The generation of a chromatogram-simplified *Streptomyces albus* S4 heterologous host and the activation and characterisation of surugamide biosynthesis in *S. albus* S4

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The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work of others.

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Abstract

The rise in antimicrobial resistance combined with the decline in antibiotic discovery has put antibiotic therapy at risk. As a result, there is an urgent need for new antibiotics. Microbial secondary metabolites are a major source of industrially and medically significant compounds. Most of the antibiotics used in the clinic are derived from secondary metabolites produced by streptomycetes. Even though streptomycetes were thought to be mined to exhaustion, advances in genome sequencing and genome mining has revealed the presence of the untapped wealth of secondary metabolite encoding biosynthetic gene clusters harboured in their genomes. Developing strategies to awaken these silent gene clusters along with understanding the biosynthesis of these natural products is therefore of great importance.

In this work, five gene clusters were targeted in *S. albus* S4 to generate a new heterologous host, which is hoped to aid in the research efforts for new antimicrobials. Activation of silent gene clusters hold great potential in the discovery of new and useful compounds. The genome of *S. albus* S4 harbours many silent gene clusters. In this study, pleiotropic approaches were used to switch on the production of an antibacterial compound(s) in *S. albus* S4. Bioinformatic analyses of the spectral data obtained from the chemical extracts identified surugamides within the chemical extract. As surugamide A was previously reported to have antibacterial activity, its bioactivity profile was further investigated. Surugamides belong to the family of non-ribosomal peptides. Non-ribosomal peptides constitute a major class of natural products with diverse activities ranging from antibacterials to immunosuppressants. Atypical to canonical non-ribosomal peptide synthetase systems, the surugamide gene cluster lacks a *cis*-acting release mechanism responsible for the release of the cyclic and linear peptides it encodes the production for. In this work, a *trans*-acting standalone release factor was identified and its *in vitro* activity was also investigated.

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Abbreviations

x *g*	Times gravity
μg	microgram
μL	microlitre
A domain	Adenylation domain
Act	Actinorhodin
ACV	L-δ-(α-aminoadipoyl)-L-cysteinyl-D-valine
AMPA	3-amino-2-methylpropionic acid
antiSMASH	Antibiotics & Secondary Metabolite Analysis Shell
Apr	Apramycin
ARC	Antibiotic remodelling compound
ATP	Adenosine triphosphate
BAC	Benzalkonium chloride
BGC	Biosynthetic gene cluster
bp	Base pair
C domain	Condensation domain
CDA	Calcium dependent antibiotic
CPK	Coelimycin
C_T domain	Condensation-like domain
CTAB	Cetyltrimethylammonium bromide
Da	Dalton
DMSO	Dimethyl sulfoxide
E domain	Epimerisation domain
EDTA	Ethylenediaminetetraacetic acid
EIC	Extracted ion chromatogram
EPL	Expected product length
ESKAPE	*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter.*
FAS	Fatty acid synthase
FIC	Fractional inhibitory concentration
g	gram
GlcNAc	*N*-acetyl glucosamine
GNPS	Global Natural Products Social Molecular Networking
GTP	Guanosine-5'-triphosphate
h	hour

HDAC	Histone deacetylase
HMW	High molecular weight
HPLC	High performance liquid chromatography
HDR	Homology directed repair
Hyg	Hygromycin
Kan	Kanamycin
kb	Kilobase pairs
LA	Lennox agar
LAL	Large-ATP binding regulators of LuxR family
LB	Lennox broth
LC-HRMS	Liquid chromatography high resolution mass spectrometry
LMW	Low molecular weight
M	Molar
MDR	Multi-drug resistance
MeOH	Methanol
MHB	Mueller Hinton Broth
MM	Minimal medium
MS	Mannitol-soya flour
MSMS	Tandem mass spectrometry
MIC	Minimum inhibitory concentration
min	Minute
mL	Millilitre
MRSA	Methicillin resistant *Staphylococcus aureus*
NaCl	Sodium chloride
NRP	Non-ribosomal peptide
NRPS	Non-ribosomal peptide synthetase
OSMAC	One strain many compounds
PBP	Penicillin binding protein
PCD	Programmed cell death
PCP	Peptidyl-carrier protein
PCR	Polymerase chain reaction
PG	Peptidoglycan
PK	Polyketide
PKS	Polyketide synthase
ppGpp	Guanosine tetraphosphate
Ppk	Polyphosphate kinase
PPT	Phosphopantetheine
PPTase	Phosphopantetheinyl transferase

R domain	Reductase domain
Red	Undecylprodigiosin
rpm	Revolutions per minute
SA	Surugamide A
SB	Surugamide B
SDR	Short chain dehydrogenase/reductase superfamily
SDS	Sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SF	Surugamide F
SNAC	*N*-acetyl cysteamine
T domain	Thiolation domain
TE	Thioesterase
TSB	Tryptic soy broth
WT	Wild type

Chapter 1 Introduction

1.1 A brief history of the discovery of antibiotics

The discovery and use of antibiotics is rightfully considered to be one of the greatest medical accomplishments of all time. The clinical availability of these therapeutics led to dramatic reductions in morbidity and mortality rates, enabling giant leaps in modern medicine. Without the treatments that we have in place through antibiotics, many of the medical achievements such as surgery and organ transplantations that we take for granted today, would not be possible (Rubin, 2007; Lewis, 2013). Though the term antibiotic is associated with the modern era, there is historical evidence of ancient civilisations using naturally available sources such as plants and moulds for the treatment of infections (Awad et al., 2012). Surprisingly, traces of the modern antibiotic tetracycline was even detected in the skeletal remains of an ancient Sudanese tribe that date as far back as 350 A.D, with the rate of infectious diseases recorded in this population as low (Bassett et al., 1980; Nelson and Levy, 2011). Detection of tetracycline in this group was not an isolated event, with skeletal samples from the Roman period in the Dakhleh Oasis in Egypt also revealing traces of tetracycline (Cook et al., 1989). Therefore, contrary to the thought that the exposure to and use of antibiotics are confined to the modern 'antibiotic era', there are historic examples of the use of antimicrobials in ancient civilisations (Bassett et al., 1980; Cook et al., 1989; Aminov, 2010; Awad et al., 2012; Gould, 2016).

The beginning of the modern 'antibiotic era' is associated with the names of Paul Ehrlich (1854 – 1915) and Alexander Fleming (1881 – 1955) (Rubin, 2007; Valent et al., 2016; Gould, 2016). Ehrlich's work on investigating pathogen specific antibacterial properties of dyes led to the discovery of arsenic-based Salvarsan, which despite its side effects, was the first modern chemotherapeutic agent to be employed and was used in the treatment of syphilis- an incurable disease at the time (Bosch and Rosich, 2008; Valent et al., 2016). Following on from Ehrlich's work on the effect of azo dyes on microbes, Josef Klarer and Fritz Mietzsch synthesised sulfonamidochrysoidine to demonstrate its antibacterial potential (Rubin, 2007). It was then through the efforts of Gerhard Domagk who demonstrated the *in vivo* efficacy of this sulfa drug in a murine model of *Streptococcus*

pyogenes systemic infection that it was marketed as Prontosil in 1935 to treat streptococcal and staphylococcal infections (Rubin, 2007). Further work carried out on Prontosil revealed its sole active component to be sulfanilamide, a known moiety at the time but not as an antibacterial, bringing sulfonamide drugs to the frontline (Rubin, 2007).

The serendipitous discovery of penicillin by Fleming from the filamentous fungus, *Penicillium notatum* in 1928, and the demonstration of its *in vivo* antibacterial efficacy by Florey and Chain and later, the industrialisation of penicillin production in the 1940s, marked the beginning of the Golden Age of antibiotic discovery (Fleming, 1929; Chain et al., 1940; Fernandes, 2006; Rubin, 2007). The discovery platform introduced by Selman Waksman, a soil microbiologist, in the 1940s led to the discovery of majority of the antibiotic classes in use today and contributed to this rich period of discovery (Lewis, 2013). Waksman's discovery platform involved screening soil derived actinomycete bacteria for antimicrobial activity against an indicator organism by looking for the presence of zones of inhibition in growth on overlay plates (Schatz et al., 1944). The term 'antibiotic' was coined in 1941 by Waksman and was used to describe any microbial product that antagonised the growth of another microbe. Today, any natural, synthetic or semi-synthetic compounds that can target and oppose microbial growth are referred to as antibiotics (Rubin, 2007; Clardy et al., 2009; Aminov, 2010; Davies and Davies, 2010). Waksman's discovery of the aminoglycoside streptomycin in 1944 from the soil dwelling actinomycete *Streptomyces griseus* and the efficacy of streptomycin in the treatment of tuberculosis spurred on research efforts worldwide for the discovery of new antibiotics (Schatz et al., 1944; Rubin, 2007). Waksman's bioactivity-guided screening platform brought the phylum Actinobacteria into the limelight as prolific antibiotic producers and as a result, they were extensively mined for new antibiotics (Zaffiri et al., 2012). The discovery of many antibacterial classes in use today such as the β-lactams, tertracyclines, aminoglycosides and glycopeptides ensued during this rich period of discovery (Davies and Davies, 2010; Nelson and Levy, 2011; Lewis, 2013).

