosphates (Mcauliffe *et al*., 1998). This is hypothesised to occur in a manner similar to nisin, in which Ltnα binds to Lipid II sequestering it and inhibiting cell wall synthesis. This binding to lipid II then induces a conformational change in Ltnα, allowing Ltnβ to bind to lipid II resulting in the formation of a transmembrane pore (Wiedemann *et al*., 2006). Haloduracin is another Type II lantibiotic, also comprised of two peptides, and early studies suggest that it functions in identical manner to lacticin 3147 (Lawton *et al*, 2007; Oman and Van Der Donk, 2009).

1.4.2 Class Ib: The head to tail cyclised bacteriocins

The head to tail cyclised bacteriocins are cyclic bacteriocins that derive their circular shape from a head to tail cyclisation of their N and C-terminal ends and should not be confused with other cyclic bacteriocins such as the sactibiotics (Perez, Zendo and Somomot, 2018). They are an ever-growing class of bacteriocins, of which enterocin AS-48 is the best characterised (Galvez *et al.,* 1991). Unlike other bacteriocins there is little sequence conservation between the members of the class and the only structurally uniform feature is the presence of a saposin fold in the mature peptide that confers stability to the bacteriocin from thermal, pH or degradative protease stress (Perez, Zendo and Somomot, 2018).

The genetic locus of many characterised circular bacteriocins has been identified and these dedicated operons always contain the prepeptide, ABC-transporter related proteins, immunity associated peptides and a SpoIIM family domain protein, and it generally appears that most if not all of these proteins are essential for biosynthesis of mature circular bacteriocins (Figure 1.1; Scholz *et al.,* 2014, Galvez *et al*., 1991). Despite the characterisation of the genetic determinants and mature peptide of circular bacteriocins, the process by which the prebacteriocin is processed into the mature, bioactive form is still a relative mystery. Some theorise that not all of the genes essential for bacteriocin processing are present in the dedicated operon and that another protein encoded elsewhere in the genome is also responsible. This is due to the difficulty in expressing some circular bacteriocins in heterologous hosts

genetically distinct from the original producing strains (Fernández *et al*., 2007). However, a more likely theory is that the accessory proteins of the circular bacteriocin biosynthetic operons are all required for correct processing and form a membrane associated protein complex that carries out leader cleavage, circularisation and secretion in a combined process, thus expressing only selected genes in heterologous hosts would result in poor recombinant protein production (Perez, Zendo and Somomoto, 2018).

The mode of action of circular bacteriocins is generally accepted to be through pore formation on the target cell, however the exact mechanisms of this is not particularly well understood. The circular bacteriocins gassericin A and enterocin AS-48, induce cell death by creating pores in the target cell in a receptor independent manner, whereas Garvicin ML induces cell death by forming pores following binding to a maltose binding surface protein (Kawai *et al*., 2004; Cebrian *et al.,* 2014). It is therefore likely that there is no unifying mechanism of action of circular bacteriocins, beyond the formation of pores in the target cell membrane.

1.4.3 Class Ic: The Sactibiotics

Sactipeptides are a class of RiPPs that contain characteristic cysteine sulphur to α-carbon thioether (sactionine) linkages resulting in a cyclic, stable mature peptide. Sactipeptides that have antimicrobial activity are classified as bacteriocins and termed the sactibiotics (Mathur and Rea, 2019). Subtilosin A, first isolated from *Bacillus subtilis* 168, is the best characterised of the sactibiotics. Although since its discovery many other promising sactibiotics have been identified, including thuricin CD a potent anti-clostridial, two component sactibiotic (Rea *et al.,* 2010). The biosynthetic machinery for sactibiotics is generally found in dedicated operons and Figure 1.1 highlights the organisation of the operon for subtilosin A production (Zheng *et al*., 1999). These operons generally contain the structural prebacteriocin gene, although as mentioned thuricin CD contains two structural genes; whereas ruminococcin C, a recently discovered sactibiotic from *Ruminococcus garvis*, encodes five prebacteriocin genes, although only two have been shown to have antimicrobial activity experimentally (Balty *et al.,* 2019). Also, present in dedicated sactibiotic operons are radical S-adenosyl-L-methioinine (SAM) enzymes also referred to as

sactisynthases, these enzymes catalyse the formation of the sactionine linkages in mature sactibiotics and are essential for function (Himes *et al.*, 2016; Grove *et al.*, 2017).

In addition to the structural gene/s and their respective SAM enzymes, the biosynthetic clusters also generally contain genes encoding for ABC-transport related proteins and dedicated immunity proteins that protect the host from the produced bacteriocin's antimicrobial activity (Zheng *et al.*, 1999; Rea *et al*., 2010). Mathur *et al.,* 2015 studied the immunity proteins of the sactibiotic thuricin CD in detail. They found that TrnI, a small membrane associated peptide with two transmembrane domains, conferred complete immunity to thuricin CD when heterologously expressed in *L. monocytogenes*. They hypothesised that TrnI functions by sequestering thuricin CD or physically blocking thuricin CD from binding to the producer cell wall (Mathur *et al*., 2015). Additionally, it was established that TrnI did not confer immunity to *L. monocytogenes* against the other sactibiotics, highlighting that immunity is highly specific, with cross immunity a rare occurrence (Mathur *et al*., 2015).

The inhibitory spectrums of sactibiotic bacteriocins are diverse amongst the class with thuricin CD being considered as a treatment for CDI due to its extremely narrow inhibitory activity against *Clostridia* and related *Bacillus* spp. (Rea *et al*., 2010). Conversely, subtilosin A has a broad spectrum of activity affecting a range of Gram-positive bacteria and uncapsulated Gram-negative bacteria (Shelburne *et al.,* 2007). The differences in ranges of activity of sactibiotics suggests that they have varying mechanisms of action, it is hypothesised that the subunits of thuricin CD act synergistically to depolarise the target cell membrane and that due to the narrow spectrum of activity they target a *Bacillus*-specific receptor molecule. Subtilosin A, however, acts in a concentration-dependant manner, binding to the lipid bilayer of the target cell resulting in membrane permeabilization (Thennarasu *et al.,* 2005). The sactibiotics are an ever-growing class of bacteriocins, which show more potential as alternative antimicrobials as more information is elucidated from them.

1.4.4 The Linear azoline containing peptides

Linear azoline-containing peptides (LAPs) are peptides that undergo post-translational modifications to form heterocyclic rings comprised of the unusual amino acids thiazole and methylthiazole (Alvarez-seirro *et al*., 2016). The biosynthetic clusters and post translational modifications involved in the production of LAPs is strikingly similar to lanthipeptides, and their similarity to the Class Ia bacteriocins is why they are currently classified as bacteriocins of Gram-positive bacteria (Montalbon-Lopez *et al.,* 2020). However, research on streptolysin A, a LAP produced by *Streptococcus pyogenes*, focuses on this peptide's function as a virulence factor of pathogenic *S. pyogenes* and its anti-mammalian cell properties with no information regarding antimicrobial activity (Molloy *et al*., 2007). Similarly, in the classification proposed by Alvarez-Seirro *et al*., (2016) only microcin B17 from *E. coli* was given as an example of a LAP with antimicrobial activity. Therefore, whilst the classification of LAPs as RiPPs related to lanthipeptides cannot be disputed, the lack of examples of LAPs from Gram-positive bacteria with antimicrobial activity suggests their inclusion in classification of bacteriocins from Gram-positive bacteria is currently redundant.

1.4.5 Class Ie: Glycocins

Glycocins are peptides that are post-translationally modified to add glycosylated residues to cysteines, serine and/or threonine residues (Montbalon-Lopez *et al*., 2020). Glycocin F was the first Class Ie bacteriocin to be discovered in Gram-positive bacteria in the LAB *Lactobacillus plantarum* (Dubois *et al.,* 2011). This bacteriocin exhibits a reversible, bacteriostatic effect on target cells in a mechanism as yet unknown (Dubois *et al.,* 2011). Sublancin 168 is another Class Ie bacteriocin, originally identified in *B. subtilis* 168 that shows a relatively narrow spectrum of activity against other *Bacillus spp.* (Paik *et al.,* 1998). When it was first characterised, sublancin 168 was classified as a Class Ia bacteriocin due to its post-translational modifications. However, it has since been reclassified as a Class Ie bacteriocin, as its structural features were elucidated (Montbalon-Lopez *et al*., 2020). The biosynthetic operon of sublancin 168 is highlighted in Figure 1.1; to achieve the active peptide a structural gene, ABC transport related gene and a gene encoding a thiol disulphide oxidoreductase enzyme are required (Dorenbros *et al.,* 2002).

1.4.6 Class If: Lassopeptides

The Class If bacteriocins – the lassopeptides – are bacteriocins that feature a characteristic lasso fold. This fold comprises a macrolactam bond between the N-terminus of the peptide and the carboxylate of aspartic acid/glutamic acid at positions +7 or +9 in the core peptide. The structure is then stabilised by threading of the C-terminus through the ring (Alvarez serirro *et al*., 2016; Montbalon-Lopez *et al*., 2020). As is the case for class I bacteriocins, lassopeptides are translated as an inactive prepeptide and processed to mature active form by the activity of two enzymes: a cysteine protease for leader cleavage and a macrolactam synthetase to form the macrolactam bond (Figure 1.1; Montalbon-Lopez *et al.,* 2020). Of the Class If bacteriocins of Gram-positive bacteria, the lassomycins and lariatan type bacteriocins have gained considerable attention as potential treatments for multidrug-resistant *Mycobacterium tuberculosis* (MDR-TB), because of their strong antagonistic activity (Zhu *et al*., 2019). Whilst the mechanisms of action of the lariatin type bacteriocins remains unknown, the target for lassomycin in *M. tuberculosis* has been identified as the ClpC1 protease, which is essential for survival in *M. tuberculosis*. Lassomycin binds to ClpC1, inducing ATPase activity and decoupling it from its protein complex (Gavrish *et al*., 2014).

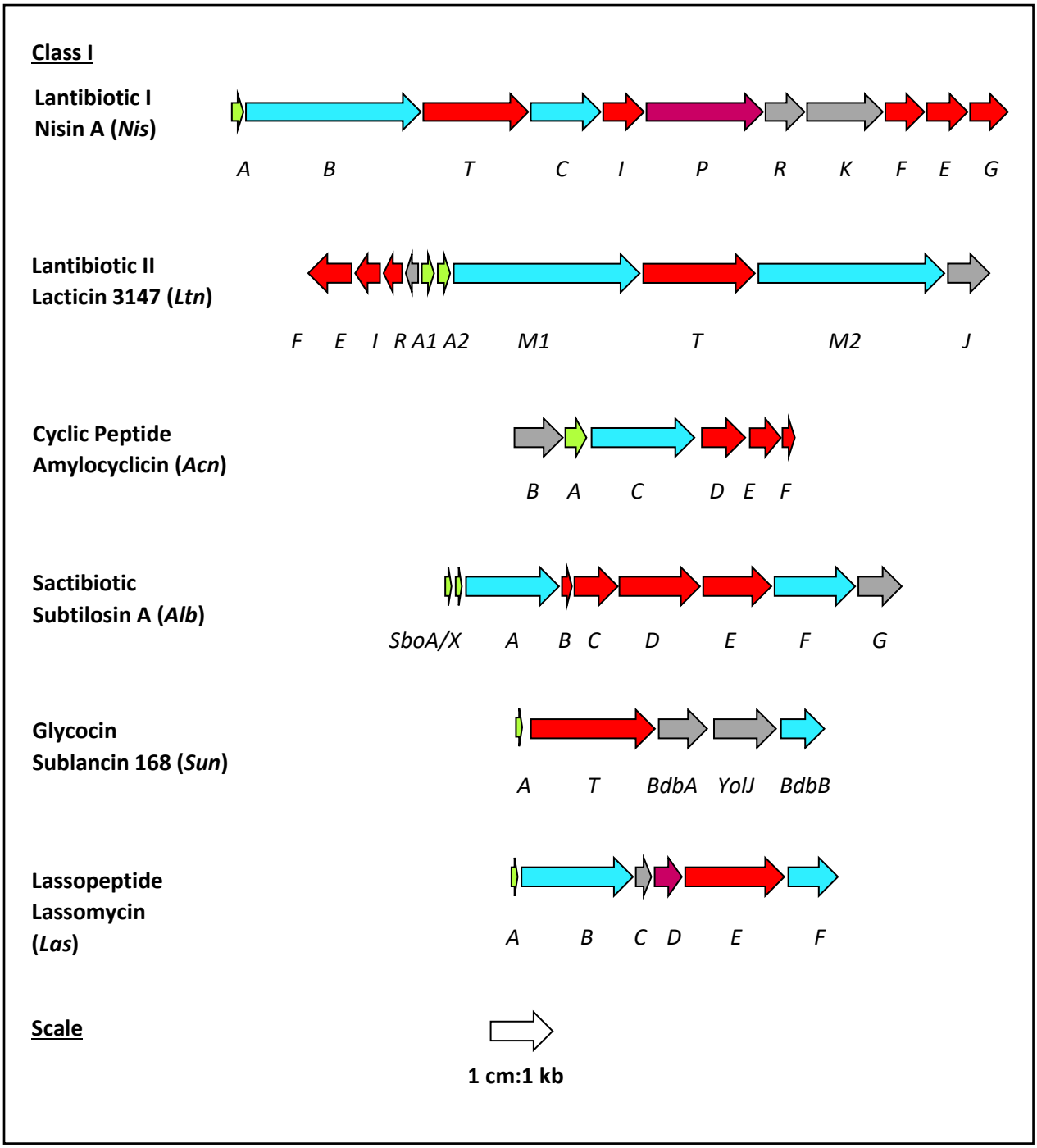

Figure 1.1: Schematic representation of the biosynthetic operons producing class I bacteriocins. Organisation of the nisin (*Nis)* production operon (Cheigh and Pyun, 2005); lacticin 3147 (*Ltn*) operon (Cotter, Hill and Ross, 2005); amylocyclicin (*Acn*) operon (Scholz *et al.*, 2014); subtilosin A (*Sbo/alb*) operon (Zheng *et al.,* 1999); sublancin 168 (*Sun*) operon (Dorenbros *et al*., 2002); lassomycin (*Las*) operon (Gavrish *et al.,* 2014). Open reading frames drawn to scale. Green arrows: Structural

genes (A); Blue: Maturation enzymes; Red: Transport and immunity related genes; Purple: proteases (P/D); Grey: Other Functions. Image based on schematics presented in bacteriocin reviews including Alvarez Seirro *et al*., (2016) and Franz *et al.*, 2007).

1.5 Class II: Unmodified bacteriocins

1.5.1 Class IIa: Pediocin like bacteriocins

The unmodified bacteriocins class (Class II) comprises the bacteriocins without extensive post translational modification such as those seen in the Class I. However, mature bacteriocin production is not devoid of processing as these bacteriocins often undergo leader cleavage and minor processing prior to secretion from the producer cell as an active peptide. The class IIa pediocin-like bacteriocins - so called because pediocin PA-1 is the best characterised member of the group – are a class of bacteriocins that are most commonly produced by members of the LAB, although they have been found to be produced by other species such as *Bacillus* spp. (Rodriguez *et al.,* 2013; Hyronimus *et al.,* 2002). The pediocin-like bacteriocins are characterised by their potent anti-listerial activity and a highly conserved, hydrophilic N-terminal region, within which is the "pediocin box", the conserved amino acid sequence YGNGVx (although there is a tolerance for slight variation) (Cui *et al.,* 2012). Their anti-listerial activity and their production by food safe LAB means that these bacteriocins are an attractive option as anti-spoilage agents, with pediocin PA-1 currently available as a commercial additive (Cotter, Hill and Ross, 2005). The genetic determinants of Class IIa bacteriocins are often encoded on mobile plasmids and their expression is determined by quorum sensing (QS), the process by which bacteria produce signalling molecules that induce a response at high cell densities (Cui *et al.,* 2012; Okada *et al.,* 2005). The biosynthetic clusters (shown in Figure 1.2) encode genes for the bacteriocin structural peptide, immunity proteins, ABC-transport related proteins and accessory proteins for translocation (Rodriguez *et al*., 2013). Secretion of the prebacteriocin is directed by a leader sequence bearing a signature double glycine motif, however, some class IIa bacteriocins like enterocin P are secreted via the general secretory pathway (Guitierrez *et al.,* 2005). Once secreted, Class IIa bacteriocins exploit a mannose phosphotransferase system on the target cells to form pores and leakage of small intracellular components (Diep *et al*., 2007). Due to the ubiquity of mannose phosphotransferase systems in Gram-positive bacteria, the inhibitory spectrum of Class IIa bacteriocins is relatively broad spectrum

against Gram-positive bacteria with activity against *Clostridia* even being reported (Jecklemen *et al*., 2020; Kjos *et al*., 2011).

1.5.2 Class IIb: Two-peptide bacteriocins

Class IIb bacteriocins are unmodified bacteriocins produced primarily by members of the LAB family, which are defined by their requirement of two synergistic peptides for activity, lactococcin G produced by *L. lactis* is the best characterised member of the class (Nissen-Meyer *et al*., 2009; Moll *et al.,* 1996). The biosynthetic machinery of Class IIb bacteriocins is comprised of two structural proteins, with the genes encoding them directly next to one another in an operon. In addition to the structural peptides, an immunity protein, ABC-transport related proteins and an accessory protein are often encoded in the bacteriocin operon (Figure 1.2). The two structural peptides of the Class IIb bacteriocins plantaricin J/K and plantaricin E/F are all located together in a single operon with the immunity proteins of the respective bacteriocins and plantaricin A, a QS pheromone responsible for induction of the plantaricins expression (Hauge *et al.,* 1998). The ABC -transport proteins and the accessory protein for these bacteriocins is located on a genetically separate, nearby operon.

Each subunit of Class IIb bacteriocins contain a GxxxG motif, which is inherent in the formation of α-helices. No receptor molecule has been identified and it has been proposed that the lactococcin G subunits undergo a structural change to the α-helix conformation upon contact with the hydrophobic lipid membrane of target cells, the α-helices of the subunits then insert into the membrane and together form a membrane-spanning pore that leads to the selective transport of intracellular ions (Nissen-Meyer *et al*., 2009). Interestingly plantaricin E/F and plantaricin J/K form pores that lead to opposite charged ions being leaked from the target cell (Moll *et al.,* 1999).

1.5.3 Class IIc: Leaderless bacteriocins

The class IIc bacteriocins are an atypical class of bacteriocins that are termed the leaderless bacteriocins as they are synthesised from a leaderless prepeptide that is bioactive immediately upon translation and to which the host strain does not have a specific immunity-associated protein (Perez, Zendo and Somomoto, 2018). Enterocin L50 a leaderless two-peptide bacteriocin is the best characterised of the class,

however there are examples of multipeptide leaderless bacteriocins with up to four subunits (Cintas *et al*., 1998).

Unlike other bacteriocins, their associated biosynthetic proteins are poorly understood, however, members of the class maintain high sequence similarity to one another and many mature leaderless bacteriocins contain a saposin fold in their structure, similar to the circular bacteriocins despite their apparent differences (Towle and Vederas, 2017). Like the circular bacteriocins, the leaderless bacteriocins also form surface pores in their target bacteria and it is thought that the saposin fold structure inherent in these two classes of bacteriocins is what confer their cell surface disrupting capabilities (Towle and Vederas, 2017).

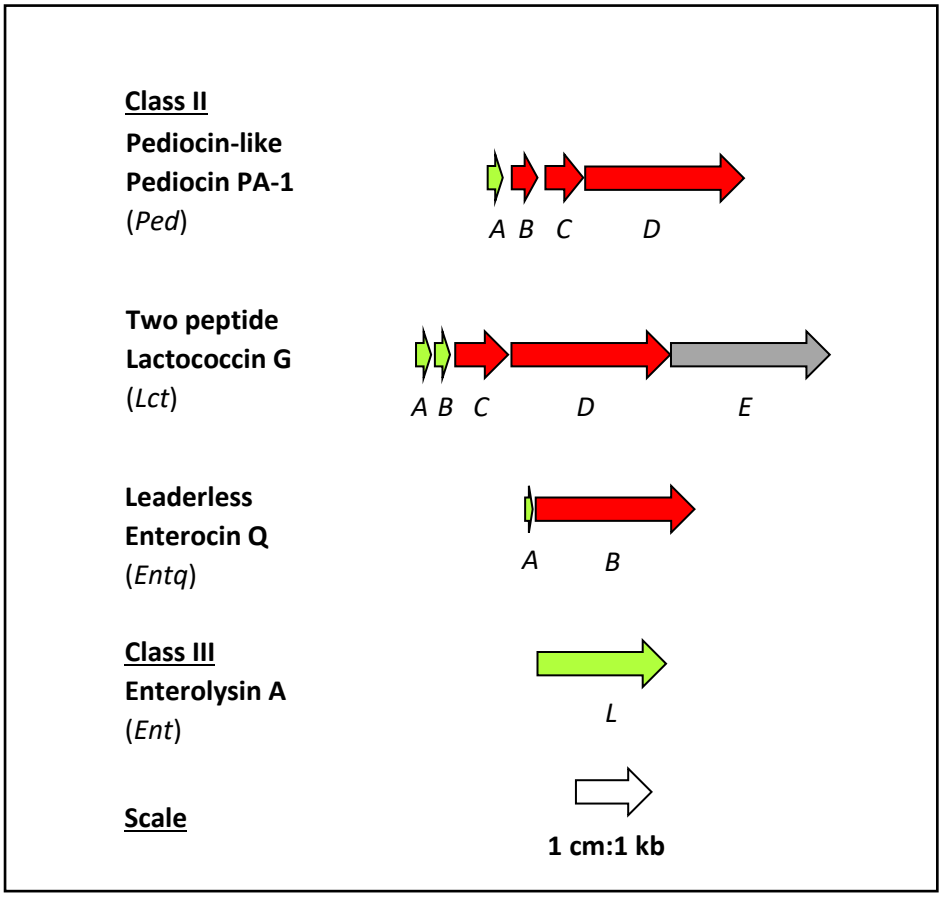

Figure 1.2: Schematic representation of the biosynthetic operons producing class II & III bacteriocins. Organisation of the pediocin (*Ped)* production operon (Rodriquez *et al.,* 2013); lactococcin G (*Lct*) operon (Oppegård *et al*, 2007); enterocin Q (*Entq*) operon (Criado *et al.*, 2006); enterolysin A (*Ent*) operon (Hickey *et al.*, 2003). Open reading frames drawn to scale. Green arrows: Structural genes (A); Red: Transport and immunity related genes; Grey: Other Functions. Image based on schematics presented in bacteriocin reviews including Alvarez-Seirro et al., (2016) and Franz *et al., (*2007).

1.5.4 Class IId: Unrelated linear single peptides

The class IId bacteriocins are a heterogenous group of unrelated bacteriocins synthesised with a leader peptide that do not conform to the other subclassifications (Alvarez-Seirro *et al*., 2016). As a diverse class there is no unifying feature of these bacteriocins and within the class there is a range of structures, genetic determinants and modes of action. The LAB are notable producers of Class IId bacteriocins with *enterococcus* spp. producing many of the currently described class IId bacteriocins (Franz *et al*., 2007).

1.6 Class III: Large, heat Labile enzymes

The Class III bacteriocins are large, heat labile proteins with multiple domains that were once termed the bacteriolysins to reflect their lytic activity against target cells (Alvaraz-Seirro *et al.*, 2016; Rea *et al*., 2011). Enterolysin A, produced by *Enterococcus faecalis* is likely one of the best characterised of the Class III bacteriocins (Franz *et al*., 2007). Enterolysin A is a 34.5 kDa polypeptide comprised of an N-terminal catalytic domain and a C terminal target recognition site, enterolysin A is encoded for by a structural gene that produces a prepeptide with a sec-dependent signal peptide for processing (Hickey *et al.,* 2003; Nilsen *et al*., 2003). Enterolysin A induces cell lysis in susceptible *Enterococcus* spp. and related LAB. Mode of action studies have shown that cell lysis is initiated through cleavage of two sites within the target cells peptidoglycan (Khan *et al*., 2013). Zoocin A is another Class III bacteriocin produced by *Streptococcus equi* ssp. *zooepidemicu*s 4881 that exerts its inhibitory action by cleaving target cell peptidoglycan (Akesson *et al.,* 2007). However, unlike enterolysin A, zoocin A activity is narrow spectrum affecting only related *Streptococcus* spp. (Akesson *et al.,* 2007).

Table 1.3: A summary of the current classification of bacteriocins, including their class defining features. Based on the classification scheme proposed by Alvarez-Seirro *et al*., (2016).

Classification	Subclassification	Title	Class-defining features
I: Small post translationally modified peptides	Ia	Lantibiotics	Lan enzymes catalyse the formation of the unsual amino acids lanthionine and β-methyllanthionine in the mature peptide
	Ib	Head to Tail cyclised	Circular shape derived from N to C-terminus linkage
	Ic	Sactibiotics	Radical SAM enzymes catalyse the formation of sactionine linkages in the mature peptide
	Id	LAPs	Contain the unusual amino acids thiazole and methylthiazole
	Ie	Glycocins	Glycosylated residues on serine/threonine
	If	Lassopeptides	Contain a lasso fold structure
II: Unmodified peptides	IIa	Pediocin-like	Contain the pediocin box consensus sequence and display potent anti-listerial activity
	IIb	Two-peptide bacteriocins	Require two peptides for activity
	IIc	Leaderless bacteriocins	Synthesised without a leader peptide
	IId	Unrelated linear, single peptides	Single, linear peptides that do not conform to current classifications
III: Large, heat labile enzymes			Large proteins with enzymatic activity

1.7 Bacteriocins as anti-clostridial agents

Whilst many bacteriocins have been shown to have activity against *C. difficile* in activity spectrum experiments, few have been the focus of research dedicated to novel treatments for CDI. The anti-clostridial potential of nisin has been well documented. A study by Bartoloni *et al. (2004)* showed that nisin had an *in vitro* MIC90 of 0.256 mg L^{-1} against *C. difficile* compared to vancomycin and metronidazole that shared an MIC90 of 0.8 mg L^{-1}, thus nisin was significantly more effective than both the recommended antibiotic treatments of *C. difficile*. Dormant *C. difficile* spores can be responsible for the relapse of infection. Lelay et al., (2016) revealed that spore viability of *C. difficile in vitro* dropped by 50% when exposed to nisin at a concentration of 25.6 µg mL^{-1} (Lelay *et al*., 2016). This inhibitory activity against *C. difficile* spores could potentially reduce cases of infection relapse if nisin was developed as a treatment. Despite the effectiveness of nisin as a potential anti-clostridial its broad-spectrum inhibitory activity is a problem, as like traditional antibiotics, it is indiscriminate in its killing activity and therefore affects essential gut commensals such as *Lactobacillus spp.* and *Bifidobacterium* spp. (Rea *et al*., 2010). Additionally, the essential nature of the target molecule of nisin, the Lipid II precursor, means there is significant selective pressure for *C. difficile* to develop resistance mechanisms. A 2011 study by McBride and Sonenshein showed that the transcriptional products of the *dlt* operon confers resistance to *C. difficile* against nisin and other cationic peptide antimicrobials via the addition of D-alanine to the teichoic acid of the cell wall, which reduces the negative charge of the bacterial cell wall and thus reduces susceptibility to nisin. Therefore, whilst nisin has shown impressive inhibitory activity against *C. difficile* and its spores, the clinical potential could be hindered by its broad-spectrum activity and the development of resistance mechanisms by *C. difficile*.

Lacticin 3147 produced by *L. lactis* has also been studied with a focus against *C. difficile*. Rea *et al*., (2007) examined the inhibitory activity of lacticin 3147 against several genetically distinct, clinical isolates of *C. difficile* (Rea *et al*., 2007). This study showed that purified lacticin 3147 had inhibitory activity comparable to that of metronidazole and vancomycin. The MIC50 of lacticin 3147 was 3.6 µg mL^{-1}, which

compared favourably to the MIC50 of metronidazole and vancomycin, which ranged from 0.5 - 4 μg mL^{-1}. However, despite its efficacy in *in vitro* studies, in a model of *C. difficile* infection of the distal colon the concentration of lacticin 3147 had to be increased to three times that of metronidazole and vancomycin to achieve the same inhibitory activity (Rea *et al*., 2011). In addition to this, the broad-spectrum activity of lacticin 3147 is problematic as a treatment option in the same ways as metronidazole and vancomycin. In *in vitro* studies lacticin 3147 was shown to be inhibitory towards Gram-positive gut commensals (Rea *et al*., 2007) and in the model of the distal colon lacticin 3147 changed the taxonomic distribution from predominately firmicutes to predominately proteobacteria, as did metronidazole and vancomycin (Rea *et al*., 2011). Therefore, although Lacticin has shown to be efficacious in inhibiting *C. difficile in vitro*, its reduced efficacy in models of the distal colon and its broad-spectrum activity reduce its potential as an alternative clinical therapy against *C. difficile*.

As mentioned, the sactibiotic thuricin CD shows promise as an anti-clostridial therapy due to its specificity against *C. difficile* and related *Bacillus* spp. (Rea *et al.*, 2010). Studies have shown that thuricin CD shows inhibitory activity favourable to that of metronidazole against all strains of *C. difficile*, including the highly virulent ribotype 027 (NAP1) strain (Rea *et al*., 2010). The narrow spectrum of activity and favourable performance compared to the traditional *C. difficile* treatments meant that thuricin CD has been patented as an alternative treatment to CDI, although public information regarding its development is now limited.

1.8 Clinical perspectives of Gram-positive bacteriocins

The bacteriocin database Bactibase currently lists over 200 characterised bacteriocins from both Gram negative and Gram-positive bacteria (Hammami *et al.,* 2010). As *in vitro* studies characterising bacteriocins almost always include analysis of the inhibitory spectrum of bacteriocins – often with a specific clinical pathogen as the target – it is therefore surprising that no bacteriocins have of yet been approved for clinical use in humans. Indeed, searching the NCBI database of clinical trials for

the keyword “bacteriocin” reveals only 7 historical and current phased trials featuring bacteriocins (https://clinicaltrials.gov/).

Despite being patented as a treatment for CDI in 2011 and its effective performance *in vivo,* the bacteriocin thuricin CD has not yet reached clinical trials phase of development. Similarly, nisin - with its long history of approval for human consumption - is not approved for medicinal use. This may in part be due to the study by Rea *et al.,* (2011), that highlighted nisin’s strong microbiota modulating action when administered in the model distal colon due to its broad spectrum of activity. Additionally, nisin has been shown to highly susceptible to proteolytic enzymes, which can possibly hinder its applications in internal medicine (Jarvis *et al*., 1969). With that in mind, the use of nisin as a topical application has been explored. Topical treatment with nisin for the treatment of staphylococcal mastitis in lactating women was examined in 2008, highlighting its effectiveness at relieving symptoms (Fernandez *et al*., 2008). Therefore, given its drawbacks in other areas of medicine the use of nisin as a topical treatment akin to the non-bacteriocin protein antibiotic bacitracin (Polyfax®, GSK – discontinued 2017), is an area worth exploring.

One area of concern when developing bacteriocins for clinical use is their effect on host cells. As bacterial peptide products, there is a fear that bacteriocins could illicit dangerous immune responses or even directly kill host tissue cells (Benitez-Chao *et al.*, 2021). The circular bacteriocin enterocin AS-48 was recently reviewed for host toxicity with a view for clinical applications (Cebrian *et al*., 2019). This study found that AS-48 was minimally haemolytic, non-toxic and non-inflammatory to human cell lines, whilst also having effective antimicrobial properties *in vivo*, highlighting the immense potential for Class Ib bacteriocins as therapeutics (Cebrián *et al*., 2019). The sactibiotic subtilosin A has also been examined for adverse effects on human tissue in its exploration as a treatment for *Gardnerella* derived bacterial vaginosis (BV). It was found that subtilosin A does not exert cytotoxic activity against the human cervical model, affirming its potential as a treatment for BV. Additionally, spermicidal activity of subtilosin A was also highlighted in this study, which opens up applications in health beyond traditional infection control (Sutyak *et al.,* 2008).

Efficacy of bacteriocins as anti-cancer agents beyond their role as antimicrobials has also been an area of research interest (reviewed in Kaur and Kaur, 2016). Nisin has been shown to exhibit anti-cancer properties, with a 2012 study showing a reduction in tumour size in a murine oral model following treatment with nisin (Joo *et al.*, 2012). Another application of bacteriocins that has received considerable attention beyond human health, is the use of bacteriocins as therapeutics in veterinary medicine (Hernandez-Gonzalez *et al*., 2021). This application of bacteriocins is an exciting opportunity for research, as not only is animal husbandry a driver of antibiotic resistance, but the approval process for veterinary medicine is less stringent than that of human health, which has an added cost benefit (Hernandez-Gonzalez *et al*., 2021). Ultimately, it appears that monetary considerations are the key element currently preventing the research and utilisation of bacteriocins as therapeutics, as the process for discovering, characterising and producing bacteriocins is intensive and costly prior to the investment required to commence preclinical and clinical assessment (Soltaini *et al.*, 2021).

1.9 Conclusion and General Aims

The emergence of the hypervirulent ribotype 027 strain of *C. difficile*, with its characteristic increased rate of recurrence and increased resistance to antibiotics commonly used to treat *C. difficile*, coupled with growing concerns of the negative impact antibiotic treatment has on human health has meant that there is increased pressure to find alternative therapies to treat *C. difficile* infection. FMT has been used successfully in clinics to treat persistent *C. difficile* infections, although, safety issues centred around the introduction of faecal matter and the potential pathogens associated with it has been a cause for concern, particularly in the case of immunocompromised patients. Bacteriocins offer a promising alternative to antibiotics as this heterogenous class of RiPPs show diversity in their antimicrobial spectrums and mode of actions, which could lead to the development of novel target therapeutics. However, despite the vast number of bacteriocins that have been discovered few have been studied with the intention of developing a novel CDI treatment and of that few, there has been little public progress on this development. This study aims to isolate novel environmental bacteria with inhibitory activity

against *C. difficile* that can be attributed to novel bacteriocins. This study then aims to characterise these anti-*C. difficile* bacteriocins through *in silico* methods and purification from the native producers/ recombinant production.

Chapter 2 Materials and Methodology

2.1 Bacterial strains and plasmids

2.1.1 Table of Strains

Bacterial strains used in this study are listed in Table 2.1.

Table 2.1: Summary of the bacterial strains used in this study, a brief description, and their source.

Bacterial Strain	Description	Source
Bacillus subtilis 168	Indicator *spp*	BBSRC/EPSRC Synthetic Biology Research Centere Nottingham culture collection, University of Nottingham
***Bacillus subtilis* 50B**	*C. difficile* inhibitory strain	This study. Environmental isolate from pig colon (University of Nottingham)
Bacillus subtilis WB800N	*nprE aprE epr bpr mpr::ble nprB::bsr Δvpr wprA::hyg cm::neo;* NeoR	MoBiTec Molecular Biotechnology
***Bacillus velezensis* B18**	*C. difficile* inhibitory strain	This study. Environmental isolate from pig colon (University of Nottingham)
Bacillus velezensis FZB42	Known bacteriocin producer	German Collection of Microorganisms and Cell Cultures (DSMZ)
Staphylococcus aureus	Indicator *spp*	BBSRC/EPSRC Synthetic Biology Research Centere Nottingham culture collection, University of Nottingham
Escherichia coli TOP 10	F- *mcrA* Δ(*mrr-hsdRMS-mcrBC)* ϕ80lacZΔM15 Δl*acX74 nupG recA1 araD139* Δ(*ara-leu*)7697 *gal*E15 *galK16 rpsL(Strr*) endA1 λ-	Invitrogen
Escherichia coli S17	Indicator *spp*	BBSRC/EPSRC Synthetic Biology Research Centere Nottingham culture collection, University of Nottingham

Pseuodomonas aeruginosa HA1	Indicator *spp*	Dr Christopher Penfold, University of Nottingham
Enterococcus faecalis	Indicator *spp*	BBSRC/EPSRC Synthetic Biology Research Centere Nottingham culture collection, University of Nottingham
Clostridium sporogenes NCIMB 10696	Indicator *spp*	BBSRC/EPSRC Synthetic Biology Research Centere Nottingham culture collection, University of Nottingham
Clostridium perfringens	Indicator spp	BBSRC/EPSRC Synthetic Biology Research Centere Nottingham culture collection, University of Nottingham
Clostridium butyricum NCTC 7423	Indicator spp	BBSRC/EPSRC Synthetic Biology Research Centere Nottingham culture collection, University of Nottingham
Clostridioides difficile* strains *630 DH196, R20291, 11205, TL178, 003, EK15, TL174, UK-1, M68. CF5, R10079 ClosDi22, CD305, CD196, R8438, 046, CDC34, 056, R9367, 075, 077, M120, EK28, 081, Liv22, R10432, 117, 126, 131	Indicator spp	BBSRC/EPSRC Synthetic Biology Research Centere Nottingham culture collection, University of Nottingham

2.1.2 Table of plasmids

Plasmids used in this study are outlined in Table 2.2.

Table 2.2: Plasmids used in this study, a brief description, and their source.

Plasmid	Description	Source
pHT43	*B. subtilis* overexpression secretory plasmid	Mobitec
pGEM T Easy	*E. coli* Plasmid	Promega
pHT43_acn	Overexpression of amylocyclicin genes	This study
pGEMK_acn	Amylocyclicin genes deletion vector	This study
pGEMK_sbo	Subtilosin A genes deletion vector	This study

2.2 Culture media and growth conditions

2.2.1 Culture media

Bacterial culture media used in this study are listed in Table 2.3. Dehydrated media were sourced from Oxoid and chemical constituents were sourced from Merck. All media and components were sterilised either by autoclaving at 121°C at 100 kPa above atmospheric pressure for 15 minutes or filtration through a 0.2 µM filter.

Table 2.3: Preparation of the culture media used in this study.

Brain Heart Infusion Supplemented (BHIS)
37 g brain heart infusion, 20 g yeast extract and 1 g L-cysteine in 1 L dH_2O. For solid plate media: 10 g L^{-1} bacteriological agar added.
BHIS Top Agar
37 g brain heart infusion, 20 g yeast extract, 1 g L-cysteine and 5 g bacteriological agar.
Lysogeny Broth (LB)
10 g Tryptone, 5 g yeast extract, 10 g sodium chloride in 1 L dH_2O. For solid plate media: 15 g L^{-1} bacteriological agar added.
M9 minimal medium
57 mM sodium phosphate dibasic, 22 mM monopotassium phosphate, 20 mM ammonium chloride, 1 mM sodium chloride, 0.1 mM calcium chloride, 1 mM magnesium sulfate, 1 mg L^{-1} manganese chloride, 1.7 mg L^{-1} zinc chloride, 0.43 mg L^{-1} copper chloride.$2H_2O$, 0.6 mg L^{-1} cobalt chloride.$6H_2O$, 0.6 mg L^{-1} sodium molybdate.$2H_2O$, 4.9 mg L^{-1} ferric chloride and 0.5% glucose in 1 L dH_2O.
HS medium for bacillus
15 mM ammonium sulfate, 80 mM dipotassium phosphate, 40 mM monopotassium phosphate, 4 mM sodium citrate, 0.5% glucose, 0.005% L-tryptophan, 0.02% casamino acids, 0.5% yeast extract, 0.8% arginine and 0.4% histidine.
LS medium for Bacillus

15 mM ammonium sulfate, 80 mM dipotassium phosphate, 40 mM monopotassium phosphate, 4 mM sodium citrate, 0.5% glucose, 0.0005% L-tryptophan, 0.01% casamino acids, 0.1% yeast extract, 2.5 mM magnesium chloride and 0.5 mM calcium chloride.
2xYT medium
16 g tryptone, 10 g yeast extract and 5 g sodium chloride in 1 L dH_2O. For solid plate media: 15 g L^{-1} bacteriological agar added.
GCHE medium
1% glucose, 0.2% potassium L-glutamate, 100 mM potassium phosphate buffer (pH 7), 3 mM trisodium citrate, 3 mM magnesium sulfate, 22 mg L^{-1} ferric ammonium citrate, 50 mg L^{-1} L-tryptophan and 0.1% casein hydrolysate
Transformation medium
15 mM ammonium sulfate, 80 mM dipotassium phosphate, 45 mM monopotassium phosphate, 35 mM sodium citrate, 1 mM EGTA, 25 mM glucose, and 30 mM magnesium chloride.

2.2.2 Growth Conditions

Bacillus strains were cultured aerobically at 30°C unless stated otherwise, broth cultures were incubated with agitation at 200 RPM. Other Gram-positive aerobes tested were incubated at 37°C on their respective medium. *E. coli* and *Pseudomonas* strains were cultured aerobically at 37°C and as before broth cultures were incubated with agitation at 200 RPM. *C. difficile* and other anaerobes were cultured in an anaerobic workstation (MG1000, Don Whitley Scientific) containing an atmosphere of 80% N_2, 10% CO_2 and 10% H_2 at 37°C. Frozen stocks of all bacteria were prepared by growing strains according to their specific growth requirements, following this cell mass was suspended in media plus 15% glycerol and stored at -80°C.

2.3 Microbiological methods

2.3.1 Isolation of environmental bacteria

Initial isolation of the bacteriocin-producing environmental bacteria was carried out by homogenising 1 g of porcine faecal sample, collected from the colon of slaughterhouse pigs (University of Nottingham), in 500 µL of sterile phosphate buffered saline (PBS). Tenfold serial dilutions of homogenised samples were prepared with PBS to 1 in 10,000 and 100 µL of each dilution was plated onto BHIS plates. All dilutions were plated in duplicate with one set being incubated aerobically and the other anaerobically at 37°C. Following incubation for 16 h, colonies with differing morphologies from both sample sets were selected for and streaked to purity on BHIS plates. Antimicrobial activity of the isolates against *C. difficile* was tested for using an adaptation to the deferred antagonism assay described by Moran *et al.,* (2016) in which the environmental isolates were stab inoculated onto BHIS plates and incubated for 16 h under their relevant growth conditions. Following incubation, the plates were overlaid with 4 mL of 0.5% BHIS agar seeded with *C. difficile* 630 at an optical density of 0.05 when read at 600 nm (OD600) and incubated for 16 h anaerobically before analysing the zones of inhibition. Colonies with strong antagonistic activity against *C. difficile* 630 were subsequently tested against *C. difficile* strains R20291 and DH1916 and those showing strong inhibitory to the three strains of *C. difficile* were selected for further study.

2.3.2 Minimum inhibitory concentration assay

The MIC of the antibiotic chloramphenicol against environmental *Bacillus* isolates was established by preparing a 5.12 mg mL^{-1} stock solution of chloramphenicol in 100% ethanol. The chloramphenicol stock solution was diluted with BHIS in a serial log_2 dilution and inoculated with the environmental strains from a broth culture to an optical density of 0.05 in 150 µL on a 99-well plate (Mayrhofer *et al*., 2007). The microtitre plate was incubated at 37°C with shaking and the optical density at 600 nm was measured every hour. The data collected was analysed and plotted using Graphpad Prizm.

2.3.3 Preparation of chemically competent *E. coli*

A single colony of *E. coli* was inoculated into LB media and grown overnight at 37°C with shaking. 1 mL of the overnight culture was then used to inoculate 100 mL of LB and the culture was grown at 37°C with vigorous shaking until it reached an optical density of 0.4- 0.7 when read at 600 nm. Following this the culture was chilled on ice for 15 min before the cells were harvested by centrifugation for 10 min at 3300 x g at 4°C and resuspended in 15 mL ice cold 0.1 M $CaCl_2$. The resuspended cells were chilled on ice for a further 30 mins before being centrifuged as before and resuspended in 2 mL 0.1M $CaCl_2$ plus 15% glycerol. At this point the cells were considered competent and were stored at -80°C until required (Green and Rogers, 2013).

2.3.4 Preparation of naturally competent *B. subtilis* WB800N

Overnight cultures of *B. subtilis* WB800N in HS medium were added at a ratio of 1:100 to 50 mL HS medium and incubated with vigorous shaking at 37°C. 10 mL samples of culture were taken every 15 minutes once the culture reached stationary phase. Sterile glycerol was added to the 10 mL aliquots at a concentration of 8% and the competent *B. subtilis* WB800N were then chilled on ice for 15 minutes before being stored at -80°C (Phan *et al*., 2006).

2.3.5 Preparation of naturally competent environmentally isolated *Bacillus spp.*

Competent environmental *Bacillus* spp were obtained following the method of Kunst and Rapoport (1995). Overnight cultures of environmental bacilli grown in LB were used to inoculate GCHE medium to an OD_{600} reading of 0.3. The culture was incubated with vigorous shaking at 37°C until it reached mid-exponential growth, wherein it was diluted with an equal volume of GC medium (GCHE medium without potassium glutamate and casein hydrolysate). The diluted culture was then incubated for a further hour under the same conditions. Following incubation, the culture was aliquoted into two equal parts and cells were harvested by room temperature centrifugation at 6000 RPM for 5 min. Cell pellets were resuspended in 200 µL of supernatant and from this point cells were considered competent and transformation was performed immediately.

2.4 Proteomics methods

2.4.1 Ammonium sulfate precipitation of potential bacteriocins

Overnight cultures of bacteriocin producers were inoculated into 400 mL of media (split into 200 mL cultures in 500 mL flasks) and grown shaking for 24 h at 30°C. Following incubation, the cell free supernatant (CFS) was harvested by centrifugation at 7,500 x g for 10 min in a Beckman Coulter Allegra X-30R centrifuge and the cell pellet discarded. Solid ammonium sulfate (Sigma Aldrich, UK) was gradually added to the CFS to a saturation of 60% and incubated with constant agitation for 4 h at 4°C before being centrifuged at 20,000 x g for 30 min at 4°C in a Thermo Scientific Lynx 6000 ultracentrifuge (Wingfield, 2001). Following centrifugation, the CFS was discarded and the precipitated protein pellet was resuspended in 1/100th of the original volume of Buffer A (50 mM Tris 20 mM NaCl, pH 8) and dialysed in the same buffer in a 3.5 KDa molecular weight cut off SnakeSkin™ Dialysis Tubing (Thermo Scientific) for 16 h with 2 buffer changes. This protein preparation was referred to as the crude bacteriocin preparation (CBP).

2.4.2 Well-diffusion assay

Initially, anti-*C. difficile* activity of the CBP was assessed via the well diffusion assay (WDA). 4 mL of 0.5% agar BHIS seeded with *C. difficile* 630 to an OD_{600} value of 0.05 was used to overlay a BHIS agar plate and allowed to solidify for 30 min. Following this, 5 mm wells were bored into the agar using sterile stainless steel corkborers, 50 µl of CBP was added to these wells and the plates were incubated anaerobically for 18 h at 37°C. Anti-*C. difficile* activity was assessed via the diameter of the zone of inhibition of *C. difficile* 630 around the well (Pingitore *et al.*, 2007).

2.4.3 Critical dilution assay

As direct measurement of activity of unpurified bacteriocins is generally not possible, antibacterial activity is often estimated using the critical dilution assay and values are expressed in arbitrary units per mL (AU mL^{-1}) (Henderson *et al.,* 1992). To estimate the anti-*C. difficile* activity of the CBP from *B. velezensis* B18 and *B. subtilis* 50B, serial two-fold dilutions of the CBP were prepared using buffer A. 10 µL of each dilution was spotted on a lawn of *C. difficile* 630, which was prepared and incubated as

described for the WDA. Following incubation, the critical dilution was defined as the highest dilution producing a zone of inhibition against *C. difficile* 630. In the case of a hazy zone the critical dilution was taken as the average of that dilution and the last that provided a clear zone. An activity value in AU mL^{-1} for the CBP was calculated as (1000/10) multiplied by the dilution factor.

2.4.4 SDS-PAGE overlay assay

Protein preparations were resolved using protein gel electrophoresis to estimate the size of the *C. difficile* inhibitory bacteriocins. 16% Tricine SDS-PAGE gels (Novex) were run at 150 Volts for 90 minutes in an XCell SureLock™ Mini-Cell Electrophoresis System (Novex) using Tricine buffer (Novex) (Schagger, 2006). The Spectra™ Multicolor Low Range Protein Ladder was used as the molecular weight marker. Following electrophoresis, one half of the gel was stained with Coomassie Brilliant Blue (CBB) and destained with a solution of 30% methanol and 7.5% acetic acid, the other half was washed in 0.1% Tween 80 for 40 minutes three times. The washed gel was overlaid with 12 mL 0.5% BHIS agar inoculated with *C. difficile* 630 at an OD*600* value of 0.05 and incubated anaerobically at 37°C for 16 h. Zones of inhibition on the overlaid gel were then compared with the complementary stained gel to estimate the size of the protein inhibiting *C. difficile*.

2.4.5 Isoelectric focussing overlay assay

The isoelectric points (pI) of the anti – *C. difficile* bacteriocins were determined by subjecting the CBP to isoelectric focusing (IEF) analysis in a method adapted from Zheng and Slavik (1998). CBP were run in duplicate on Novex™ pH 3-10 IEF Protein Gels in an XCell SureLock™ Mini-Cell Electrophoresis System using Novex™ pH 3-10 IEF Buffer Kit at 100 V for 1 h, 200 V for 1 h and 500 V for 0.5 h. Following electrophoresis, half of the gel was fixed with 12% trichloroacetic acid (TCA) for 30 min, washed extensively with distilled water before being stained and destained as previously described for SDS-PAGE gels. The other half of the gel was washed with distilled water before being overlaid with *C. difficile* and incubated anaerobically for 16 h at 37°C. As before, zones of inhibition on the overlaid gel were then compared

with the complementary stained gel to estimate the pI of the protein inhibiting *C. difficile.*

2.4.6 Part purification of bacteriocin using anion exchange chromatography

A CBP was prepared as described in 2.4.1 from an 800 mL culture of the bacteriocin producers in M9 medium. This CBP was then applied to a 5 mL Q-sepharose FF anion exchange column (GE Healthcare, UK) previously equilibrated with buffer A at a flow rate of 1 mL min^{-1}. Following washing with buffer A to remove unbound proteins the column was connected to an AKTA prime and bound proteins were eluted with a 0-50% gradient of 1 M NaCl in buffer A in 1.5 mL fractions. The anti-*C. difficile* activity of the fractions was assessed using the spot-on lawn method as described for the critical dilution assay. Fractions with inhibitory activity against *C. difficile* were desiccated in a freeze drier and resuspended in x1 tricine SDS-PAGE buffer and the SDS-PAGE overlay assay was carried out as before (2.4.4).

2.4.7 Mass spectrometry

MALDI-TOF MS was carried out on protein bands excised from 16% tricine SDS-PAGE gels, and transported on dry ice to The Centre of Excellence In Mass Spectrometry (University of York).

2.4.8 Heat, pH and proteolytic stability of potential bacteriocins

To test the effect of heat, proteolytic digestion and pH on the activity of the potential bacteriocins a CBP was prepared from both strains as described (2.4.1). To evaluate heat stability, 150 µL of the CBP was heated at 80°C or 100°C for 30 min and anti-*C. difficile* activity was assessed via the CDA, untreated sample was considered 100%. To assess bacteriocin activity after proteolytic degradation 150 µL of the CBP was treated with 500 U mL^{-1} trypsin in 50 mM acetic acid (Pierce™), 40 U mL^{-1} α-chymotrypsin in 1 mM HCl (Merck), 500 U mL^{-1} pepsin in 10 mM HCl pH 7 (Roche) and 1 mg mL^{-1} (30 U) proteinase K (Thermo Scientific™). Enzyme treated samples were incubated at 37°C for 3 hours, following incubation the proteolytic enzymes were denatured at 100°C for 5 min and anti-*C. difficile* activity was assessed via the

CDA as described (2.4.3). Enzymes without CBP added were tested for anti-*C. difficile* activity as a negative control.

2.4.9 Overexpression induction in *B. subtilis* WB800N pHT43_acn

An overnight culture of *B. subtilis* WB800N harbouring the pHT43_acn plasmid was inoculated into fresh 2xYT medium to an optical density of 0.15 when read at 600 nm. This inoculate was incubated at 37°C until it reached an optical density of 0.7 – 0.8, at which point the culture was split and one half was induced with 1 mM IPTG, both cultures were then incubated for a further eight hours at 37°C. Both cultures were centrifuged at 6,000 x g at 4°C for 10 min and the supernatant was collected for ammonium sulfate precipitation (2.4.1). The cells were washed and resuspended in 50 mM sodium phosphate buffer (pH 7) to an OD_{600} value of 10. Resuspended cells were then disrupted via sonication, with 6 x 15 s pulses at 12 W, and samples were taken of the disrupted cells and the supernatant at this stage cleared of cell debris by centrifugation. The cell-debris free supernatant was then centrifuged again at 8,200 x g for 10 min at 4°C. All samples collected were analysed via Tricine SDS-PAGE overlay assay (2.4.4) using the amount of protein corresponding to 0.025 OD_{600}. Controls included the uninduced half of culture, *B. subtilis* WB800N without the plasmid and *B. velezensis* B18.

2.5 Molecular methods

2.5.1 DNA extraction

DNA required for routine PCR was prepared using the Sigma-Aldrich GenElute™ Bacterial Genomic DNA Kit. This method required an overnight culture of bacterial cells to be separated from media via centrifugation for 2 min at 16,000 × g and lysed with 2.115 × 106 unit mL^{-1} lysozyme for 30 min at 37°C. Following lysis, proteinase K and lysis solution C (provided in kit) were added to the sample and the mix was incubated for 10 min at 55°C. This lysate was then loaded onto a pre-prepared GenElute Miniprep Binding Column, centrifuged at 6,500 x g for 1 min and the flow through discarded. The column with the bound lysate was then washed twice with Wash solution (provided in kit) before the bound DNA was eluted in 10 mM Tris-HCl, 0.5 mM EDTA, pH 9.0 buffer.

Bacterial genomic DNA required for whole genome sequencing of the isolates was prepared using the phenol-chloroform extraction method, which entails separating the cells of an overnight bacterial culture from the media via centrifugation. Following this the cells were lysed with 10 mg lysozyme in PBS and incubated for 30 min at 37°C. Following incubation, 4 µL of RNase A was added to the lysate and the solution was incubated for a further 30 min at room temperature. Proteinase K, dH_2O and 10% sodium dodecyl sulfate (SDS) were then added to the lysate and incubated for 30 min at 65°C. After incubation, an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the lysate where it was then transferred to a phase lock tube and centrifuged at maximum speed for 3 minutes. The top phase was collected following centrifugation and 400 µL of phenol:chloroform:isoamyl alcohol was added and centrifugation was repeated. This step was repeated twice before the final top phase was collected and resuspended in 40µl of 3M sodium acetate and 800µl of ice cold 100% ethanol and placed on ice for 30 minutes. At this point the DNA preparation is insoluble and so the pellet was collected with a glass spool, washed in ethanol before being air-dried and resuspended in dH_2O. The concentration and purity of the DNA was measured using a QUBIT (Invitrogen).

2.5.2 Polymerase chain reaction

Routine Polymerase Chain Reactions (PCR) were carried out in 20 µL volumes using DreamTaq Green PCR Master Mix (2X) (Thermo Scientific), 1 µL of each of the relevant primers (Table 2.6), 50 ng of template DNA and dH_2O were added to a 20 µL total volume. The manufacturer's Thermal cycling conditions for Dreamtaq polymerase are outlined in Table 2.4. For colony PCR the template DNA in the reaction set up was replaced with a small sample of colony mass and the initial denaturation step was extended for 10 mins to lyse cells.

Table 2.4: Thermocycling conditions for DreamTaq Green PCR Master Mix.

Step	Temperature °C	Time	Number of cycles
Initial Denaturation	95	1-3 min	1
Denaturation	95	30 s	25 - 40
Annealing	Tm-5*	30 s	
Extension	72	1 min/kb	
Final Extension	72	5 – 15 min	1

*Annealing temperatures for specific primer pairs was calculated using NEB Tm calculator (https://tmcalculator.neb.com/#!/main).

PCR for cloning and proof-reading purposes were carried out in 50 µL volumes comprising 10 µL X2 Q5 Reaction Buffer (NEB), 1 µL 10 mM dNTPs (NEB), 2.5 µL of the relevant primers (Table 2.6), 50 ng of template DNA, 0.5 µL of Q5 High-Fidelity DNA Polymerase (NEB) and dH_2O to 50 µL. The manufacturer's thermal cycling conditions are outlined in Table 2.5.

Table 2.5: Thermocycling conditions for Q5 High Fidelity DNA Polymerase (NEB).

Step	Temperature °C	Time	Number of cycles
Initial Denaturation	98	30 s	1
Denaturation	98	5 – 10 s	25 – 35
Annealing	50 – 72	10 – 30 s	
Extension	72	20 – 30 s/kb	
Final Extension	72	2 min	1
Hold	4 – 10	-	-

2.5.3 Plasmid extraction

Plasmid extraction was carried out using the NEB Monarch™ Plasmid Extraction Kit according to manufacturer's instructions.

2.5.4 Restriction digests

Restriction endonucleases were sourced from NEB and stored at -20°C prior to and following use. Restriction digests were set up in 50 µL reactions containing 1 µg of plasmid DNA or PCR product, 5 µL of 10X CutSmart buffer, 20 U of the relevant restriction endonuclease and dH_2O to 50 µL. The reaction was then incubated at 37°C for 2 hours for PCR products and 4 hours for plasmids. In order to prevent plasmid self-ligation, 2.5 µL of Antarctic phosphatase (NEB) and 6 µL of Antarctic phosphatase reaction buffer (NEB) were added to the plasmid reaction following digestion and incubated for a further 30 mins at 37°C. Digested PCR products and digested, dephosphorylated plasmids were then visualised on agarose gels.

2.5.5 Agarose gel electrophoresis

DNA samples were diluted with 6 x gel loading dye (NEB) before being applied to 1% agarose gels in 1x TAE containing 1 x SybrSafe (Invitrogen). For DNA fragment size estimation the 1 kb plus DNA Ladder (NEB) was loaded with DNA samples. Gels were then subject to a voltage of 100 V for 1 h before being visualised and imaged under UV light in a Gel Doc XR system (Biorad).

2.5.6 DNA extraction from agarose

When required, DNA fragments were excised from agarose gels using the Monarch® DNA Gel Extraction Kit (NEB) according to the manufacturer's instruction.

2.5.7 PCR production purification

PCR products were purified using the Monarch® PCR & DNA Cleanup Kit according to the manufacturer's instructions.

2.5.8 Ligation

Ligation reactions were carried out in 20 µL volumes using T4 DNA ligase (NEB), T4 DNA ligase buffer 10X (NEB) and purified vector and insert DNA at a molar ratio of

1:3 vector to insert. Reactions were chilled overnight in ice water before being transformed into chemically competent *E. coli* (2.3.3)

2.5.9 HiFi assembly

Primers for HiFi assembly were designed with 30 nucleotide overlapping 5' end overhangs (Table 2.7). PCR products obtained using the HiFi primers and appropriately linearised vectors at a molar ratio of 1:1 were combined with NEBuilder HiFi DNA Assembly master mix (NEB) and dH_2O to a reaction volume of 20 µL. The reaction was incubated for 1 hour at 50°C before being transformed into chemically competent *E. coli* (2.3.3).

2.5.10 Transformation of chemically competent *E. coli*

Chemically competent *E. coli* were thawed and 50 µL were aliquoted into a prechilled 1.5 mL microfuge tube and 5-10 µL of a ligation mixture (or 5 ng of plasmid DNA) was added to the competent cells. The mixture was chilled on ice for 30 min before being heat shocked at 42°C for 90 s and then immediately returned to ice for 2 min. Following this 800 µL of SOC media was added to the cells and they were incubated for 1 h at 37°C before being plated onto LB plates containing the relevant antibiotics. (EMBL Transformation).

2.5.11 Transformation of *B. subtilis* WB800N

1 mL aliquots of naturally competent *B. subtilis* WB800N were thawed at 37°C before being inoculated into 20 mL LS medium and incubated for 2 h a 30°C with gentle agitation. Following incubation, 10 µL of 0.1 M EGTA was added to a 1 mL aliquot of culture and incubated for 5 minutes at room temperature. 1 ug of plasmid DNA was then added to the culture aliquot and the culture was then incubated for 2 h at 37°C with vigorous shaking before being plated onto 2xYT agar plates containing 5 µg mL^{-1} of chloramphenicol (Phan *et al.*, 2006).

2.5.12 Transformation of environmental *Bacillus* spp.

The competent cells prepared previously (2.3.5) were diluted in 2 mL transformation buffer and 1 µg of plasmid DNA was added and the culture was then incubated for 20 min at 37°C with gentle shaking (75 rpm). Following this, cells were diluted with 1

mL of LB containing the appropriate antibiotic at 0.1 μg mL^{-1} and incubated for a further 90 min at 37°C with vigorous shaking before being plated onto selective agar plates and grown overnight at 37°C (Kunst and Rapoport, 1995).

2.6 Bioinformatics methods

2.6.1 Genome assembly and annotation

Genomic DNA from the isolates prepared using phenol chloroform extraction were used as the templates to generate a draft whole genome sequence. Genomic DNA was sequenced using the Illumina MiSeq and assembled into contigs by Microbes NG (Birmingham). The largest three contigs for each sample were entered into NCBI BLAST and the top hit for each environmental sample was chosen as the reference genome to align the contigs against. The contigs obtained from sequencing were then aligned against the chosen reference genome for each strain using Mauve (Darling Lab, University of Technology Sydney), which reorders the contigs against the reference to generate a *de novo* sequence for all samples. Open reading frames (ORFs) of the *de novo* sequences were annotated using the online annotation programme RAST (Version 2.0; http://rast.nmpdr.org/rast.cgi). Additional, manual genome annotation was carried out using ARTEMIS (Carver *et al*., 2012).

2.6.2 Digital DNA-DNA Hybridization

The relatedness of the environmental isolates to one another was established using the Genome-to-Genome Distance Calculator 2.1 (GGDC) a web-based service hosted by the DSMZ (http://ggdc.dsmz.de/). DNA-DNA hybridization (DDH) is currently regarded as the taxonomic 'gold standard' for establishing species delineation in bacteria. The GGDC is an *in silico* alternative to DDH, in which genome sequences (complete or partial) can be uploaded and rapidly compared and the relatedness of the organisms established. GGDC values are comparable to the values obtained using DDH and it has also been shown to consistently outperform Average Nucleotide Identity (ANI) in the delineation of species (Meier-Kolthoff *et al*., 2013). The GGDC tool estimates a digital DDH value using three separate equations, for the purpose of this study the recommended equation was used, in which the digital DDH value is calculated by dividing the identical base pairs of the compared genomes with the length of intergenomic matches.

2.6.3 Homology searches in the bacteriocin database BAGEL4

Annotated genomes were uploaded to the online bacteriocin identification tool BAGEL4 (http://bagel4.molgenrug.nl/index.php), which scans the genome for any putative/known bacteriocin precursors and any accessory proteins including those involved in bacteriocin transport, modification and immunity (Van Heel *et al.*, 2018). Initial protein alignments were carried out using the EMBL needle pairwise alignment webserver (https://www.ebi.ac.uk/Tools/msa/clustalo/). Further protein alignments were carried out using Clustal X2 2.0 software (Larkin *et al.,* 2007).

2.6.4 Homology searches in antibiotic database antiSMASH

The annotated genomes of the organisms of interest were uploaded to the 'antibiotic and secondary metabolites analysis shell (antiSMASH) 3.0 to identify the presence of non-bacteriocin antibiotics (https://antismash.secondarymetabolites.org/#!/start). This online tool functions in much the same way as BAGEL4 does, searching the genome for conserved enzymes indicative of biosynthetic gene clusters (BGCs) associated with the production of antibiotics (Weber *et al.*, 2015).

2.6.5 Sanger sequencing

Sanger sequencing of short reads was carried out by Source Bioscience (Nottingham/Oxford) and results were analysed using Benchling (https://www.benchling.com/).

2.7 Oligonucleotides

The oligonucleotides used in this study were designed in the program Benchling, unless otherwise stated, and synthesised by Sigma-Aldrich. Table 2.7 outlines the oligonucleotides used in this study and their respective functions.

Table 2.6: The function, source and sequence of oligonucleotides used in this study. Restriction sites are underlined, overhanging non-annealing regions are denoted in lowercase.

Oligonucleotide	Sequence 5' – 3'	Function	Source
27 F	AGAGTTTGATCATGGCTCA	16 S rRNA sequencing forward primer	Eurofins
1429 R	TACGGTTACCTTGTTACGACTT	16 S rRNA sequencing reverse primer	Eurofins
Amy F	atatatTCTAGAATGATAGGTCAATTGAGC	Amylocyclicin overexpression forward primer	This study
Amy R	atatatCCCGGGTTAAATACCGAAATAAAGAGC	Amylocyclicin overexpression reverse primer	This study
AcnB F	TTTTAGATCATCGGGAAACCCG	Sequencing primer for amylocyclicin operon	This study
AcnA R	GACTATCATACTTTCAATCCTTACATATG	Sequencing primer for amylocyclicin operon	This study
AcnC F	GCAGCTGCTTGGTAAAATCG	Sequencing primer for amylocyclicin operon	This study
AcnC R	GTCAATCATGACGCTGCCTG	Sequencing primer for amylocyclicin operon	This study
AcnD F	GTCATGTTTCTGTTTCTTCGCAG	Sequencing primer for amylocyclicin operon	This study
AcnF R	GTTGAAATGGAGCAGGATCTG	Sequencing primer for amylocyclicin operon	This study
AcnBU F	ggccgccatggcggccgcgggaattcgattCACAATATGATGATACGGC	Forward HiFi primer for region upstream of *acnB*	This study
yjcK R	attgtctaaatcaattttattaaagttcatGTAGGTATTTTCTCCGACGG	Reverse HiFi primer for region upstream of *acnB*	Scholz *et al* 2014
CatPA F	tattattcttccgtcggagaaaatacctacATGAACTTTAATAAAATTGATTTAGACAATTG	Forward HiFi Primer for chloramphenicol resistance cassette	This study
CatPA R	agagatatagtcctgccaccccaaaagtaaTTATAAAAGCCAGTCATTAGG	Reverse HiFi Primer for chloramphenicol resistance cassette	This study

Guac F	tcagataggcctaatgactggcttttataaTTACTTTTGGGGTGGCAGG	Forward HiFi primer for region downstream of *acnF*	Scholz *et al* 2014
Guac R	ctgcaggcggccgcgaattcactagtgataATTGTGGACAGCCGATCTG	Reverse HiFi primer for region downstream of *acnF*	Scholz *et al* 2014
SboUP F	ggccgccatggcggccgcgggaattcgattCCATCATTGCTCATCAGATTTGA	Forward HiFi primer for *sboA*	This study
SboUP R	attgtctaaatcaattttattaaagttcatTCCGATCGAGCATGTTGCACAA	Reverse HiFi primer for *sboA*	Phelan *et al* 2013
CatPS F	aacaaaggttgtgcaacatgctcgatcggaATGAACTTTAATAAAATTGATTTAGACAATTG	Forward HiFi primer for chloramphenicol resistance cassette	This study
CatPS R	cgcaaaaaaatcactgtacagattgcggccTTATAAAAGCCAGTCATTAGG	Reverse HiFi primer for chloramphenicol resistance cassette	This study
AlbF F	tcagataggcctaatgactggcttttataaGGCCGCAATCTGTACAGTG	Forward HiFi primer for intergenic region of *albF*	This study
AlbF R	ctgcaggcggccgcgaattcactagtgataAAAACGGCAGCTGGTGAG	Reverse HiFi primer for intergenic region of *albF*	This study

Chapter 3: Isolation and genetic characterisation of environmental isolates with inhibitory activity against *C. difficile*

3.1 Chapter Introduction

The research on bacteriocins focusses mainly on their potential to be exploited as antimicrobial agents, however, the true role of bacteriocins is in providing a competitive advantage in diverse species rich environments (Riley, 2011). As mediators of bacterial competition their production is ubiquitous with all lineages of prokaryotes being shown to produce bacteriocins and with an estimated 99% of bacteria encoding at least one bacteriocin (Riley, 2011; Klaenhammer *et al*., 1993). Given the extensive diversity in microbial communities this equates to a vast arsenal of undiscovered antimicrobial peptides in a range of environments that can potentially be exploited as alternative therapies against CDI. Conversely, the diversity of microbial communities poses the question of where to begin in the search for novel bacteriocins?

3.1.1 Screening for environmental bacteria producing bacteriocins

When examining the literature, it becomes apparent that the choice of environment to screen for bacteriocin producers is often reflective of the chosen indicator species. For example, many of the anti-listerial Class IIa bacteriocins have been successfully discovered from *Lactobacillus spp.* isolated from fermented, spoilage-resistant foods (Nissen-Meyer *et al*., 2010). The rationale being that the foods that naturally inhibit *L. monocytogenes* contamination contain bacteria producing anti-listerial bacteriocins. Similarly, the Lantibiotics epidermin and gallidermin which have activity against the acne causing bacteria *Propionibacterium acnes* were discovered by screening *Staphylococcal spp.* that belong to the normal human skin microflora (Gotz *et al*., 2014). The anti-clostridial sactibiotic thuricin CD was isolated from an anaerobic *B. thuringiensis* gut commensal under the rationale that a non-disrupted gut flora would contain commensals producing anti-*C. difficile* bacteriocins (Rea *et al*., 2010).

For this work the microbiota of healthy pigs was an environment of interest for isolating bacteria producing anti-*C. difficile* bacteriocins. This is due to pigs also being susceptible to CDI, although infection presents as neonatal enteritis and diarrhoea in piglets under seven days old and adult pigs are rarely, if ever, affected (Debast *et al*.,

2009). However, a 2019 study by Jurburg *et al*., showed that adult pigs could be made susceptible to *C. difficile* colonisation following antibiotic treatment, this suggests that an established, mature microflora in pigs is enough to resist *C. difficile* colonisation, similar to that seen in humans. Colonization resistance could possibly be due to the healthy microflora of mature pigs containing bacteria that produce anti-*C. difficile* bacteriocins. For this reason, the intestinal microflora of healthy adult pigs was chosen as the environment from which to isolate anti-*C. difficile* bacteriocin producing bacteria in this study.

3.1.2 Lab-based methods for bacteriocin production screening

Once an environment has been chosen, the bacteria from this environment are often cultured on nutrient plates to establish single colonies, these isolated bacteria are then grown and overlain with a semi-solid agar seeded with a selected indicator strain in the deferred antagonism assay (Molbculaire, Pasteur and Roux, 2019). Zones of inhibition against the indicator species indicate the production of an inhibitory compound and so these bacteria would be selected for further study. The process involved in the lab-based identification and downstream analysis of bacteriocins will be detailed in subsequent chapters. One common bioinformatical approach to identifying bacteriocins is to sequence the whole genome of identified inhibitory organisms and analyse for encoded antimicrobials.

3.1.3 I*n silico* tools for bacteriocin identification

Twenty years ago, the procurement of bacterial whole genome sequences was a labour-intensive endeavour, however modern advancements have allowed for the fast, affordable whole genome sequencing of bacteria. The ease with which genetic information can be sequenced from bacteria, and the existence of databases harbouring all deposited genomic information has allowed for the development of *in silico* tools for the purposes of bacteriocin identification. For example, the bacteriocin database BACTIBASE is a compiled resource of the physiochemical characteristics of bacteriocins, compiling the PubMed bacteriocin publications with genomic data banked in NCBI (Hammami *et al*., 2010). Additionally, BACTIBASE features online based tools for the identification of bacteriocins, alignment with known bacteriocins

and structure prediction in user-uploaded genomic data (Hamami *et al*., 2010). The genome mining tool BAGEL4 (Formerly BAGEL3) is also of great use when identifying novel bacteriocins. BAGEL4 is a tool also linked to the NCBI database that searches through user uploaded genomic data to identify open reading frames associated with bacteriocin production, based on key motifs and homology to described bacteriocins in the NCBI databases (Van Heel *et al*., 2018). BAGEL4 highlights these bacteriocin related open reading frames as AOIs (Areas of Interest) and presents the open reading frames as a user accessible diagram with features allowing further analysis, particularly alignments to other known bacteriocin-related genes (Figure 3.1). Additionally, the online tool antiSMASH is useful when analysing the genomic data of strains. antiSMASH functions almost identically to BAGEL4, except this tool is specialised at finding motifs associated with the production of secondary metabolites, although some RiPPs can also be identified by antiSMASH (Weber *et al*., 2015).

These tools have been used successfully in the literature to identify novel bacteriocins from environmentally isolated species. For example, the operon encoding the lantibiotic subtilomycin was identified using BAGEL3 from a strain of *B. subtilis* MMA7 isolated from sea sponge that had shown inhibitory activity against a range of indicator species (Phelan *et al*., 2013). Similarly, the two component lantibiotic formicin was also identified by BAGEL3 as being encoded in the genome of *Bacillus paralicheniformis* which had been isolated from fish guts and had shown inhibitory activity against a range of indicator organisms (Collins *et al.*, 2016). Utilising BAGEL3 to analyse the genomic data of the strains in these cases both led to the identification of the bacteriocins responsible for the activity seen against the indicator strains. However, as these databases rely on homology to described bacteriocins in order to identify novel bacteriocins, there is always a possibility that novel bacteriocins with low identity to known clusters could be missed by the algorithms of these *in silico* tools.