With the discovery and implementation of antibiotics, significant progress was made in managing infectious diseases (Clardy et al., 2009). Most antibiotics in use today are derived from natural products or secondary metabolites produced by bacteria and fungi (Davies and Davies, 2010;

Lewis, 2013). These small molecules are termed secondary metabolites as they are considered to be non-essential for the survival of the producing organism but often confers upon the organism many survival advantages such as by increasing the nutrient availability (e.g. siderophores) or providing the organism with competitive or defensive advantages (e. g. via antibiotics) (Davies and Davies, 2010). The evolution of these secondary metabolites with high specificity for single targets makes them highly medically desirable. These natural products have been the primary source of majority of the drugs in the clinic today (Clardy et al., 2006; Butler and Buss, 2006; Newman and Cragg, 2012).

1.2 Actinobacteria

Actinobacteria represent one of the largest taxonomic units among the major lineages currently recognised within the domain Bacteria. They are Gram-positive, high G+C, filamentous bacteria (Ventura et al., 2007; Barka et al., 2016). *Actinobacteria* are mostly comprised of environmental bacteria and are found in a wide range of habitats: soils, marine, rhizosphere and highly arid environments (Barka et al., 2016). Bacteria belonging to this phylum are a major component of the microbial population within the soil. (Hopwood, 2007; Ventura et al., 2007; Miao and Davies, 2010; Thaker et al., 2013; Liu et al., 2013; Barka et al., 2016). Many secondary metabolites produced by actinomycetes have a broad spectrum of activities that are clinically and economically relevant. Their bioactivities range from antibacterial (streptomycin, chloramphenicol), to antifungal (nystatin) to antiviral (tunicamycin) to anticancer (doxorubicin, bleomycin), to antiparasitic (avermectin) to immunosuppressive (rapamycin) properties (Chaudhary et al., 2013). Genera of *Actinobacteria* such as *Streptomyces*, *Salinispora, Amycolatopsis* and *Saccharopolyspora* are talented producers of natural products and account for over two-thirds of all the clinically relevant antibiotics and anticancer drugs in use today (Ventura et al., 2007; Miao and Davies, 2010; Davies and Davies, 2010; Barka et al., 2016). Within the *Actinobacteria* phylum, streptomycetes account for over 70% of all the antibiotics produced (Bibb, 1996; Awad et al., 2012). Only the *Streptomyces* genus will be further explored in this chapter.

1.3 Streptomyces

Streptomycetes are sporulating saprophytes, which are ubiquitous in soils worldwide and are competitive inhabitants of aquatic and marine ecosystems (Challis and Hopwood, 2003). They are non-motile, and as a consequence, are exposed to many stressful conditions (Challis and Hopwood, 2003). The production of complex secondary metabolites that possess antibacterial, antifungal, anti-parasitic, antiviral or other bioactive properties for self-preservation are believed to contribute to their remarkable success in the soil (Bibb, 1996; Challis and Hopwood, 2003; Awad et al., 2012). These developmentally complex bacteria generally produce the antibiotics in a growth phase dependent manner when they switch from their vegetative growth phase to aerial growth phase in response to detection of signals such as nutrient depletion or other environmental stress signals (Figure 1) (Bibb, 1996; Challis and Hopwood, 2003; Chater, 2006; Hopwood, 2007; de Lima Procópio et al., 2012).

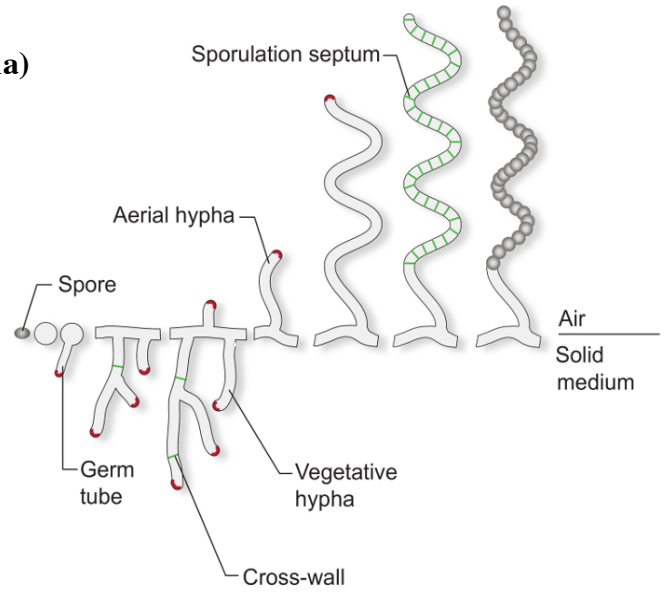
Under favourable conditions and nutrient availability, a spore germinates to give rise to germ tubes (Figure 1a). The germ tubes grow by tip-extension and branching hypha via mycelial growth to form the vegetative mycelia (Flärdh and Buttner, 2009). The branching thread-like hyphae that form the vegetative mycelium in the soil coupled with the secretion of hydrolytic enzymes such as cellulases and xylanases aid them in scavenging for and accessing the nutrients in soil (Flärdh and Buttner, 2009). In response to signals such as nutrient limitation, the production of secondary metabolites is initiated along with morphological differentiation, whereby the vegetative hyphae are broken down and non-branching aerial hyphae break the surface tension and grows into the air. The aerial hyphae then form aseptate tip compartments, which then septate synchronously to form unigenomic spore chains (Chater, 2006; Chater and Chandra, 2006; Flärdh and Buttner, 2009). They produce complex secondary metabolites such as antibiotics in the oldest vegetative mycelium while exospores are produced from the specialised aerial hyphae to aid dispersal and colonization of new environments (Bibb, 1996; Flärdh and Buttner, 2009; Liu et al., 2013).

A further refined model of development has been reported for the stages prior to the formation of aerial mycelium and sporulation in streptomycetes (Figure 1b) (Yagüe et al., 2013). A transitory compartmentalised mycelium

(MI) initiates development where a fraction of the MI undergoes highly ordered programmed cell death (PCD) (Manteca et al., 2006; Yagüe et al., 2013). The remaining viable cells differentiate into multinucleated second mycelium (MII) where septa are scarce. MII forms the vegetative substrate mycelia and undergoes another round of PCD (Yagüe et al., 2013; Yagüe et al., 2016). The remaining viable MII hyphae continues to grow until the rodlet layer is assembled providing the surface hydrophobicity necessary for its escape into air as aerial mycelium (Figure 1b) (Yagüe et al., 2013). Secondary metabolism typically occurs in the MII phase (Yagüe et al., 2013; Rioseras et al., 2014).

In nature, streptomycetes produce the antibiotics in small amounts during their transition stage into the aerial phase at the expense of the nutrients available from the breakdown of the vegetative hyphae (Bibb, 1996; Flärdh and Buttner, 2009). It is likely that the antibiotics produced help defend their food source from other soil microbes (Chater, 2006; Flärdh and Buttner, 2009). Both morphological differentiation and secondary metabolism are also governed by the availability of nutrients such as carbon, phosphorous, nitrogen and trace elements and thus, many of the genes that are involved in both processes are often interlinked (McCormick and Flärdh, 2012; Yagüe et al., 2013). Therefore, morphological differentiation and the production of antibiotics in streptomycetes are intrinsically linked (Chater, 2006; Flärdh and Buttner, 2009; Liu et al., 2013). For example, sigma factors and regulatory components encoded by the *bld* and *whi* genes are known to be involved in both the regulation of morphological differentiation and secondary metabolism (Bibb, 1996; Chater, 2006; Flärdh and Buttner, 2009; Rodríguez et al., 2013).

1a)



1b)

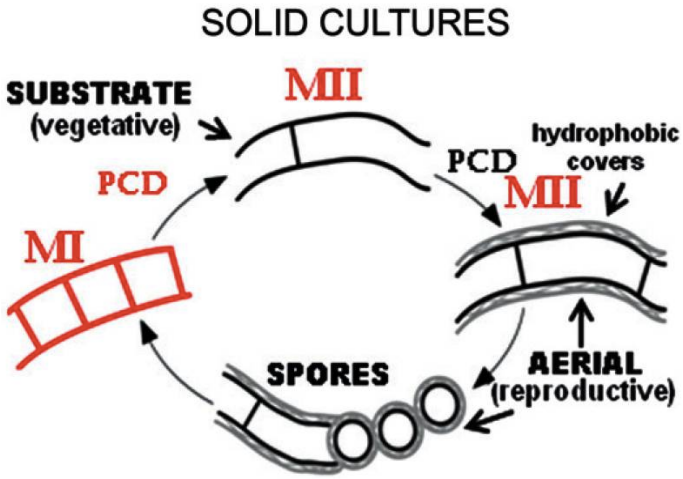


Figure 1 The developmental life cycle of streptomycetes. 1a) Under favourable conditions, a spore germinates to give rise to germ tubes. The germ tubes grow by tip-extension and branching hypha via mycelial growth to form the vegetative mycelia. Morphological differentiation and the production of secondary metabolites are initiated upon detection of some signals including the A-factor, SapB and SapT. The vegetative hyphae are then broken down and multi-genomic aerial hyphae are produced along with the production of secondary metabolites. The aerial hyphae then grow by tip extension and septate synchronously to give rise to uninucleiodal spores. The spores are then dispersed again and the process repeats (Bibb, 1996; Flärdh and Buttner, 2009). 1b) A refined model for the development stages prior to the formation of aerial mycelia on solid medium. MI, first compartmentalised mycelium; PCD, programmed cell death and MII, second multinucleated mycelium (Figure 1a- (Schlimpert et al., 2016); Figure 1b-(Yagüe et al., 2013)).

This relationship between morphological differentiation and secondary metabolite production is well studied and understood in *Streptomyces griseus.* The autoregulatory factor or A-factor, a hormone like γ-butyrolactone signalling molecule, produced in *S. griseus* in a growth-phase dependent manner, induces streptomycin production and sporulation (Horinouchi and Beppu, 1993; Ohnishi et al., 2005). The γ-butyrolactones are widely produced in streptomycetes, but their roles are species-specific (Takano, 2006; Flärdh and Buttner, 2009). For example, in the model organism of the genus, *Streptomyces coelicolor*, γ-butyrolactones play a key role in regulation of antibiotic biosynthesis but do not seem to be associated with differentiation (Takano, 2006; Flärdh and Buttner, 2009). *S. griseus* lacking A-factor is unable to produce streptomycin or spores, but with the addition of A-factor at a concentration of 10^{-9} M, is able to regain its wild-type phenotype (Horinouchi and Beppu, 1992; Flärdh and Buttner, 2009). The A-factor in *S. griseus* exerts its effect on differentiation via the master regulator, AdpA- a transcriptional activator of the AraC family. During the vegetative growth phase, ArpA (the A-factor receptor protein) blocks *adpA* transcription by acting as a repressor leading to the accumulation of A-factor (Yamazaki et al., 2004; Ohnishi et al., 2005). This accumulation of A-factor leads to the de-repression of expression of *adpA* and the activation of the AdpA regulon sub-sequentially. AdpA directly targets genes that encode important proteins involved in morphological differentiation (Yamazaki et al., 2004; Ohnishi et al., 2005). These proteins include SSgA- a protein with essential roles in sporulation and septation, σ^{AdsA}- an exocytoplasmic sigma factor that is key in the formation of aerial mycelium and the response regulator AmfR, which initiates the SapB biosynthetic operon expression. The induced AdpA also activates a number of genes involved in the biosynthesis of streptomycin via the activation of *strR* encoding a pathway-specific activator of biosynthetic genes involved in streptomycin production (Yamazaki et al., 2004; Ohnishi et al., 2005; Flärdh and Buttner, 2009). Both differentiation and the production of *S. griseus* characteristic secondary metabolite streptomycin is blocked upon the loss of A-factor (Yamazaki et al., 2004; Flärdh and Buttner, 2009).

Early genetic mapping in the model actinomycete, *S. coelicolor* A3(2), gave evidence that the biosynthetic genes involved in the production of any specific secondary metabolite are clustered together on the chromosome or plasmids (Kirby et al., 1975; Rudd and Hopwood, 1979). The molecular

analyses, which followed revealed these clusters to be large (often tens of kb) and that they usually include several operons (Bentley et al., 2002; Chater, 2006). This knowledge along with the development in technology allowed the study of antibiotic biosynthesis regulation at the molecular level, shedding further light on the interplay between antibiotic production and morphological differentiation (Bentley et al., 2002; Chater, 2006).

Most of the antibiotics in use today are derived from the secondary metabolites produced by this remarkable genus of microorganisms (Barka et al., 2016). These compounds exert their antimicrobial activities in many ways. The common mechanisms of antibacterial action are discussed in the next section.

1.4 Common mechanisms of action of antibacterial compounds

Antibacterial compounds work by either killing the bacteria (bactericidal) or by the arresting their growth (bacteriostatic). They achieve this by targeting and interfering with the bacterial cell wall synthesis, cell membrane, DNA synthesis, transcription, translation or the folate pathway (Figure 2) (Wright, 2010). These mechanisms of action are discussed in this section.

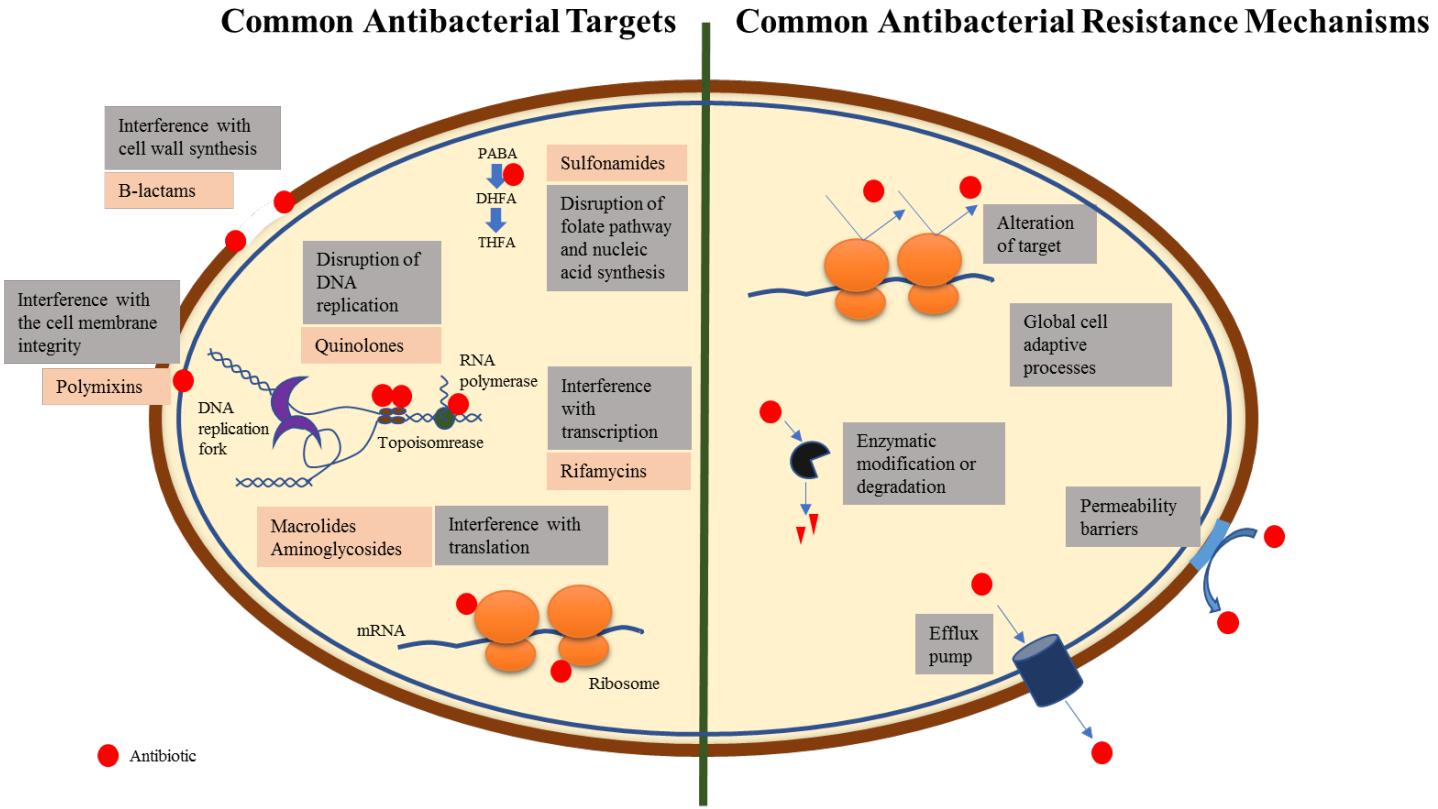


Figure 2. Common antibacterial targets and antibacterial resistance mechanisms.

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1.4.1 Interference with cell wall synthesis

Agents that interfere with the bacterial cell wall synthesis work by inhibiting peptidoglycan (PG) synthesis (Kohansky et al., 2010). Bacterial cells are enclosed by PG layers. The mechanical rigidity conferred by this layer of cell wall is critical for the bacterium's survival in environmental conditions that may result in the alteration of the existing osmotic pressures (Scheffers and Pinho, 2005). The latter steps in the biosynthesis of the cell wall include the synthesis of PG subunits made of *N*-acetylglucosamine linked to *N*-acetylmuramic acid with an attached pentapeptide. The subunit is transferred across the cytoplasmic membrane to the cell exterior or the periplasmic space (Scheffers and Pinho, 2005). The polymerisation of these subunits along with their incorporation into the PG layer is achieved via the action of the penicillin binding proteins (PBPs), which are responsible for the transglycosylation and transpeptidation reactions (Scheffers and Pinho, 2005). Transglycosylases are involved in the formation of glycosidic bonds between the sugars to extend the glycan strands of existing PG monomers while the transpeptidases are involved in the cross-link formation between adjacent peptide chains while cleaving the two terminal D-alanines in the process to form the mature lattice-like PG layer (Scheffers and Pinho, 2005).

The cell wall targeting antibacterial agents work by inhibiting the transport of peptidoglycan monomers in the cytosol across the cytoplasmic membrane, blocking the formation of peptide cross links by inhibiting the transpeptidase or by blocking both the transpeptidase and transglycosylases enzymes (Kohansky et al., 2010; Bakheet and Doig, 2015). Two antibacterial classes that work by interfering with the cell wall synthesis are β-lactams and glycopeptides (Kohansky et al., 2010). β-lactams function by blocking the transpeptidation activity of the PBPs. The β-lactam antibiotics share a common core made up of a 4-membered cyclic amide ring, which shares structural similarity with the PBP substrate D-Ala-D-Ala backbone. As a result, the β-lactams are able to bind to the transpeptidase active site and block its ability to form the peptide cross-links (Kohansky et al., 2010; Zeng and Lin, 2013). Glycopeptides, on the other hand, work by binding to the D-Ala-D-Ala at the carboxy terminus of the growing PG thereby blocking transglycosylation and transpeptidation through steric hinderance (Kang and Park, 2015).