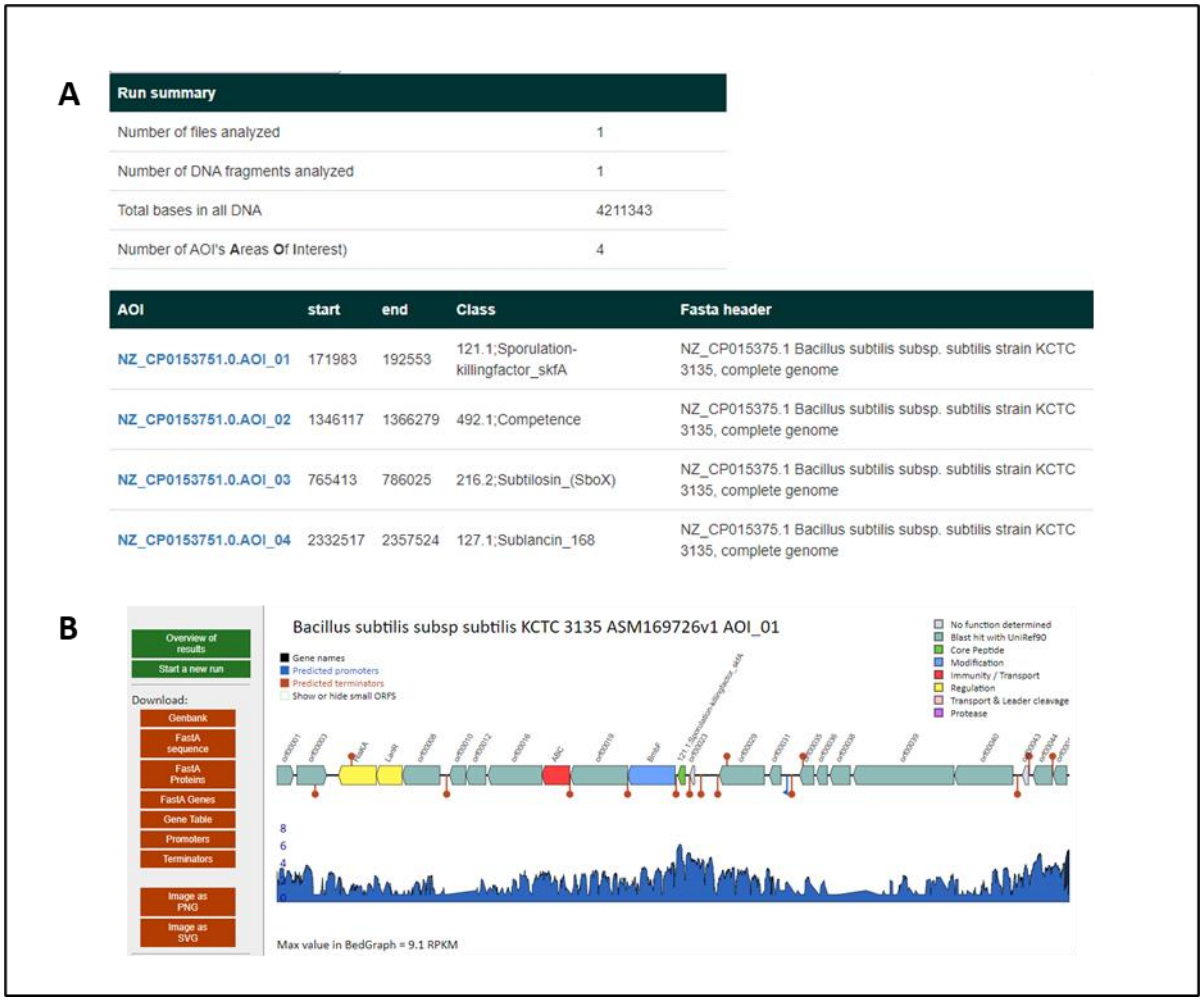

Figure 3.1: An example of the output from bacteriocin mining tool BAGEL4, using the *B. subtilis* 168 whole genome example input (Van Heel et al, 2018). A) Summary of analysis and a table of identified AOIs. B) Schematical representation of the clusters containing AOIs and options for further analysis.

3.1.4 Alternative *in silico* methods for bacteriocin identification

The methods of isolating bacteria based on their inhibition against an indicator-organism and then identifying the bacteriocins produced is generally utilised when the bacteriocin search is targeted for a specific indicator organism. However, there are those whose research focuses entirely on the identification of bacteriocins for the purposes of developing the understanding of the classes or expanding the classification schemes. For these purposes, the targeted bacteria identification stage is omitted and instead the vast catalogue of microbial genome sequences deposited in the NCBI database can instead be exploited in the search for novel bacteriocins. The two component bacteriocin lichenicidin from *B. licheniformis* ATTC 1580 is an example that this approach was utilised for (Begley *et al*., 2011). Due to the conserved nature of the LanM synthetase enzyme and its essentiality in producing Subclass II lantibiotics, the amino acid sequence for LtnM1 (a LanM synthetase involved in the production of lacticin 3147) was used as a driver sequence against all bacterial genomes deposited in NCBI (Begley *et al*., 2011). Following identification of LtnM1 homologous enzymes in the deposited genomes, the genomic regions harbouring these enzymes were analysed for other lantibiotic related genes, namely those encoding the structural prepeptide and transport and immunity related proteins. Ultimately the operon encoding lichenicidin was identified, the strain was procured and active lichenicidin was produced and proven to be a Class II lantibiotic (Begley *et al*., 2011).

This approach was also utilised by Murphy *et al.,* (2011) using the amino acid sequence of TrnC and TrnD, the radical SAM enzymes involved in production of thuricin CD. By using these enzymes as the driver sequence against the NCBI database, 15 putative sactibiotic clusters were identified within three phyla of bacteria (the firmicutes, the bacteroides and the thermotagae) (Murphy *et al*., 2011). Whilst none of these putative sactibiotics were shown to have antibacterial activity due to the strictly *in silico* nature of this work, it was fundamental in identifying the conserved nature of the YGGEPL amino acid motif in radical SAM enzymes (Mathur and Rea, 2019).

Similarly, the circular bacteriocin pumilarin- produced by *B. pumilis* – was discovered by analysing the publicly available bacterial genomes on the NCBI database via BAGEL3, with a specific selection for the SpoIIM (Formerly Duf95) motif associated with circular bacteriocin biosynthetic clusters (Heel *et al.,* 2017). Ultimately the cluster responsible for the production of pumilarin was identified, the strain was procured and bioactive pumilarin was purified (Heel *et al*., 2017).

A more recent *in silico* study used the prepeptide (minus the signal peptide) amino acid sequences of the previously experimentally confirmed, circular bacteriocins as driver sequences against the NCBI database of bacterial genomes (Vezina *et al*., 2020). In doing so 94 previously undescribed putative circular bacteriocin clusters were identified. This study also corroborated the essentiality of SpoIIM family proteins in the production of circular bacteriocins as all putative circular bacteriocin clusters contained a protein of this family (Vezina *et al.*, 2020). Whilst the antimicrobial activity of these putative bacteriocins was not confirmed experimentally, this work was successful in confirming class defining characteristics of the circular bacteriocins. Interestingly, the authors of this study attribute their success to the omission of the strain specific leader peptides in the driver sequences and also the decision to not use BAGEL4 in their bioinformatic mining (Vezina *et al*., 2020). This is due to BAGEL4 including a search for signal peptides within the algorithm, however, the signal peptide of circular bacteriocins is not conserved to reflect the strain-specific nature, thus resulting in low identities to known bacteriocins and oversights of the clusters by BAGEL4 (Van heel *et al*., 2018). Highlighting a key drawback of the homology-based antimicrobial mining tools.

This study hoped to employ the traditional indicator species driven approach to isolate bacteria of the porcine microflora with inhibitory activity against *C. difficile*, which could potentially be attributed to the production of bacteriocins.

Additionally, this study aims to whole genome sequence any potential candidate bacteria and take advantage of the repertoire of *in silico* tools to identify encoded bacteriocins.

3.2 Results

3.2.1 Environmental bacteria with inhibitory activity against *C. difficile*

In order to isolate potential anti-*C. difficile* bacteriocin producers, 1 g of the colon contents from recently slaughtered (<2 hours), six-month-old male pigs (University of Nottingham, School of Veterinary Medicine) was serially diluted 1 in 10 in PBS. The dilutions were then plated onto BHIS nutrient agar in duplicate with one set being incubated anaerobically and one set aerobically, at 37°C for 16 hours. This isolating step was repeated four times with the colon contents of separate pigs. Over one thousand bacterial colonies were isolated in this manner and were initially screened for antagonistic activity against *C. difficile* strains R20291, DH1916 and 630 using the overlay described in 2.3.1. Six bacteria isolated produced large, clear zones of inhibition against *C. difficile* strains and were selected for further analysis. These *C. difficile* inhibiting bacteria were all strictly aerobic and they were designated Y5, Y6a, Y46, B18, 50B and 50A. Figure 3.2 represents the inhibitory activity of these six aerobic isolates following their growth on BHIS for 16 h aerobically at 37°C and the subsequent overlay with *C. difficile* 630 and 16 h of anaerobic incubation at 37°C. The image used was a representative of an experiment completed in triplicate. To identify the species of these aerobic isolates their genomic DNA was isolated and the DNA of the 16s rRNA subunit was amplified by PCR, the PCR product was then purified and sequenced by sanger sequencing (Source Bioscience, Nottingham UK). All the isolates were shown to be members of the *B. subtilis* species complex, however, this complex is composed of 15 highly similar species that are indistinguishable through 16S rRNA DNA sequencing alone (Fan *et al.,* 2017) and thus whole genome sequencing was performed.

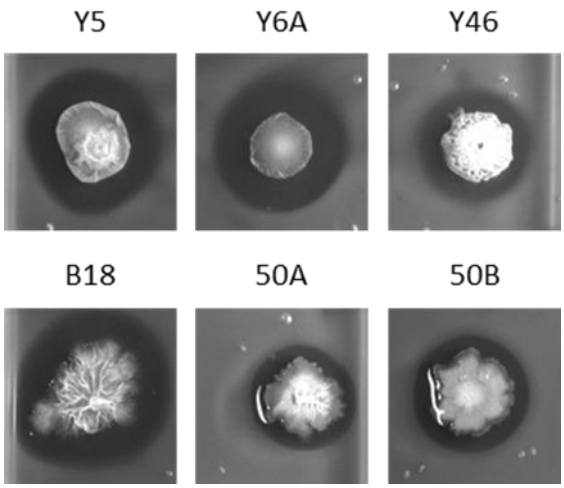

Figure 3.2: Representative images of the aerobic isolates' inhibitory activity against *C. difficile* 630. Aerobic bacteria were cultured for 16 h at 37°C on BHIS nutrient agar. Following this *C. difficile* 630 was overlaid and a further 16 h anaerobic incubation at 37°C was carried out. Experiment was repeated in triplicate and a representative image was chosen.

3.2.2 Species Identification of the *C. difficile* inhibiting environmental isolates

Genomic DNA from all isolates was prepared using phenol-chloroform extraction and sequenced using Illumina Miseq by MicrobesNG (Birmingham, UK). Table 3.1 outlines the data regarding the sequences obtained from MicrobesNG for all environmental isolates.

Table 3.1: Summary of the whole genome sequence data obtained for each isolate following Illumina MiSeq sequencing performed by MicrobesNG (Birmingham, UK).

Environmental Isolate	Species	Number of Contigs	Genome Length (bp)
Y5	*B. velezensis*	62	4086774
Y6a	*B. velezensis*	66	4088097
Y46	*B. velezensis*	53	4083144
B18	*B. velezensis*	54	4042183
50A	*B. subtilis*	65	4043695
50B	*B. subtilis*	52	4040613

Following analysis of the largest three contigs from each bacterial isolate using the National Centre for Biotechnology Information's (NCBI) BLASTn tool setting parameters for bacteria (Taxid:2) and highly similar sequences, a reference genome was chosen to align the contigs against (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The reference genomes chosen for each isolate as follows: *B. velezensis* AGVL-005 (CP024922.1) as the reference genome for Y5, Y6A and Y46; *B. velezensis* YJ11-1-4 (CP011347.1) as the reference genome for B18; *B. subtilis* HJ0-6 (CP016894.1) as the reference genome for 50A and 50B. The reference genome for each of the isolates was obtained in FASTA format from the NCBI website and subsequently used to align the contigs against using the program MAUVE (Darling Lab, University of Technology Sydney). These aligned genomes were annotated automatically using RAST and any additional, manual annotation was carried out using ARTEMIS (Version 2.0; http://rast.nmpdr.org/rast.cgi; Carver *et al.*, 2012). These final assembled and annotated genomes in FASTA format were then used for all further bioinformatical analysis. From the information obtained in identifying a reference genome for the isolates it was evident that *B. velezensis* Y5, Y6A and Y46 most likely originated from the same strain. Similarly, this was thought to be the case for *B. subtilis* 50A and 50B.

Interestingly, *B. velezensis* B18 had presented with a distinguishing colony morphology with the appearance similar to a biofilm on agar plate. This colony morphology was not seen in the other three isolates of *B. velezensis* and the alignment of the contigs appeared to suggest this isolate was genetically as well as phenotypically distinct from the other three strains of *B. velezensis*. Further analysis was required to confirm this.

3.2.3 Relatedness of the environmental isolates

The relatedness of the *Bacillus spp.* to one another was calculated using GGDC 2.1 (https://www.dsmz.de/services/online-tools/genome-to-genome-distance-calculator-ggdc). This estimates a digital DNA-DNA hybridization (DDH) value by comparing genomes, this value is comparable to results obtained in wet lab DDH experiments in which a DDH similarity of >70% implies organisms are the same species and a value of >79% indicates they are the same sub-species (Kolthoff *et al*., 2013). When compared, the *B. velezensis* isolates had DDH values of >90% when compared to one another and all had a nearly identical DDH value of around 28% when compared to the two *B. subtilis* strains (Table 3.2). This suggested that the *B. velezensis* were all derived from the same subspecies, although this was unusual as *B. velezensis* B18 had required a separate reference genome for alignment based on BLAST searches and exhibited a different phenotype. A separate DDH comparison of the two *B. velezensis* reference genomes (*B. velezensis* YJ11-1-4 and *B. velezensis* AGVL-005) to *B. velezensis* B18 showed DDH estimates of 83.03%, with a 93.08% likelihood that they are the same sub-species (data not shown). Therefore, it was not likely that *B. velezensis* B18 was genetically distinct from the other three *B. velezensis* isolates. Variation in the contigs may explain why a different, but still closely related reference genome was suggested based on the BLAST alignments. The difference in colony morphology was likely due to differences in gene expression, *B. subtilis* species complex members can naturally undergo a change to biofilm morphology and some members have been shown to have more potent biocontrol and antimicrobial activity in the biofilm morphology (Caulier *et al.,* 2019). Therefore, *B. velezensis* B18 was chosen as the representative of the *B. velezensis* isolates for further study due to its interesting morphology.

Similarly, *B. subtilis* 50A and 50B showed a DDH similarity of 98.29% (Table 3.2) which makes it highly likely these are identical isolates of the same organism and so *B. subtilis* 50B was chosen at random to be the representative of the *B. subtilis* species isolated in this study. All further work was carried out on these chosen representative strains alone.

Table 3.2: Comparison of the relatedness of the environmental bacteria using DDH estimates calculated by GGDC 2.1 (https://www.dsmz.de/services/online-tools/genome-to-genome-distance-calculator-ggdc).

Organism	*B. velezensis* Y5	*B. velezensis* Y6A	*B. velezensis* Y46	*B. velezensis* B18	*B. subtilis* 50A	*B. subtilis* 50B
B. velezensis Y5		100%	100%	93.6%	28.1%	28.1%
B. velezensis Y6A			100%	93.6%	28.1%	28.1%
B. velezensis Y46				93.5%	28.2%	28.2%
B. velezensis B18					27.90%	28%
B. subtilis 50A						98.29%
B. subtilis 50B						

3.2.4 *B. velezensis* B18 encodes the known bacteriocins amylocyclicin and LCI

Having used the whole genome sequence of the six isolates to determine their species, this data was next used for *in silico* identification of encoded bacteriocins. To achieve this, the annotated whole genome sequences in FASTA format were uploaded to the BAGEL4 webserver (http://bagel4.molgenrug.nl/index.php). BAGEL4 analysis of the draft genome of *B. velezensis* B18 revealed that this strain encoded the previously described, circular bacteriocin amylocyclicin, which was originally isolated from *B. velezensis* FZB42 (Scholz *et al*., 2014). The amylocyclicin operon spans roughly 4.2 kb and encodes six proteins (Figure 3.3) including the amylocyclicin precursor (AcnA), the enzyme responsible for its maturation (AcnC), two ABC transporters (AcnD and AcnE), a protein associated with immunity (AcnF) and a protein of unknown but essential function (AcnB). *B. velezensis* encodes all the necessary proteins for production of amylocyclicin with near 100% amino acid identity to the published amylocyclicin operon as shown in Table 3.3.

Table 3.3: Percentage identity of amylocyclicin operon proteins of *B. velezensis* B18 with the *B. velezensis* FZB42 reference sequence. Alignments carried out using the EMBL pairwise needle online tool (https://www.ebi.ac.uk/Tools/msa/clustalo).

Protein (Reference accession no.)	Function	Identity
AcnB (ABS75255.1)	Unknown function	99.5%
AcnA (ABS75254.1)	Amylocyclicin precursor	100%
AcnC (ABS75253.1)	Bacteriocin maturation enzyme	99.5%
AcnD (ABS75252.1)	ABC-Transporter	99.1%
AcnE (ABS75251.1)	Transport/ immunity	99.3%
AcnF (ABS75250.1)	Immunity	100%

Also encoded by *B. velezensis* B18 is the 279 bp gene (Figure 2b) required to produce the unclassified and unmodified bacteriocin LCI originally isolated from *B. subtilis* A014 (Gong et al., 2011). The protein product of *LCI* encoded for by *B. velezensis* B18 has 98.9% identity and 100% similarity to the published LCI protein sequence and a schematic of this gene is featured in Figure 3.3 (WP_101669965.1).

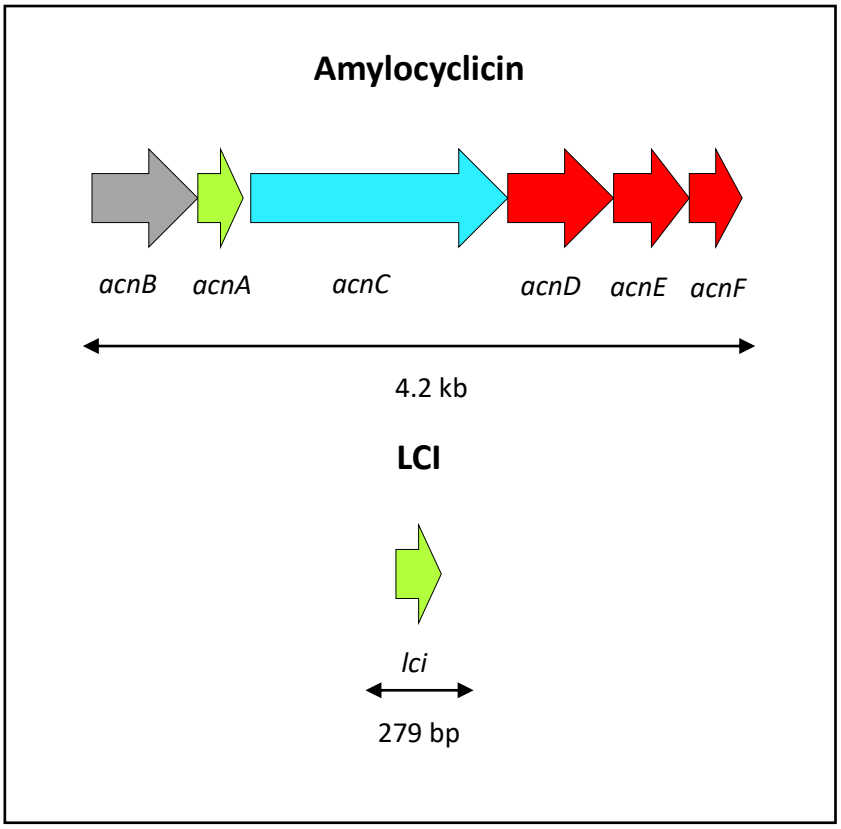

Figure 3.3: Schematic representation of the amylocyclicin and LCI operons encoded for by *B. velezensis* B18. Open reading frames are represented by arrows and colour coded according to protein function using the colour scheme of BAGEL4 (Van Heel et al, 2018). Green represents bacteriocin precursor proteins. Blue represents modification enzymes. Red represents transport and immunity proteins. Grey represents other functions. ORFs drawn to scale wherein 1 cm equates to 1 kb.

3.2.5 *B. velezensis* B18 encodes the two-peptide lantibiotic amyloliquecidin

Analysis of the *B. velezensis* B18 genome also identified the 18 kb biosynthetic gene cluster required to produce the putative two-component lantibiotic amyloliquecidin (Figure 3.4). This gene cluster was previously identified in *B. amyloliquefaciens* AD2 (Du Preez van Staden, 2015) and has since been shown to be present in *B. velezensis* spp. (Gerst *et al*., 2017). Mature amyloliquecidin functions via the synergistic activity of two mature peptides AmyAα (ANA04695.1) and AmyAβ (ANA4696.1), which are encoded by *amyA1* and *amyA2* respectively. *B. velezensis* B18 encodes the alpha and beta subunit prepeptides with high protein identity (98.5% and 97.2% respectively) to the published sequences. The reference sequences for the associated genes in the operon are not publicly available on NCBI, therefore direct comparison was not possible. However, directly downstream of *amyA1* is a gene encoding a LanM-like dehydratase, AmyM1, which shares 36.2% identity with HalM1 (BAB04174.1) the lantibiotic synthetase of haloduracin and 35.1% identity with LicM1 (AAU42941.1) the equivalent protein in the Lichenicidin operon. The beta subunit is also followed by a LanM-like synthetase (AmyM2) that shares 31.3% and 32.7% identity with HalM2 and LicM2 respectively. These identities are similar to those reported in Du Preez van Staden (2015). The operon in *B. velezensis* B18 also contains a LanT-like ABC-transporter (AmyT), a lantibiotic specific serine protease (AmyP), six additional genes encoding proteins associated with transport and immunity (AmyEFG1EFG2), two proteins thought to act as a two-component regulatory system and another protein thought to act as a putative lantibiotic transcription regulator (AmyR), all of which were described in the original operon. It is therefore highly likely that *B. velezensis* B18 has the capacity to produce the two- peptide lantibiotic amyloliquecidin.

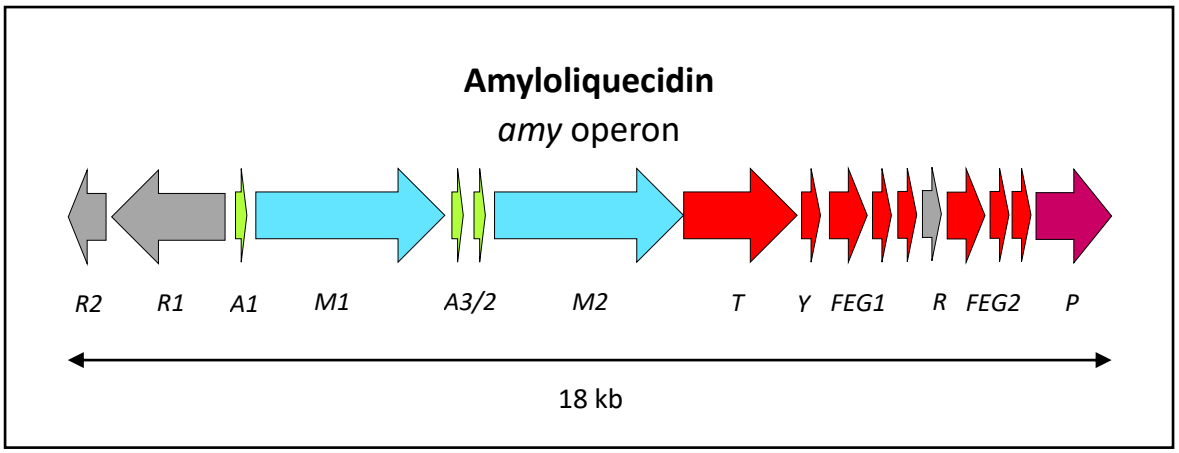

Figure 3.4: Schematic representation of the amyloliquecidin operon encoded for by *B. velezensis* B18. Open reading frames are represented by arrows and colour coded according to protein function using the colour scheme of BAGEL4 (Van Heel *et al.*, 2018). Green represents bacteriocin precursor proteins. Blue represents modification enzymes. Red represents transport and immunity proteins. Purple represents lantibiotic specific serine protease. Grey represents other functions.

Interestingly, closer inspection of the intergenic region between *amyM1* and *amyA2* revealed a 222 bp open reading frame, which was not described in the original publication (Figure 3.4). This putative open reading frame, *amyA3,* appears to encode another lantibiotic prepeptide (AmyAβ2). This 73-amino acid protein has low protein identity to AmyAα (17.4%) with higher identity to AmyAβ (46.1%). Additionally, it contains a cysteine-free region followed by a GA motif suggestive of a 39 amino acid leader peptide that is likely cleaved during processing, a characteristic feature of lantibiotic precursors (Begley *et al*., 2009). The shared identity with AmyAβ implied this putative precursor could be another, undescribed beta subunit of amyloliquecidin. For comparative purposes the core peptide of AmyAβ2 was aligned against the core peptides of other β-subunits of two-component lantibiotics (Figure 3.5). Comparison showed that AmyAβ2 shares some conserved cysteine, threonine and serine motifs with the β-subunits of amyloliquecidin, lichenicidin (Cotter, Hill and Ross, 2009), haloduracin (Oman and Van Der Donk, 2009), plantaracin W (Holo *et al*., 2001) and lacticin 3147 (Mcauliffe *et al*., 1998). However, AmyAβ2 lacks a key threonine/serine motif at the C-terminus that is essential for lantibiotic functionality. These residues are subject to dehydration catalysed by LanM to form the amino acids

Dhb (threonine) or Dha (serine), which subsequently form Lan/MeLan through a cyclisation reaction with cysteine thiols also catalysed by LanM (Montbalon-Lopez *et al*., 2020). AmyAβ2 also lacks this key C-terminal cysteine for cyclisation and formation of the mature lantibiotic. Therefore, it appears unlikely that *amyA3* currently encodes a novel, functional, alternative beta subunit for amyloliquecidin.

Figure 3.5: Alignment of core peptide of AmyAβ2 with related peptides using the Clustal X2 2.0 software. AmyAβ (ANA04696.1), LchAβ (WP_003186379.1), HalAβ (WP_010896631.1), PlnWAβ (AAG02566.1) and LtnAβ (087237.1). Conserved residues are highlighted grey. The leader cleavage site of AmyAβ2 is highlighted yellow.

3.2.6 Non-ribosomally synthesised protein antibiotics encoded by *B. velezensis* B18

Bacteriocins are not the only inhibitory molecules produced by bacteria to antagonise the growth of competing organisms and so it was of importance to also identify the non-bacteriocin antimicrobials the strains encode. The genomes of the strains were uploaded to the antiSMASH server (2.6.4), which identifies antibiotic biosynthetic clusters in the genomes (https://antismash.secondarymetabolites.org). *B. velezensis* B18 was shown to encode multiple complete biosynthetic clusters for non-ribosomally synthesised protein antibiotics with 100% similarity to published sequences. These include the complete clusters for the lipopeptides surfactin and fengycin (Medeot *et al*., 2020; Theatre *et al*., 2021). *B. velezensis* B18 also encodes the antibiotic siderophore bacillibactin, which inhibits the growth of competing bacteria by chelating iron sources and thus starving them of iron (May, Wendrich and Marahiel, 2001). Additionally, *B. velezensis* B18 encodes the biosynthetic cluster for bacilysin, a protein antibiotic containing the non-proteinogenic amino acid L-anticapsin responsible for inhibiting peptidoglycan synthesis in competing bacteria (Kenig, Vandamme and Abraham, 1976).

3.2.7 Polyketide antibiotics encoded by *B. velezensis* B18

B. velezensis B18 was also shown by antiSMASH to encode the biosynthetic clusters for the polyketide antibiotics bacillaene and difficidin with 100% similarity to the published sequences (Wilson *et al*., 1987; Patel *et al*., 1995).

3.2.8 *B. subtilis* 50B encodes the known bacteriocins subtilomycin and subtilosin A

Like *B. velezensis* B18, *B. subtilis* 50B was shown to encode known bacteriocins following BAGEL4 analysis, the first to be discussed here is the Class I lantibiotic subtilomycin, which had previously been isolated from *B. subtilis* MMA7 (Phelan *et al*., 2013). Six genes spanning an 8 kb operon are required for the production, maturation and secretion of subtilomycin, a schematic representation of this operon is shown in Figure 3.6. *B. subtilis* 50B encodes the entirety of the subtilomycin operon, with all but one of the proteins encoded for in the operon having 100% identity to the published sequences, the exception being SubI, a protein hypothesised to play a role in producer-immunity to subtilomycin (Table 3.4). The

lower identity in the immunity protein could potentially reflect the strain specific nature of bacteriocin immunity proteins.

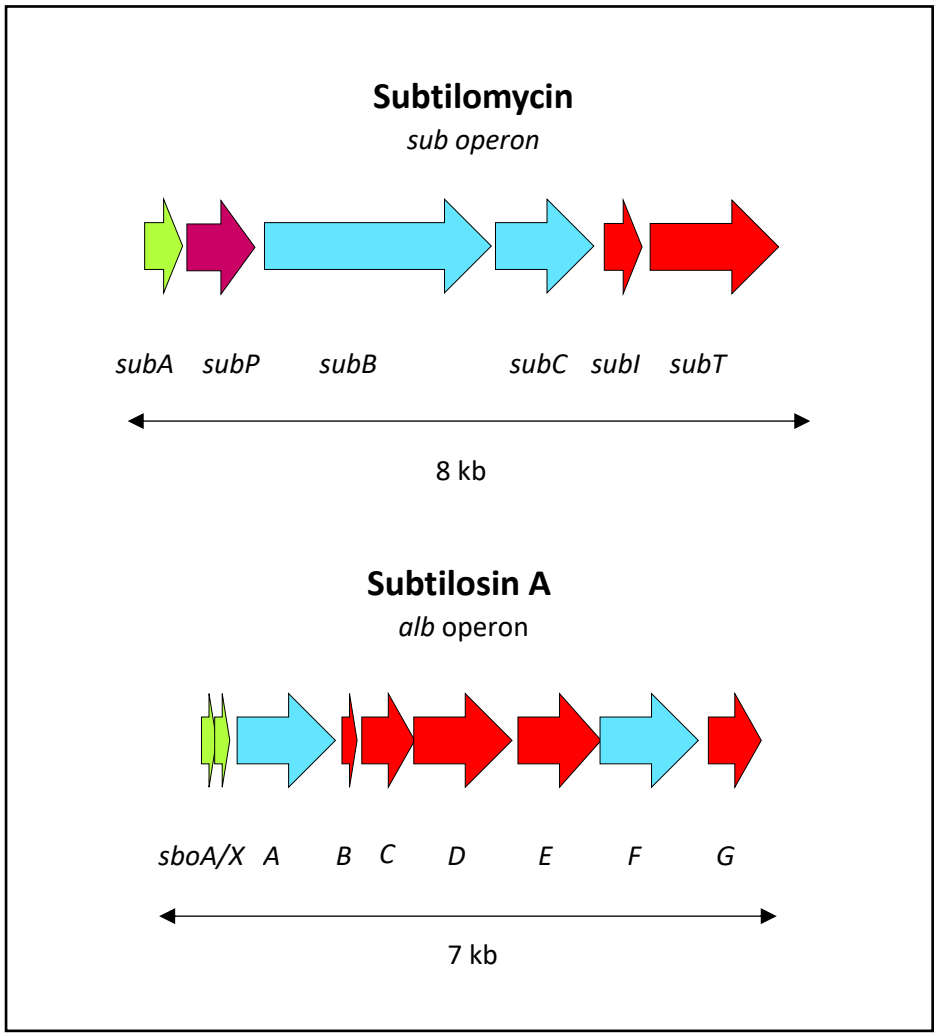

Figure 3.6: Schematic representation of the subtilomycin and subtilosin A operons encoded for by *B. subtilis* 50B. Open reading frames are represented by arrows and colour coded according to protein function using the colour scheme of BAGEL4 (Van heel *et al.,* 2018). Green represents bacteriocin precursor proteins. Blue represents modification enzymes. Red represents transport and immunity proteins. Purple represents lantibiotic specific serine protease.

Table 3.4: Percentage identity of subtilomycin operon proteins of *B. subtilis* 50B with the *B. subtilis* MMA7 reference sequences (JX912247.1). Alignments carried out using the EMBL pairwise needle online tool (https://www.ebi.ac.uk/Tools/msa/clustalo).

Protein	Function	Identity
SubA	Subtilomycin prepeptide	100%
SubP	Extracellular serine protease	100%
SubB	LanB like dehydratase	100%
SubC	LanC like cyclase modification	100%
SubI	Immunity Associated	87.8%
SubT	ABC-Transporter	100%

BAGEL4 also identified that *B. subtilis* 50B encodes the genes necessary for the production of the sactibiotic subtilosin A. Subtilosin A was first isolated from an anti-listerial strain of *B. subtilis* 168 (Babasaki *et al*., 1985), but has since been shown to be widespread in many strains of *B. subtilis* (Velho *et al*., 2013). The genes required for subtilosin A production and maturation are arranged in the 7 kb *sbo-alb* operon (Zheng *et al*., 1999) (Figure 3.6), of the nine genes in the operon eight are essential for optimum production of subtilosin A. The function of each of the protein products of the *sbo-alb* operon are detailed in Table 3.5, alongside the protein identity of the corresponding protein to the reference sequence. *B. subtilis* 50B encodes all of the proteins of the *sbo-alb* operon with high sequence identity, including retaining the use of TTG as the start codon for *albA* and *albE*, which was described by Zheng and colleagues (2000).

Table 3.5: Identity of subtilosin A operon proteins of *B. subtilis* 50B to the reference sequences from *B. subtilis* 168 (Zheng et al, 2000). Alignments carried out using the EMBL pairwise needle online tool (https://www.ebi.ac.uk/Tools/msa/clustalo).

Protein (Reference accession no.)	Function	Identity
SboA (NP_391616.1)	Subtilosin A precursor	100%
sboX (YP_054594.1)	Bacteriocin-like prepeptide	100%
AlbA (NP_391617.1)	Modification of presubtilosin SAM superfamily	99.8%
albB (NP_391618.1)	Membrane bound immunity associated protein	100%
albC (NP_391619.1)	ABC-Transporter associated with export and immunity of mature subtilosin A	100%
albD (NP_391620.1)	Integral membrane protein involved in subtilosin production and immunity	97.5%
albE (NP_391621.1)	Processing Protease	97.9%
albF (NP_391622.1)	Modification of presubtilosin SAM superfamily	99.1%
albG (NP_391623.1)	Membrane bound Immunity associated protein	97%

3.2.9 Non-ribosomally synthesised protein antibiotics encoded by *B. subtilis* 50B

Analysis of the genome of *B. subtilis* 50B using antiSMASH showed that this strain also encoded multiple biosynthetic clusters for non-ribosomally synthesised protein antibiotics with 100% similarity to the published sequences. These include, the lipopeptides fengycin and surfactin, the siderophore bacillibactin and bacilysin.

3.2.10 Polyketide antibiotics encoded by *B. subtilis* 50B

B. subtilis 50B also encoded the biosynthetic cluster for the polyketide antibiotic bacillaene with 100% similarity to the published sequence.

3.3 Chapter discussion

Whilst looking for bacteriocin producing bacteria from pig faeces, six organisms with strong inhibition against three different isolates of *C. difficile* were discovered. These organisms were identified as members of the *B. subtilis* species complex via 16S rRNA gene sequencing, however, differentiation of the individual species within this complex was not possible through 16S rRNA sequencing alone due to the highly conserved nature of the gene (Fan *et al*., 2017). Whole genome sequencing revealed that the six isolates were in fact representing identical isolates of only two distinct species. Therefore, *B. velezensis* B18 and *B. subtilis* 50B were chosen as the representative strains and all further work was carried out on these two strains. Bacteria of the genus *Bacillus* are a diverse group of Gram-positive, rod-shaped, endospore forming aerobes (Abriouel *et al*., 2011). It is unsurprising that the organisms inhibitory to *C. difficile* isolated in this study were identified as *B. subtilis* complex members as this group of bacteria are notorious producers of antimicrobials, with an estimated 5% of their core genome dedicated to the production of antimicrobials such as phospholipids, non-ribosomally synthesised peptides and bacteriocins (Stein, 2005).

However, the fact that only aerobic species of *Bacillus* showed inhibitory activity was surprising. The anaerobic environment of the pig colon and the subsequent anaerobic incubation of the sample was specifically chosen under the assumption that bacteria likely to encounter *C. difficile* in the environment would produce anti-*C. difficile* bacteriocins (Riley, 2011). This was demonstrated by the identification of thuricin CD when anaerobic spore formers were selected for, yet it was not the case for this study (Rea *et al.,* 2010).

Upon analysis of the genome sequences using BAGEL4 it was shown that *B. velezensis* B18 encodes all the necessary genes to produce the 6.4 kDa circular bacteriocin amylocyclicin, discovered in 2014 in *B. velezensis* FZB42 (formerly *B. amyloliquefaciens*) (Scholz *et al*., 2014). Amylocyclicin has been shown to have a relatively narrow spectrum of activity affecting primarily the Gram-positive organisms *Bacillus spp*., *Paenibacillus spp*. and *Clavibacter spp.* However,

amylocyclicin has no inhibitory activity against other Gram-positive bacteria including *S. aureus* and *Arthrobacter*. Amylocyclicin has also never been shown to be inhibitory against *C. difficile,* although specific activity against *C. difficile* has never been tested (Scholz *et al*., 2014). The activity against *Bacillus* spp. and related genera suggests that amylocyclicin may be the bacteriocin responsible for the anti-*C. difficile* activity seen from *B. velezensis* B18, as even the bacteriocin thuricin CD produced by *B. thuringiensis* DPC 6431 has strong inhibitory activity against a number of *Bacillus* species including *B. cereus*, despite it being referred to as a "*C. difficile* specific" bacteriocin by Rea *et al*., (2010).

B. velesensis B18 was also shown to encode the operon for the two-component lantibiotic amyloliquecidin. The alpha and beta precursor structural genes for amyloliquecidin are present in the genome of *B. velezensis* B18 with high sequence identity to the described bacteriocin (Du Preez van Staden, 2015). The alpha and beta subunits of two-component lantibiotics act in a synergistic manner with the mature alpha protein binding to Lipid II on the target cell membrane, the mature beta subunit is then recruited to this complex wherein it acts to form pores in the target cell membrane, allowing efflux of intracellular phosphate and potassium ions causing rapid cell death (Wiedemann *et al*., 2006; Oman and Van Der Donk, 2009). The activity spectrum of amyloliquecidin encompasses a range a of Gram-positive bacteria including *S. aureus*, *L. monocytogenes* and *Enterococcus* spp. (Du Preez van Staden, 2015; Van Staden *et al*., 2016). Whilst amyloliquecidin has not been described as being active against *C. difficile*, it cannot be ruled out that this lantibiotic is responsible, at least in part, for the inhibitory activity seen against *C. difficile* as many lantibiotics have a broad spectrum of activity, which generally includes *Clostridia* (Collins *et al*., 2016; Bartoloni *et al*., 2004; Rea *et al*., 2007).

Interestingly, *B. velezensis* B18 also encodes a third lantibiotic structural gene with 46.1% identity to the beta subunit of amyloliquecidin. It was therefore theorised that this additional structural gene could encode an alternative beta subunit of amyloliquecidin, however, in depth analysis of the prepeptide highlighted that this protein lacks key cysteine and threonine/serine residues essential for the activity of lantibiotics and so it is most likely that this additional structural gene is currently non-

functional and likely the product of a gene duplication mutation (Montbalon-Lopez *et al.,* 2020). Whilst this additional subunit is unlikely to undergo maturation to a functional lantibiotic due to the absence of these key residues, the potential for it to be used as a protein scaffold to produce an alternative beta subunit to amyloliquecidin is not inconceivable (Bedard and Biron, 2018). Indeed, the four C-terminus amino acids of the likely non-functional β-subunit encoded in the amyloliquecidin operon are as follows "Cysteine – Asparagine – Isoleucine – Tyrosine". A single bp substitution in the nucleotides encoding asparagine would result in a mutation to serine. Likewise, a single bp substitution in the nucleotides encoding for tyrosine would result in a mutation to cysteine. Therefore, whilst currently perceived to be non-functional the introduction of two point-mutations could potentially result in a semi-synthetic β subunit of amyloliquecidin, although that work would be beyond the scope of this current study.

B. velezensis B18 also encodes the 5.5 kDa unmodified bacteriocin LCI, originally isolated from *B. subtilis* strain A104, although homologs have previously been identified in *B. velezensis* FZB42 (Gong *et al*, 2011; Liu *et al*., 1990). LCI is unlikely to be responsible for the inhibitory activity against *C. difficile* as activity has only been described against Gram-negative phytopathogens including *Xanthomonas campestris pv. oryzae* and *Ralstonia solanacearum* with no activity described against Gram-positive organisms (Liu et al, 1990). Additionally, the mode of action of LCI is hypothesised to involve the lipopolysaccharide of Gram-negative bacteria, thus activity against *C. difficile* is highly unlikely to occur (Gong *et al*, 2011).

Analysis of the *B. subtilis* 50B genome showed that it encodes all the relevant genes to produce two known bacteriocins subtilosin A and subtilomycin. Subtilosin A is a well characterised, 3.9 kDa sactibiotic that is found in many strains of *B. subtilis* (Velho *et al*., 2013). The inhibitory spectrum of subtilosin A has been relatively well established and it displays a broad spectrum of activity, inhibiting mostly Gram-positive *Streptococcus* spp. and related species with activity also described against uncapsulated Gram-negative bacteria (Shelburne *et al.*, 2007). Subtilosin A has thus far not been described as having activity against *C. difficile* and in the original publication for thuricin CD the authors stated that they had not found subtilosin A to

exhibit anti-*C. difficile* activity, however, no supporting experimentation was presented in this study (Rea *et al.,* 2010). Thuricin CD itself and the recently characterised ruminococcin C are both sactibiotics that display potent anti-Clostridial (and related species) activity, which could suggest sactibiotics as a class display anti-Clostridial activity. It is therefore not unreasonable to assume, without contradictory evidence, that subtilosin A produced by *B. subtilis* 50B could be responsible at least in part for the inhibitory activity seen against the *C. difficile* strains tested.

Subtilomycin is a 3.25 kDa, broad spectrum, Class I lantibiotic first described in *B. subtilis* strain MMA7 that is encoded in the genome of *B. subtilis* 50B. Subtilomycin has been shown to have inhibitory activity against members of *Clostridia* spp., including weak activity against *C. difficile* (Phelan *et al*., 2013) and so it is possible subtilomycin is responsible at least in part for the activity against *C. difficile* seen in *B. subtilis* 50B.

Through analysis of their draft genomes via antiSMASH it was shown that both *B. velezensis* B18 and *B. subtilis* 50B encoded the biosynthetic clusters required to produce a range of non-ribosomally synthesised protein antibiotics. Both strains encode the potent antifungal lipopeptide class fengycins, the activity of fengycins is generally restricted to filamentous fungal phytopathogens (Vanittanakom *et al.,* 1986). Any antibacterial activity thus far described by fengycins has been against Gram-negative phytopathogens (Medeot *et al*., 2020). Therefore, it is unlikely that fengycins are responsible for the anti-*C. difficile* activity seen in these strains.

Both *B. subtilis* 50B and *B. velezensis* B18 also encode the catecholic siderophore bacillibactin, which sequesters iron and antagonises the growth of competing bacteria by making the iron unavailable to them (May, Wendrich and Marahiel, 2001). The activity spectra of bacillibactin appears to be limited, again, to fungal phytopathogens (Yu *et al*., 2011). Whilst there is little literature focusing on the siderophores antimicrobial activity, the research thus far suggests bacillibactin is an unlikely candidate for the anti-*C. difficile* activity seen in these strains.

Bacilysin, a potent antibiotic first discovered in 1949 (Abraham and florey, 1949) is also encoded by both strains. Bacilysin has a broad spectrum of activity and proves

antagonistic against Gram-negative and Gram-positive bacteria, including other *Bacillus spp.* and some yeast such as *Candida albicans* (Kenig, Vandamme and Abraham, 1976). At this stage bacilysin could not be discounted as being responsible for some of the anti-*C. difficile* activity seen in both strains.

Both *Bacillus* isolates also encoded the biosynthetic cluster for surfactin, a family of cyclic lipopeptide antibiotics produced by *Bacillus spp*. inhibitory against a broad range of bacterial pathogens including other causes of neonatal swine diarrhoea *C. perfringens* and *Brachyspira hyodysentenae* (Horng *et al*., 2019). Surfactin production in *Bacillus spp.* is controlled by density dependant quorum sensing (Ongena and Jacques, 2008), interestingly ComX, the pheromone simultaneously associated with competence and the expression of surfactin, was highlighted as a potential bacteriocin prepeptide by BAGEL4 (data not shown). Whilst there is no evidence that ComX has antimicrobial capacities there is evidence of other bacterial pheromones having direct inhibitory activity (Gilmore *et al*., 2015). Additionally, at this stage surfactin could also not be ruled out as being responsible for the inhibitory activity against *C. difficile* seen from both strains of *Bacillus.*

Both strains of *Bacillus* isolated in this study also encode the polyketide antibiotic bacillaene, which could be responsible in part for the anti-*C. difficile* activity exhibited. Bacillaene has a very broad spectrum of activity against both Gram-positive and Gram-negative bacteria and therefore has the potential to inhibit *C. difficile* (Patel *et al.,* 1995). *B. velezensis* B18 also encoded the polyketide antibiotic difficidin, which could also be responsible in some degree for the anti-*C. difficile* activity displayed by this isolate, as this antibiotic was named for its potent activity against *C. difficile* (Wilson *et al.,* 1987).

Assembly and analysis of the genomes of the *C. difficile* inhibiting *Bacillus* spp. isolated in this study revealed they each encode a plethora of antimicrobial compounds, any number of which could be responsible for the activity seen against *C. difficile*. However, whilst BAGEL4 and antiSMASH identify antimicrobial compounds encoded in the genomes of strains they cannot identify if these antimicrobials are expressed in specific circumstances. Additionally, the data collected from these databases is based on homology of sequences to described

antimicrobials, therefore novel or genes with low homology are easily overlooked. This coupled with potential sequencing or assembly errors of the draft genomes means there is still a possibility that the anti-*C. difficile* substances produced by both species of *Bacillus* spp. are novel bacteriocins. Therefore, it is imperative that the inhibitory substances produced by these *Bacillus* spp. are identified definitively through experimental techniques.

Chapter 4 Towards recombinant bacteriocin production and bacteriocin deficient-native strains

4.1 Chapter Introduction

The ribosomal origin of bacteriocins ensures that the biosynthetic machinery for bacteriocins is encoded within the genomic data. This was seen for the genomic analysis of *B. velezensis* B18 and *B. subtilis* 50B (3.2), in which four bacteriocins between the two strains were hypothesised to be responsible in part for the anti-*C. difficile* activity. These were the lantibiotics amyloliquecidin and subtilomycin; the circular bacteriocin amylocyclicin and the sactibiotic subtilosin A (3.3). Identification of the genetic locus of the biosynthetic machinery is highly advantageous in the study of bacteriocins as it allows for the possibility of genetically engineering the original producing strain, as well as the opportunity to produce recombinant bacteriocins and their biosynthetic machinery in non-native strains. The production of recombinant bacteriocins in non-native hosts has a number of advantages over native hosts including controlled overexpression of bacteriocin genes, which can lead to higher protein yields than the native strains (Zhang *et al.,* 2018). Additionally, bacteriocins are often isolated from pathogenic or antibiotic resistant bacteria such as the enterocins of the *Enterococcus* spp. (Franz *et al*., 2007), heterologous overexpression could then be carried out in a safe, food-grade species of bacteria to mitigate harm.

Despite its initial advantages, heterologous expression of bacteriocins is complicated by many factors, including the toxic nature of bacteriocins and the potential for the toxic product to accumulate in the host cell cytoplasm, exerting its bactericidal affect (Mesa-Pereira *et al.,* 2018). Aside from the obvious lethality, the bacteriocins of Gram-positive bacteria are a heterogenous group of protein toxins with many classes requiring extensive post translational modifications by specific enzymes such as leader cleavage, addition of unusual amino acids and circularisation before an active peptide is produced. This complicated maturation process causes many additional problems in heterologous overexpression, including differences in codon usage that impair initial translation to differences in the general host cell physiology and biochemistry that could lead to improper disulphide bond formation and improper folding of the mature peptides (Zhang *et al.*, 2018. Mesa-Pereira *et al.*, 2018).

Manipulation of the biosynthetic machinery in the native host strains, such as in deletion of the prebacteriocin gene or other essential genes, is also not a straightforward process with problems arising at all stages including DNA uptake and presence of native genes destroying non-native DNA or proteins (Mesa-Pereira *et al.*, 2018). Nevertheless, there are many examples in the literature of these methods being used successfully in the study of bacteriocins from Gram-positive bacteria.

4.1.1 Gene Deletion for bacteriocin identification

Once a biosynthetic operon for bacteriocins has been identified in an inhibitory strain it is common practice to carry out deletions of genes in the operon to determine their importance in the production of the mature peptide. The biosynthetic operon for the circular bacteriocin amylocyclicin was originally proven to be responsible for the inhibitory activity of *B. velezensis* FZB42 by a homologous recombination deletion of the operon and replacing it with a kanamycin cassette (Scholz *et al.,* 2014). Subsequently, the roles of each of the genes in the operon (besides *acnB*) were deciphered by single gene deletions and analysis of the amylocyclicin peptide (or lack of) that was produced (Scholz *et al.,* 2014). The role that each of the genes in the biosynthetic operon for the sactipeptide bacteriocin subtilosin A was also elucidated by homologous recombination and subsequent replacement of the genes with an antibiotic cassette (Zheng *et al*., 2000). Most bacteriocins are primarily identified from environmental samples and there is sometimes the issue that these environmental bacteria are not as amenable to genetic manipulation as common lab strains of bacteria due to a myriad of factors including restriction systems present in the cell, inability to uptake large DNA fragments (e.g plasmids) and other general features of the bacterial cell physiology. In these situations, heterologous overexpression of the target bacteriocins and their associated genes is an alternative approach to assess their activity.

4.1.2 Heterologous expression of Class Ia bacteriocins from Gram-positive bacteria

The lantibiotic class is an example of bacteriocins of Gram-positive bacteria that require extensive post translational modifications before a mature peptide is produced. The prebacteriocin of the lantibiotics undergo extensive processing by

dedicated LanB and LanC (subclass I) or LanM (subclass II) enzymes, in which the unusual amino acids lanthionine, methyllanthionine, dehydroalanine and dehydrobutyrine are incorporated into the structure (Montbalon-Lopez *et al.,* 2020). The subclass I lantibiotic nisin is one of the few bacteriocins available as a commercial product, however, commercial production still relies on batch fermentation of the native producer strain *L. lactis* rather than heterologous production in a surrogate host strain (Younes *et al*., 2017). Nevertheless, heterologous expression of bioactive nisin has been achieved in other species of Lactic Acid bacteria such as in the nisin deficient strain *L. lactis* MG1363 (Kong and Lu, 2014). The nisin precursor peptide, NisA has also been expressed in *E. coli* under the control of the T7 promoter, however, this heterologously produced nisin precursor was unprocessed and untested for biological activity (Sen and Narbad, 2012). Kayalvizhi *et al*., (2016) and colleagues heterologously expressed the precursor for the subclass I lantibiotic, mersacidin, in *E. coli* using a similar method to Sen and Narbad (2012). However, they reported that their recombinant and unprocessed mersacidin bacteriocin had activity against a range of Gram-positive indicator organisms (Kayalvizhi *et al*., 2016).

Recombinant lichenicidin, a subclass II lantibiotic requiring two peptides for activity has also been produced in *E. coli*. Kuthning *et al*., (2015) and colleagues sequentially constructed two pRSFduet plasmids (Figure 4.1) containing each prepeptide of lichenicidin and its corresponding modification machinery. Upon transformation of *E. coli* BL21 (DE3) with these plasmids, the mature lichenicidin subunits were expressed, purified and confirmed using LC/MS data. However, as this study focussed on strain engineering, the biological activity remained untested (Kuthning *et al*., 2015). Despite the complicated processing required to produce a mature bioactive lantibiotic it has been shown that recombinant lantibiotics can successfully be produced in non-native strains including other LAB family members and to a lesser extent *E. coli*.

4.1.3 Heterologous expression of Class Ib bacteriocins from Gram-positive bacteria

The Class Ib group of bacteriocins are referred to as the circular bacteriocins due to undergoing head to tail cyclization following leader cleavage in the processing of the mature bioactive peptide. The biosynthetic machinery to achieve this circularisation

is located in the same operon as the structural gene for circular bacteriocins, however, there are many issues reported when attempts have been made to produce recombinant circular bacteriocins in heterologous hosts. Fernandez *et al*., (2007) attempted to clone the biosynthetic operon for enterocin AS-48, a circular bacteriocin originally produced by pathogenic *E. faecalis* S-48, into food safe strains of *Lactobacillus* spp*.*. In their study they found that despite the presence of the complete biosynthetic machinery on vectors in *Lactobacillus* strains, enterocin AS-48 was not produced by these strains. However, those same vectors cloned into strains of *Enterococcus* spp. that did not naturally produce enterocin AS-48 resulted in them producing mature bioactive enterocin AS-48 and also gaining immunity to the effect of exogenous enterocin AS-48 (Fernandez *et al.,* 2007). Similarly, attempts to produce recombinant novel circular bacteriocin carnocyclin, originally produced by *Carnobacterium maltaromaticum* were only successful when the biosynthetic machinery was cloned into another strain of *C. maltaromaticum*, which did not naturally produce carnocyclin A (Van Belkum *et al*., 2010). More recently, Kurata *et al*., (2019) produced recombinant and bioactive amylocyclicin in *E. coli* by cloning the structural gene*, acnA*, for amylocyclicin into a pET101DTOPO plasmid without its leader sequence. Although, bioactive recombinant amylocyclicin was produced in *E. coli*, it lacked its circularization and activity was minimally achieved only from the cell debris fraction (Kurata *et al*., 2019). The difficulty in producing recombinant circular bacteriocins with sufficient activity in acceptable hosts may be due to the requirement of all the proteins present in the biosynthetic operon for processing of circular bacteriocins (Perez, Zendo and Somomoto, 2018). Nevertheless, examples from the literature have shown that recombinant circular bacteriocin production is possible, even if it requires the use of a host strain closely related to that of the native producer.

4.1.4 Heterologous expression of Class Ic bacteriocins from Gram-positive bacteria

The sactipeptides are a relatively new class of bacteriocins of which, subtilosin A produced by *B. subtilis* is the best characterised. The sactipeptides are defined by their extensive post translational modification by the SPASM-domain containing radical SAM enzymes, which leads to the formation of intramolecular thioether

bridges between cysteine sulphurs and unreactive α-carbons of bridging partner amino acids (Mathur and Rea, 2019). The radical SAM enzymes are often located in the same operon as their substrate precursor bacteriocins, thus knowing the genetic location and their role in bacteriocin maturation has allowed for informed experimental approaches to produce recombinant sactibiotic bacteriocins. Himes *et al*., (2016) utilised two methods of heterologous expression of the definitive sactibiotic, subtilosin A, and its dedicated radical SAM enzyme, AlbA, in *E. coli* BL21 (DE3). The first method was by cloning the subtilosin A precursor gene, *sboA* into the vectors pMCSG7 and pET28c with alternating N or C-terminal histidine tags. Concurrently, the *albA* gene was overexpressed in *E. coli* according to Fluhe *et al*., (2013). Incubation of Nickel-column purified AlbA peptide with the presubtilosins and subsequent LC-MS analysis confirmed that mature subtilosin A had been produced using this method. Himes *et al.,* 2016 then used the additional method of generating mature subtilosin A by inserting the *sboA* gene and the *albA* gene sequentially into the pET-Duet system (Figure 4.1) and thus expressing the two simultaneously in *E. coli* BL21 (DE3). Whilst the mature subtilosin A peptide was confirmed by Himes and colleagues to be produced in this manner through LC/MS analysis, antimicrobial activity of recombinant subtilosin A was never demonstrated in their study, therefore it is unknown whether this recombinant subtilosin A retains its antimicrobial activity.

Ruminococcin C is a recently discovered sactibiotic produced by the human gut commensal *R. gnarvus* and traced to a dedicated sactibiotic biosynthetic operon containing 5 structural prepeptides and 2 radical SAM enzymes (Balty *et al*., 2019). One of these prepeptides (C1) was successfully heterologously produced in *E. coli* to an active, mature form (RumC1) by co-expressing with its allocated radical SAM enzyme in the pRSF-Duet vector (Figure 4.1) in *E. coli* BL21 (DE3). Interestingly, antimicrobial activity was only achieved when the processed peptide was digested by trypsin, which could be either the natural process for activation of this bacteriocin or an additional step required due to improper processing of RumC1 in the *E. coli* host (Balty *et al*., 2019). Therefore, despite the extensive post translational

modifications, it has been demonstrated that recombinant sactibiotics can successfully be produced in *E. coli.*

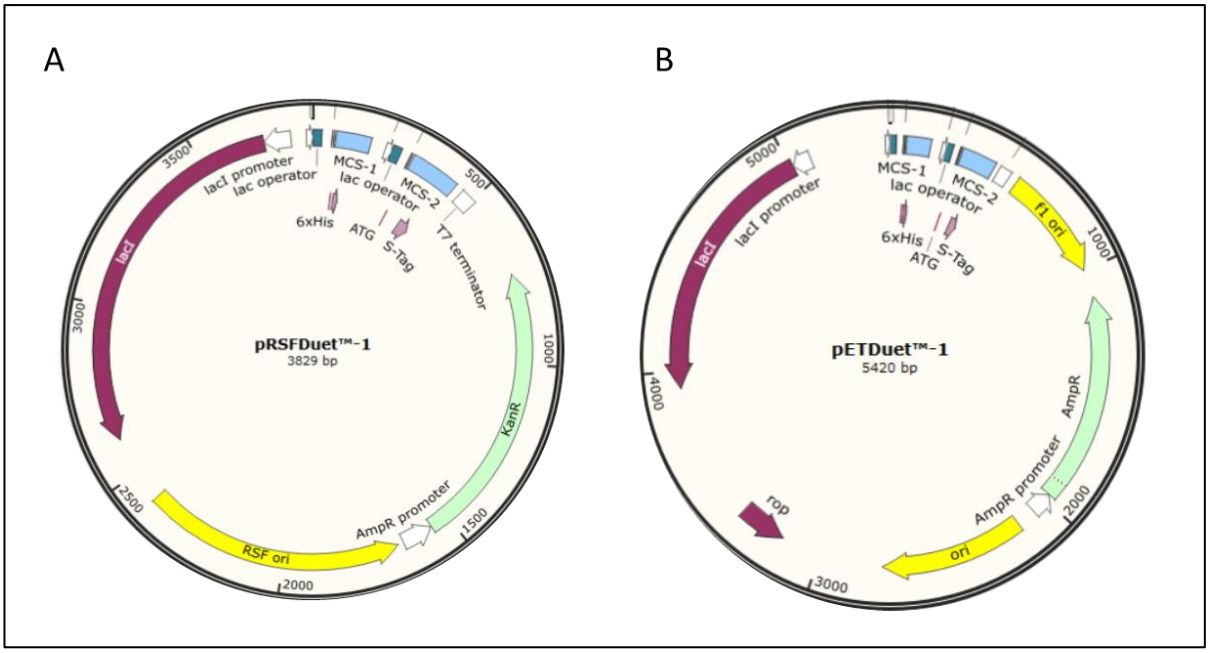

Figure 4.1: Double protein expression vectors utilized in the production of recombinant bacteriocins in *E. coli*. A) pRSFDuet™-1 vector (Novagen), featuring two multiple cloning sites (Blue) inducible by the Lac system (purple) under the control of T7 promoters, the *E. coli* RSF origin of replication (yellow) and a kanamycin resistance gene (Green). B) pETDuet-1 vector (Novagen) featuring two multiple cloning sites, inducible by the Lac system under the control of T7 promoters, The *E. coli* ORI (yellow) and an ampicillin resistance cassette (green). Plasmids visualised on Snapgene viewer software 3.3.4 (Insightful Science).

4.1.5 *Bacillus* spp. as host for recombinant bacteriocin production

Despite the wealth of literature available outlining protocols for heterologous production of the major classes of bacteriocins available, most studies opted for *E. coli* or members of the LAB family as their hosts. There seemed to be little exploration into the use of *B. subtilis* as a host for recombinant bacteriocin production, even in the case of bacteriocins originally produced by *Bacillu*s spp. The justification in the literature for the use of members of the LAB as hosts for bacteriocin production was due to the GRAS status of many members of the LAB, in particular lactobacillus. However, members of the *B. subtilis* family complex have also been recognised as GRAS species, suitable for use in food production (Sewalt *et al*., 2016), also *B. subtilis* species complex members have a history in the production of commercial enzymes with *Bacillus* being the primary source for many commercial enzymes (Miendi *et al*., 2014). Similarly, *E. coli* was justified as a host for bacteriocin production due to its extensive history and the development of dedicated lab strains and vectors for protein expression. However, in that regard *B. subtilis* is the Gram-positive equivalent of *E. coli* with an equally extensive use in genetic engineering and recombinant protein production (Harwood, 2003). Use of *B. subtilis* as a heterologous host in the case of bacteriocins produced by other *Bacillus* spp., as is the case for this study, also has additional benefits including potentially eliminating problems caused by differences in codon usage, cell machinery, promoters, and improper folding of the mature peptide. Therefore, the use of *B. subtilis* as a heterologous host for the production of bacteriocins is worth investigating.

This study, therefore, aimed to utilise a *B. subtilis* protein expression system to produce recombinant anti-*C. difficile* bacteriocins identified in the genomes of *B. velezensis* B18 and *B. subtilis* 50B.

Additionally, this study aimed to carry out deletions of the biosynthetic machinery for the candidate bacteriocins identified in the genomes of *B. velezensis* and *B. subtilis* 50B to identify the bacteriocin responsible for the anti-*C. difficile* activity.

4.2 Results

4.2.1 Construction of the pHT43_*acn* overexpression vector

Although the anti-*C. difficile* activity seen from *B. velezensis* B18 could not at this stage of the study be attributed to any of the candidate antimicrobials identified during genome mining, amylocyclicin was a likely candidate due to this bacteriocins described potent activity against Gram-positive bacilli (Scholz *et al*., 2014). To investigate whether amylocyclicin was indeed responsible for the anti-*C. difficile* activity seen from *B. velezensis* B18, purification was required and so heterologous overexpression and purification of amylocyclicin was to be carried out using the *B. subtilis* WB800N overexpression system (MobiTec).

The amylocyclicin operon was PCR amplified (2.5.2) from the genomic DNA of *B. velezensis* B18 using the primer pair Amy F and Amy R (2.7), which annealed to the start of the *acnB* gene and the end of the *acnF* gene respectively. Figure 4.2A shows the agarose gel visualisation and size confirmation of the 4,173 bp amylocyclicin insert obtained following PCR. Correct sequence of the insert was confirmed using sanger sequencing (Source Bioscience, Nottingham) and primer sets AcnB F/ AcnA R; AcnC F/ AcnC R and AcnD F/ AcnF R, which spanned the entire amylocyclicin operon. Following sequence confirmation, the amylocyclicin insert was digested (2.5.4) with the restriction enzymes *XbaI* and *XmaI* and ligated into the *XbaI* and *XmaI* sites of digested, dephosphorylated pHT43 (MobiTec) generating the overexpression vector pHT43_*acn* (2.5.8), a schematic of which is shown in Figure 4.3.

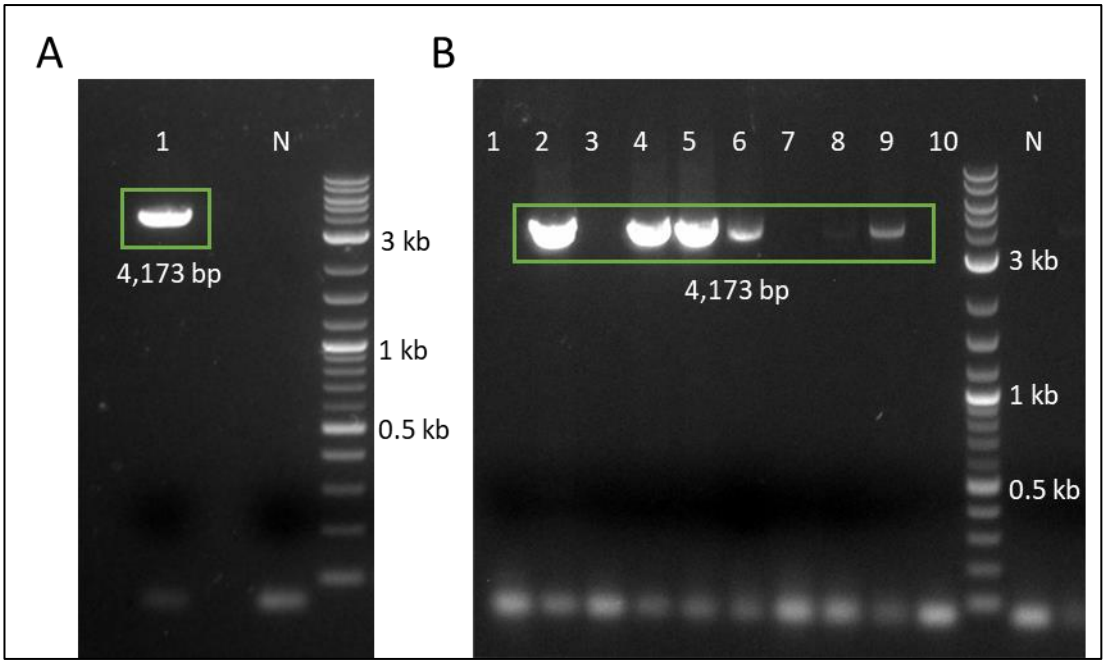

Figure 4.2: 1% agarose gel visualisation of the amylocyclicin operon using the primer pair Amy F and Amy R. A) The 4,173 bp amplified amylocyclicin operon (1) from the genomic DNA of *B. velezensis* B18. B) The 4,173 bp amplified amylocyclicin operon from *E. coli* Top10 (2, 4, 5, 6 and 9) transformed with the pHT43_acn vector. The negative control in lane 'N' and the 1 kb plus DNA ladder (NEB) for size reference.

The ligated pHT43_*acn* vector was then transformed into chemically competent *E. coli* Top10 and transformants were screened for the amylocyclicin operon through colony PCR (2.5.2) and the primer pair Amy F and Amy R (2.7). Ten *E. coli* Top10 transformants were PCR screened for the presence of the amylocyclicin operon and mobilised on agarose gel (2.5.5; Figure 4.2B). The *E. coli* colonies designated 2, 4, 5, 6 and 9 all showed a band corresponding to just above the 4 kb band on the ladder, the expected size of the complete amylocyclicin operon. As they showed the most vivid bands the PCR products from colonies 2, 4 and 5 were purified (2.5.7), and correct sequence was confirmed as before with the amylocyclicin operon primer pairs and sanger sequencing (Source Bioscience, Nottingham).

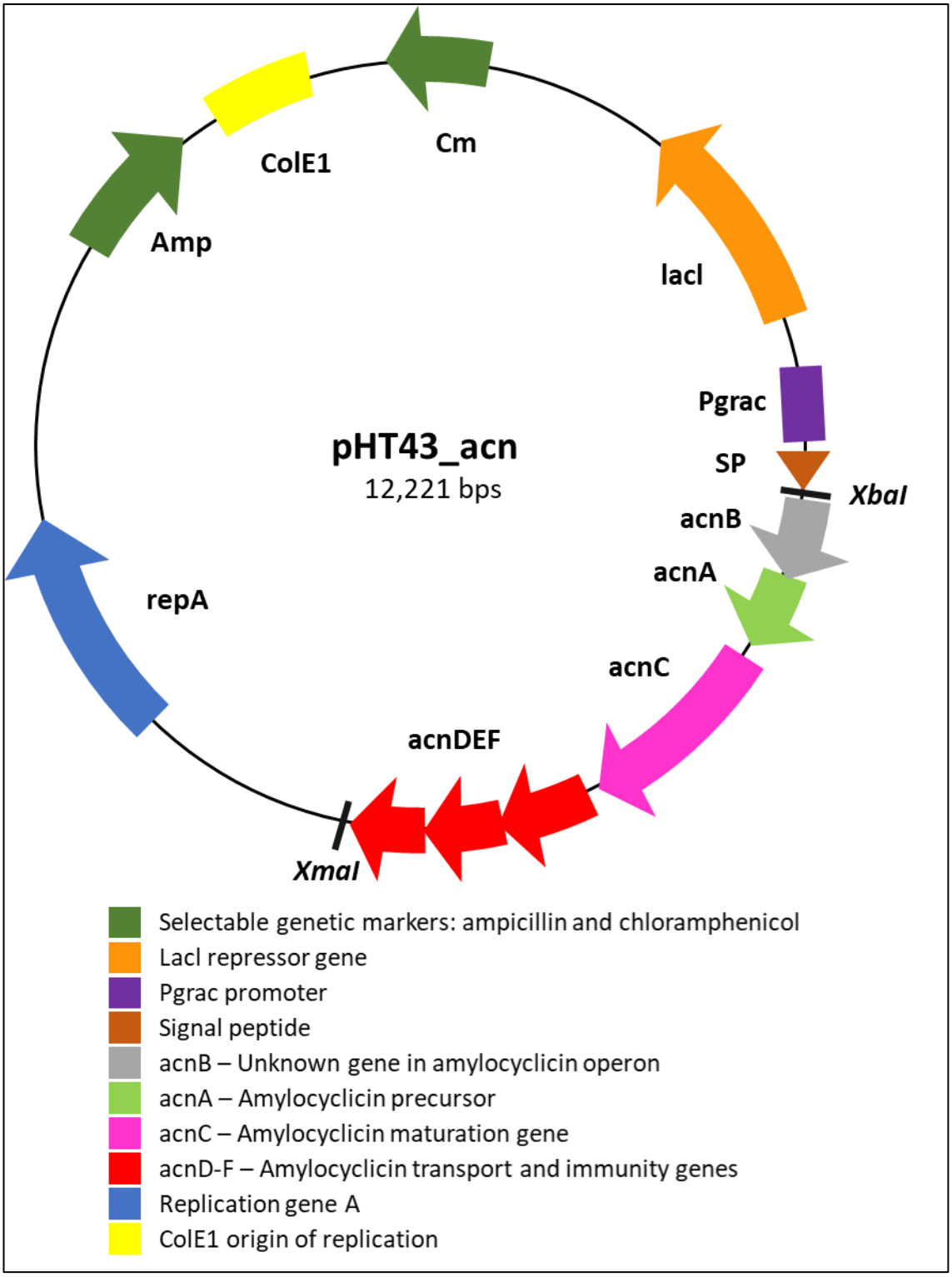

Figure 4.3: Schematic representation of the pHT43_acn vector constructed for the overexpression of the amylocyclicin operon in *B. subtilis* WB800N. Image adapted from a vector constructed *in silico* using the Benchling online tool (https://www.benchling.com/).

4.2.2 Transformation of *B. subtilis* WB800N with pHT43_*acn*

Having confirmed via sanger sequencing that the intact amylocyclicin operon was present in the *E. coli* colonies, pHT43_*acn* was prepared from colony two as described in the methods. 1 µg of this plasmid preparation was transformed into competent *B. subtilis* WB800N, the dedicated overexpression strain for use with the secretion vector pHT43. Transformed *B. subtilis* WB800N was then plated onto agar plates containing 5 µg of chloramphenicol for vector selection. Following incubation, the *B. subtilis* WB800N transformation yielded only one colony, which was colony PCR screened for the amylocyclicin operon using the Amy F and Amy R primer pair (2.7; 2.5.2). The visualisation of the colony PCR product (Figure 4.4) showed a band corresponding to just above the 4 kb marker on the ladder in the *B. subtilis* WB800N transformant, which was not present in the negative control. Due to smearing of the PCR product the distinct band was excised from the gel and purified before having its sequence confirmed as previously described in 2.5.6. Sanger sequencing showed that this band was the intact amylocyclicin operon, confirming this strain of *B. subtilis* WB800N now contained the amylocyclicin overexpression vector pHT43_*acn.*

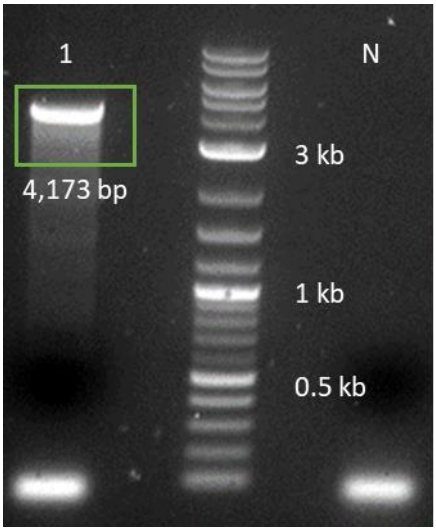

Figure 4.4: 1% agarose gel visualisation of the amylocyclicin operon using the primer pair Amy F and Amy R. The 4,173 bp amplified amylocyclicin operon (1) from *B. subtilis* WB800N transformed with the pHT43_*acn* vector (1). The negative control in lane 'N' and the 1 kb plus DNA ladder (NEB) for size reference.

4.2.3 Heterologous overexpression of amylocyclicin in *B. subtilis* WB800N

Having obtained the strain of *B. subtilis* WB800N that encoded the amylocyclicin operon, the strain needed to be tested for production of overexpressed amylocyclicin. *B. subtilis* WB800N encoding amylocyclicin was induced and protein fractions were harvested for SDS-PAGE analysis and anti-*C. difficile* activity. Protein fractions were also collected for uninduced *B. subtilis* WB800N, wild type *B. subtilis* WB800N and *B. velezensis* B18 as controls. These protein fractions were separated by SDS-PAGE and tested for anti-*C. difficile* activity (2.4.4). The SDS-PAGE gels and their corresponding *C. difficile* overlays are shown in Figure 4.5.

B. velezensis B18 produced a zone of inhibition against *C. difficile* that corresponded to bands slightly above the 4.6 kDa marker on the stained gel in the ammonium sulfate precipitated fractions only (Figure 4.5C), although at this stage there was no evidence to confirm that this active band was amylocyclicin. Analysis of induced *B. subtilis* WB800N pHT43_*acn* showed no bands indicative of protein overexpression (Figure 4.5A). However, analysis of the *C. difficile* overlaid gel showed inhibitory activity in all protein fractions collected with the strongest inhibitory activity seen in the ammonium sulfate fractions, which did not correspond to any distinct bands on the stained gel. Analysis of uninduced and wild type *B. subtilis* WB00N showed the same inhibitory activity against *C. difficile* in all fractions with increasing activity in the ammonium sulfate precipitated fractions. This anti-*C. difficile* activity seen from wild type *B. subtilis* WB800N coupled with the negative PCR result for the amylocyclicin operon in this strain suggests that the inhibitory activity seen in all *B. subtilis* WB800N samples was due to an antimicrobial product naturally produced by the strain and not due to any heterologous overexpression of the amylocyclicin operon.