1.4.2 Interference with the cell membrane integrity

Compounds that target the cytoplasmic membrane cause disruption of the bacterial cell membrane and as a result, alter the permeability of the cell components (Hancock and Chapple, 1999). The bacterial cell membrane plays a key role in the survival of the bacteria as it provides selective permeability to maintain cellular homeostasis (Epand et al., 2016). Gram-negative bacteria possess an additional outer membrane rich in lipopolysaccharides that are unique to them, imposing an additional layer of protection (Epand et al., 2016). The inner monolayer of the outer membrane of these bacteria as well as both layers of the cytoplasmic membrane in both Gram-positive and Gram-negative bacteria contain the lipid components: phosphatidylglycerol, phosphatidylethanolamine and cardiolipin (Epand et al., 2016). The polymyxin group of antibiotics exert their antibacterial activity against Gram-negative bacteria by binding to lipid A, which is the anchor for lipopolysaccharides in the outer membrane, thereby disrupting the permeability barrier (Galizzi et al., 1975; Yu et al., 2015; Epand et al., 2016).

1.4.3 Disruption of DNA replication

Modulation of chromosomal supercoiling through type II topoisomerase mediated strand breakage and re-joining is critical for DNA synthesis (van Eijk et al., 2017). During DNA replication, two bacterial topoisomerase II enzymes- DNA gyrase and topoisomerase IV play essential roles in modifying the topology of DNA by controlling the negative and positive supercoiling of DNA or by removing knots and tangles during the replication. Both DNA gyrase and topoisomerase IV are tetramers composed of two subunits each (two GyrA and two GyrB subunits for DNA gyrase and two ParC and two ParE subunits for topoisomerase IV) (van Eijk et al., 2017). Both DNA gyrase and topoisomerase can modulate the DNA topology by generating a transient double stranded break in the double helix, which they can then re-ligate. Even though both enzymes follow a similar mechanistic way of functioning, their physiological functions vary. DNA gyrase is capable of introducing negative supercoils into the DNA while topoisomerase IV plays a role in decatenating and removing the knots in DNA (van Eijk et al., 2017). The quinolone class of antibacterial compounds work by targeting DNA gyrase and topoisomerase IV by forming a DNA-topoisomerase-quinolone complex and strand breaks. If the SOS response and other DNA repair mechanisms fail to repair the breaks, it leads to eventual cell death (Hooper, 2001; Aldred et al., 2014).

1.4.4 Interference with transcription and translation

The process of synthesising RNA from its template DNA is mediated by the enzyme RNA polymerase. In bacteria, a single type of RNA polymerase is responsible for all transcription (Ma et al., 2016). Agents that target transcription exert their mode of action by binding to the RNA polymerase and inhibiting transcription (Kohansky et al., 2010). The rifamycin group of antibacterial compounds work by binding to the actively subscribing DNA bound RNA polymerase and inhibiting the DNA dependent transcription (Floss and Yu, 2005; Kohansky et al., 2010). Many antibacterial compounds also work by binding to the bacterial ribosomal subunits and interfering with the translation process. The bacterial ribosome is composed of two ribonucleoprotein subunits, the 30S subunit, which contains 20 proteins as well as the 16S rRNA chain and a large 50S subunit, which consists of 34 proteins and the 23S and 5S rRNA chains (Mccoy et al., 2011). The ribosome together with various other factors are responsible for mRNA translation over three steps: initiation, elongation and termination. Antibacterial compounds that target the ribosome targets one the following three sites: the 30S decoding site, which is responsible for the codon-anticodon recognition, the peptidyl transferase centre on the 50S subunit or the peptide exit channel on the 50S subunit (Kohansky et al., 2010; Mccoy et al., 2011). For example, macrolide antibiotics work by blocking the exit tunnel of the peptide on the 50S subunit. Aminoglycosides on the other hand bind to the 30S decoding site and interfere with codon recognition and translocation, and allow the incorporation of incognate amino acids into the growing peptide chain (Kohansky et al., 2010; Mccoy et al., 2011).

1.4.5 Disruption of the folate pathway

Key steps of the folate synthesis pathway are targeted by antibiotics such as sulfonamides, which in turn affect nucleotide synthesis, the building blocks of DNA and RNA (Murima et al., 2014). Folate is a crucial precursor in the biosynthesis of purines, pyrimidines and amino acids. Dihydrofolate reductase has an important role in maintaining the cellular pool of tetrahydrofolate, an important cofactor required for the synthesis of amino acids, purines, S-adenosylmethionine and formyl-methionine. Dihydrofolate reductase can therefore act as a target (Murima et al., 2014; Bourne, 2014). Another enzyme involved in the folate pathway is dihydropteroate synthase. Dihydropteroate synthase is responsible for the production of 7,8-dihydropteroate by catalysing the condensation of 6-hydroxymethyl-7,8-

dihydropterin pyrophosphate with para-aminobenzoic acid (pABA). Sulfonamide compounds are analogues of pABA and can act as alternative substrates and competitive inhibitors of pABA and result in dead-end products (Bourne, 2014; Murima et al., 2014) .

Use of any effective therapeutic agent in the clinic is compromised by the potential development of resistance to that agent (Davies and Davies, 2010). The evolution of resistance in microbes to most of the front-line antibiotics is an urgent matter at hand. The widespread use of antibiotics combined with poor stewardship has led to an antibiotic resistance crisis (Davies and Davies, 2010). The resistance crisis has in turn put antibiotic therapy in great jeopardy (Wright, 2010; Silver, 2011; Livermore, 2011).

1.5 Mechanisms of antibacterial resistance

Resistance to antibacterial compounds is achieved mostly via the following general mechanisms: i) modifications of the compound itself, ii) target modification and/or bypass, iii) global cell adaptive processes and iv) prevention from reaching the target either by preventing entry or through efflux (Figure 2) (Dever and Dermody, 1991; Tenover and Georgia, 2006; Wright, 2010; Munita and Arias, 2016). Alteration of the compound is achieved by enzymes that can modify it in such a fashion that it is no longer able to interact with the target, or by destroying the compound itself (Munita and Arias, 2016). There are many kinds of modifying enzymes that have been reported. Regardless of what they are, the modifications are often correlated with a higher bacterial minimal inhibitory concentration (MIC) usually owed to steric hindrances that lower the avidity of the compound for its target (Munita and Arias, 2016). For example, aminoglycoside modifying enzymes are able to covalently modify the amino or hydroxyl groups of an aminoglycoside molecule and is reportedly the predominant mechanism of aminoglycoside resistance worldwide (Munita and Arias, 2016). Destruction of the compound to completely render it ineffective is another highly successful bacterial resistance strategy. β-lactamases are an important example of this where these enzymes are able to hydrolytically cleave the β-lactam ring, which is essential for the antibacterial action of the compound (Dever and Dermody, 1991; Wright, 2010; Munita and Arias, 2016).

Changes in target sites are achieved through target protection or target modification. An example of target protection is the tetracycline resistance determinants TetM and TetO, where both of these proteins are able to interact with the ribosome in a GTP-dependent manner to dislodge tetracycline to allow protein synthesis to resume (Donhofer et al., 2012; Munita and Arias, 2016). Modifications of the target sites may be owed to i) mutations in the target site encoding gene (e.g. rifampin resistance), ii) bypass or replacement of the target so that new targets now exist to achieve a function similar to that of the original target (e. g. vancomycin resistance in *enterococcus*), and/or iii) enzymatic modification of the target (e.g. macrolide resistance through methylation of the ribosome) (Wright, 2010; Munita and Arias, 2016). The rifamycin antibiotic rifampin works by binding to a rifampin binding pocket on the RNA polymerase (encoded by the gene *rpoB)* blocking elongation of the RNA. Rifampin resistance is observed through single point mutations in the *rpoB* gene, which modifies the target and results in a decreased affinity of rifampin for its target (Campbell et al., 2001; Munita and Arias, 2016). The glycopeptide vancomycin works by interfering with the bacterial cell wall synthesis by binding to the terminal D-Ala-D-Ala residues of the PG chain preventing further incorporation of the PG subunits leading to cell death. Vancomycin resistance is primarily achieved through the *van* gene cluster that is capable of encoding the production of proteins, which can remodel PG by replacing the D-Ala-D-Ala to D-Ala-D-lactate/D-serine, thereby lowering the affinity of vancomycin to its target (Miller et al., 2014; Munita and Arias, 2016). Macrolide resistance is observed through a biochemical change via the ribosomal methylation by an enzyme encoded by the *erm* genes (Leclercq, 2002).

Global cell adaptations enable the bacterium to cope with and adapt to stressful conditions whereby complex mechanisms are developed in order to maintain pivotal cellular process. An example of bacterial resistance that is achieved through global cell adaptations is against daptomycin (Munita and Arias, 2016). The calcium dependent lipopeptide antibiotic daptomycin exerts it antibacterial activity by modifying the bacterial cell membrane homeostasis by interfering with its associated phospholipids (Tran et al., 2015). Daptomycin shares functional and structural similarity to the cationic antimicrobial peptides that are produced by the innate immune system. Resistance to daptomycin is associated with critical changes in the bacterial cell physiology brought about by adaptive changes in the cell wall and cell

membrane homeostasis (Tran et al., 2015). Limiting the influx of compounds to prevent it from reaching its target is another mechanism of antibacterial resistance. This is especially important in Gram-negative bacteria where the compounds have to penetrate the outer membrane and/or the inner cytoplasmic membrane to exercise its bioactive properties (Wright, 2010; Munita and Arias, 2016). Efflux pumps are a mechanism of resistance, which affects a wide array of antibacterial classes. They are bacterial machineries that are able to extrude the antibacterial compounds out of the cell, which in turn results in antibacterial resistance (e. g. tetracycline resistance in *E. coli*) (Wright, 2010; Munita and Arias, 2016).