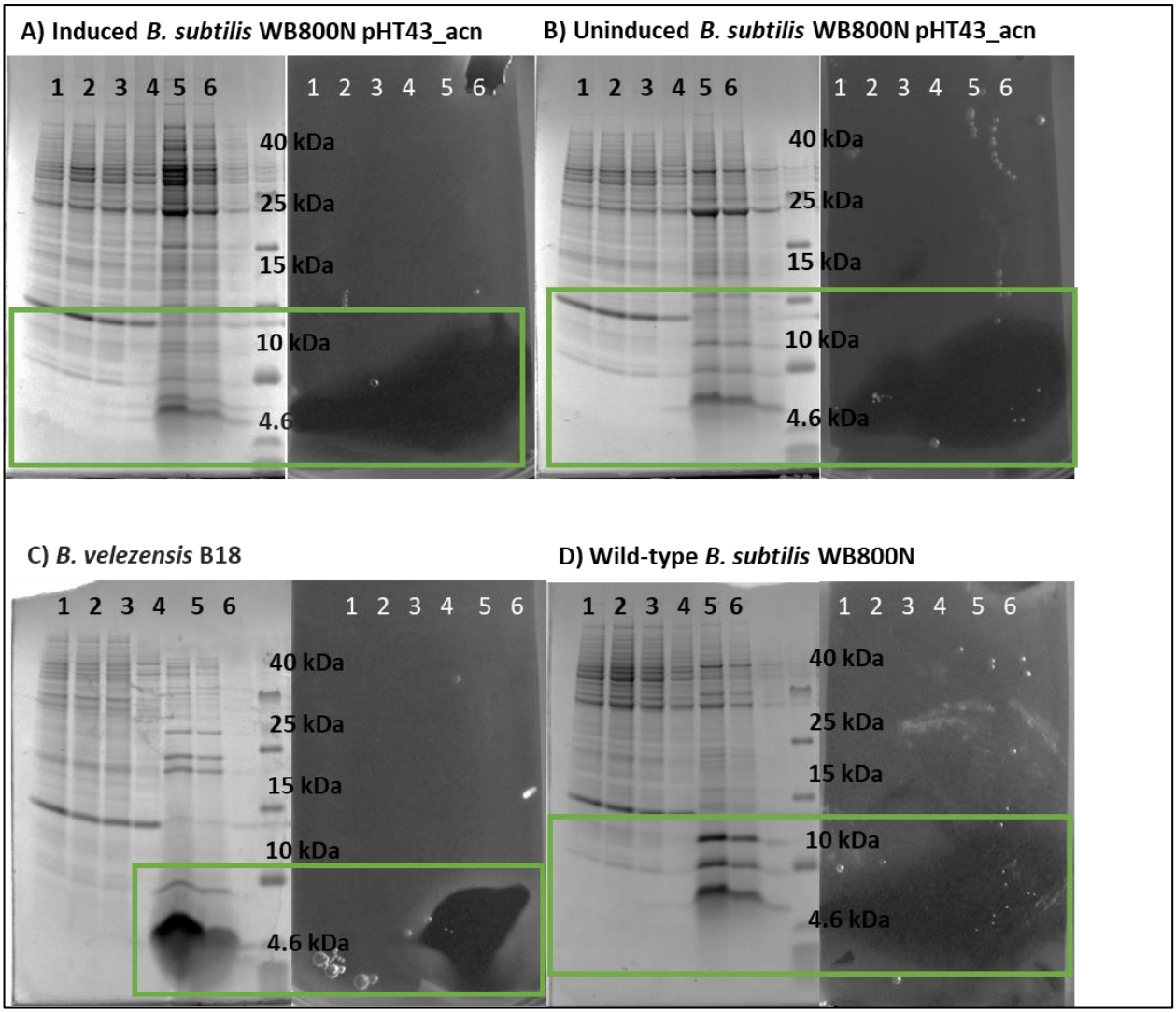

Figure 4.5: 16% Tricine SDS-PAGE (Novex) separation of protein fractions harvested during purification and the corresponding bioassay (2.4.4). SDS-PAGE Gel overlaid with *C. difficile* 630 and incubated anaerobically for 16 h shown on the right. Gel stained with Coomassie featuring the Spectra low range protein ladder shown on left. Lane 1, total protein harvested; lane 2, total protein with cell debris removed; lane 3, the insoluble protein fraction; lane 4, the soluble protein fraction; lane 5, ammonium sulfate precipitated culture proteins; lane 6, ammonium sulfate precipitated culture proteins diluted by half. A) Induced *B. subtilis* WB800N pHT43_acn. B) Uninduced *B. subtilis* WB800N pHT43_acn. C) *B. velezensis* B18. D) Wild Type *B. subtilis* WB800N.

4.2.4 Identification of known antimicrobials encoded by *B. subtilis* WB800N

The whole genome sequence of *B. subtilis* WB800N was obtained in FASTA format from the NCBI database (NZ_CP032310.1), and was then analysed using BAGEL4 and antiSMASH, as previously described in 2.6.3 to identify genes encoding antimicrobials. Table 4.1 gives an overview of the antimicrobial agents encoded by *B. subtilis* WB800N that had the potential to be responsible for the anti-*C. difficile* activity. Among these were the bacteriocins sublancin 168 and subtilosin A; the non-bacteriocin protein antibiotics fengycin, bacillaene, bacillibactin and bacilysin; and the lipopeptide antibiotic surfactin. A bacteriocin overexpression system would ideally not produce interfering antimicrobials with activity against the indicator species being investigated. Whilst the overexpression of amylocyclicin failed in *B. subtilis* WB800N, the naturally occurring, anti-*C. difficile* activity from this strain highlighted it was an unsuitable system for the purposes of attributing the anti-*C. difficile* activity from *B. velezensis* B18 to amylocyclicin. Therefore, other molecular methods would have to be explored.

Table 4.1: The antimicrobial products encoded for in the *B. subtilis* WB800N genome (NZ_CP032310.1) and their similarity to known sequences, as identified by antiSMASH and BAGEL4 webservers.

Antimicrobial	Class	Similarity to known clusters
Surfactin	Lipopeptide antibiotic	82%
Bacillaene	Polyketide antibiotic	100%
Fengycin	NRP	100%
Sublancin 168	Lantipeptide	100%
Bacillibactin	NRP	100%
Subtilosin A	Sactipeptide	100%
Bacilysin	Bacteriolysin	100%

4.2.5 Chloramphenicol susceptibility of *B. velezensis* B18

As the *B. subtilis* WB800N overexpression system was shown to have limited potential in the context of this study, deletion of the operon responsible for the production of amylocyclicin was to be explored. Operon deletion was to be carried out using homologous recombination and before a vector could be designed to achieve this, the antibiotic sensitivity of *B. velezensis* B18 had to be determined.

An MIC assay was carried out for *B. velezensis* B18 against the bacteriostatic antibiotic chloramphenicol in concentrations ranging from 256 µg mL^{-1} to 0.1 µg mL^{-1} (2.3.2). Following 12 hours of incubation the optical density was measured at 600 nm to assess bacterial growth and the results plotted to determine the MIC. Figure 4.6A highlights that *B. velezensis* B18 was susceptible to inhibition by chloramphenicol, with bacterial growth less than half of the untreated control in even the lowest concentrations of chloramphenicol. Although, upon analysis of the data it appeared that *B. velezensis* B18 showed an increase in bacterial density at 32 µg mL^{-1} of chloramphenicol, an MIC value that would suggest this strain was resistant to chloramphenicol. However, the OD_{600} value at this concentration was 0.02 (+/- 0.01), which was less than the initial bacterial density of 0.05, suggesting no increase in growth had occurred. Based on Figure 4.6 it appeared that this trend of "increasing bacterial density" continued in the 16 and 8 µg mL^{-1} concentrations of chloramphenicol, however as before inspection of the OD_{600} values at these concentrations showed that they were also below the initial OD_{600} value of 0.05, which showed that *B. velezensis* B18 was susceptible to chloramphenicol at these concentrations. *B. velezensis* B18 had a bacterial density equal to an OD_{600} value of 0.1 (+/- 0.005) at 4 µg mL^{-1}, which was twice the initial OD_{600} value. As the literature recommends a chloramphenicol concentration of between 4 and 8 µg mL^{-1} for use as a selective marker in *Bacillus* spp., this was of some concern. However, The bacteriostatic nature of chloramphenicol means that in MIC tests, slight growth above the starting bacterial density - such as that seen in the 4 µg mL-1 sample- is not taken into consideration and the strains are deemed sensitive. Therefore, chloramphenicol was selected as a selective genetic marker in the design of a

homologous recombination vector for deletion of the amylocyclicin operon in *B. velezensis* B18.

4.2.6 Chloramphenicol susceptibility of *B. subtilis* 50B

The previously characterised bacteriocin subtilosin A, identified in the genome of *B. subtilis* 50B had the potential to be responsible for the anti-*C. difficile* activity seen from *B. subtilis* 50B and so this bacteriocin would also be targeted for deletion via homologous recombination. As before, the antibiotic sensitivities of *B. subtilis* 50B had to be established via MIC assay before deletion vectors could be designed.

Figure 4.6B shows the plotted data for bacterial density of *B. subtilis* 50B when challenged with the range of chloramphenicol concentrations. As with *B. velezensis* B18, the OD_{600} value for untreated *B. subtilis* 50B was twice that of the lowest concentration of chloramphenicol. However, *B. subtilis* 50B density began to increase at 8 µg mL^{-1} chloramphenicol with the OD_{600} value twice the starting value at 0.1 (+/- 0.006). However, as before this was likely a reflection of the bacteriostatic nature of chloramphenicol and this increase in growth was deemed negligible. Therefore, chloramphenicol was chosen as the selective genetic marker for the construction of the subtilosin A deletion vector in *B. subtilis* 50B.

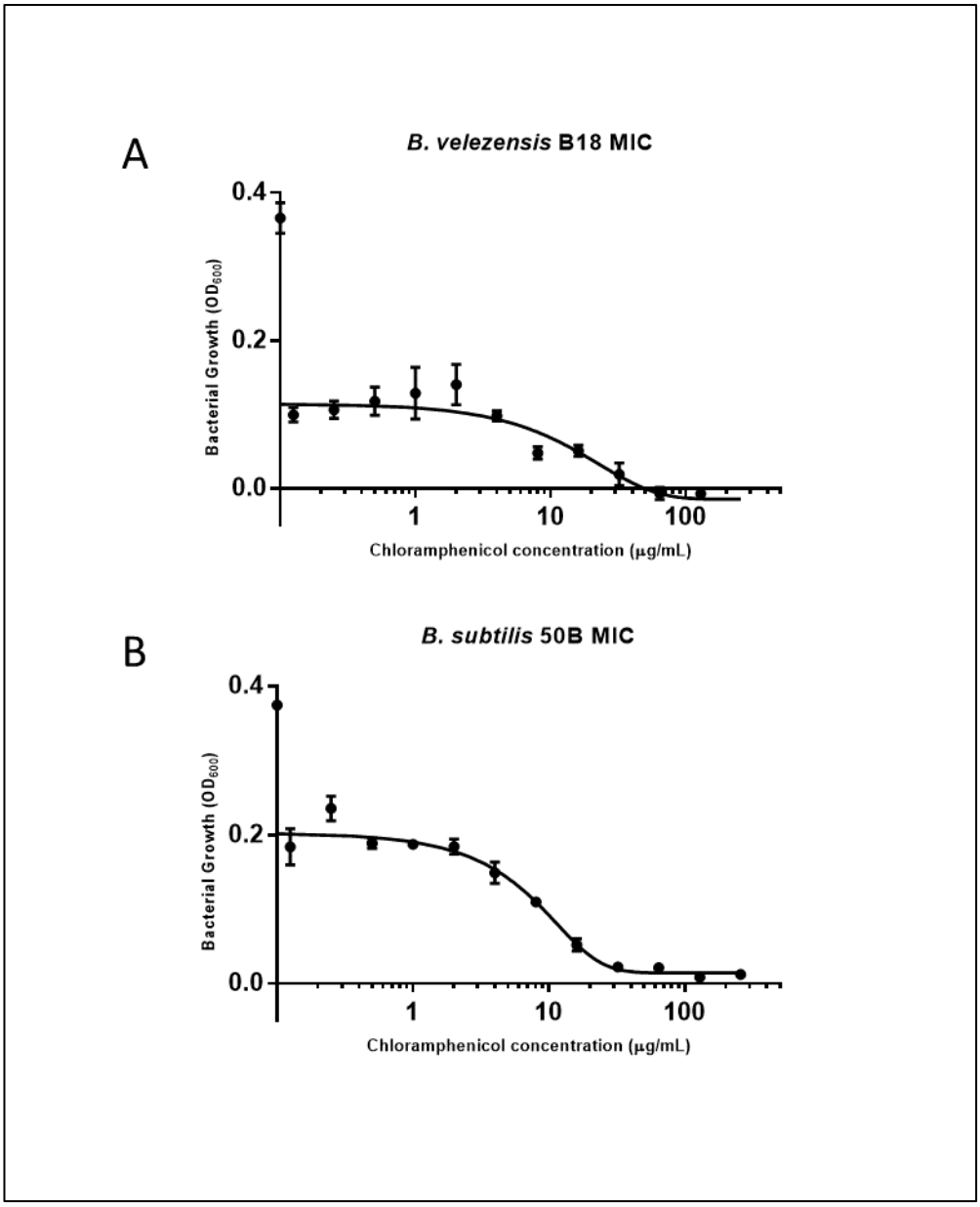

Figure 4.6: Graphic representation of *B. velezensis* B18 (A) and *B. subtilis* 50B (B) susceptibility to the antibiotic chloramphenicol. OD_{600} measurements of *B. velezensis* B18 and *B subtilis* 50B following 12 h incubation in chloramphenicol concentrations ranging from 0 – 252 µg mL^{-1}. Plotted values are mean +/- Standard deviation of three experimental repeats calculated and plotted using Graphpad PRIZM™.

4.2.7 Construction of the candidate bacteriocin deletion vectors

Deletion of the candidate bacteriocin operons from each strain was to be carried out using double homologous recombination using vectors consisting of a pGEM-T Easy (Promega) backbone with regions of DNA homologous to the producing strains flanking a chloramphenicol resistance cassette.

For the construction of the amylocyclicin deletion vector a 1,147 bp fragment encompassing the region directly upstream and the first 138 bp of the *AcnB* gene in the amylocyclicin operon was PCR amplified using the primer pair AcnBU F/Yjck R and the genomic DNA of *B. velezensis* B18 as template (2.5.2; 2.7). Overhangs homologous to the chloramphenicol cassette and pGEM-T easy vector were added to the PCR products by AcnBU F and Yjck R, respectively. A 1,121 bp fragment encompassing the final 131 bp of the *AcnF* gene of the amylocyclicin operon and the region directly downstream of the operon was amplified from the genomic DNA of *B. velezensis* B18 using the primer pair Guac F and Guac R, which added overhangs homologous to pGEM-T easy and the chloramphenicol cassette at each end of the PCR product. A 641 bp chloramphenicol resistance cassette was amplified from the pHT43 vector (Mobitec) using the primer pair CatPA F and CatPA R with overhangs homologous to the *B. velezensis* B18 homology arms.

Subtilosin A, as the most well characterised bacteriocin encoded for by *B. subtilis* 50B, was selected for deletion in this strain. For the construction of the subtilosin A deletion vector, the primer pair SboUP F and SboUP R were used to amplify a 284 bp fragment from the genomic DNA of *B. subtilis* 50B. This fragment contained the first 54 bp of the essential *sboA* gene and 230 bp upstream of the subtilosin A operon. Additionally, 30 nucleotide overhangs were added to the fragment using primers SboUP F and SboUP R, which were homologous to pGEM-T easy and the chloramphenicol cassette from pHT43. A 284 bp fragment containing an intergenomic region of *albF* from the subtilosin A operon was amplified by the primer pair AlbF F and AlbF R, which added 30 nucleotide overhangs to the fragment homologous to the chloramphenicol cassette from pHT43 and pGEM-T easy,

respectively. As before the 641 bp chloramphenicol resistance cassette was amplified from the pHT43 vector using the primer pair CatPS F and CatPS R, which added 30 nucleotide overhangs to the PCR product homologous to the *B. subtilis* 50B homology arms.

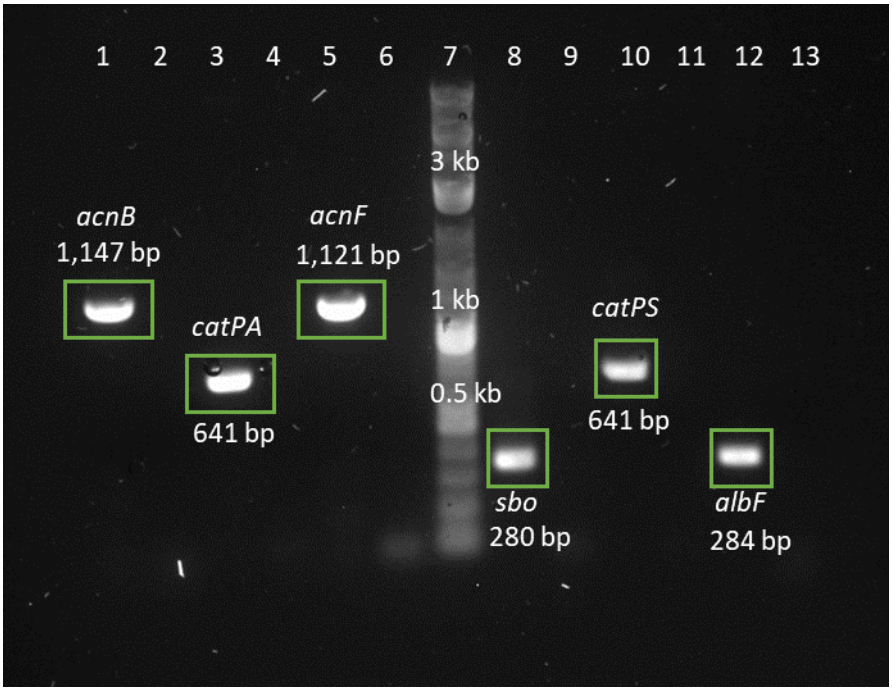

Figure 4.7: 1% agarose gel visualisation of the PCR fragments required for the construction of the pGEMK_sbo and pGEMK_acn deletion vectors. Lane 1, 1,147 bp *acnB* fragment from the amylocyclicin operon amplified from *B. velezensis* B18 genomic DNA using primer pair AcnBU F/Yjck R. Lane 3, 641 bp chloramphenicol resistance cassette amplified from the pHT43 vector using the primer pair CatPA F and CatPA R. Lane 5, 1,121 bp *acnF* fragment from the amylocyclicin operon amplified from *B. velezensis* B18 genomic DNA using primer pair Guac F and Guac R. Lane 7, the 1 kb plus DNA ladder (NEB) for size reference. Lanes 8, 280 bp *sbo* upstream fragment amplified from *B. subtilis* 50B genomic DNA using the primer pair SboUP F and SboUP R; Lane 10, 641 bp chloramphenicol resistance cassette amplified from the pHT43 vector using the primer pair CatPS F and CatPS R. Lane 12, 284 bp *albF* fragment amplified from *B. subtilis* 50B genomic DNA using the primer pair AlbF F and AlbF R. All negative controls directly follow their respective reaction.

The PCR amplified fragments were run on a 1% acrylamide gel for visualisation and size confirmation (2.5.5; Figure 4.7). Following size confirmation, the fragments were subjected to PCR clean-up (2.5.7), the sequences were confirmed using sanger sequencing (Source Bioscience, Oxford UK) with the corresponding primer pairs. Fragments were ligated to one another and the pGEM-T easy backbone using the NEB HiFi Assembly kit (2.5.9) to generate the plasmids pGEMK_*sbo* and pGEMK_ *acn* for the purposes of deletion of the subtilosin A and amylocyclicin operons, respectively.

4.2.8 Transformation of *B. subtilis* 50B with pGEMK_*sbo*

The HiFi ligated pGEMK_*sbo* was transformed into competent *E. coli* Top 10 (2.5.10) and following incubation on chloramphenicol selection plates, 20 *E. coli* Top 10 transformants were screened for the presence of the pGEMK_*sbo* vector using the primers Sbo UP F and Alb F R without the overhangs. Figure 4.8A shows the agarose visualisation of this colony PCR. Colony's 2, 5, 6 and 10 to 18 all showed a band that corresponded to the 1.2 kb marker on the ladder, the expected size of the homology arms ligated to the chloramphenicol cassette in the vector. Additionally, colonies 3, 8, 9 and 19 showed weak bands also corresponding to the 1.2 kb marker. Plasmids were prepared from the PCR positive colonies 10 and 11 and the undigested plasmids run on a gel for size confirmation (Figure 4.8B). The pGEMK_*sbo* vector from *E. coli* Top10 colonies 10 and 11 sized correctly at the 4 kb marker and so both were sent for sequencing using the aforementioned primer pair (Source Bioscience, Oxford UK). The vector from Colony 10 was shown through sequencing to encode the complete, correct chloramphenicol cassette between the *B. subtilis* 50B homology arms. 1 µg of the purified plasmid from *E. coli* Top 10 colony 10 was transformed into competent *B. subtilis* 50B (2.5.10) in addition to 1 µg of the purified PCR product obtained using the primers Sbo UP F and Alb F R. The plasmid pHT43 was also transformed into competent *B. subtilis* 50B as a positive control and all plates were incubated for 24 h with 5 µg mL^{-1} chloramphenicol.

Despite multiple attempts and using both *Bacillus* transformation protocols described in the methods (2.5.12; 2.5.11), no *B. subtilis* 50B transformants were isolated from the pGEMK_*sbo* vector, PCR product or pHT43 positive control

transformations. Additional, attempts utilising pGEMK_*sbo* linearised with *Sac*I were also unsuccessful. The fact that no transformants were obtained using the positive control and multiple methods suggested that the issue stemmed from the DNA uptake stage of the transformation or issues with competency of *B. subtilis* 50B.

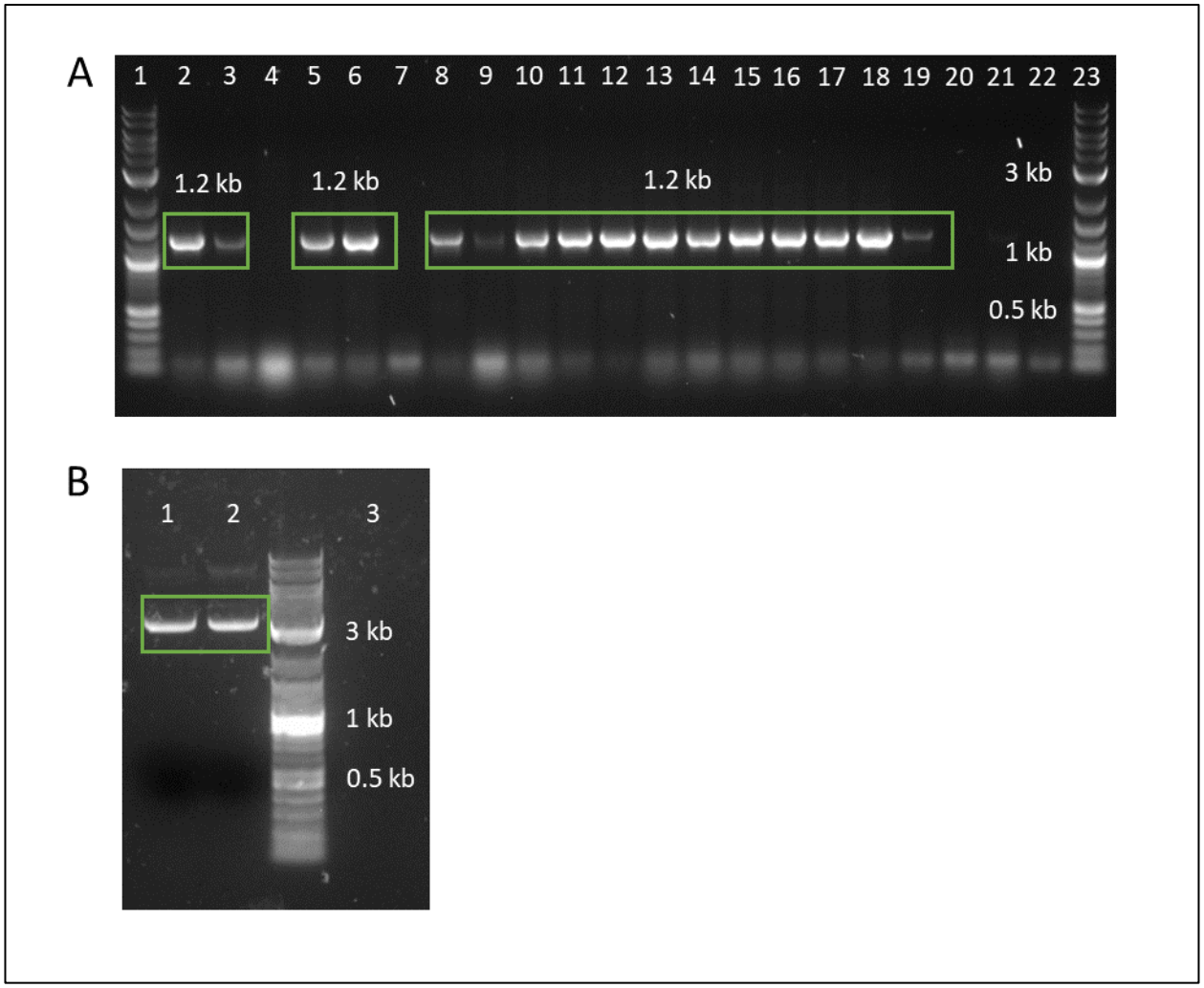

Figure 4.8: 1% agarose gel visualisation of the PCR products using primer pair SboUP F and SboUP R from *E. coli* Top10 colonies transformed with pGEMK_*sbo*. A) lanes 1 and 23 contain the 1 kb plus ladder and lanes 2 – 21 represent *E. coli* colonies 1 – 20. With colonies 1,2,4,5 and 7-18 showing the 1.2 kb expected size of the insert. B) Visualisation of the 3 kb purified pGEMK_*sbo* vector harvested from *E. coli* colonies 10 and 11.

4.2.9 Transformation of *B. velezensis* B18 with pGEMK_*acn*

The HiFi ligated pGEMK_*acn* vector was transformed into competent *E. coli* Top 10 (2.5.10) and following selection on chloramphenicol selection plates, 20 *E. coli* Top 10 transformants were screened for the presence of the pGEMK_*acn* vector using the primers AcnBU F and Guac R without the overhangs. Figure 4.9A shows the agarose gel visualisation of these colony PCRs. *E. coli* colonies 1, 2, 3, 7, 8 and 10 all showed a band of roughly 3 kb, the expected size of the amylocyclicin operon homology arms and the chloramphenicol resistance cassette. However, these colonies also showed the amplification of a small 400 bp fragment, of unknown origin. Colonies eight and ten showed the strongest amplification of the expected band size and also the weakest amplification of the unknown band, therefore plasmids were prepared from these colonies. Figure 4.9B shows the agarose gel visualisation of the undigested plasmids harvested from *E. coli* Top10 transformants eight and ten. Both plasmids appeared to show the correct 6 kb expected size of the pGEMK_*acn* vector and so the sequence was confirmed using the AcnBU F and Guac R (Source Bioscience, Nottingham). Through sanger sequencing it was confirmed that *E. coli* colony ten contained the intact insert. Colony eight, however, contained a nearly intact insert with some mismatching of bases. The origin of the 400 bp PCR product was not elucidated using sequencing of the purified plasmid. It was therefore decided that since the pGEMK_*acn* vector from colony ten was intact and showed no possible origins of the unknown fragment, that this vector would be used to transform *B. velezensis* B18. Competent *B. velezensis* B18 was transformed with 1 µg of purified plasmid form colony ten and incubated on 5 µg chloramphenicol plates for 24 h (2.5.12). As was the case for *B. subtilis* 50B, no transformants were obtained from *B. velezensis* B18 transformed with the pGEMK_*acn* vector, the 3 kb PCR insert, the pHT43 positive control or the vector linearised with the *SapI* restriction enzyme using either of the methods for *Bacillus* transformation outlined in 2.5.11 or 2.5.12. As before this was suggestive of an issue at the DNA uptake stage of transformation.

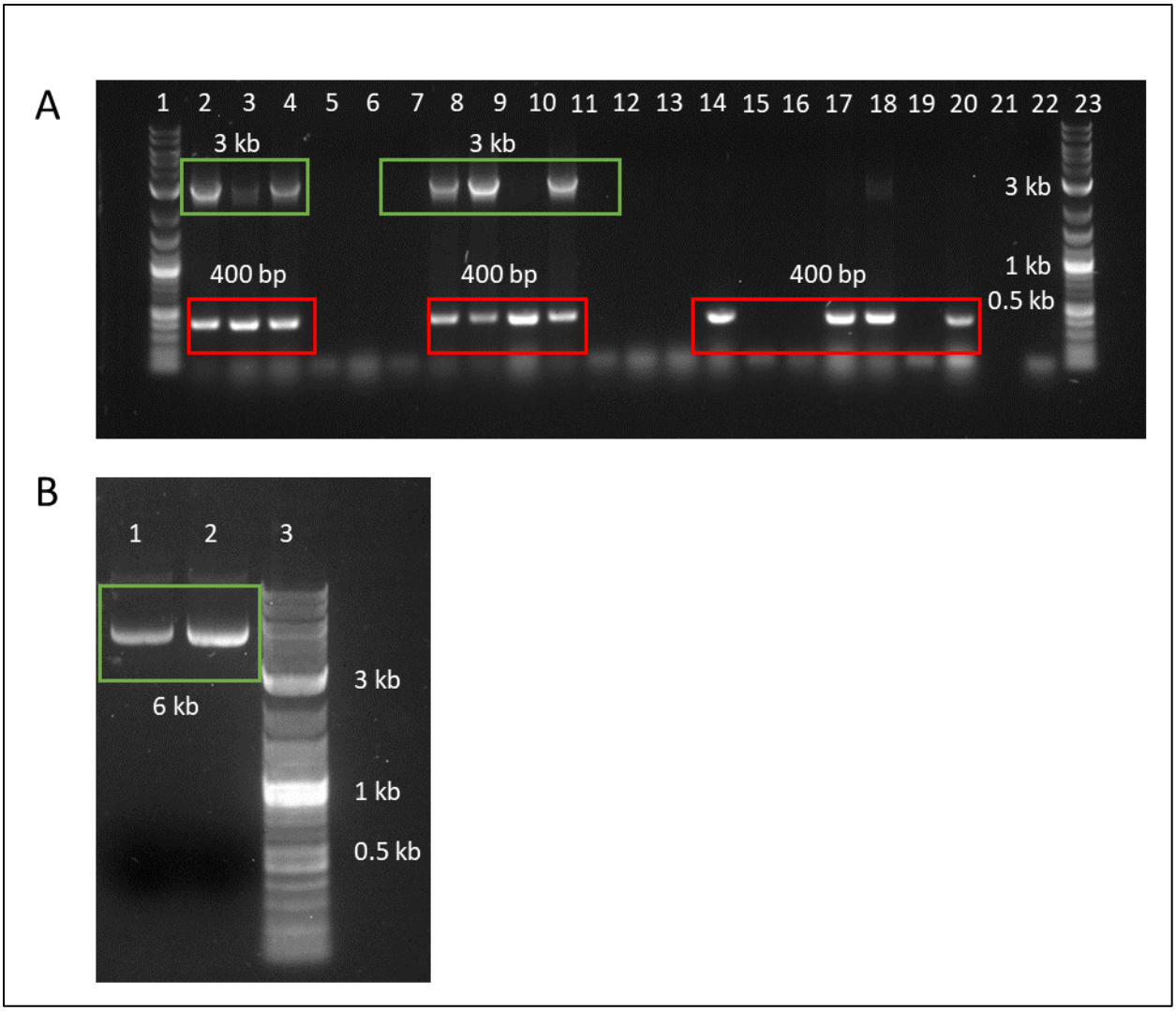

Figure 4.9: 1% agarose gel visualisation of the PCR products using primer pair AcnBU F and Guac R from *E. coli* Top10 colonies transformed with pGEMK_*acn* vector. A) lanes 1 and 23 contain the 1 kb plus ladder and lanes 2 – 21 represent *E. coli* colonies 1 – 20. With colonies 1, 2, 3, 7, 8 and 10 showing the 3 kb expected size of the insert and an additional unknown 400 bp PCR product. B) Visualisation of the 6 kb purified pGEMK_*acn* vector harvested from *E. coli* colonies 8 (1) and 10 (2).

4.2.10 Analysis of environmental genome isolates for competence and transformation related proteins

To identify whether the environmental *Bacillus spp.* isolates encoded the necessary genes to reach the competent state and subsequently uptake extracellular DNA, the genomes were analysed for the presence of key genes involved in *Bacillus* spp. competence and transformation. The whole genome sequence of *B. velezensis* and *B. subtilis* 50B were analysed in NCBI protein BLAST for the presence of homologs of key proteins related to competence and DNA uptake (Com) using the amino acid sequence of these proteins obtained from the NCBI database as driver sequences. Table 4.2 shows the key Com proteins, their functions and the sequence identity to the published driver sequences, if present, in the genome of *B. velezensis* B18. *B. velezensis* B18 encoded the gene necessary to produce the master regulator of competence, ComK, which had 82.3% identity to the published protein sequence. The genes encoding the ComX (16.7%) pheromone and its dedicated sensor histidine kinase, ComPA (56.7%) were found in the *B. velezensis* B18 genome in an operon with related competence proteins including the maturation enzyme (ComQ) of the ComX pheromone precursor. Interestingly, due to the ComX precursors sequence similarity to bacteriocins, this operon was highlighted previously by BAGEL4, as being encoded by *B. velezensis* B18, as it was not a bacteriocin it was previously discounted. The ComS regulator encoding gene was found in the *B. velezensis* B18 genome in the surfactin biosynthesis operon with 51.9% identity to the driver sequence. The genes encoding the ComG proteins that form the extracellular DNA uptake pseudopillus in competent *B. subtilis* species complex members were found clustered in a dedicated operon in the genome of *B. velezensis* B18 with 71%, 64.4% and 69.4% protein identity to the ComGA, ComGB and ComGC driver proteins, respectively. The ComE and ComF encoding genes were also located in dedicated operons in the *B. velezensis* B18 genome, with 54% and 56% protein identity to the driver sequences of ComEA and ComEC and 61.1% identity to ComFA. The recombinases responsible for the fate of incoming exogenous DNA in *Bacillus* spp. were also present in the genome of *B. velezensis* B18 with high sequence identity of 96.8%, 89% and 100% to the driver sequences of RecA, RecO and RecU.

Table 4.2: Key proteins involved in competence and transformation in *Bacillus* spp. (Accession no.), their function and the protein identity of the homologs encoded by *B. velezensis* B18 and *B. subtilis* 50B.

Com protein (Accession number)	Function	*B. velezensis* B18 Identity	*B. subtilis* 50B Identity
ComK (WP_003245329.1)	Master regulator	82.3%	99.6%
ComS (WP_010886404.1)	Competence regulator	51.9%	97.8%
ComX (WP_003242801.1)	Competence pheromone	16.7%	18.7%
ComPA (WP_003242894.1)	Sensor histidine kinase	56.7%	56.2%
ComGC (ADM38485.1)	DNA uptake pseuodpillus component	69.4%	99%
ComGB (QRQ50885.1)	DNA uptake pseuodpillus component	64.4%	99.4%
ComGA (WP_004399124.1)	DNA uptake pseuodpillus component	71.0%	100%
ComEA (WP_004398514.1)	DNA protection	54%	98%
ComFA (WP_003243962.1)	DNA uptake	61.1%	96.2%
ComEC (WP_009967776.1)	DNA uptake	56.5%	97.1%
RecA (WP_003245789.1)	DNA recombinase	96.8%	100%
RecU (WP_004399067.1)	DNA recombinase	100%	100%
RecO (WP_003230055.1)	DNA repair	89%	100%

Table 4.2 highlights the protein identity of the key Com proteins identified as being encoded by *B. subtilis* 50B. The master transcriptional regulator ComK, and the regulator ComS were identified as being encoded by *B. subtilis* 50B with high sequence identity of 99.6% and 97.8% identity to the published sequence. As before, the ComX pheromone precursor, and its related proteins ComPA were encoded together in an operon in *B. subtilis* 50B with 18.7% and 56.2% identity to the driver sequences. The ComX pheromone precursor utilised valine as a start codon, and due to this and the low identity, it was not identified during the BAGEL4 mining of the *B. subtilis* 50B genome despite the cluster being identified in *B. velezensis* B18 at that stage. The ComG protein encoding genes were also located together in an operon in *B. subtilis* 50B with high protein identity to the driver sequences of 100%, 99.4% and 99% respectively for ComGA, ComGB and ComGC. The ComE and ComF encoding genes were also located together in dedicated operons in the *B. subtilis* 50B genome, with 98% and 97.1%% protein identity to the driver sequences of ComEA and ComEC and 96.2% identity to ComFA. The recombinases responsible for the fate of incoming exogenous DNA in *Bacillus* spp. were also present in the genome of *B. subtilis* 50B with 100% identity to the driver sequences of RecA, RecO and RecU.

Identifying the presence of genes encoding the key Com proteins in both environmental *Bacillus* isolates suggests that these strains are naturally competent and capable of transforming extracellularly sourced DNA. Therefore, the failure to achieve transformants with the homologous recombination plasmids was likely due to other aspects of the environmental *Bacillus* spp. cell biochemistry and physiology such as the restriction endonucleases harboured by them or simply other experimental problems.

4.3 Chapter Discussion

This study had hoped to utilise *B. subtilis* as a host for production of recombinant amylocyclicin, the best characterised of the bacteriocins encoded by *B. velezensis* B18 as there was an apparent absence in its utilisation in the literature despite *B. subtilis* being commonly used in industrial biotechnology and protein production (Harwood, 2003). The *B. subtilis* WB800N overexpression system with the dedicated pHT43 secretory plasmid (Mobitec) was selected as this system offered a number of advantages including deletion/disruption of genes for key extracellular and intracellular proteases which would protect the recombinant protein (Jeong *et al.*, 2018). Additionally, the dedicated secretory plasmid directing recombinant proteins for export into the culture media could potentially eliminate issues in protein recovery such as formation of inclusion bodies (Phan *et al.,* 2005).

Initial crude cloning of the whole amylocyclicin operon into the MCS of pHT43 and subsequent transformation into *B. subtilis* WB800N yielded a transformant that encoded the amylocyclicin operon. Despite this, upon induction of overexpression and subsequent SDS and bioassay analysis of the protein fractions collected there was no discernible overexpression of recombinant proteins. Instead, it was highlighted that WT *B. subtilis* WB800N, without the amylocyclicin operon was highly inhibitory against *C. difficile,* and analysis of the genome of *B. subtilis* WB800N confirmed that this strain naturally encoded seven antimicrobial substances including several bacteriocins.

When selecting a host for recombinant bacteriocin production other bacteriocins produced by the host is a key consideration as multibacteriocinogenic hosts can hinder the purification and identification processes by obscuring inhibitory activity from the recombinant bacteriocin or attributing natural activity to recombinant proteins (Mesa-Pereira *et al*., 2018). The natural inhibitory activity that *B. subtilis* WB800N possessed against *C. difficile* therefore meant that this strain was unsuitable for the production of recombinant anti-*C. difficile* bacteriocins. The reputation *B. subtilis* has as a prolific producer of antimicrobials is likely the reason that this species has been underutilised in the production of recombinant bacteriocins (Caulier *et al*.,

2019). Despite this, there are still advantages to using a Gram-positive overexpression system in the production of recombinant Gram-positive bacteriocins and so there would be merit in developing a non-bacteriocinogenic strain of *B. subtilis* for the dedicated purpose of producing recombinant bacteriocins, although this was beyond the scope of this study.

The separation of protein fractions via SDS-PAGE and the bioassay carried out against *C. difficile* also for the first time showed an estimated size of the anti-Clostridial agent produced by *B. velezensis* B18 as corresponding to an aggregated mass of proteins at >4.6 kDa on the gel. Only culture media protein fractions from *B. velezensis* B18 showed activity against *C. difficile*, which suggested that the anti-clostridial agent produced by *B. velezensis* B18 was released into the extracellular matrix and not cell/surface bound. The secreted nature and predicted size of just above 4.6 kDa reaffirmed the belief that amylocyclicin was the most likely candidate to be responsible for the anti-clostridial activity seen in *B. velezensis* B18 as despite amylocyclicin's confirmed size being 6.3 kDa, circular bacteriocins are known to migrate on a gel as smaller than the actual size (Kawai *et al*., 2004, Scholz *et al*., 2014). All protein fractions collected from *B. subtilis* WB800N inhibited *C. difficile* in the bioassay, with the greatest activity being seen in the cell supernatant fractions. This inhibitory activity, like *B. velezensis* B18, was localised to an estimated size of 4.6 kDa on the SDS-PAGE gel. However, unlike *B. velezensis* B18, the inhibitory activity from *B. subtilis* WB800N did not appear to directly correspond to bands on the gel.

Analysis of the *B. subtilis* WB800N identified that it encoded five antimicrobials in common with *B. velezensis* B18 (surfactins, fengycins, bacillibactin, bacilysin and bacillaene) and it was a fear that one of these shared antimicrobials could be responsible for the anti *C. difficile* activity seen in both strains. As discussed previously the anti *C. difficile* activity in *B. velezensis* B18 was unlikely to be due to fengycins due to their strict inhibitory activity against fungi (Medeot *et al.,* 2020). Likewise, it was also unlikely to be due to bacillibactin as this siderophore is not responsible for direct antagonistic activity as was seen in these strains (May, Wendrich and Marahiel, 2001). The small size (>4.6 kDa) of anti-clostridial agent also ruled out bacilysin, which has a molecular weight of 24.5 kDa (Kenig, Vandamme and

Abraham, 1976). Similarly, the >4.6 kDa size ruled out bacillaene as this antibiotic has a molecular weight of 580 Da (Butcher *et al.,* 2007). The surfactin lipopeptide has a molecular weight of 1.2 kDa, however on SDS-PAGE gels this antibiotic can aggregate and have a perceived mass of above 3 kDa (Alajani *et al*., 2016), raising the possibility that the anti-clostridial activity seen in *B. velezensis* B18 could be due to surfactin and not amylocyclicin as suspected. However, *B. subtilis* WB800N also encoded the bacteriocins subtilosin A and sublancin 168, which have similar molecular weights to aggregated surfactin, and as the inhibitory activity of *B. subtilis* WB800N was not strictly bound to the extracellular fractions it is possible that these bacteriocins and not one of the encoded bacteriocins shared with *B. velezensis* B18 were responsible for the anti-*C. difficile* activity (Sharma *et al*., 2020; Velho *et al*., 2013).

Whilst it was decided that *B. subtilis* WB800N was unsuitable for the purposes of this study, a fatal flaw in experimental design must still be addressed. The pHT43 plasmid includes the signal peptide for the α-amylase, AmyQ, preceding the MCS that would then direct the recombinant protein for export via the general secretory pathway (Phan *et al.,* 2005; Meyer *et al*., 1999). As this was an initial, exploratory experiment the whole amylocyclicin operon was cloned into the MCS of pHT43 in an effort to include the entire biosynthetic machinery for amylocyclicin, however, this oversight led to the affixation of the signal peptide to *acnB*, the unknown yet essential first protein of the amylocyclicin operon (Scholz *et al*., 2014). Due to this error, it was likely that during induction of the plasmid in the *B. subtilis* WB800N host, transcription of *acnB* with the signal peptide would be carried out and then terminate at the stop codon of *acnB* resulting in the other operon members - including the prepeptide – not being transcribed. However, analysis of the SDS-PAGE gels of induced *B. subtilis* WB800N did not indicate an overproduction of AcnB, as a 20 kDa band of overexpression would be expected if induction had been successful. Therefore, if a non-bacteriocinogenic strain of *B. subtilis* WB800N was to be developed, rational experimental redesign would be essential.

As the production of recombinant bacteriocins did not achieve the desired results in attributing the anti-*C. difficile* activity seen in *B. velezensis* B18 to the bacteriocin amylocyclicin, gene deletion of bacteriocin biosynthetic machinery was to be carried

out. For *B. velezensis* B18 the amylocyclicin biosynthetic machinery was to be replaced by an antibiotic resistance gene utilising double homologous recombination, in the method of Scholz *et al*., (2014). In the case of environmental *B. subtilis* 50B, subtilosin A was the most likely candidate for the anti-*C. difficile* activity and so the biosynthetic machinery for subtilosin A as to be knocked out in this strain utilising the previously successful method of Phelan *et al*., (2013). Upon successful construction of the pGEMK_*sbo* and pGEM_*acn* double homologous recombination vectors, subsequent attempts to transform *B. velezensis* B18 and *B. subtilis* 50B with them did not result in transformants. Despite the use of the traditional natural competence protocol of *B. subtilis* and a slightly modified version which was utilized by Scholz et al., (2014) (Anagnotopolous and Spizizen, 1961; Kunst and Rapoport, 1995).

Before further troubleshooting could be carried out it was integral to know whether the environmental *Bacillus* spp. could actually reach competence. Competence in *Bacillus* spp. is a natural physiological state, that subpopulations of the culture enter during stationary phase of growth as opposed to chemical or electroporation induced competence (Maier, 2020). The processes involved in reaching the competent state, or K-state, are tightly regulated at the transcriptional and post translational levels with more than 100 genes upregulated to achieve the K-state (Maier, 2020). The *Com* genes are especially upregulated when populations enter the K-state, and the transcription of these genes is controlled by ComK, the essential master regulator of competence, which can induce its own transcription by binding to the *ComK* promoter (Maier, 2020; Van Sindermen, 1995). The presence of a ComK encoding gene in the genomes of both *B. velezensis* B18 and *B. subtilis* 50B with high protein product identity suggests that these strains possess the ability to induce the competent state and that the ComK protein is highly conserved within members of the *B. subtilis* family complex.

Post translationally, ComK concentrations in the bacterial cell can be increased by quorum sensing. High concentrations of the quorum sensing pheromone ComX externally activates the sensor histidine kinase ComPA, starting a molecular chain of events that culminates in the protein ComS inhibiting degradation of cellular ComK

by dedicated proteases, thus increasing the ComK concentration in the cell and prolonging the K-state (Magnuson *et al*., 1994; Weinrauch *et al.*, 1990; Turgay *et al*., 1998). Both environmental *Bacillus* spp. B18 and 50B encode homologs of the ComS competence regulator within the biosynthetic machinery of surfactin (Hamoen *et al.,* 1995), and both also encode homologs of the ComX pheromone, and the ComPA sensor histidine kinase located together in an operon with other competence related genes (Tran *et al.*, 2000). The ComX pheromone homologs in both strains of *Bacillus* had limited protein identity to the published driver sequence of ComX (WP_003242801.1), which is likely a reflection of the fact that ComX must be specific for each producer in order to provide a fitness benefit on the producing bacteria. The ComX pheromone shares sequence similarity with some bacteriocins and for this reason the ComX operon of *B. velezensis* B18 had already been identified by BAGEL4 during genome mining. However, despite the presence of a ComX pheromone homolog in *B. subtilis* 50B this was not highlighted by BAGEL4, which is likely due to the open reading frame being overlooked by BAGEL4 due to its short length, low sequence similarity and use of valine as the start codon. This highlights that BAGEL4 is not infallible when searching for bacteriocins and confirms that potentially novel bacteriocin encoding genes can be overlooked due to their inherent features.

The proteins involved in DNA uptake are also upregulated during K-state, the *ComG* operon proteins (ComGA-GC) are involved in the construction of a membrane spanning pseudopillus, which binds to extracellular DNA (ComGC) and actively uptakes it through the activity of a cytosolic ATPase (ComGA) and an integral membrane protein (ComGB) (Chung and Dubnau, 1998). Internalised DNA is protected by ComEA and the integral membrane protein ComEC transports a single strand of DNA into the cytosol supported by ComFA (Draskovic and Dubnau, 2005; Londono-Vallejo and Dubnau, 1994a). Following this the internalised DNA can then be recombined into the chromosome of the host *Bacillus* spp. by RecA or annealed into a self-replicating plasmid using RecO (Yadav *et al*., 2013). Bioinformatic analyses confirmed that both environmental *Bacillus* spp. encoded all of the essential proteins required for uptake and fate of extracellular DNA with relatively high protein identity to the driver sequences and it can be assumed that these isolates are capable of

reaching the K-state and successfully transforming DNA. However, transformation of both environmental *Bacillu*s spp. yielded no transformants despite multiple attempts and differing experimental conditions.

Having confirmed through genome mining that *B. velezensis* B18 and *B subtilis* 50B were likely able to achieve the competent state it was thought that perhaps the pGEMK plasmids were the cause of the failure to achieve transformants from the environmental *Bacillus* spp. Plasmid size is known to influence transformation efficiency in *B. subtilis*, however, the double homologous recombination plasmids at 4.2 kb and 5.9 kb were not large enough to reduce transformation efficiency (Ohnse *et al*., 1995). There are examples in the literature of the linearised plasmids being used successfully to transform *B. subtilis* and so the pGEMK plasmids were linearised (Wu *et al*., 2019). Despite attempts to transform the environmental bacillus with linearised plasmid this method was also unsuccessful. Attempts to transform the *Bacillus* spp. with the linear PCR product containing the chloramphenicol resistance gene flanked by the homologous DNA regions also resulted in no transformants, despite a similar method being utilised successfully in *B. subtilis* in the literature (Wu *et al.,* 2019).

Restriction endonucleases are enzymes involved in restriction modification systems of bacteria. Part of the primitive bacterial immune system, they function by destroying viral DNA by recognising and cutting specific target sites of unmethylated DNA (Bron, Murray and Trautner, 1988). Unfortunately, DNA derived from PCR is also unmethylated and thus is at the risk of attack by host cell endonucleases if there is a target site within the region of DNA. When designing the pGEMK vectors care was taken to ensure the sites homologous to the bacteriocin operons in *B. subtilis* 50B and *B. velezensis* B18 did not contain target sites of known *B. subtilis* family restriction endonucleases. However, as environmental isolates of bacteria and not dedicated lab strains, the restriction modifications systems harboured by the environmental *Bacillus* spp. were unknown, especially considering that restriction modification systems have a fitness benefit to bacteria and so are widely shared by horizontal gene transfer (Jeltschand Pingoud, 1996). Therefore, *B. subtilis* 50B and *B. velezensis* B18 could possibly produce endonucleases not native to *Bacillus* spp with

the potential to destroy the double homologous recombination inserts, thus resulting in no transformants. A comprehensive review of the genomes of *B. velezensis* B18 and *B. subtilis* 50B to identify restriction modification systems they harbour would be beneficial before repeating the bacteriocin operon knock out experiments. In relation to restriction endonucleases and their activity, non-competent *B. subtilis* show increased cell surface endonuclease activity and exogenous DNA is rendered inactive and unable to be transformed upon exposure to non-competent *B. subtilis* (Joenje and Venema, 1975). Additionally, during stationary phase of growth the non-competent subpopulation far outweighs the competent subpopulation with only 5-20% of the culture reaching K-state (Maier, 2020). Therefore, the failure to achieve transformants of *B. subtilis* 50B and *B. velezensis* B18 with disrupted bacteriocin biosynthetic operons could simply be due to experimental errors resulting in a higher population of non-competent, exogenous DNA-destroying cells in the culture media. Therefore, optimization of the competence and transformation protocols for the environmental *Bacillus* spp. would also need to be investigated, however, time constraints meant this could not be implemented in this study.

Although the initial aim of this study had been to produce recombinant amylocyclicin in the *B. subtilis* WB800N expression system and to then assess the anti-clostridial activity of this recombinant bacteriocin, complications arose. Primarily, the strong, natural anti-clostridial activity of *B. subtilis* WB800N highlighted that this strain would be unsuitable for expression/identification of recombinant bacteriocins without extensive genome editing. However, these initial experiments allowed a size estimation for the anti-clostridial agent produced by *B. velezensis* B18 and highlighted the most likely candidates from the list of encoded antimicrobials. Attempts to create mutants of *B. velezensis* B18 and *B. subtilis* with disrupted biosynthetic clusters for amylocyclicin and subtilosin A were also complicated by difficulty in achieving transformation in these environmental isolates. Although as before, a benefit of this was that it confirmed that BAGEL4 can often overlook bacteriocin-like clusters due to low similarity. Therefore, as the genetic manipulation methods required extensive troubleshooting beyond the scope of this study, it was

decided that purification of the anti-clostridial agents from the environmental *Bacillus* spp. was the only appropriate course of experimentation in order to definitively identify them.

Chapter 5: Part purification and physiochemical characterisation of the anti-*C. difficile* bacteriocins

5.1 Chapter Introduction

Bioinformatic analysis of the genomes of *B. velezensis* B18 and *B. subtilis* 50B revealed that both strains encoded multiple antimicrobial compounds that could be responsible for the anti-*C. difficile* activity seen from these strains (3.2). Attempts to produce recombinant bacteriocins from the candidates and similar attempts to delete the genes encoding the candidate bacteriocins from the native strains required optimization beyond the scope of this study (4.2). Therefore, purification of the anti-*C. difficile* compounds was the only way to definitively identify them. As this study was focussed on the isolation of novel bacteriocins, only purification methods for bacteriocins were explored, whilst purification methods for non-protein antimicrobials were not taken into consideration. Table 5.1 highlights some well-characterised bacteriocins that have been discovered within the *B. subtilis* species complex and the purification methods used for them. As can be seen from Table 5.1, purification of bacteriocins is no easy feat with the diversity seen in bacteriocins reflected in their purification, no one process is suitable for all bacteriocins. Despite this, there is a general structure of the purification processes of bacteriocins, although a myriad of experimental approaches to achieve this.

Table 5.1: Bacteriocins produced by the *B. subtilis* species complex and the associated purification/identification methods. This list is not comprehensive and is reflective of well characterised bacteriocins from *B. subtilis* species complex members.

Bacteriocin	Classification	Producing species	Purification/Identification method	Source
Amylocyclicin	Class Ib	*B. velezensis* FZB42	Ammonium sulfate precipitation, methanol extraction, RP-HPLC, MALDI-TOF MS.	Scholz *et al.*, 2014
Entianin	Class Ia	*Bacillus subtilis* subsp. *spizizenii* DSM 15029T	Methanol extraction, solid phase extraction, RP-HPLC and MALDI-TOF MS.	Fuchs *et al*, 2011
Ericin A	Class Ia	*Bacillus subtilis* A1/3	RP-HPLC, MALDI-TOF MS.	Stein *et al.*, 2002
Ericin S	Class Ia	*Bacillus subtilis* A1/3	RP-HPLC, MALDI TOF MS.	Stein *et al.*, 2002
lichenin	Class IId	*Bacillus licheniformis* 26 L-10/3RA	Ammonium sulfate, gel filtration, ion exchange. Edman sequencing.	Pattnaik *et al*, 2001
Lichenicidin	Class Ia	*B. licheniformis*	Isopropanol extraction, RP-HPLC, MALDI-TOF MS.	Begley *et al.,* 2009
Sonorensin	Class IId	*Bacillus sonorensis* MT93	Methanol extraction, ion exchange, gel filtration, RP-HPLC.	Chopra *et al*, 2014
Subtilosin A	Class Ic	*B. subtilis* 168	Ion exchange, butanol extraction, gel filtration.	Zheng *et al.*, 1999
Subtilomycin	Class Ia	*B. subtilis* MMA7	Ammonium sulfate, RP-HPLC, MALDI-TOF MS.	Phelan *et al.*, 2013

5.1.1 Salt precipitation of bacteriocins from culture media

The most common first stage of any bacteriocin purification process is the concentration of the proteins from culture media into an aqueous sample. This stage is critical as bacteriocins are generally low in abundance in the culture media with concentrations ranging from 10 – 100 mg L^{-1} (Parrente and Ricciardi, 1999). Salting out culture media proteins using ammonium sulfate is generally favoured. And entails removing the bacterial cell mass from the culture and saturating the supernatant with ammonium sulfate. At high salt concentrations the solubility of the culture proteins decreases, and precipitation occurs allowing the proteins to be separated from the supernatant through centrifugation, resuspended in buffer and dialysed to remove any remaining ammonium sulfate (Wingfield, 2001). Ammonium sulfate precipitation is used regularly in the purification of bacteriocins as the "salting out" method is not denaturing to proteins and they generally retain their biological activity (Wingfield, 2001) as opposed to other "harsh" precipitation methods.

5.1.2 Solvent Precipitation of bacteriocins from culture media

Another method for initial precipitation of bacteriocins from culture media that is often employed is the use of organic solvents. Many well-known novel bacteriocins such as thuricin CD from *B. thuringiensis* (Rea *et al.,* 2010) and lichenicidin from *B. licheniformis* (Begley *et al.,* 2009) were purified by incorporating propan-2-ol precipitation into their purification processes. Interestingly, it appears solvent precipitation is most effectively utilised in the case of cell-bound bacteriocins as was the case for the bacteriocins formicin and uberolysin (Collins *et al.,* 2016; Wirrawan *et al*., 2007). Although less common, acetone has also been used instead of alcohol in organic solvent precipitation. The bacteriocin lacticin Q from *L. Lactis* was purified following acetone precipitation of the culture media (Fujita *et al*., 2007). Whilst it has been shown that organic solvent precipitation of bacteriocins is possible, there is an increased risk of loss of activity when using these methods due to the denaturation of the bacteriocins by the said solvents.

5.1.3 Adsorption/desorption to precipitate bacteriocins

Yang *et al*., (1992) suggested a method for selective precipitation of bacteriocins produced by lactic acid bacteria (LAB), which has been adapted and used successfully by utilized for various classes of bacteriocins (Tulini and De Martinis, 2010). This method exploits the adsorption of bacteriocins to their producer cells at a specific pH and the subsequent release of the bacteriocins following a drastic change in pH. Whilst this method has been shown to generate high yields, it depends on pH extremes that can denature some bacteriocins and so it is not an appropriate method in all cases. Additionally, this method was tailored specifically for LAB and there is no evidence for its use in isolating novel bacteriocins from *Bacillus* spp.

5.1.4 Spot on lawn assay and well diffusion assay to identify precipitated bacteriocins

Regardless of the initial method used for bacteriocin precipitation the antimicrobial activity of the precipitated fraction must be tested to ensure the bacteriocin is present. The well diffusion assay (WDA) and the spot-on lawn assay are common techniques used to detect bacteriocin activity that are adapted from the deferred antagonism assay (Pingitore *et al*., 2007). Both methods rely on agar-diffusion, and both involve a layer of agar seeded with the indicator strain of bacteria. In the spot-on lawn assay a small volume, usually 10 µL, of bacteriocin preparation is spotted on to the agar layer containing the indicator species. In the well diffusion assay, however, small wells are prepared on the agar layer containing the indicator species and bacteriocin preparation is applied to the wells. Following incubation under conditions favourable to the indicator species, antimicrobial activity of the bacteriocin fraction is assessed as zones of inhibition around the well (Pingitore *et al*., 2007).

5.1.5 Quantifying bacteriocin activity

Accurate direct quantification of bacteriocin concentration within protein preparations is not possible until purification due to contaminating proteins, therefore, the WDA and spot-on lawn assay can be adapted as a means of semi quantifying bacteriocin activity in a protein preparation (Henderson *et al*., 1992). The critical dilution assay adapts the spot-on lawn assay to assess bacteriocin activity by serially diluting two-fold the bacteriocin preparation. The dilution series is then

spotted on a lawn of indicator and the critical dilution (CD) is defined as the highest dilution still providing distinct antimicrobial activity. Bacteriocin activity is then calculated as (1000/Original Volume) x CD and expressed in arbitrary units per millilitre (AU mL^{-1}) (Parente and Ricciardi, 1999). The WDA can be adapted in a similar manner to provide a critical dilution expression of bacteriocin activity. In this method the dilution series is applied to wells in the indicator lawn and the CD is defined as the last dilution to exhibit a zone with a diameter larger than the diameter of the well. Although quantitative, dose-dependent expressions of bacteriocin activity that adapt the well diffusion assay are also available (Parente and Ricciardi, 1999).

5.1.6 SDS-PAGE overlay assay to detect bacteriocins

Another method for the detection of bacteriocins, which has the added benefit of allowing size estimations of the bacteriocin even in crude samples, is the SDS-PAGE overlay assay. First developed by Bhunia *et al*., (1987) the bioassay relies on SDS-PAGE separation of protein sample containing bacteriocin, the proteins are then fixed in place with solvents and overlaid with indicator species. Zones of inhibition on the overlaid gel are then compared to a reference gel to determine size of the inhibitory bacteriocin. The bioassay relies on bacteriocin inhibitory activity to confirm its presence, however, sample preparation for SDS-PAGE requires the proteins be reduced and linearised, which can result in loss of activity for many bacteriocins (Laemlii *et al.,* 1970). Additionally, the solvent fixation step has the potential to further denature bacteriocins leading to loss of activity.

In an effort to minimise these drawbacks, variations of the bioassay exist, in which the solvent fixation step is omitted and instead the gel is washed with a low concentration of Tween 80 to remove residual SDS from the gel before being washed in dH_2O and overlaid with indicator species (Yamamoto *et al*., 2003). However, these variations still include the denaturing preparation of the protein samples and, although uncommon, it is possible to replace the SDS-PAGE gel in the overlay assay with a Native-PAGE gel under non-reducing conditions (Wang, Chen and Hsu, 2012). The SDS-PAGE overlay assay is a powerful method for both size estimation and confirmation of activity of bacteriocins, with variations in execution reflecting the variation of bacteriocin sensitivities to denaturants.

5.1.7 Ion exchange chromatography for part purification of bacteriocins

Following bacteriocin separation from culture media and confirmation of activity in an aqueous solution, the traditional purification strategy utilizes chromatographic methods to separate bacteriocins from contaminating proteins in the sample. Initially bacteriocins are part-purified using a combination of Fast protein liquid chromatography (FPLC) methods that each exploit different properties of the bacteriocins in order to achieve maximum bacteriocin purity. Ion exchange chromatography (IXC) is one such FPLC method commonly used in the purification process of bacteriocins, which separates proteins based on net surface charge. A column is chosen with a matrix of either positively charged ligands (AXC) or negatively charged ligands (CXC), which selectively bind proteins based on their charge (Cummins, Rockfort and O'Connor, 2016). Bacteriocin containing samples can be applied to IXC columns and bound proteins can be eluted with a gradient of increasing ionic strength, typically high concentration NaCl buffers. The Na^+ or Cl^- ions of the buffer displace the proteins bound to the column and those proteins with the lowest affinity to the ligands are eluted first in the lowest concentrations of NaCl (Cummins, Rockfort and O'Connor, 2016). The eluant can then be tested for bacteriocin presence using the described detection methods. IXC was successfully used in the recovery of the bacteriocins subtilosin A and licnenin (Table 5.1).

5.1.8 Hydrophobic interaction chromatography for part purification of bacteriocins

Hydrophobic interaction chromatography (HIC) is another FPLC method often employed in place of IXC or when IXC has failed to give sufficient separation of the bacteriocin from the protein preparation. HIC relies on the reversible binding of hydrophobic regions of proteins to non-ionic hydrocarbon or aromatic ligands packed in a column (O'connor and Cummins et al, 2016). To maximise the surface tension of proteins and thus improve hydrophobic binding of protein and ligands, proteins are prepared in high concentration salt buffers and eluted in a decreasing salt concentration (O'connor and Cummins et al, 2016). The necessity of a high salt sample buffer means HIC is particularly suited to bacteriocin purification as bacteriocins prepared using ammonium sulfate precipitation can be directly applied to HIC columns without additional dialysis steps. Additionally, the reversible

hydrophobic interaction means that eluted proteins generally retain their biological activity. For these reasons, HIC is widely used in the purification of bacteriocins and often when HIC is omitted from the purification strategy it is replaced by the addition of hydrophobic resins directly to the culture media which facilitate hydrophobic interaction separation of the bacteriocins from contaminating proteins (McAuliffe et al, 1998; Zendo et al, 2003).

5.1.9 Gel filtration Chromatography for part purification of bacteriocins

Another FPLC method often used is size exclusion chromatography (SEC; also known as gel filtration chromatography). To utilize SEC in bacteriocin purification a bacteriocin containing sample is applied to a column packed with beads of a porous matrix (dextran or agarose). Large molecules are unable to permeate the pores of the matrix and so are eluted from the column early; whereas small molecules first permeate the pores and so are eluted later in the purification process (O'Fagain *et al*., 2016). This exploits the fact that bacteriocins are generally less than 10 kDa in size to separate them from larger contaminating proteins in the sample and has been used successfully in the purification processes of many bacteriocins including subtilosin A and sonorensin (Table 5.1). Whilst SEC has successfully been used in the purification of novel bacteriocins it is best suited to late-stage purification applications in combination with other described FPLC methods. This is due to the matrix having an inherently low sample handling capacity and so diverse protein samples with high concentrations will result in irregular separation of proteins (O'Fagain *et al*., 2016). Regardless of the FPLC method/methods used to generate partly purified bacteriocins, fractions collected that show bacteriocin activity are almost always pooled in preparation for final purification.

5.1.10 Reverse phase high pressure liquid chromatography for final purification of bacteriocins

Following the traditional strategy for bacteriocin purification, the final purification stage almost always involves separation of the bacteriocin from other proteins using Reverse phase high pressure liquid chromatography (RP-HPLC). The principle of RP-HPLC is similar to HIC, in that they both separate proteins based on hydrophobic interactions of amino acids with a hydrocarbon ligand matrix (Josic and Kovak, 2010). However, the hydrophobic interactions formed in RP-HPLC are much stronger and therefore proteins are both applied to the matrix and eluted from the matrix using high concentrations of water-miscible organic solvents (Josic and Kovak, 2010). These harsh conditions frequently lead to denaturation of proteins and loss of biological activity, despite this its use as the final purification step for bacteriocins is ubiquitous and is usually required for definitive identification of novel bacteriocins (Table 5.1).

5.1.11 Mass Spectronomy identification of novel bacteriocins

Identification of novel bacteriocins can be done at any stage in the purification strategy, however it is most reliable following final RP-HPLC purification, due to the compatibility of RP-HPLC with mass-spectrometry (MS). MALDI-TOF MS is the most widely used method for definitive identification of novel bacteriocins. To accomplish MALDI-TOF MS identification, protein samples are fragmented and crystalised in an energy absorbent matrix, before being ionised with a laser to generate single protonated ions. The mass to charge ratio (m/z) of the ions is then calculated using the time it takes each ion to travel the flight tube and a peptide mass fingerprint (PMF) is generated (Singhal *et al*., 2015). The PMF of protein fragments are then compared to the predicted mass of other peptides and an amino acid sequence is identified. Bioinformatical methods can then be used to identify the presence of genes encoding the amino acid fragments identified leading to the identification of novel bacteriocins and the genes responsible for their production. MALDI-TOF MS is considered the gold standard of bacteriocin identification and has been used successfully to identify the novel bacteriocins amylocyclicin, subtilomycin and lichenicidin produced by members of the *B. subtilis* family (Scholz *et al.,* 2014., Begley *et al*., 2009., Phelan *et al.,* 2013).

5.1.12 Alternative methods for bacteriocin purification

Whilst the traditional purification strategy has been effectively utilized in purifying and identifying most well-known bacteriocins, the drawbacks are substantial. The lengthy nature and multiple sample preparation stages lead to low recovery rates of bacteriocins from culture media. Additionally, many methods for sample processing have the potential to denature the bacteriocin and eliminate biological activity and so lengthy optimization is required for each individual bacteriocin. To combat these issues, some have developed novel methods of purifying bacteriocins. Suarez *et al*., (1997) developed a one-step protocol for purification of nisin A with bacteriocin-specific immunoglobulins. However, this method requires the identity of the bacteriocin to be known prior to purification and is unsuitable for purifying novel, unknown bacteriocins. Bacteriocin recovery using surfactants has also been demonstrated (Wong *et al*., 2017), however as with the other "simplified" purification strategies this method also entails lengthy optimisation.

The first aim of this study was to employ the traditional methods of bacteriocin precipitation to generate an aqueous crude sample of the proteins from *B. velezensis* B18 and *B. subtilis* 50B that retained inhibitory activity against *C. difficile*.

The second aim of this study was to use these crude extracts from *B. velezensis* B18 and *B. subtilis* 50B to elucidate physicochemical characteristics of the potential bacteriocins, which would then inform downstream chromatographic purification.

The final aim of this study was to utilise MS to identify the potential bacteriocins from *B. velezensis* B18 and *B. subtilis* 50B.

5.2 Results

5.2.1 Crude bacteriocin preparation from *B. velezensis* B18 and *B. subtilis* 50B results

Previous experimentation had implied that the inhibitory substance produced by *B. velezensis* B18 was secreted into the extracellular matrix and activity could successfully be recovered by ammonium sulfate precipitation (4.2). Therefore, ammonium sulfate precipitation was chosen as the precipitation method to generate aqueous CPB from *B. velezensis* B18 and *B. subtilis* 50B. Proteins were precipitated from 200 mL of BHIS culture medium (following 16 h of aerobic growth and 30°C) with 40%, 60% and 80% saturation of ammonium sulfate as described (2.4.2). The 30°C growth temperature was chosen for the *Bacillus* spp. to suppress the production of surfactin non-bacteriocin antibiotics encoded by both strains that had the potential to be responsible for anti-*C. difficile* activity displayed, temperatures ≥37°C favour the production of surfactin antibiotics (Caulier *et al.*, 2019). The anti-*C. difficile* activity of the CBP was tested using the well-diffusion assay (2.4.2). In the case of *B. velezensis* B18, precipitation with 60% ammonium sulfate produced the largest diameter zone of inhibition against *C. difficile* 630 and confirmed that the anti-*C. difficile* potential bacteriocin could be recovered from the extracellular matrix using ammonium sulfate (Figure 5.1). 60% was chosen as the optimum saturation for precipitation of the potential bacteriocin from *B. velezensis* B18 due to the negligible activity seen in the 80% fraction. The 40% and 60% fractions from *B. subtilis* 50B produced identical diameter zones of inhibition against *C. difficile* 630 (Figure 5.1), and again the 80% fraction produced negligible zones of inhibition. To ensure maximum bacteriocin precipitation, 60% was also chosen as the optimum saturation for precipitation of the potential bacteriocin from *B. subtilis* 50B.

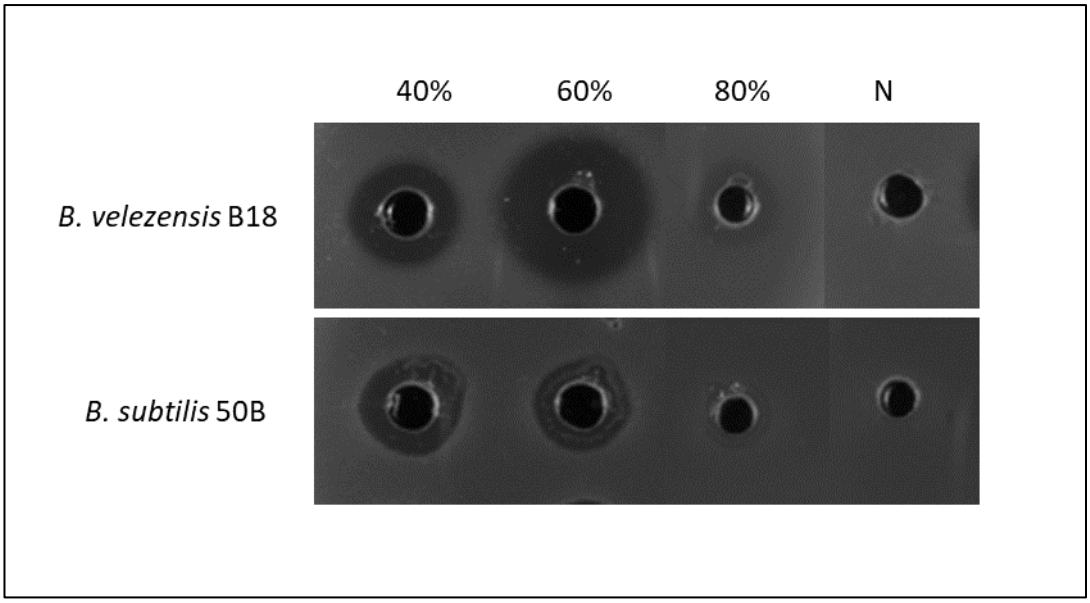

Figure 5.1: Inhibition of *C. difficile* 630 by the CBP of *B. velezensis* B18 (top) and *B. subtilis* 50B (bottom). 50 µL of the 40%, 60% and 80% ammonium sulfate precipitated fractions were applied to wells in a *C. difficile* 630 indicator lawn. Images were taken following 16 of anaerobic incubation at 37°C and a representative image of three replicates was chosen, negative control denoted 'N'.

5.2.2 Size determination of the potential bacteriocin from *B. subtilis* 50B

The bioactive 40% and 60% fractions of the CBP obtained from *B. subtilis* 50B were separated by 16% tricine SDS-PAGE and overlaid with *C. difficile* 630 to estimate the size of the potential bacteriocin responsible for the anti-*C. difficile* activity (2.4.4). The results showed a zone of inhibition against *C. difficile* 630 in both the 40% and 60% fraction on the overlaid gel that corresponded to two distinct bands of roughly 5 kDa in size (Figure 5.2). These bands were subsequently excised from the stained gels and underwent LC MS/MS analysis (University of Cambridge). Unfortunately, no candidate was identified as the potential bacteriocin produced by *B. subtilis* 50B due to contamination of the samples. The LC MS/MS data predominately consisted of mammalian proteins, specifically bovine proteins, the origins of which were most likely the BHIS medium. Therefore, BHIS was deemed an unsuitable medium to continue using for the purification and identification of the 5 kDa potential bacteriocin from *B. subtilis* 50B due to the interference of the medium-derived proteins.

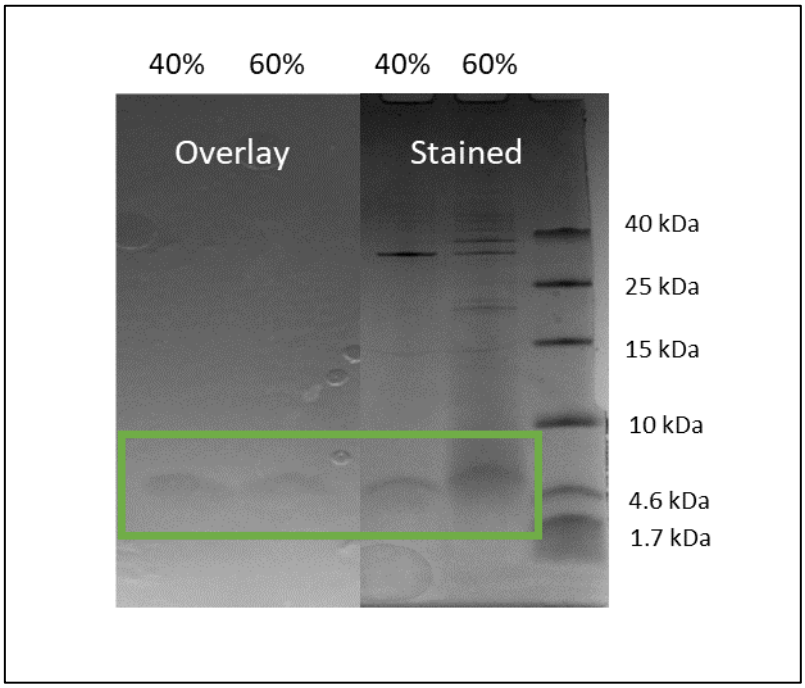

Figure 5.2: SDS-PAGE overlay assay of the 40% and 60% fractions of the Crude bacteriocin preparation from *B. subtilis* 50B. SDS-PAGE Gel overlaid with *C. difficile* 630 and incubated anaerobically for 16 h shown on the left. Gel stained with Coomassie featuring the Spectra low range protein ladder shown on right. Zones of inhibition and corresponding bands excised for LC MS/MS analysis shown within the white rectangle.