Antibiotic-producing microbes in nature have always been inherently resistant to the antibiotics they themselves produce (Davies and Davies, 2010). However, the man-made selection pressure in the environment for antibiotic resistance has been so strong that the magnitude of resistance found in the environment is rapidly changing. The antibiotic resistance gene pool, as a result of this selective pressure, has become more accessible to microbes (Levy and Marshall, 2004; Alekshun and Levy, 2007; Munita and Arias, 2016).

1.6 The emergence and impact of antibiotic resistance

A microbiological point of view defines resistance as the state in which an isolate has a mechanism of resistance making it less susceptible to an antimicrobial agent when compared to other members of the same species that lack resistance mechanisms (Cantón and Morosini, 2011). In a clinical setting, antibacterial resistance generally refers to the pathogenic bacterial population that was originally susceptible to the compound of interest but has now become resistant and as a result, the clinical criteria of cure is not reached (Turnidge and Paterson, 2007; Munita and Arias, 2016). The acquisition or evolution of resistance in microbes to most of the front-line antibiotics is a pressing matter that is acknowledged by the highest levels of government as a significant threat to global health. The World Health Organization considers this crisis to be ‘one of the biggest threats to global heath today’ (Davies and Davies, 2010; WHO, 2018).

The overuse and misuse of antibiotics since its deployment has played a major role in the evolution of antibiotic resistance (Ventola, 2015). Horizontal

gene transfer between bacteria on mobile genetic elements such as plasmids have contributed to this greatly where bacteria are able to transfer the resistance encoding genes between different species (Ventola, 2015). Resistance can also arise through spontaneous mutations (Ventola, 2015; Hwang and Gums, 2016). Under the increased selection pressure the resistance genes and the hosts that are selected for thrive and multiply and spreads it to other hosts and locations (Davies and Davies, 2010). Inappropriate prescription and the extensive use of antibiotics in agricultural purposes have also contributed significantly to the emergence of resistance (Davies and Davies, 2010; Ventola, 2015; Hwang and Gums, 2016). Infections caused by microbial agents that were once under control are now increasing in incidence due to multi-drug resistance. The most alarming examples, and arguably the most costly in regards to morbidity and mortality, concern bacteria (Levy and Marshall, 2004; Davies and Davies, 2010).

The increase in multi-drug resistant (MDR) pathogenic bacteria have compromised the treatment of many infectious diseases (Tanwar et al., 2014). MDR bacteria disrupt disease control by increasing the possibility of the dissemination of the resistant pathogens while lowering the efficiency of the treatment itself leading to a prolonged infection time in the patient (Tanwar et al., 2014). Hospitals across both the developed and the developing world are faced with many challenges, epitomized especially by a group of pathogens referred to as the ‘ESKAPE’ pathogens (Louis B. Rice, 2008; Boucher et al., 2009). The ESKAPE group of pathogens include *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* species (Louis B. Rice, 2008). These pathogens are common causes of life-threatening nosocomial infections. They are becoming more and more prevalent and becoming increasingly resistant to most or all of the antibiotics used in the clinic limiting therapeutic options while increasing morbidity and mortality rates (Louis B. Rice, 2008; Boucher et al., 2009).

Enterococci are opportunistic pathogens that can cause highly infectious diseases. They are a leading cause of bacteraemia and urinary tract infections. The plasticity of their genomes allow them to acquire resistance to many antibacterial compounds, which is concerning, particularly vancomycin resistant enterococci (Santajit and Indrawattana, 2016; Esposito

and De Simone, 2017). The vancomycin resistance in enterococci is primarily associated with *E. faecium* with the prevalence of vancomycin resistant *E. faecium* being especially worrisome. Aminoglycoside resistance among enterococci worldwide has also been a cause of concern (Santajit and Indrawattana, 2016; Esposito and De Simone, 2017). *Staphylococcus* species caused infections have traditionally responded well to penicillin, however resistance emerged soon after the introduction of penicillin in the clinic with the synthesis of penicillinases enzymes in *S. aureus*. The production of β-lactamases in *S. aureus* strains increased drastically in the following years (Green et al., 2012). To counteract this resistance, methicillin, a semi-synthetic penicillin derivative, was introduced into the clinic. However, as with penicillin, resistance against methicillin followed soon after with the appearance of methicillin resistant *S. aureus* (MRSA) (Green et al., 2012). MRSA is a leading cause of skin and soft tissue associated infections acquired in hospitals, placing a significant burden on the health care system globally (Green et al., 2012).

Klebsiella pneumoniae is a member of the of the *Enterobacteriaceae* family and is major causative agent of both community and hospital derived infections (Santajit and Indrawattana, 2016). The infections they cause include sepsis, pneumonia and urinary tract infections and are associated with high rates of morbidity and mortality. The rapidly accumulating multi-drug resistance determinants against β-lactams, aminoglycosides and fluoroquinolones in *K. pneumoniae* is a cause of major concern worldwide (Santajit and Indrawattana, 2016). *A. baumanii* is another major causative agent of nosocomial infections including ventilator associated infections and urinary tract infections. The emergence of carbapenemase producing *A. baumanii* that can resist colistin and imipenem has been concerning as the combination of the resistance encoding genes allow them to evade the activity of most antibacterial compounds (Santajit and Indrawattana, 2016). *P. aeruginosa* is an opportunistic pathogen that is found in the normal gut microbiota. Even though the carriage rate of *P. aeruginosa* is fairly low in the general population, in health care settings, they can cause life threatening nosocomial infections, especially in immunocompromised patients. *P. aeruginosa* resistant to carbapenems, aminoglycosides and fluoroquinolones adds to resistance crisis (Santajit and Indrawattana, 2016). The opportunistic infections caused in immunocompromised patients by MDR *Enterobacter* spp add further concern as these strains are resistant to almost all of the

available antibiotics (Santajit and Indrawattana, 2016; Esposito and De Simone, 2017).

The rise in the number of hospital infections caused by *P. aeruginosa, A. baumannii* and *K. pneumoniae* that are resistant to all or most antibiotics is particularly worrisome as they are Gram-negative bacteria (Levy and Marshall, 2004; Slama, 2008; Davies and Davies, 2010). The outer membranes of Gram-negative bacteria act as a semi-impermeable barrier to amphipathic molecules, which is problematic as most drugs tend to be amphipathic in order to cross the cytoplasmic membrane and be soluble (Lewis, 2013). Thus, the treatment of Gram-negative bacteria especially with the rise of multi-drug resistance has placed a significant burden on healthcare systems globally. The sheer importance of antimicrobials in the treatment of infections cannot be understated and with multi-drug resistance on the rise, continued discovery and development of new antimicrobial compounds is of utmost importance (Davies and Davies, 2010; Lewis, 2013).

Furthermore, it was put forward by researchers that soil might be an exhausted antibiotic source and the many different antibiotics discovered during the Golden Age of antibiotic discovery were termed the ‘low hanging fruit’ (Wright, 2010; Silver, 2011; Livermore, 2011; Katz and Baltz, 2016). The repeated rediscovery of the same antibiotics contributed significantly to this bottleneck (Silver, 2011). The withdrawal of big pharmaceutical companies from antibiotic discovery due to the failures in combinatorial chemistry, target based screening from genome sequences for new antimicrobial compounds combined with the poor profitability of antibiotics led to the closure of many drug discovery programmes, which has served to exacerbate the antimicrobial resistance crisis (Projan, 2003; Fernandes, 2006; Li et al., 2009; Coates et al., 2011). As a result, the world is now facing the possibility of a pre-antibiotic era highlighted especially with the emergence of resistant strains on the rise (Hopwood, 2007).

1.7 Secondary metabolites: a continuing source of antimicrobials

Tailoring of existing antibiotic classes that allowed the production of improved versions of antibiotics has proved to work to a certain extent (Silver, 2011). One such example is Augmentin, which is a penicillin antibiotic made by the addition of clavulanic acid, a β-lactamase inhibitor, to amoxicillin (White et al., 2004). During the last 30 years, there was interest in the pharmaceutical companies to develop analogues of the antibiotic as tailoring of existing antibiotics is much cheaper and less risky than finding novel classes (Coates et al., 2011). In any case, natural products remain and continue to be the lead molecules required for the development of novel antibiotics. Their complexity, structural diversity and notably, their selective and specific bioactivity along with the untapped wealth of secondary metabolites awaiting to be discovered emphasise this (Cragg and Newman, 2013).