5.2.3 Size determination of the potential bacteriocin from *B. velezensis* B18

Earlier experimentation had highlighted that the *C. difficile*-inhibitory substance produced by *B. velezensis* B18 had an estimated size of 4.6 kDa (4.2). In order to confirm this, the CBPs generated from the 40% and 60% ammonium sulfate precipitation were subjected to the SDS-PAGE overlay assay (2.4.4). Strangely, no inhibitory activity against *C. difficile* 630 corresponding to a protein band was observed using these samples (data not shown). As this approach had previously been shown to be successful it was theorised the concentration of the inhibitory substance was too low for bioactivity and so the ammonium sulfate precipitation was repeated with a fresh 200 mL culture at 60% precipitation only (2.4.1). This fresh CBP in both neat and half diluted with 50 mM tris concentrations was analysed using the SDS-PAGE bioassay. The overlaid gel showed a zone of inhibition of *C. difficile* 630 in both the neat and half diluted samples that corresponded to a "smear" of protein on the stained gel that began just under the 4.6 kDa marker, confirming what was seen previously (Figure 5.3). The smearing on the gel could possibly be due to other low molecular weight proteins obscuring the view of a distinct band responsible for the zone of inhibition. It was possible these other low molecular weight proteins could originate from BHIS or be a product of *B. velezensis* B18. With this possibility and the interfering role BHIS derived proteins can play on bacteriocin identification that was seen for *B. subtilis* 50B, it was decided that production media other than BHIS could be explored to maximise likelihood of identifying the potential anti-*C. difficile* bacteriocin produced by both species.

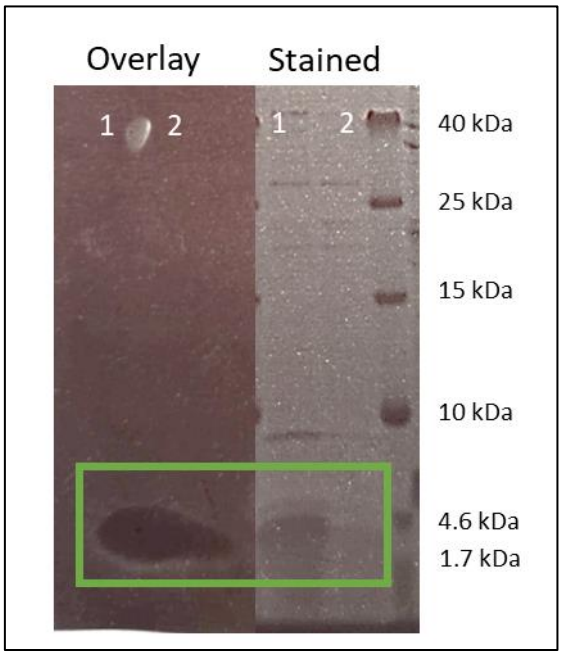

Figure 5.3: SDS-PAGE overlay assay of 60% ammonium sulfate precipitated crude bacteriocin preparation from *B. velezensis* B18. Lanes 1 and 2 contain the crude bacteriocin preparation serially diluted by 2 in tris buffer separated via SDS-PAGE. SDS-PAGE Gel overlaid with *C. difficile* 630 and incubated anaerobically for 16 h shown on the left. Gel stained with Coomassie featuring the Spectra low range protein ladder shown on right

5.2.4 Alternative media for use in bacteriocin purification

The previous experiments showed that protein contaminants present in BHIS medium hindered the identification of the potential bacteriocins from both strains and so alternative, less protein-rich media had to be explored. Due to their small size and relatively low abundance in the culture media, bacteriocins are often obscured by protein additives in the media and so it is not uncommon for mineral media with no protein additives to be used for bacteriocin identification. In some cases, these nutrient low media can even improve bacteriocin production (Pingitore *et al*., 2009).

B. velezensis B18 was grown in 200 mL of LB and 200 mL of the minimal mineral M9 medium for 24 h (Harwood and Cutting), the CBP was prepared from each of these cultures as in 2.4.1. BHIS comparison was omitted as the LC-MS data from *B. subtilis* 50B had highlighted that the protein contaminants interfere with downstream processes. The anti-*C. difficile* activity of these CBP were assessed via the critical dilution assay and the anti-*C. difficile* activity expressed as AU mL^{-1} (2.4.3). Figure 5.4 shows the respective activity of the CBP prepared from each media. The CBP from LB culture medium was shown to have the strongest anti-*C. difficile* at 14,400 AU mL^{-1}, although it was less reproduceable than the M9 comparison with a large error margin. Analysis of the protein profile of the CBP via SDS-PAGE showed that despite greater anti-*C. difficile* activity there was visually a greater overabundance of low molecular weight proteins than was seen in CBP from M9 (Figure 5.4). It was again likely that many of these low molecular weight proteins were media derived contaminants that would interfere with downstream purification processes and so despite the potent anti-*C. difficile* activity, LB was decidedly too protein rich to be a suitable media for purification and identification of the potential bacteriocin from *B. velezensis* B18. In the context of this study the CBP from M9 medium appeared to have 61.1% of the inhibitory activity of the LB comparison with 8800 AU mL^{-1} on average, however this activity is not dissimilar to activity of unpurified bacteriocins reported in the literature (Schirru *et al*., 2014; Pilasombut *et al*., 2015). Analysis of the protein profile also showed more defined separation of the low molecular weight proteins than the LB comparison. Whilst there was still smearing of similarly sized proteins it was not possible these potential contaminants were introduced in the

media, and instead are produced by *B. velezensis* B18. Due to the reproducibility and potency of the anti-*C. difficile* activity of the CBP prepared from M9 mineral media and the elimination of culture media contaminants, M9 was chosen as the medium for purification of the potential bacteriocin from *B. velezensis* B18. Although time constraints meant that an experimental comparison for *B. subtilis* 50B was not available, M9 media was also chosen as the purification media for the potential bacteriocin of *B. subtilis* 50B as M9 is a general-purpose media for the *B. subtilis* species complex.

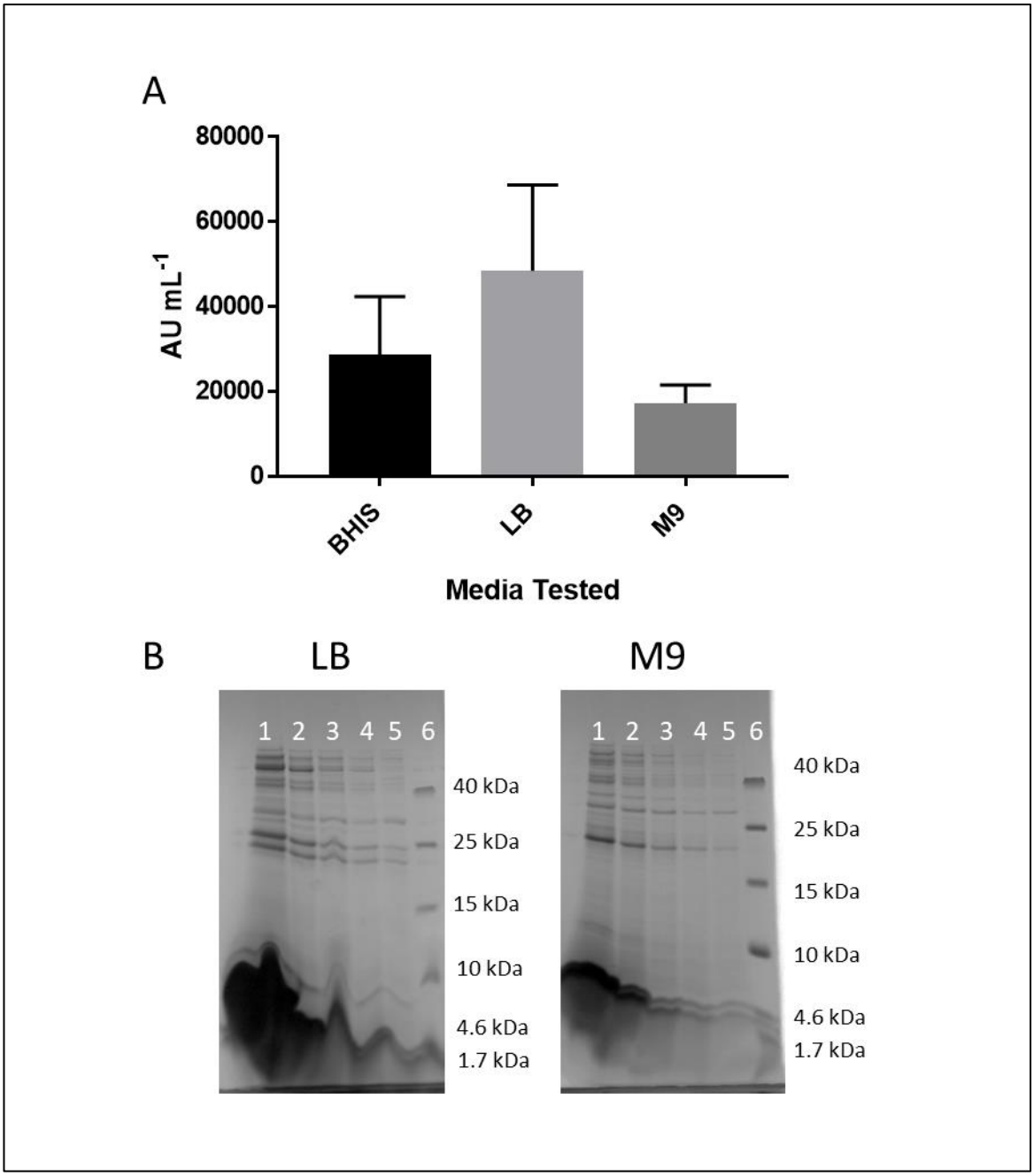

Figure 5.4: Comparison of crude bacteriocin preparation of *B. velezensis* B18 grown in different media. A) **Anti-*C. difficile* activity of the crude bacteriocin preparation from *B. velezensis* B18 grown in LB, BHIS and M9 media. Quantified via critical dilution assay and expressed in AU mL^{-1}**. Data from three replicates was included to calculate the mean and standard deviation (error bars) on Graphpad PRIZM. B) Coomassie stained SDS-PAGE gel visualising the protein profile of the crude bacteriocin preparation from *B. velezensis* B18 grown in LB or M9 media. Lanes 1 – 5 show the protein profile of the crude bacteriocin preparation serially diluted by half.

Lane 6 contains the Spectra low range protein ladder for size estimation. 16% Tricine SDS-PAGE electrophoresis was carried out according to manufacturer's instructions (Novex) and stained with Coomassie brilliant blue (Novex).

5.2.5 Proteolytic and heat stability of the potential bacteriocins from *B. velezensis* B18 and *B. subtilis* 50B

In addition to size, it was important to know other physicochemical characters of the potential bacteriocin from *B. velezensis* B18. To accomplish this, the CBP from *B. velezensis* B18 was prepared from M9 culture as described and subject to proteolytic degradation and heat treatment (2.4.8). The activity of the CBP against *C. difficile* 630 following these treatments was assessed via the CDA and results are shown as a percentage loss of activity (FIGURE 5.5). A 61% (+/-9.5%) decrease in activity was seen following tryptic degradation of the CBP. Similarly, treatments with the broad-range proteases proteinase K and pepsin resulted in a 54.1% and 52.7% reduction in activity, respectively. However due to the large error margin in the experimental data for pepsin this result is likely not to be statistically negligible. The potential bacteriocin also appeared to be highly sensitive to heat treatments with 5 minutes at 100°C resulting in a 59.5% (+/-8.4%) reduction in activity. Treatment with the enzyme α-chymotrypsin had no effect on the activity of the potential bacteriocin. The sensitivity of the potential bacteriocin to heat, proteinase K, and to a lesser extent pepsin, was confirmation that the molecule responsible for anti-*C. difficile* activity was proteinaceous in nature and likely a heat-labile bacteriocin.

The CBP from *B. subtilis* 50B was prepared and subjected to heat and proteolytic degradation as before. The activity of the CBP against *C. difficile* 630 following these treatments was assessed via the CDA and results are shown as a percentage loss of activity (FIGURE 5.5). The potential bacteriocin from *B. subtilis* 50B was shown to be heat stable with no change in activity against *C. difficile* 630 observed following exposure to temperatures of 80°C and 100°C. Conversely, the potential bacteriocin was extremely susceptible to α-chymotrypsin or proteinase K degradation with a complete loss of activity against *C. difficile* 630 following treatment with these enzymes. The potential bacteriocin showed slight susceptibility to pepsin and trypsin with 38.67% (+/- 9.8%) and 30.33% (+/- 4.6%) reductions in activity, respectively. The varying degrees of susceptibility of the potential bacteriocin from *B. subtilis* 50B to all of the proteases tested confirm that the inhibitory molecule responsible for the

inhibition of *C. difficile* was proteinaceous in nature and likely a heat-stable bacteriocin.

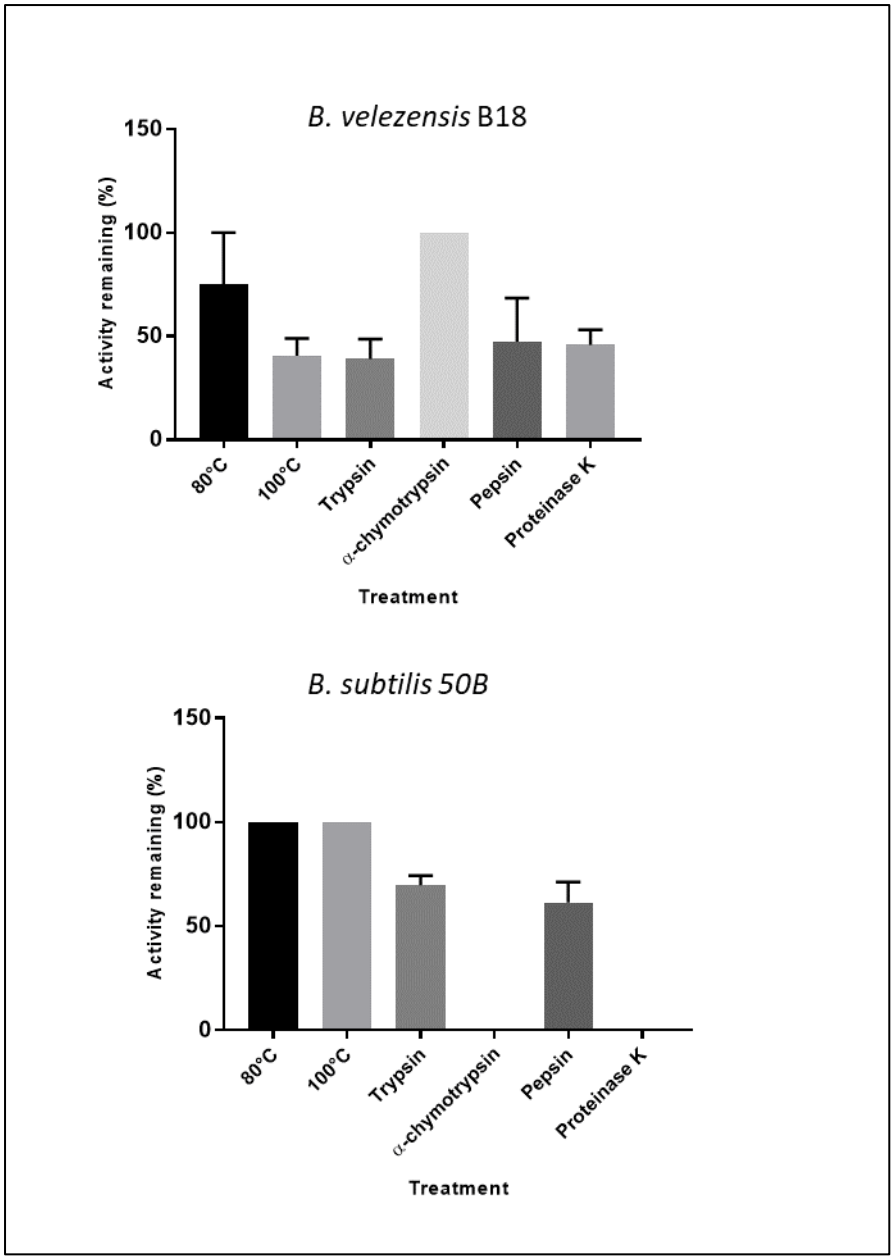

Figure 5.5: Remaining activity of the Crude bacteriocin preparation from *B. velezensis* B18 and *B. subtilis* 50B following heat and protease treatment. Activity was calculated in AU mL^{-1} via the Critical dilution assay against *C. difficile* 630 and reduction in activity defined as a percentage. Plotted values are mean +/- standard deviation from 3 experimental data sets as calculated by GraphPad PRIZM™.

5.2.6 Isoelectric point identification of the potential bacteriocins from *B. velezensis* B18 and *B. subtilis* 50B

Analysis of the protein profile from the CBP from *B. velezensis* B18 grown in M9 medium showed there was still an abundance of low molecular weight proteins of a similar size to the potential bacteriocin. As it was likely many of these proteins could be obscuring identification of a single band responsible for activity, size-based purification processes would not be an appropriate purification strategy for the potential bacteriocin. A charge-based purification strategy was deemed the most viable option and so it was imperative that the isoelectric point (pI) of the potential bacteriocin was elucidated. The pI was established using the CBP from *B. velezensis* B18 subjected to the Isoelectric focusing (IEF) bioassay (2.4.5), the results of which are shown in Figure 5.6. The IEF bioassay showed a zone of inhibition on the *C. difficile* overlaid gel that corresponded again to a trailing band of protein on the stained gel. These peptides that appeared to be responsible for the inhibition of *C. difficile* had apparent pIs ranging from 4.2 – 6.0. Although the "smeared" appearance could be the result of the degradation of the inhibitory substance, it was also possible that more than one inhibitory substance was present in the CBP obtained from *B. velezensis* B18 that had not been adequately resolved from one another using the IEF bioassay. This is due to the apparent two, distinct overlapping zones, which are clearer in the CBP sample half diluted with 50 mM tris (Figure 5.6). Nevertheless, knowing the narrow pI range of the potential bacteriocin from *B. velezensis* B18 informed the choice of charge-based purification.

CBP from *B. subtilis* 50B grown in M9 was also used to establish the pI of the potential bacteriocin from this strain using the IEF bioassay (2.4.5) (Figure 5.6). Interestingly, the *C. difficile* overlaid IEF gel showed multiple potential zones of inhibition against *C. difficile*. The first zone of inhibition appeared to correspond to a region on the stained gel with a pI ranging between 5.2 and 6.0, a second zone of inhibition seen only in the neat and half-diluted sample had an apparent pI range of 4.5 – 5.2 and a final zone of inhibition had an apparent pI of 4.2. The IEF bioassay highlighted that the CBP from *B. subtilis* 50B appeared to produce three potential bacteriocins with

inhibitory activity against *C. difficile* whilst also informing downstream purification processes.

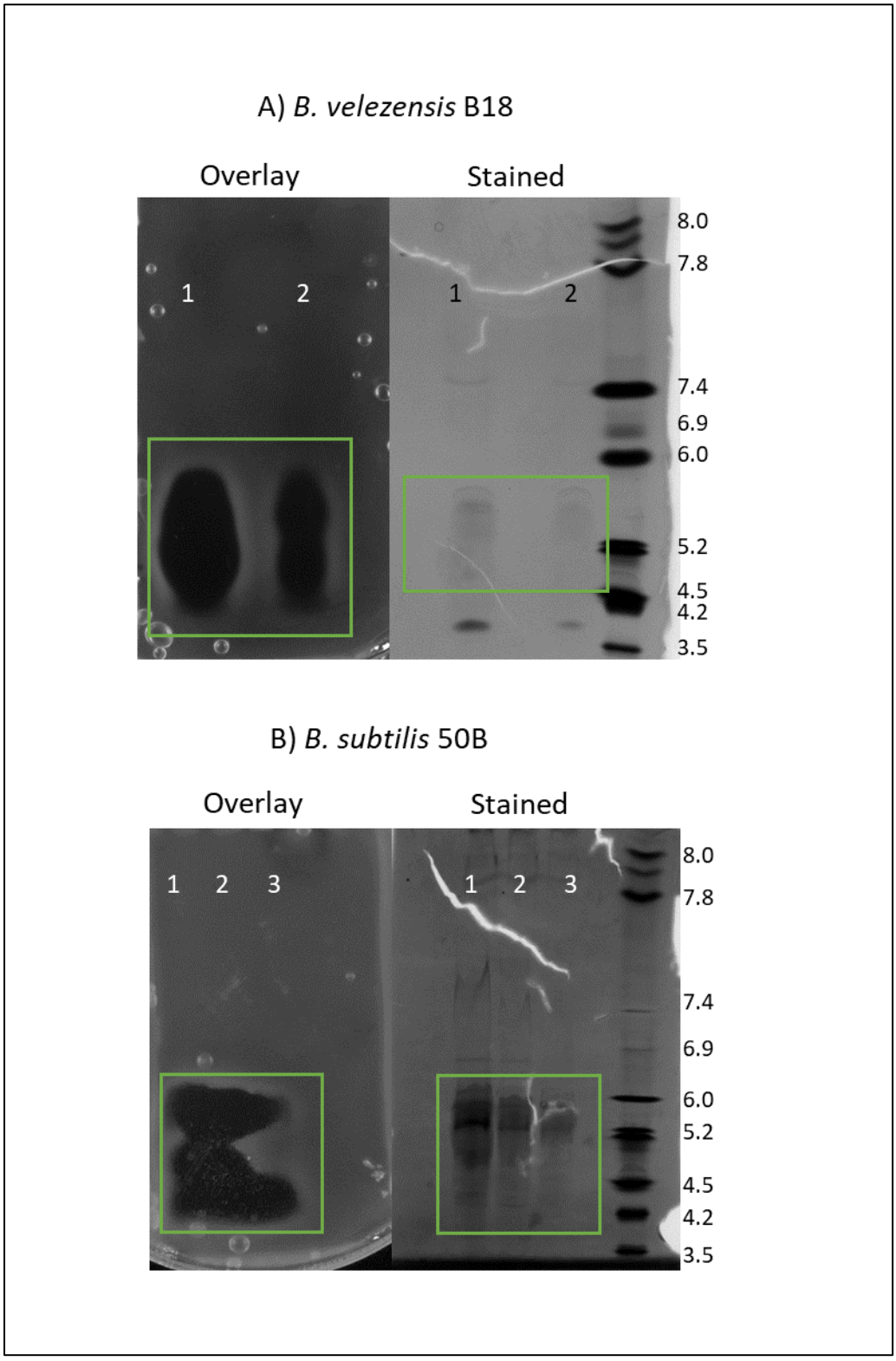

Figure 5.6: IEF overlay assay of the crude bacteriocin preparation from *B. velezensis* B18 and *B subtilis* 50B. A) Lanes 1 and 2 show the protein profile and corresponding overlay gel of the crude bacteriocin preparation from *B. velezensis* B18 serially diluted by half. B) Lanes 1 -3 show the protein profile and corresponding overlay gel of the

crude bacteriocin preparation from *B. subtilis* 50B serially diluted by half. The IEF 3 – 10 marker (Serva) was used for pI estimation. Zones of inhibition and corresponding protein bands highlighted in green. IEF electrophoresis was carried out according to manufacturer's instructions (Novex). Gels overlaid with *C. difficile* 630 and incubated anaerobically shown on the left. Gels stained with Coomassie brilliant blue (Novex) shown on the right.

5.2.7 Part purification of the potential bacteriocin from *B. velezensis* B18 using anion exchange chromatography

IEF showed that the potential bacteriocin from *B. velezensis* B18 had a maximum pI of 6 and so in the pH 8 tris buffer used to dialyse the CBP following precipitation the potential bacteriocin would have a net negative charge and so anion exchange chromatography was chosen as the purification strategy. Following application of the CBP from *B. velezensis* B18 to the positively charged anion exchange column the bound proteins were eluted with an NaCl gradient (2.4.6), this elution profile is shown in Figure 5.7. From this elution, 81 1.5 mL fractions were collected in addition to two 10 mL 1 M NaCl washes, and their anti-*C. difficile* activity was tested using the spot-on lawn assay, Figure 5.7 shows the inhibitory activity of the fractions. As can be seen in Figure 5.7, weak inhibitory activity against *C. difficile* was displayed from fraction 26 until fraction 31, following which the fractions were strongly inhibitory until fraction 55 were the activity again visibly decreased to a faint haze. Interestingly, very weak inhibitory activity was observed again from Fractions 71 to 80, however fraction 81 and the high salt washes have no inhibitory activity. The loss and subsequent return of weak inhibitory activity strengthens the belief that the CBP of *B. velezensis* B18 contains more than one protein with inhibitory activity against *C. difficile*.

As fractions 26 to 58 had shown the strongest inhibitory activity against *C. difficile* 630, they were chosen for further analysis in the hopes of identifying one of the possible two bacteriocins produced by *B. velezensis* B18. These fractions were individually desiccated by freeze-drying overnight. Following which, each desiccated sample was resuspended in 30 µL of Tricine SDS-PAGE buffer (NOVEX) and the resuspended samples were applied in duplicate to SDS-PAGE gels in preparation for the SDS-PAGE overlay assay. The SDS-PAGE bioassay showed zones of inhibition of *C. difficile* 630 in the samples of fractions 50 – 58 corresponding to a region between the of 3 kDa on the ladder that contained 2 protein bands, either of which could be responsible for the inhibitory activity (Figure 5.8). This 3 kDa size is not so dissimilar to the earlier estimated size of 4.6 kDa, especially considering the lower concentration in these samples allowed greater resolution of the smaller size

proteins. It is therefore not unreasonable to assume this 3 kDa protein contributed in part to the earlier inhibition of *C. difficile* in the SDS-PAGE overlay assay.

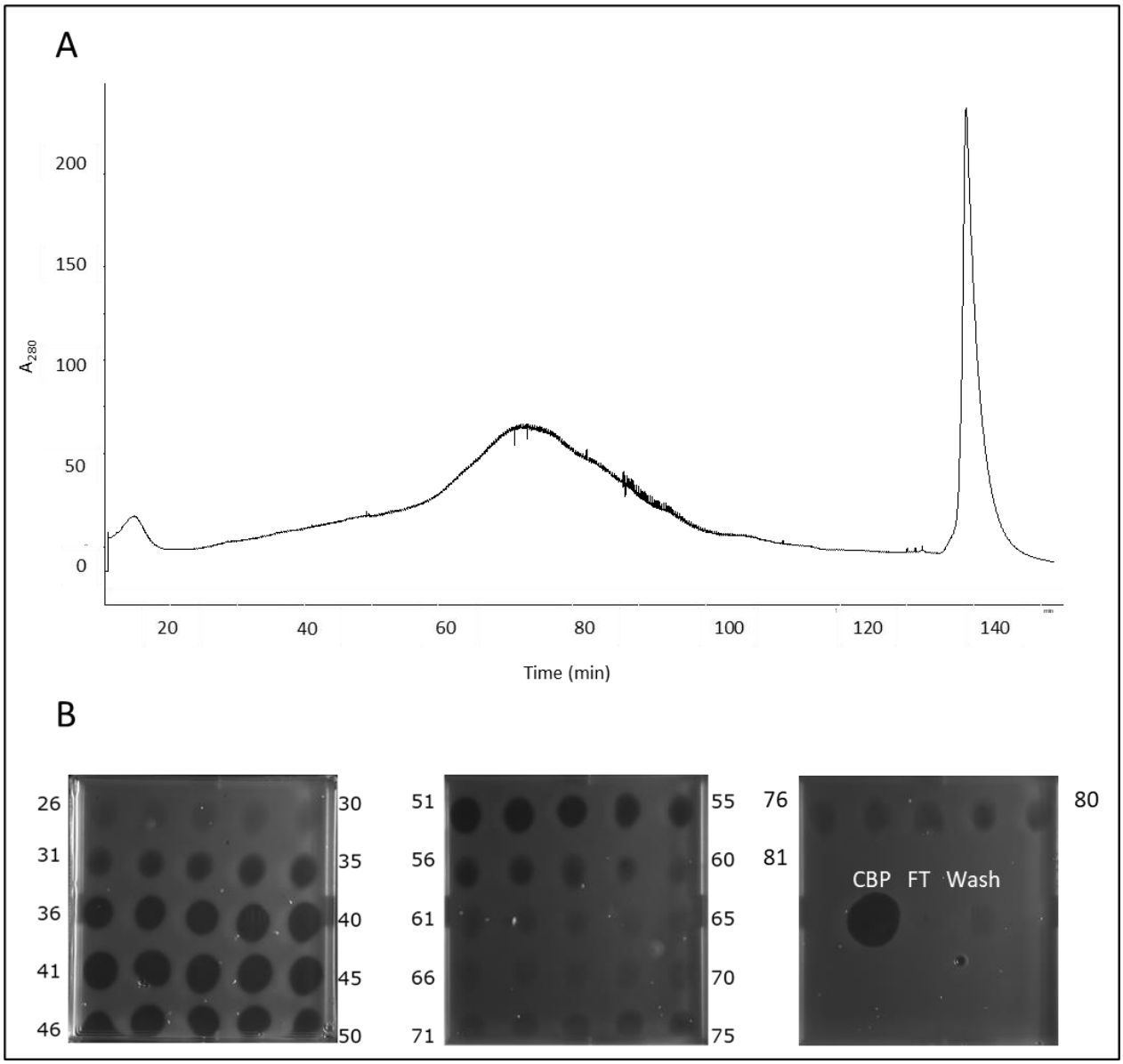

Figure 5.7: Anion exchange chromatography elution profile of the Crude bacteriocin preparation from *B. velezensis* B18 (A) and antimicrobial activity of a 10 µL sample of the 1 mL fractions collected during elution against *C. difficile* 630 (B). CBP of *B. velezensis* B18 obtained as before and tested for anti-*C. difficile* activity, following dialysis it was applied to Q Sepharose column. The flow-through was collected and tested for antimicrobial activity as was the column wash. 1 mL fractions were collected during elution by an increasing gradient of NaCl to 500 mM (with a final 10 mL fraction collected following elution with 1 M Nacl).

Interestingly, no activity was seen in the bioassay of fractions 31 – 49, despite the strong anti-*C. difficile* activity of the fraction when tested using the spot-on lawn assay (Figure 5.8). Of fractions 50 to 58, fractions 51 and 52 showed the strongest visible activity in the *C. difficile* bioassay and so both potential protein bands were excised from the stained gels for MALDI-TOF MS analysis (University of York, UK). Unfortunately, MALDI-TOF MS analysis failed to detect any peptide signals from any of the four bands sent for analysis and only self-digestion of trypsin used in peptide fragmentation was detected. Additionally, the remaining 750 µL volume of fractions 50 – 58 were pooled and applied to a C_{18} RP-HPLC and bound proteins were eluted in an acetonitrile gradient (data not shown). Fractions that showed potential protein elution from the chromatograph were tested against *C. difficile* 630 in the spot-on-lawn assay as single fractions and in combination with one another under the possibility that two synergistic peptides were required for activity, however no fraction or combination exhibited anti-*C. difficile* activity. The failure of MALDI-TOF and RP-HPLC implied the concentration of inhibitory substance in the single bands and combined fractions was not adequate for identification of the potential bacteriocin from *B. velezensis* B18 that had eluted first from ion exchange and so scale up would be required. Attempts to increase initial culture volume of *B. velezensis* B18 encountered its own issues and attempts to optimize continued until experimentation was required to cease due to the ongoing SARS-coV-2 pandemic. Attempts to purify the anti-*C. difficile* bacteriocin from *B subtilis* 50B using IEX were complicated by aggregation of proteins on the column and no elution of active fragments. Due to the end of experimentation, optimisation for this strain could not be further explored.

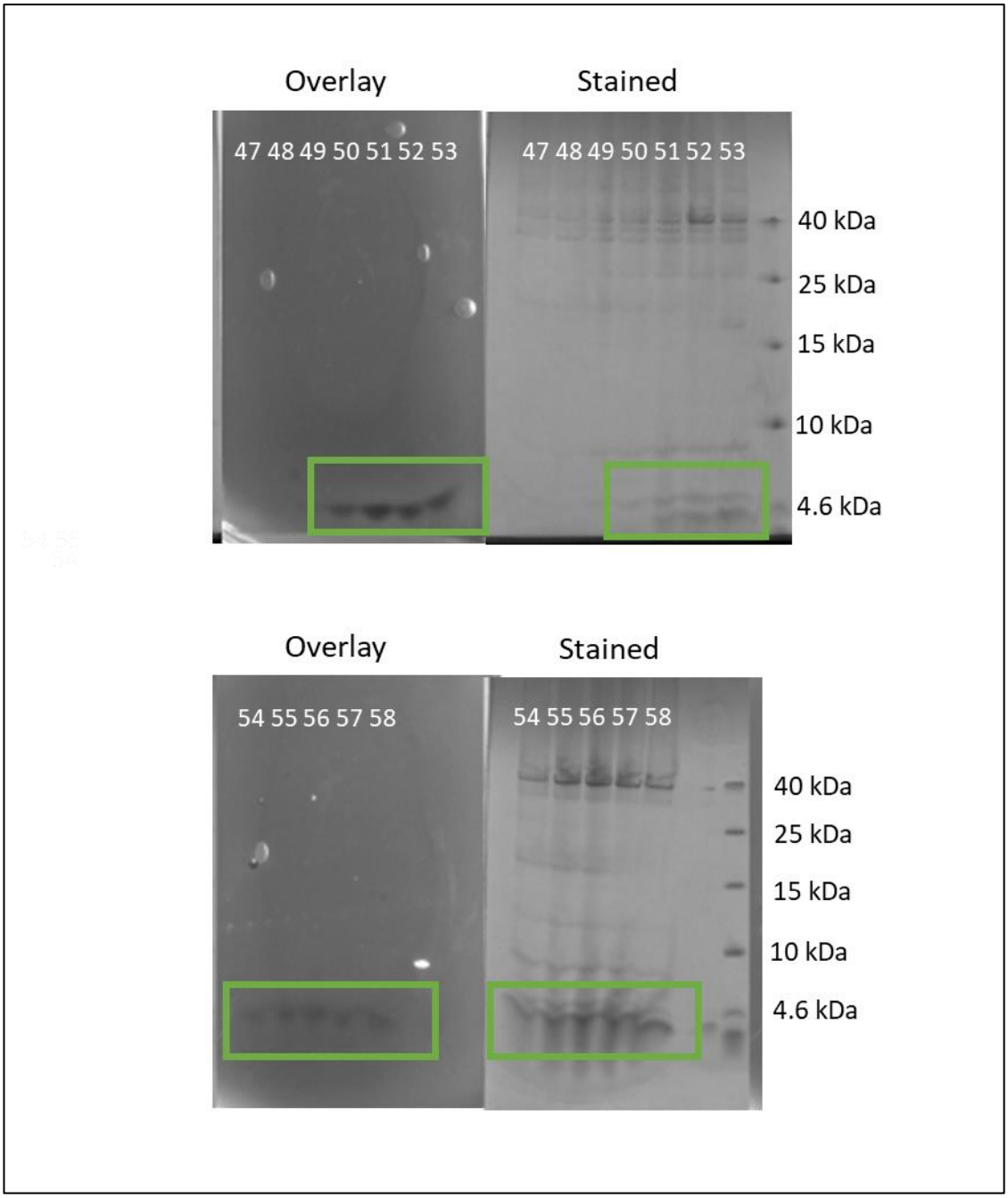

Figure 5.8: SDS-PAGE overlay assay of the desiccated then resuspended fractions collected from the CBP of *B. velezensis* B18 following anion exchange separation. Fractions 26 – 58 were selected based on their strong anti-*C. difficile* activity. 750 µL of the 1.5 mL fractions selected were desiccated for 16 h and resuspended in Tricine SDS-PAGE sample buffer (Novex) and separated via SDS-PAGE in duplicate. Gels overlaid with *C. difficile* 630 shown on left and gels stained with coomassie shown on right.

5.2.8 Inhibitory spectrum of the CBP from *B. velezensis* B18 and *B. subtilis* 50B against *C. difficile* ribotypes.

Due to the circumstances of this study further investigation into purification of the anti-*C. difficile* bacteriocins from both strains was not possible. However, other characteristics of the bacteriocins could be explored. An essential feature in an anti-*C. difficile* therapeutic would be the capability to inhibit most *C. difficile* strains and ribotypes. To ensure that the potential bacteriocins present in the CBP from *B. velezensis* B18 and *B. subtilis* 50B had activity against a wide range of *C. difficile* ribotypes, the anti-*C. difficile* activity was tested using the spot-on lawn assay against 29 additional strains of *C. difficile* (Table 5.2). In general *B. velezensis* B18 inhibited all the strains of *C. difficile* tested against in this study. However, only weak activity was seen against the two ribotype 078 strains tested against. Similarly, *C. difficile* R10079 (ribotype 020) and *C. difficile* 126 (ribotype 126) were only weakly inhibited by the CBP of *B. velezensis* B18.