1.7.1 Genome mining and silent gene clusters

Sequencing and annotation of the *S. coelicolor* A3(2) genome paved the way for its rational mining and allowed the identification of many silent or cryptic pathways (Bentley et al., 2002). *S. coelicolor* was only known to produce four or five specialized metabolites prior to its genome being sequenced. However, upon obtainment of its genome sequence, a further 18 biosynthetic gene clusters (BGCs) were identified (Bentley et al., 2002). The isolation of a novel natural product following this discovery, the iron chelator coelichelin, in *S. coelicolor* ushered in a new era of genome mining for chemical entities in microbes (Bibb, 1996; Challis and Ravel, 2000; Bentley et al., 2002). Genome mining is based on the identification of secondary metabolite BGCs in the genome sequence based on their homology to known secondary metabolite BGCs (Bachmann, 2014). With the next generation sequencing technologies available, and the relatively easy and inexpensive access to genome sequence data, it was revealed that many microbes contain blueprints for synthesising many more secondary metabolites. As a result, genomics and genome mining has revolutionised the search for novel secondary metabolites (Bachmann, 2014; Genilloud, 2018).

Bioinformatic tools are able to detect and predict the secondary metabolite BGCs with homologies derived from known secondary metabolite BGCs based on detection strategies such as the type of pathway, hidden Markov models and conserved motifs. Computational tools such as antiSMASH (antibiotics & Secondary Metabolite Analysis Shell) can identify and predict known classes of secondary metabolite BGCs in the genome (Medema et al., 2011). AntiSMASH is also able to provide comprehensive functional annotations of non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) systems identified and can predict the possible chemical structures of the compounds predicted to be produced by these megaenzymes (Medema et al., 2011). Non-ribosomal peptides (NRPs) and polyketides (PKs) are two large families of structurally diverse compounds with biologically important activities (Fischbach and Walsh, 2006). The more in-depth understanding of the biochemical programming and the substrate specificity on a molecular basis of these two systems have made powerful tools in making these predictions for novel modular NRPS and PKS system products as well as paving way towards the rational engineering of such systems (Challis, 2008a; Challis, 2008b). Being able to predict the structural features and physio-chemical properties of the secondary metabolites encoded by the pathways help avoid the rediscovery of known compounds along with shedding light on ways to engineer these pathways (Ziemert et al., 2016).

As with *S. coelicolor,* many microbes that were otherwise thought to be mined to exhaustion were shown to have the potential to produce many more secondary metabolites (Bachmann, 2014). These silent BGCs identified are not expressed under standard laboratory growth conditions (Rutledge and Challis, 2015). Bioinformatics predicted several of these gene clusters likely to encode novel products with complex structural features (Challis, 2008a; Rutledge and Challis, 2015). Genome mining of numerous *Streptomyces* spp. has revealed the presence of many such silent pathways with the potential for the biosynthesis of new and complex chemical entities. On average, *Streptomyces* genomes harbour about 30 secondary metabolite BGCs and most of these are silent under standard laboratory conditions (Ziemert et al., 2016). Many of these silent BGCs are predicted to encode the production of new and complex chemical entities (Chaudhary et al., 2013; Harvey et al., 2015). Therefore, these silent pathways and their

potential for the production of novel antibiotics point towards an exciting new age for antibiotic discovery (Challis, 2008b; Rutledge and Challis, 2015).

The activation of silent BGCs has therefore attracted significant interest from researchers across the globe. The two main approaches in trying to awaken these silent pathways are by using either pleiotropic approaches or cluster-specific approaches (both of which are explored further in Chapters 3 and 4). Pleiotropic approaches include strategies such as varying growth conditions, co-cultivation, addition of elicitor molecules while gene cluster-specific approaches include overexpression/deletion of cluster-specific regulators, gene knockout studies and heterologous expression. While pleiotropic approaches are largely consisted of trial and error efforts, cluster-specific approaches utilise the genome sequencing information to provide a more directed and controlled approach towards activating these BGCs. The exponential growth in bioinformatics has aided researchers in the efforts in the discovery of new and useful natural products by prioritising silent gene clusters (Rutledge and Challis, 2015). As opposed to the traditional bioactivity guided screening, genome mining has the theoretical potential to eliminate chances of rediscovering the same compounds (Bachmann, 2014). Genome mining has also played a role in furthering the knowledge in the divergence of BGCs across various lineages (Genilloud, 2018). Additionally, it has also shed light into understanding the roles of secondary metabolites in their endogenous contexts with their roles in interspecies, intergeneric as well as inter-kingdom associations such as their roles in communication, antibiosis and symbiosis (Bachmann, 2014).

1.8 Aims and objectives

Heterologous expression of BGCs is an effective strategy in the activation of silent BGCs, over-expression of BGCs as well as in engineering natural product variants (Ongley et al., 2013). Therefore, having a readily available suite of heterologous hosts is invaluable in the continued discovery and development of new and useful natural product derived compounds. One of the aims of this study is to generate a chromatogram-simplified *Streptomyces albus S4* heterologous host. *S. albus* is one of the most frequently used heterologous hosts because of its fastidious growth and genetic tractability (Seipke, 2015). These properties along with the potential *S. albus* S4 possess to produce a wide array of secondary metabolites motivated the endeavour to create an *S. albus S4* heterologous host strain.

S. albus S4 was originally isolated from the nests of the higher attine ant species *Acromyrmex octospinosus,* an unexplored niche (Seipke, Crossman, et al., 2011; Seipke, Barke, et al., 2011). These leaf cutting ants employ antibiotic producing actinomycetes to protect their fungal cultivar from other invading fungi (Barke et al., 2010; Seipke, Barke, et al., 2011). *S. albus* S4 is known to produce the antifungal compounds candicidin and antimycin. However, it does not produce any antibacterial agents when cultivated under standard laboratory conditions, but bioinformatic analysis of its genome sequence revealed that it has the potential to produce compounds with antibacterial properties (Seipke, Crossman, et al., 2011; Seipke, 2015). This study aims to use pleiotropic approaches to activate the production of an antibacterial compound(s) in *S. albus* S4, identify and chemically characterise the activated antibacterial compound and to further investigate its antimicrobial profile.

NRPs are a large family of structurally complex and diverse natural products, often with remarkable biologically and therapeutically relevant activities. They are synthesised by large multifunctional mega enzymes called NRPSs with a modular, assembly line like synthetic logic (Martínez-Núñez and López, 2016; Süssmuth and Mainz, 2017). Each module plays a role in the peptide elongation and usually, a C-terminally located thioesterase (TE) domain facilitates the hydrolytic release or macrocyclisation of the mature peptide (Süssmuth and Mainz, 2017). The surugamide (*sur*) BGC encodes the production of the cyclic octapeptide surugamide A (SA) and the linear decapeptide surugamide F (SF) (Ninomiya et al., 2016). The *sur* BGC is made up four NRPSs (*surABCD*) arranged in an operon where *surAD* encodes the production of SA and *surBC* encodes the production of SF (Ninomiya et al., 2016). Contrary to the norm, this NRPS system lacks a *cis*-acting TE domain responsible for the release of these peptides from the assembly line (Takada et al., 2013; Ninomiya et al., 2016; Kuranaga et al., 2018). Therefore, this study also aims to understand the assembly line release of SA and SF. As NRPs are an important source of medically relevant compounds, understanding alternative mechanisms of release will be important in expanding the synthetic biology toolbox available for engineering NRP synthesis.

Chapter 2 Materials and Methods

2.1 Strains and plasmids

Bacterial strains, plasmids/cosmids used and constructed in this study are listed in Table 2.1.1 and 2.1.2 respectively.

Table 2.1.1 Strains used and generated in this study

Strain	Description	Reference
***E. coli* strains**		
XL10-Gold	General cloning host, stocks normally maintained in XL10-Gold cells. Tet^{r}Δ(*mcrA*)183 Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F´ *proAB lacI^{q}ZΔM15* Tn*10* (Tet^R) Amy Cam^R].	Agilent Technologies
ET12567/pUZ8002	Non-methylating host to allow the conjugal transfer of DNA into *Streptomyces* (*dam*, *dcm*, *hsdM*); Kan^R, Cam^R)	(MacNeil et al., 1992)
GB05-red	Recombineering proficient host; *araC*-BAD-γβαA: lambda *red* operon and *recA* under P_{BAD} promoter inserted at the *ybcC* locus	(Fu et al., 2012)
BL21(DE3)	Host for heterologous protein expression; F$^-$ *ompT dcm lon hsdS$_B$* (r_B^-, m_B -) *gal λ*(DE3 [*lacI ind1 sam7 nin5 lacUV5-T7* gene 1])	Agilent Technologies
E. coli BW25113	Derivative of *E. coli* K12 strain BD792; indicator strain used in antibacterial assays	(Baba et al., 2006)

***Staphylococcus* strains**		
S. aureus SH1000	Derivative of the *Staphylococcus* strain 8325-4; indicator strain used in antibacterial assays	(Horsburgh et al., 2002)
***Micrococcus* strains**		
M. luteus	Indicator strain used in antibacterial assays	(Wieser et al., 2002)
Candida strains		
C. albicans	*Candida albicans* CA-6; indicator strain used in antifungal assays	(Martel et al., 2010)
***Streptomyces* strains**		
S. albus S4	*Streptomyces albus* S4 wildtype	(Barke et al., 2010)
S4 Δ*ant*Δ*can*	*S. albus* S4 mutant in the production of antimycin and candicidin	(Seipke, Barke, et al., 2011)
S4 Δ*ant*Δ*can*Δ*sur*	*S. albus* S4 mutant in the production of antimycin, candicidin and surugamide A	This study
S4 Δ*5*	*S. albus* S4 mutant with a complete deletion of the antimycin BGC and mutations within the candicidin, surugamide, albaflavenone and fredericamycin BGCs	EH, Seipke, This study
S4 Δ*5*-cin2	*S. albus* S4 *Δ5* heterologous expression strain for cinnamycin	Seipke, unpublished
S4 Δ*surE*	*S. albus* S4 *surE* mutant, Apr^R	This study
S4 Δ*surF*	*S. albus* S4 *surF* mutant, Apr^R	This study
S4 ∆*surE attB* φBT1::pIJ10257-*surE*	*S. albus* S4 *surE* mutant complemented with *surE* expressed from the *ermE** promoter; Apr^R, HygR	This study

S4 Δ*surF attB* φBT1::pIJ10257-*surF*	*S. albus* S4 *surF* mutant complemented with *surF* expressed from the *ermE** promoter; Apr^R, Hyg^R	This study

Table 2.1.2 Cosmids and plasmids used and created in this study

Cosmid/plasmid	Description	Reference
Cosmids		
Supercos1	Cosmid backbone for *S. albus* S4 Cos994; $Carb^R$, Kan^R	Stratagene
Cos994	Supercos1 derivative spanning the surugamide gene cluster from *surF* to *surB*; $Carb^R$, Kan^R; (DT159, DT160)	This study
Cos994-*ΔsurE*	Cos994 derivative with *surE* deletion (DT161, DT162); $Carb^R$, Kan^R, Apr^R	This study
Cos994-*ΔsurF*	Cos994 derivative with *surF* deletion (DT167, DT168); $Carb^R$, Kan^R, Apr^R	This study
Plasmids		
pCRISPomyces-2	*Streptomyces* CRISPR/Cas9 editing vector, Apr^R	(Cobb et al., 2015)
pCRISPomyces-2-*sur*	Surugamide spacer sequence (BbsI site, EHS9, EHS10) and HDR arms (XbaI site; EHP19, EHP31; EHP27,EHP28)	EH, unpublished
pCRISPomyces-2-*alb*	Albaflavenone spacer sequence (BbsI site, EHS4, EHS5) and HDR repair (XbaI site; EHP7, EHP8; EHP9, EHP10)	EH, unpublished
pCRISPomyces-2-*fdm*	Fredericamycin spacer sequence (BbsI site, EHS7, EHS8) and HDR repair arms (XbaI site; EHP15, EHP16; EHP17, EHP18)	EH, unpublished
pIJ773	REDIRECT PCR template for the	(Gust et al.,

	aac(3)IV + *oriT* cassette	2002)
pET28a	Commercial protein expression vector; Kan^R	Novagen
pET30a	Commercial protein expression vector; Kan^R	Novagen
pDB-His-MBP	Commercial protein expression vector; Kan^R	Gifted by Dr Mike Webb
pET28a-*surE*	pET28a derivative containing *surE* cloned into the NdeI and XhoI sites or the NdeI and HindIII sites; Kan^R; (DT206, 213)	This study
pET28a-*surE*-sub	pET28a derivative containing *surE* subcloned from pIJ10257-*surE* into the NdeI and HindIII sites; Kan^R	This study
pET30a-*surE*	pET30a derivative containing *surE* cloned into the NdeI and XhoI sites; Kan^R; (DT206, DT207)	This study
pDB-His-MBP-*surE*	pDB-His_6-MBP derivative containing *surE* cloned into the NdeI and XhoI sites; Kan^R (DT206, DT213)	This study
pDB-His-MBP-J1074-*surE*	pDB-His_6-MBP derivative containing *S. albus* S4 *surE* cloned into the NdeI and XhoI sites; Kan^R (DT206, DT213)	This study
pIJ10257	pMS81 derivative containing *ermE**p, integrates into the φBT1 *attB* site in *Streptomyces*; Hyg^R	(Hong et al., 2005)
pIJ10257-*surE*	pIJ10257 derivative containing the *surE* coding sequence cloned into the NdeI and HindIII sites; Hyg^R; (DT210, DT211)	This study
pIJ10257-*surF*	pIJ10257 derivative containing the *surF* coding sequence cloned into the NdeI and HindIII sites; Hyg^R; (DT208, DT209)	This study

pIJ10109	Cinnamycin BGC containing vector, integrates into the Φc31 *att* site	(Widdick et al., 2003)
pUZ8002	Encodes the conjugation machinery for the mobilisation of plasmids and cosmids from *E. coli* to *Streptomyces*; Kan^R	(MacNeil et al., 1992)

2.2 Growth media, antibiotics and culture conditions

E. coli strains were cultivated on Lennox agar (LA) or broth (LB) media (1000 mL dH_20, 10 g tryptone, 5 g yeast extract, 5 g NaCl ± 15 g agar) for 16 h at 37 °C. *Streptomyces* strains were propagated on mannitol-soya flour (MS) medium (1000 mL tap water, 20 g mannitol, 20 g soya flour, 20 g agar) for 7 days at 30 °C unless otherwise stated. Antibacterial agents were added to the media when necessary at the following concentrations: carbenicillin, 100 µg/mL (Formedium); nalidixic acid, 25 µg/mL (Applichem Panreac); apramycin, 50 µg/mL (Sigma); kanamycin, 50 µg/mL (Sigma); chloramphenicol, 25 µg/mL (Formedium); hygromycin B, 50 µg/mL (Invitrogen). LA or LB with no NaCl was used with hygromycin B.

2.3 Molecular biology techniques

2.3.1 Extraction of plasmid and cosmid DNA

A bacterial cell pellet was obtained by the centrifugation of 3 - 5 mL of the bacterial culture that had been incubated while shaking at 220 rpm at 37 °C for 16 h. Cosmid or plasmid DNA extraction was carried out according to the manufacturer's instructions using either the QIAprep miniprep kit (Qiagen) or the Monarch® plasmid miniprep kit (New England Biolabs). The concentration and purity of DNA was assessed using a P300 nanophotometer (NanoPhotometer Pearl; Implen, Munich, Germany).

2.3.2 Genomic DNA isolation from *Streptomyces*

A bacterial cell pellet was obtained by the centrifugation of 10 mL of *Streptomyces* culture that had been incubated while shaking at 220 rpm in LB for 48 h at 30 °C. Genomic DNA extraction was carried out according to established protocols (Kieser et al., 2000). Essentially, the bacterial cell pellet was resuspended in 200 µL of buffer (50 mM Tris-Cl, pH 8.0, 10 mM EDTA, 100 µg/mL RNase A) and incubated with lysozyme (Sigma) at a final

concentration of 10 mg/mL for 1.5 – 3 h at 37 °C. Following the incubation, SDS and NaCl were added to a final concentration of 1% and 1.25 M, respectively and incubation continued on ice for a further 10 mins. The resultant sample was extracted twice with an equal volume of phenol: chloroform: isoamylalcohol 25: 24: 1 (Applichem Panreac). The sample was ethanol precipitated by adding ethanol (Sigma) at 2.5 times the volume of the sample followed by incubation at -20 °C for at least 30 mins. The sample was then centrifuged for 15 mins at 16,000 x *g* and the resulting DNA pellet washed with 500 µL of 70% ethanol. The pellet was air dried for 30 – 60 seconds before resuspending in 100 µL of water. The concentration and purity of DNA was assessed using a nanophotometer.

2.3.3 Amplification of DNA by polymerase chain reaction (PCR)

Oligonucleotide primers were purchased from Integrated DNA Technologies (Leuven, Belgium) (Appendix Table A1). PCR reactions were carried out in a T100 Thermal Cycler (Bio-Rad). Q5 High-Fidelity DNA Polymerase (New England Biolabs) was used for the PCR amplification of restriction site containing inserts used in this study. Thermal cycling conditions consisted of initial denaturation at 95 °C for 30 seconds followed by 26 cycles of: denaturation at 98 °C for 10 seconds, annealing for 30 seconds and extension at 72 °C for 30 seconds/kb, which was then followed by a final extension cycle at 72 °C for 2 mins. Optimal annealing temperature was calculated using the NEB Tm calculator. GoTaq G2 DNA polymerase (Promega) was used in diagnostic PCR reactions and the thermal cycling conditions consisted of: initial denaturation at 95°C for 2 mins followed by 26 cycles of denaturation at 95 °C for 30 seconds, annealing for 30 seconds, and extension at 72 °C for 60 seconds/kb, which was then followed by a final extension cycle at 72 °C for 5 mins. Optimal annealing temperatures were calculated using the Thermo Fisher Scientific Tm calculator. The resultant amplicons from the PCR reactions were visualised using agarose gel electrophoresis (2.3.4).

2.3.4 Agarose gel electrophoresis and DNA extraction

Agarose gel electrophoresis was used to determine the presence and size of PCR products or to separate and purify DNA. Samples were run alongside a 1 kb plus DNA ladder (New England Biolabs). Generally, 1% agarose gels made up in 1 x TAE buffer were used. GoTaq buffer containing PCR

reactions or DNA samples containing 1/5 volume 6x loading buffer were generally run at 100 V for 30-45 mins. To image the gels, Gene-Genius Bio-Imaging-System (Syngene) using the GeneSnap Program (Syngene) was used. For extraction of DNA, agarose gels were visualised using a UV transilluminator. The DNA bands of interest were cut out and transferred to a microcentrifuge tube. DNA extraction was carried out using the QIAquick gel extraction kit (Qiagen) according to the manufacturer's instructions.