The CBP from *B. subtilis* 50B was also generally inhibitory against all the strains of *C. difficile* tested against in this study, except for *C. difficile* 003 (ribotype 003) in which no inhibitory activity was seen. As with *B. velezensis* B18, the CBP of *B. subtilis* 50B had only weak activity against the two ribotype 078 strains tested against as well as the ribotype 020 and 126 strains.

The CBP from *B. velezensis* B18 and *B. subtili*s 50B proved to be inhibitory against the majority of *C. difficile* strains and ribotypes tested against in this study, suggesting that the potential bacteriocins from *B. velezensis* and *B. subtilis* 50B could be possible therapeutic candidates. The weak inhibitory activity against ribotype 078 strains poses some concern as it is a clinically relevant ribotype (Rupnik et al, 2018). Ideally future MIC assays would be conducted against ribotype 078 strains of *C. difficile* using purified bacteriocins from *B. velezensis* B18 and *B. subtilis* 50B.

Table 5.2: Inhibitory activity of the CBP from both *Bacillus* spp. against a spectrum of *C. difficile* strains and ribotypes*. CBP from both strains prepared according to 2.4.1 and 10 µL spotted onto a BHIS agar plate seeded with the *C. difficile* indicator strains. Activity was assessed following 16 h anaerobic incubation. All tests were completed in triplicate.

***C. difficile* strain**	**Ribotype**	***B. velezensis* B18 CBP**	***B. subtilis* 50B CBP**
11205	001	++	++
TL178	002	++	++
003	003	++	-
CD630	012	++	++
EK15	014	++	++
TL174	014	++	++
UK-1	015	++	++
M68	017	++	++
CF5	017	++	++
R10079	020	+	+
ClosDi22	023	++	++
CD305	023	++	++
R20291	027	++	++
Dh1916	027	++	++
CD196	027	++	++
R8438	029	++	++
046	046	++	++
CDC34	053	++	++
056	056	++	++
R9367	070	++	++

075	075	++	++
077	077	++	++
M120	078	+	+
EK28	078	+	+
081	081	++	++
Liv22	106	++	++
R10432	106	++	++
117	117	++	++
126	126	+	+
131	131	++	++

- ++ indicates strong inhibitory activity, + indicates weak inhibitory activity and – indicates no activity.

5.2.9 Activity spectrum of the bacteriocins from *B. velezensis* B18 and *B. subtilis* 50B

Knowing that the CBP from both strains was active against a wide range of *C. difficile* strains, it was also important to know if this activity was specific to *C. difficile*. The CBP from *B. velezensis* B18 and *B. subtilis* 50B were tested against a range of Gram-positive and Gram-negative bacteria to analyse the activity spectrums of the bacteriocins produced by both strains. The CBP of *B. velezensis* B18 showed inhibitory activity against all of the Gram-positive strains tested (Table 5.3) against, however, in the case of *Clostridium butyricum* and *Enterococcus faecalis* the activity was limited. The CBP showed no inhibitory activity against the Gram negatives *Pseudomonas aeruginosa* and *E. coli* Top 10, however, *E. coli* S17 was weakly inhibited. This data suggests that the bacteriocin produced by *B. velezensis* B18 is active primarily against Gram-positive bacteria, however, weak activity against Gram- negative bacteria is possible. Although, the susceptibility of *E. coli* S17 may be a reflection of this strains history as a laboratory-use genome reduced strain (Choe *et al*., 2019). The CBP of B. subtilis 50B strongly inhibited the Gram-positive anaerobes *Clostridium perfringens, Clostridium sporogenes* and *C. butyricum* (Table 5.3). However, again *E. faecalis* was only weakly inhibited. When tested against Gram-positive aerobes the CBP of B. subtilis 50B strongly inhibited *B. subtilis* 168 and *B. velezensis* FZB42 but showed no inhibitory activity against S. aureus. No activity was observed against *E. coli* Top10*, E. coli* S17 or *P. aeruginosa*, suggesting that the bacteriocin/s produced by *B. subtilis* 50B is active against Gram-positive bacteria only.

Table 5.3: Inhibitory activity of the CBP from *B. velezensis* B18 and *B. subtilis* 50B against a range of indicator organisms. * CBP from both strains prepared according to 2.4.1 and 10 µL spotted onto a nutrient agar plate seeded with the indicator strains. Activity was assessed following 16 h incubation under indicator species relevant growth conditions. All tests were completed in triplicate.

Indicator species	*B. velezensis* CBP	*B. subtilis* 50B
C. butyricum NCTC 7423	+	++
C. perfringens	++	++
E. faecalis	+	+
C. sporogenes NCIMB 10696	++	++
B. subtilis 168	++	++
B. velezensis FZB42	++	++
S. aureus	++	-
E. coli Top 10	-	-
E. coli S17	+	-
P. aeruginosa HA1	-	-

- ++ indicates strong inhibitory activity, + indicates weak inhibitory activity and – indicates no activity.

5.3 Chapter Discussion

Two *Bacillus* spp. had been isolated from porcine colons that showed remarkable biocontrol potential, particularly against *C. difficile*. Bioinformatic analyses had identified a list of candidate antimicrobials that could potentially be responsible for the activity seen from these two strains. Earlier attempts to identify if bacteriocins encoded by these strains were responsible for the anti-*C. difficile* activity had proven unsuccessful in the context of this study and so purification of the antimicrobials responsible was decidedly the best course of action. Although both species were known to harbour the biosynthetic operons for non-bacteriocin antimicrobials, only bacteriocin purification processes were utilised, in order to increase the likelihood of identifying a bacteriocin with novel activity against *C. difficile*.

The beginning of any purification process generally involves the generation of an aqueous protein sample obtained from the producing strains that retained biological activity (Pingitore *et al*., 2007). Often this stage comes with intense optimization as not all precipitation methods are tolerated by all bacteriocins. This study found that the inhibitory substances produced by both *Bacillus* spp. were effectively precipitated from cell-free culture media when using ammonium sulfate saturation and resuspension in Tris buffer. Suggesting that the *C. difficile* inhibitory substances produced by both strains was secreted into the extracellular matrix and not cell-surface bound, as is the case for other bacteriocins such as formicin and uberolysin (Collins *et al*., 2016; Wirrawan *et al.,* 2007).

The generation of the bioactive aqueous sample from the *Bacillus* spp. meant that downstream purification processes could be explored, and it was here that many of the issues associated with bacteriocin purification and identification were highlighted. One of the first issues was the highly sensitive nature of mass spectrometry. Despite the presence of clear protein bands, which had remained bioactive after resolution on an SDS-PAGE gel, identification of the *C. difficile* inhibitory substance from *B. subtilis* 50B was obscured by bovine proteins originating from the BHIS medium. As previously outlined, bacteriocin concentration in culture media is low and bacteriocins can often be obscured by the more abundant proteins

present (Parrente and Ricciardi, 1999). Therefore, a minimal medium without additional protein additives was selected for use as the culture media for both *Bacillus* spp. (Harwood and Cutting, 2003).

A further issue was highlighted in attempts to chromatographically separate the proteins present in the CBP of *B. velezensis* B18, in order to partly purify the *C. difficile* inhibitory substance. The multi-stage process involved in the purification and identification of bacteriocins, inevitably results in the loss of bioactive protein at each stage (Yang *et al.*, 1992). Precipitation of culture media proteins by ammonium sulfate is never 100% and proteins are lost at this stage, further loss of proteins occurs during dialysis, preparation for chromatography and then during chromatography itself, and the end recovery of bacteriocins is often poor (Pingitore *et al*., 2007; Sidooski *et al*., 2018; Guyonett *et al*., 2000). Anion exchange chromatographic separation of the CBP from *B. velezensis* B18 resulted in the recovery of fractions retaining inhibitory activity against *C. difficile* 630, subsequent analysis of these samples through SDS-PAGE revealed distinct 3 kDa bands that corresponded to *C. difficile* 630 inhibition. However, MALTi-TOF MS analysis of these bands showed no peptide fragments. Additionally, HPLC separation of the pooled active fractions obtained from AEX showed no inhibitory activity against *C. difficile* 630. This suggested the concentration of the *C. difficile* inhibitory substance following the bacteriocin purification process was too low for identification, which is reflective of the multi-stage process required. Attempts to resolve this issue highlighted yet another issue in the purification and identification of novel bacteriocins.

To potentially increase the concentration of the *C. difficile* inhibitory substance from *B. velezensis* B18 and thus increase the yield, a scale up of the initial culture volume was required. Despite optimizations being made in order to replicate the conditions of the original culture methods such as ensuring the aerated space in the flasks was the same ratio and increasing the length of incubation time, no bioactive CBP could be recovered from scaled-up *B. velezensis* B18 cultures. Issues in scale-up of bacteriocin purification is not unheard of, and the difficulties in achieving cost-effective, successful large-scale bacteriocin production is one of the primary reasons bacteriocins are not utilised effectively for their biocontrol potential, especially in a

medical context (Luna et al, 2021). Likewise, issues in achieving successful separation of bioactive fractions of the CBP from *B. subtilis* 50B hindered further purification/identification of the *C. difficile* inhibitory substance produced by this species. Ultimately, all optimisations of chromatography were required to end prematurely as a reflection of the ongoing SARs-CoV-2 pandemic.

Whilst a definitive identification of the anti-*C. difficile* substances produced by the *Bacillus* spp. was not possible due to unprecedented events, prior to the end of experimentation a great deal of work had been done to elucidate physiochemical features of the *C. difficile* inhibitory substances. Using which, candidate antimicrobials could rationally be eliminated. For *B. velezensis* B18, several of the encoded antimicrobials had already been deemed an unlikely candidate for the anti-*C. difficile* activity seen in the strains and the most likely candidates thus far were amylocyclicin, amyloliquecidin, difficidin and surfactin (1.3).

The lipoprotein antibiotic surfactin had earlier seemed a likely candidate for the activity seen from *B. velezensis* B18, due to the 3 kDa size of aggregated surfactin when run on SDS-PAGE gels (Alajani *et al*., 2016). To hopefully suppress surfactin production, *B. velezensis* B18 cultures were grown at 30°C (Caulier et al, 2019). Additionally, the ammonium sulfate precipitation method is recommended as a method for reducing lipoprotein contamination of protein samples, which would therefore also lead to a lower concentration of surfactin in the CBP. Suggesting that surfactin contamination is not likely to be responsible for the inhibitory activity seen from this strain against *C. difficile.* The polyketide antibiotic difficidin was also a likely candidate for the anti-*C. difficile* activity seen from this *B. velezensis* B18 due to its potent activity against *C. difficile* (Wilson *et al*., 1987). However, proteolytic digestion of the *C. difficile* inhibitory CBP of *B. velezensis* B18 using trypsin, pepsin and proteinase K, revealed this substance was highly susceptible to proteolytic degradation, confirming its proteinaceous nature and likely ruling out the possibility of difficidin being the causative agent.

The susceptibility of the *C. difficile* inhibitory protein from *B. velezensis* B18 to trypsin also implies that amylocyclicin is not responsible for the displayed activity. Amylocyclicin's circular shape means it resists attack by endopeptidases including

trypsin, so much so that trypsin digest before MS identification was fraught with optimisations in the original publication (Scholz *et al.,* 2014). Similarly, amylocyclicin and the other Class Ib bacteriocins are notoriously stable under heat stress (Scholz *et al.,* 2014; Perez, Zendo and Somomoto, 2018). However, the CBP of *B. velezensis* B18 lost 60% of its activity following a 30 min incubation 100°C, suggesting that the *C. difficile* inhibitory peptide is not heat-stable. Additionally, elucidation of the pI of the *C. difficile*-inhibitory peptide from *B. velezensis* B18 suggested again that amylocyclicin was unlikely to be responsible for the displayed activity. At a pH of 7 two peptides with pIs of ~5.6 and 4.5 were shown to be *C. difficile* inhibitory, whereas amylocyclicin is known to have a predicted pI of between 9.02 and 10.41 at ph 7 (Scholz *et al*., 2014 Perez, Zendo and Somomoto, 2018).

The estimated pI values obtained for the two *C. difficile* inhibitory proteins produced by *B. velezensis* B18 did, however, suggest that amyloliquecidin could be the most likely encoded candidate. Both subunits of amyloliquecidin have predicted pIs of 6.03 and 6.75 for the α and β-subunits respectively (Du Preez van Staden, 2015). Although the pIs obtained for the peptides produced by *B. velezensis* B18 in this study of 5.6 and 4.5 do not match exactly the predicted pIs of the amyloliquecidin subunits this could likely be due to errors used in the programmes to predict the pIs or the experimental conditions utilised in this experiment. Similarly, over 60% of the anti-*C. difficile* activity of the CBP from *B. velezensis* B18 was lost following 30 min exposure to 100°C, which is consistent with the reduction of antimicrobial activity of amyloliquecidin following prolonged exposure to 121°C (Du Preez van Staden, 2015). Similarly, the estimated size of ~3 kDa of the anti-*C. difficile* peptides produced by *B. velezensis* B18 was also suggestive of amyloliquecidin being responsible for the activity seen from this strain as the predicted size of the subunits of amyloliquecidin are 2 and 3 kDa in size, matching the size seen on the SDS-PAGE overlay assay (Du Preez van Staden, 2015).

For *B. subtilis* 50B characterization of physiochemical features of the bacteriocin allowed rational elimination of potential candidate bacteriocins. Following earlier eliminations of the anti-fungal fengycins and bacillibactin (3.3), the remaining candidates for the activity seen from *B. subtilis* 50B were subtilosin A, subtilomycin,

bacilysin, bacillaene, and surfactins (3.3). The prediction of the 4.6 kDa size of the anti-*C. diffiicile* substance from *B. subtilis* 50B using SDS-PAGE suggested that as before it was unlikely bacilysin and bacillaene were responsible for the activity seen from *B. subtilis* 50B, due to the 24.5 kDa size of bacilysin and 580 Da size of bacillaene (Butcher *et al*., 2007; Kenig, Vandamme and Abraham, 1976). Additionally, surfactins were unlikely to be responsible for growth due to the 30°C culture temperature and the ammonium sulfate precipitation selection (Caulier *et al.,* 2019; Ion exchange Handbook, GE Healthcare). Therefore, the bacteriocins subtilosin A (3.3 kDa) and subtilomycin (3.2 kDa) were the most likely candidates for anti-*C. difficile* activity, although size estimation did little to differentiate the two (Phelan *et al*., 2013; Zheng *et al*., 1999). The proteinaceous nature of the anti-*C. difficile* substance produced by *B. subtilis* 50B was confirmed due to its susceptibility to proteolytic enzyme degradation, furthering suggestion that a bacteriocin was the likely candidate.

Interestingly, the inhibitory protein from *B. subtilis* 50B completely lost biological activity following treatment with proteinase K and α-chymotrypsin, subtilomycin, however, has been reported as being resistant to degradation by both proteinase K and α-chymotrypsin (Phelan *et al*., 2013). Whilst this may be due to differences in experimental set up, it cast doubt on the role subtilomycin had to play in the anti-*C. difficile* activity. Conversely, the mature peptide of subtilosin A, which was also a candidate for the activity seen, has many hydrophobic residues, including key phenylalanine residues essential for activity and so is likely highly susceptible to α-chymotrypsin and proteinase K treatment (Zheng *et al*., 1999). Additionally, the bacteriocin from *B. subtilis* 50B proved to highly heat stable, which is another feature of the sactibiotics (Mathur and Rea, 2019).

pI identification of the *C. difficile*-inhibitory peptide from *B. subtilis* 50B, surprisingly suggested that three peptides were contributing to the activity seen against *C. difficile* 630. A *C. difficile* inhibitory peptide with an estimated pI of 4.8 further suggested that subtilosin A produced by *B. subtilis* 50B was responsible, at least in part, for the activity seen, as subtilosin A has a predicted pI 4.78 (Zheng *et al*., 1999). Subtilosin A has never been shown in the literature to have activity against *C. difficile* and it has before been suggested that it does not display any anti-*C. difficile* activity

(Rea *et al*., 2010). However, many sactibiotics exhibit anti-clostridial and related species activity and this result tentatively implies that subtilosin A is also one of a growing number of sactibiotics displaying anti-clostridial activity (Balty *et al*., 2019, Mathur and Rea, 2019). The apparent two other *C. difficile* inhibitory substances identified had predicted pIs of 4.5 and 4.2, neither of which were suggestive of subtilomycin being responsible for activity as this bacteriocin has a predicted pI of 9.07 (Phelan *et al*., 2013). Although, as before there could be a difference between the predicted pI of subtilomycin and the experimentally achieved value, it is not unreasonable to assume that these other peptides are previously unidentified peptide antimicrobials that have little homology to known bacteriocins and therefore have been overlooked in genome mining.

Regardless of definitive identification, the CBP prepared from both *Bacillus* spp. showed remarkable inhibitory activity against a range of *C. difficile* ribotypes. *B. velezensis* B18 was inhibitory to all the ribotypes of *C. difficile* tested for, although inhibition was weaker in 027 and 126 ribotypes. This is perhaps one drawback of the inhibitory substance produced by *B. velezensis* B18 as ribotype 027 is clinically relevant and has been responsible for historical nosocomial outbreaks with higher-than-average mortality (Valiente, Cairns and Wren, 2014). Ribotype 126 is also an increasingly clinically relevant ribotype in europe and beyond (Couturier *et al*., 2018; Hung *et al*., 2016; Azimirad *et al*., 2021). However, as the purified anti-*C. difficile* compound was not obtained in this study these results can be considered the basis for further investigation. For example, if the *C. difficile* inhibitory substance produced by *B. velezensis* B18 was purified to homogeneity more accurate MIC assays could be carried out to truly assess the anti-*C. difficile* capabilities of the peptide produced by *B. velezensis* B18 (Perales-Adan *et al*., 2018).

The CBP of *B. subtilis* 50B also inhibited all of the *C. difficile* ribotypes tested, with the exception of ribotype 003. More so, *B. subtilis* 50B was strongly inhibitory to ribotype 027, which implies clinical relevance. Although it also had weaker activity to ribotype 126. If subtilosin A is the bacteriocin responsible for this activity from *B. subtilis* 50B it highlights great potential as an anti-clostridial agent. Likewise, if unidentified

bacteriocins are responsible it highlights the need for definitive identification, in order to explore their therapeutic potential.

The CBP of both *Bacillus* spp. was also analysed to tentatively assess the antimicrobial spectrum of the bacteriocins of both strains. The CBP of *B. velezensis* B18 exerted strong inhibitory activity against most of the Gram-positive bacteria tested with only weak inhibitory activity seen against *E. coli* S17. Whilst the drawbacks of not purifying the inhibitory compound to homogeneity still apply, this result suggested that the inhibitory activity of the bacteriocin produced by *B. velezensis* B18 is primarily active against Gram-positives. Interestingly, *B. velezensis* B18 strongly inhibited *B. velezensis* FZB42, the original producer of amylocyclicin (Scholz *et al*., 2014). This again suggested that amylocyclicin was not responsible for the activity seen from the CBP as both of these *B. velezensis* strains encode the immunity protein for amylocyclicin with 100% identity to one another. The strong inhibitory activity exerted on *S. aureus* by the CBP of *B. velezensis* B18 further suggests that amyloliquecidin was present and responsible for the *C. difficile* inhibition as amyloliquecidin is currently being investigated as a staphylococcal therapeutic (Van Staden *et al.,* 2016).

The activity of the *B. subtilis* 50B antimicrobial peptides also appeared to be strictly towards Gram-positive bacteria, with no activity seen against Gram-negative bacteria tested. *B subtilis* 50B was highly inhibitory to *B. subtilis* 168, the original producer of subtilosin A, despite both encoding the immunity protein (Zheng *et al*., 2000). Although this inhibitory activity could be due to subtilomycin, which is not encoded by *B. subtilis* 168 and has been shown to be active against other *Bacillus* spp. (Phelan *et al.,* 2013). Additionally, this activity could also be due to the possibly unidentified peptide antimicrobials that *B. subtilis* 50B produces.

In following the bacteriocin purification protocols and elucidating physciochemical characteristics of the potently anti-*C. diffiicle* bacteriocins produced by *B. velezensis* B18 and *B. subtilis* 50B, The results of this study tentatively suggest novel anti-clostridial activities of the known bacteriocins amyloliquecidin and subtilosin A. In addition to implying further activity due to unidentified anitmicrobial peptides produced by *B. subtilis* 50B.

Chapter 6 General Discussion

6.1 Future perspectives and conclusions

6.1.1 Future perspectives

In the hopes of identifying novel bacteriocins with therapeutic potential against *C. difficile*, the microbiota of healthy pigs was screened. Through the screening process and subsequent species elucidation two members of the *B. subtilis* species complex, *B. velezensis* B18 and *B subtilis* 50B, were identified with strong antagonistic activity against *C. difficile*. Due to the resistance adult pigs show to *C. difficile* colonization (Jurberg *et al*., 2018), it was thought there would be diversity in the bacterial species isolated that were inhibitory to *C. difficile*, however, this was not the case in this study. Genome mining to identify antimicrobials produced by these strains revealed that in addition to their displayed anti-*C. difficile* activity, both strains had incredible biocontrol potential beyond that. *B. subtilis* 50B encoded the biosynthetic clusters for seven previously identified antimicrobials with described activity against a range of bacteria (subtilosin A, subtilomycin, bacillaene and surfactin) and also fungi (fengycins and bacillibactin). *B. velezensis* B18 encoded nine previously identified antimicrobials with activity that encompassed Gram-positive and Gram-negative bacteria (amylocyclicin, amyloliquecidin, LCI, bacillaene, surfactin and difficidin) and fungi (fengycins and bacillibactin).

The incredible antimicrobial potential of the *Bacillus* spp. isolated in this study is likely a reflection of the species rich and diverse environments that members of this genus inhabit, driving the evolution of antimicrobial weapons to provide a competitive advantage (Polonca, 2020). The results of this study reaffirm the results of previous studies that highlight the genomic emphasis placed on antimicrobial production displayed in *B. subtilis* species complex members (Caulier *et al*., 2019; Liu *et al.*, 2017). In addition to the biosynthetic clusters identified, there is the exciting potential that these clusters are just a snapshot of the true antimicrobial capabilities of these strains. As mentioned, the bacteriocin and secondary metabolite genome mining tools are based on identity to previously characterised classes of antimicrobials (Van Heel *et al*., 2018; Weber *et al*., 2015). Whilst there has been stagnation in the identification of novel classes of secondary metabolite antibiotics, the bacteriocin class of peptide antimicrobials are continually evolving, with new

classes featuring unusual motifs and novel enzymes catalysing their maturation being characterised regularly (Coates, Hall and Hu, 2011; Montbalon-Lopez *et al*., 2021). Therefore, there was always the potential that as the understanding of RiPPs and bacteriocins develops, previously unrecognised clusters within the *Bacillus* spp. strains will be highlighted as encoding novel bacteriocins.

Whilst genome mining had identified a versatile repertoire of antimicrobials encoded in both strains, the focus of this study was bacteriocins with activity against *C. difficile* specifically, and so attempts were made to identify which, if any, of the encoded antimicrobials were responsible for the *C. difficile* inhibition seen from both strains. Attempts to purify the bacteriocins from both strains in a sufficient concentration for identification were ultimately unsuccessful due to an unexpected end to experimentation. The main issue in identification appeared to be concentration-dependant problems. For *B. subtilis* 50B the 4.6 kDa active peptide was not successfully identified by MS analysis due to the overabundance of medium-derived peptides. A switch to a minimal media was intended to overcome this issue, however, for *B. velezensis* B18 the bacteriocin concentration following chromatographic separation was too low to identify the 3 kDa *C. difficile* inhibitory peptide. The M9 minimal medium for *Bacillus* spp. had been chosen as the routine culturing medium for bacteriocin production of the two *Bacillus* spp. based on being devoid of peptide additives and the acceptable semi-quantified bacteriocin concentration obtained from it (5.2). However, unlike reported cases of minimal media improving production this was not the case in this study and it appeared that the reduction in activity may have hindered identification of the *B. velezensis* B18 bacteriocin (Pingitore *et al*., 2009).

An in-depth analysis of the chemical constituents of M9 medium highlighted that two of the primary components have been associated with reduced antimicrobial activity in *Bacillus* spp. Using glucose as a carbon source, as is the case in M9 medium, has before been shown to reduce antibiotic production in *Bacillus* spp. and Tabbene *et a*l., (2009) highlighted that lactose as a carbon source can lead to a four-fold increase in antimicrobial titres in *B. subtilis* B38 (Haavik, 1974). The use of lactose as the carbon source has also been shown to improve bacteriocin production, with nisin

production improved in *L. Lactis* when using lactose as a carbon source, although for LAB this could reflect their naturally preferred carbon source (Cheigh *et al.*, 2002). In M9 medium ammonium chloride is used as the nitrogen source, however, Tabbene *et al*, (2009) highlighted that ammonium chloride led to lower titres of antimicrobial activity and they proposed that ammonium succinate be used as a nitrogen source to stimulate antimicrobial production in *Bacillus* spp. Finally, $CaCl_2$, which is one of many inorganic salt additives in M9 medium was also shown to lead to a decrease in antimicrobial titre and Tabbene *et al.* (2009) suggested that manganese ions enhance bacteriocin production compared to other inorganic salts tested.

Therefore, it is proposed that in any subsequent culturing of the *C. difficile*-inhibitory *Bacillus* spp., modifications be made to the M9 medium in the hopes of increasing concentration of the *C. difficile* inhibitory bacteriocins. Including the use of lactose instead of glucose as a carbon source, the use of ammonium succinate as the nitrogen source and the omission of $CaCl_2$ as an inorganic salt additive. The initial culture volume of only 800 mL could still be an issue if the modification of M9 medium is successful. Issues arose when trying to upscale the culture volume from 800 mL, which was split 200 mL of culture per 500 mL flask, to 1.6 L (split into 400 mL of culture per 1 L flask). Therefore, to combat this, the labour-intensive original culture method should be applied in which the 1.6 L culture would be divided into eight 500 mL flasks for initial culturing. Whilst this approach is not suitable for large scale industrial production and future applications of the bacteriocins from the *Bacillus* spp., it is likely the route to take to ensure initial identification of the *C. difficile* inhibitory bacteriocins.

As discussed, two *C. difficile* inhibitory peptides were being produced by *B. velezensis* B18 and based on the evidence, it was likely to be due to the two subunits amyloliquecidin. During chromatographic separation of the CBP from *B. velezensis* B18, there was a suggestion that two peptides with inhibitory activity against *C. difficile* were eluting from the column, one with a weak ionic interaction to the ligand that was eluted early in the NaCl gradient, and one with a strong ionic interaction to the ligand that was eluted late in the NaCl gradient. As this was considered a proof-of-concept experiment prior to the end of research due to SARS CoV-2, only one of

these was analysed in detail. The 3 kDa size of the earliest eluted protein with *C. difficile* inhibition was suggestive of the alpha subunit of amyloliquecidin (Du Preez Van Staden, 2015). It would therefore be interesting to analyse the peptide eluted later in the separation and identify if it shared characteristics with the beta subunit of amyloliquecidin or if it was an aggregate of the first peptide (Ionic Exchange handbook). Although ideally, the concentration of the peptides would be higher if the above modifications were implemented, and so definitive identification would be possible.

The suggestion based on physiochemical characteristics that amyloliquecidin was responsible, at least in part, for the activity seen from *B. velezensis* B18 was surprising, as amylocyclicin had been thought to be most likely to be active against *C. difficile* (3.3). So much so, that the original experimental design to attempt to produce recombinant anti-*C. difficile* bacteriocins had been focussed on amylocyclicin solely. With this new understanding and the difficulties faced in utilising the *B. subtilis* WB800N expression system, a new experimental rationale should be proposed for further work. The two component bacteriocin lichenicidin was expressed recombinantly in *E. coli* BL21 using multigene assembly (Kuthning *et al*., 2015). Wherein, the alpha structural peptide gene (*licA1*) and that proteins maturation enzyme gene *(licM1*) were cloned separately into pET24 a(+) vectors and subsequently cloned from these vectors with their T7 promoters into a pRSF-Duet-1 vector containing the lantibiotic transport associated protein gene (*licT*) in the first MCS. An additional pRSF-Duet-1 plasmid was constructed in the same way for the beta subunit containing *licA2*, *licM2* and the lantibiotic associated serine protease gene *licP* (in addition to *LicT*). These genes were then expressed in *E. coli* and subsequently purified yielding, MALDI-MS confirmed mature lichenicidin (Kuthning *et al*., 2015). A similar method has since been utilised to produce recombinant mature mersacidin from *E. coli*, with the exception that the structural gene (*mrsA)* and the LanM enzyme gene (*mrsM*) were co-expressed on a duet vector and an additional plasmid encoding a LanD enzyme gene was expressed concurrently with the duet vector (Viel *et al*., 2021). The similarity in biosynthetic machinery of amyloliquecidin to lichenicidin suggests that the approach utilised by Kuthning *et al.*

(2015) could successfully be employed to produce recombinant amyloliquecidin in *E. coli* and is worth pursuing.

Additionally, an ORF was identified in the amyloliquecidin biosynthesis operon that was thought to encode a non-functional alternate beta subunit. As previously mentioned, two point-mutations introduced in the C-terminus of this non-functional lantibiotic subunit could theoretically confer activity by introducing key serine and cysteine residues (3.3). It is then proposed that any experimentation involving the production of recombinant amyloliquecidin carried out in the future will include the introduction of these two point-mutations to the alternative beta subunit using QuikChangeTM site-directed mutagenesis and subsequent coexpression with *amyM2* and *amyP* required for maturation of the beta subunit (Kuthning *et al*., 2015; Wang and Malcolm, 1998). Whilst AmyM2 has been shown to be the dedicated post translation modification enzyme of the already characterised beta subunit, there is the possibility that this approach would work as LanM enzymes have been shown to have a degree of promiscuity in regards to similar substrates (Montbalon-Lopez*,* Van Heel and Kuipers*,* 2017).

As previously discussed, three peptides appeared to be being produced by *B. subtilis* 50B with antagonistic activity against *C. difficile*. As was the case for *B. velezensis* B18, premature end of experimentation meant that a definitive identification of these peptides was not possible. However, elucidation of their physiochemical characteristics suggested that the bacteriocin subtilosin A and two other unknown peptides that did not fit the characteristics of the candidates encoded by *B. subtilis* 50B were responsible for the anti-*C. difficile* activity. It is therefore imperative that the above modifications to improve bacteriocin yield be implemented for *B. subtilis* 50B to allow future definitive identification of these peptides and thus exploitation of their anti-*C. difficile* potential. As for the anti-*C. difficile* potential of subtilosin A, it had been suspected from the initial genome mining that subtilosin A was in part responsible for the activity seen from *B. subtilis* 50B and the implication from the characterisations also suggested so. The earlier attempts to knock out essential elements of the sbo-alb operon from *B subtilis* 50B, following the method of Phelan *et al.* (2013), had proven unsuccessful due to an apparent experimental issue in *B.*

subtilis 50B exogenous DNA uptake. Himes *et al*. (2016) produced MS confirmed recombinant subtilosin A in *E. coli* by utilising a pETDuet-1 vector to coexpress presubtilosin and its dedicated radical SAM-enzyme gene *albA*. However, in a proof-of-concept experiment also outlined in their paper, presubtilosin was cloned into a pMCSG7 vector and subsequently nickel column purified. In tandem, *albA* was cloned into a pET28 a (+) vector, purified and reconstituted (Himes *et al.*, 2016; Fluhe *et al.*, 2013). Upon incubation of purified AlbA and presubtilosin, mature subtilosin A was produced (Himes *et al*., 2016). It is therefore suggested that subtilosin A from *B. subtilis* 50B be produced recombinantly in *E. coli* in this manner in any future experiments in order to fully explore the anti-*C. difficile* potential of subtilosin A.

In regards to the suggestion that future experiments to produce recombinant anti-*C. difficile* bacteriocin be carried out in *E. coli*, this is due to the strong anti-*C. difficile* activity exhibited by the expression strain *B. subtilis* WB800N. Genome mining using BAGEL4 highlighted this strain encoded seven antimicrobials, some of which are potentially active against *C. difficile* and therefore could obscure recombinant bacteriocin production in *B. subtilis* WB800N. It is therefore suggested that a dedicated *B. subtilis* bacteriocin production workhorse be developed. *B. subtilis* WB800N is itself a derivative of *B. subtilis* 168, with deletions in genes for extracellular proteases, a reflection of its lab-based amenability (Jeong *et al.*, 2018). The first step in producing a *B. subtilis* bacteriocin-production workhorse should aim to achieve a non-bacteriocinogenic derivative of *B. subtilis* WB800N. It is therefore recommended that a Δ*sbo* variant of *B. subtilis* WB800N, perhaps utilising the method outlined by Phelan *et al*. (2013), be produced to stop subtilosin A production. *B. subtilis* WB800N also encodes the bacteriocin sublancin 168 and it recommended this potentially *C. difficile* active bacteriocin be deleted. Dubois *et al.,* (2009) utilised double homologous recombination to delete the *sunI* peptide gene, required for immunity to sublancin 168, and perhaps this method could be adapted to delete the *sunA* structural peptide gene to produce a *B. subtilis* WB800N variant devoid of both subtilosin A and sublancin 168 production. Obviously following generation of a *B. subtilis* WB800N Δ*sbo*Δ*sunA* mutant, transformation efficiency and protein production of this strain would have to be reassessed as well as retesting the anti-*C.*

difficile capabilities (Phan *et al*., 2005). Throughout this study, surfactins have always been thought likely to cause some *C. difficile* inhibition, however, generation of *a B. subtilis* WB800N mutant lacking the ability to produce surfactin is unlikely to be successful in generating a dedicated bacteriocin-producing workhorse, as the gene encoding the essential ComS regulator is located in the surfactin biosynthetic operon and disruption of this operon could lead to low transformation efficiency, which would not be ideal for a protein expression strain (Mesa-Pereira *et al*., 2018).

6.1.2 Conclusions

Despite the resistance adult pigs show to colonisation by *C. difficile*, there was little diversity in the species isolated from this environment that were inhibitory to *C. difficile* - with only *B. subtilis* and *B. velezensis* isolates identified. However, upon genome mining *B. subtilis* 50B and *B. velezensis* B18, the representative isolates this study focussed on, showed remarkable biocontrol potential (beyond just *C. difficile*), encoding seven and nine antimicrobial clusters respectively.

Attempts to produce recombinant bacteriocins based on the candidates identified in the genomes of the *Bacillus* spp., highlighted key difficulties in the use of bacteriocinogenic expression strains - primarily the obstruction of candidate bacteriocin activity. Whist also highlighting an opportunity to create a dedicated *B. subtilis* bacteriocin production workhorse.

Definitive identification of the anti-*C. difficile* bacteriocins produced by the *Bacillus* spp. isolates was not possible due to unforeseen circumstances. However, the suggestion of novel anti-*C. difficile* activities from the sactibiotic subtilosin A and the two component lantibiotic amyloliquecidin means this study can be considered the groundwork into exploring the novel anti-*C. difficile* potential of these bacteriocins. Additionally, the presence of a redundant alternate subunit of amyloliquecidin gives the opportunity for rational design of a semi synthetic lantibiotic subunit.

Additionally, the suggestion that *B. subtilis* 50B produced an additional two peptides inhibitory to *C. difficile* with physiochemical features not in line with the other identified antimicrobials implied that the *C. difficile* biocontrol potential of this strain is far greater than the genome mining suggested.

This study has shown the incredible biocontrol potential of the environments around us and lays the groundwork for potentially novel anti-*C. difficile* applications of the bacteriocins identified.

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Appendix 1

As part of my BBSRC funded doctoral program, I had the opportunity to undertake a personal industrial placement (PIP) to further personal development. Before beginning my PhD I had worked in the private sector for one year and so I wanted to use the opportunity to gain experience in a new area and so I chose to carry out my PIP in a third sector charitable organisation: The Grantham Canal Society (GCS).

The Grantham Canal Society is a charity based locally in the Nottinghamshire area. It is dedicated to restoring the Grantham Canal, which connects Nottingham to Grantham, to full working order. There are numerous benefits to restoring waterways in Britain, but the primary reason for my interest was environmental, as free flowing, maintained canal environments are beneficial to the UK's natural ecosystem.

As part of my experience in working with the grantham canal society I had the opportunity to conduct my own analysis of water quality to assess the diversity of the enviroment, utilising equipment provided by The Canal Trust. Additionally, this provided the opportunity to collect aquatic samples for the purposes of a public-orientated open day. This experience also allowed me to further my development in scientific communication to the general public. In a time where public trust in science and research is dwindling and misinformation is rife, I believe the skill to thoughtfully explain science to the general public is key.

During my time at the Grantham Canal Society I also gained key skills in people management. As well as insight into the benefits and disadvantages of working with a volunteer labour force. A primary benefit being an enthusiastic workforce; but a main drawback being a lack of people management and organisation that inevitably led to issues with the day to day running of the organisation.

Also during my time at the GCS, I became more aware of the issues that third sector charitable organisations face, primarily in the case of fund acquisition. As many causes, which are each as deserving as one another, have to compete for scarce

grants when they present themselves. Additionally, a reliance on public donations for smaller charities is a hindrance.

I am thankful for the BBSRC for allowing me the opportunity to undertake a PIP, and this experience allowed me to gain and develop key skills, benficial to career advancements.