2.3.5 Cosmid screening

Cos994 was identified by screening a previously constructed *S. albus* S4 cosmid library (Seipke et al., 2014) using the primer set DT159 and DT160, which targeted an internal fragment of *surF.* To establish the sequence of the insert, the cosmid was insert-end sequenced using primers RFS184 and RFS185 and the resulting reads were manually mapped to the *S. albus* S4 genome using Artemis (Seipke, Crossman, et al., 2011; Seipke, Barke, et al., 2011; Carver et al., 2012).

2.3.6 Sanger sequencing

The integrity of cloned DNA, cosmid inserts and confirmatory PCR products was verified by DNA sequencing using Genewiz (Takeley, Essex, UK) or GATC Biotech (Eurofins). The sequence data was then aligned with the reference sequence using the 'A plasmid editor' tool (ApE; Davies, University of Utah Biology Department).

2.3.7 Restriction digests and ligation reactions

Restriction enzymes were purchased from New England Biolabs and the digests were carried out according to the manufacturer's protocols. Following restriction digest of the vector, an alkaline phosphatase, CIP (New England Biolabs) was used to dephosphorylate the 5´ and 3´ ends of the digested vector as specified by the manufacturer. Ligations were carried out with the restricted and gel purified vector and insert using T4 DNA ligase (New England Biolabs) following the manufacturers instructions. Typically, ligations were carried out at room temperature overnight before transforming chemically competent XL10-Gold cells.

2.3.8 Transformation of *E. coli*

Transformation of chemically competent *E. coli* was achieved by adding ~100 ng of DNA or an entire ligation mix to 100 μL of chemically competent cells followed by incubation on ice for 20 min before heat shocking at 42 °C for 30 – 60 seconds and a further 2 min of incubation on ice. Eight hundred microliters of LB was added to the transformation mix followed by 1 h recovery incubation at 37 °C. Electroporation of *E. coli* was achieved by adding ~100 ng of DNA and 100 μL of electro-competent cells to a 2 mm gap electroporation cuvette (GeneFlow) followed by electroporation with a 2.5 kV pulse for 5 ms using the Gene Pulser Xcell (Biorad) electroporation system. Following electroporation, 800 μL of LB was added, transferred to a microcentrifuge tube and incubated whilst shaking at 37 °C for 1 h before plating onto LA with the appropriate antibiotics.

2.3.9 Intergeneric conjugation of *Streptomyces*

Intergeneric conjugation of *Streptomyces* was carried out according to an established protocol (Kieser et al., 2000). Essentially, the plasmid or cosmid of interest was first transformed into the de-methylating *E. coli* strain ET12567/pUZ8002. A single transformant was then grown overnight in 10 mL LB with the appropriate antibiotics. Two hundred microlitres of the overnight culture was used to make a 20 mL LB subculture containing all the antibiotics and allowed to grow until OD_{600nm} was ~0.4. The 10 mL overnight culture and the 20 mL subculture were then pooled together and centrifuged to obtain the cell pellet. The pellet was washed twice with LB to remove antibiotics before resuspending in 1 mL of LB and mixing it with 200 μL of *Streptomyces* spores. The mixture was pelleted and resuspended in 300 μL of LB and then plated on MS agar with no selection and incubated at 30 °C for 16 – 18 h. Following this period of incubation, the plates were overlaid with 1 mL of sterile water containing nalidixic acid (500 μg/mL final concentration) and the desired antibiotic (final concentration: apramycin, 2500 μg/mL; hygromycin, 3750 μg/mL)

2.3.10 Generating spore stocks

Transconjugants picked from the conjugation plate were colony purified to generate single colonies on MS agar containing the appropriate antibiotics. Single colonies were then picked to cultivate a sporulated lawn. Following 7 days of growth at 30 °C, 2 mL of 20% glycerol was added to the plate and a

sterile cotton bud was used to dislocate the spores and another 1 mL of 20 % glycerol was added. The resulting spores were collected and stored at -80 °C.

2.3.11 ReDirect Recombineering

The *Streptomyces* mutants S4 Δ*surE* and S4 Δ*surF* were generated following the ReDirect PCR targeting protocol (Gust et al., 2002). The ReDirect apramycin cassette was amplified from pIJ773 using DT161, DT162 for the replacement of *surE* on Cos994 to generate Cos994-*ΔsurE*; DT167and DT168 were used for the replacement of *surF* on Cos994 to generate Cos994-Δ*surF*. Mutant cosmids were mobilised into *S. albus* S4 WT by *E. coli*-to-*Streptomyces* conjugation as described in section 2.3.9. Transconjugants were replica plated on MS containing apramycin or kanamycin to identify apramycin resistant and kanamycin sensitive isolates, a phenotype consistent with double cross over event and deletion of region of interest. S4 Δ*surE* was verified by PCR using the primers DT163a and 164a and S4 Δ*surF* was verified using the primers DT171 and DT172.

2.4 Generation of chemical extracts and analysis

2.4.1 Chemical extract preparation from minimal media agar plates and LC-HRMSMS analysis

Minimal media (MM) agar was prepared following an established recipe (Kieser et al., 2000), except 25 mM mannitol was used instead of glucose. Sporulated lawns of the *S. albus S4* strains were grown on MM agar at 30 °C for 7 days. Following the incubation period, the sporulated lawn containing agar was cut up into small pieces and extracted with ethyl acetate (Sigma). Five petri dishes worth of agar was extracted with 50 mL of ethyl acetate (volumes were scaled up accordingly as number of plates increased). Extraction was done at room temperature with periodic shaking for approximately 3 h. The extract was then dried in vacuo and resuspended in 500 μL of methanol. The methanolic extract was centrifuged for 10 mins at 16,000 x *g* to remove any insoluble material. Two microliters of the extract was injected into a Bruker MaXis Impact time of flight mass spectrometer equipped with a Dionex UltiMate 3000 high-performance liquid chromatography apparatus with conditions as described previously for analysis by LC-HRMSMS (Liu et al., 2015).

2.4.2 Chemical extract preparation and LC-HRMS analysis of surugamides

LB seed cultures (10 mL) of the *S. albus* S4 strains were cultivated while shaking at 220 rpm at 30 °C for 3 days, at which point the entire culture was added to ISP2 broth (50 mL in a 250 mL flask; 1000 mL dH_2O: 4 g yeast extract, 10 g malt extract, 4 g dextrose; pH 7.2) and incubated at 30 °C with shaking at 180 rpm for 4 days. Following the incubation period, bacterial cells were removed by centrifugation and metabolites were extracted from the supernatant twice with two volumes of ethyl acetate. Chemical extracts were then pooled and concentrated in vacuo. The remaining residue was resuspended in 0.7 mL of 100% methanol (Thermo Fisher) and centrifuged at 16,000 x *g* for 15 mins to remove any insoluble material. LC- HRMS was carried out in the mass spectrometry facility in the Department of Chemistry at the University of Warwick by Dr Lijiang Song. Two microliters of the methanolic extract were injected into a Bruker MaXis II coupled with a Dionex 3000 RS UHPLC equipped with an Agilent C18 column (2.1 x 100 mm). The solvents used for elution of the column were as follows: solvent A, 0.1% formic acid in water; solvent B, 0.1% formic acid in acetonitrile and the following gradient was used: 0-5 mins, 5% B; 5-20 mins, 5% B to 100% B; 20-25 mins, 100% B; 25-27 mins, 100% B to 5% B. The flow rate was 0.2 mL/min. Calibration was achieved with 1 mM sodium formate at the beginning of each run. Bruker Data Analysis Software (version 4.1) was used to analyse the data.

2.4.3 *S. albus* S4 and *S. albus* Δ5 chemical extract preparation and HPLC analysis

S. albus S4 and *S. albus* Δ5 were cultured in 50 mL of liquid GYM (1000 mL dH_2O: 4 g glucose, 4 g yeast extract, 10 g malt extract, pH 7.2) and MS medium whilst shaking at 220 rpm in a 250 mL flask at 30 ºC for 7 days. Bacterial cells were then removed by centrifugation and 45 mL of supernatant was extracted with 90 mL of ethyl acetate. The extract was then evaporated to dryness in vacuo and resuspended in 500 µL of 100% methanol. The chemical extract was then centrifuged for 10 minutes at 16,000 x *g* to remove any insoluble material prior to analysis. Ten microliters of the methanolic extract was injected into a Dionex HPLC comprising a P680 pump, an ASI100 autosampler and a PDA100 detector with diode array capability running the Chromeleon software. Compounds were separated on a Phenomenex Luna C18 column (150 x 4.6 mm, 100 Å, pore

size 5 µm) with a C18 guard cartridge (Phenomenex). The solvents used for elution of the column were as follows: solvent A, 5% acetonitrile, 0.1% formic acid in water; solvent B, 95% acetonitrile, 0.05% formic acid. The following gradient was used: following a 3 min wash with 100% A, samples were eluted with a gradient of 0 to 100% B over 40 mins followed by holding at 100% B for 3 mins before returning to 0% B to re-equilibrate for 9 mins. The flow rate was 1 mL/min.

2.4.4 Chemical extract preparation and LC-HRMS analysis of cinnamycin production

S. albus Δ5 and *S. albus* Δ5-cin2 strains were cultured in 50 mL TSB (Sigma) cultures at 30 ºC for 7 days. Bacterial cells were pelleted and the supernatant discarded. The cell pellets were extracted with 45 mL of methanol and incubated shaking at room temperature for 5 h. The extracts were evaporated to dryness in vacuo and resuspended in 500 µL of methanol. Equal volumes of methanolic extracts for three independent replicates for each *Streptomyces* strain were pooled together and the extract was then centrifuged for 10 mins at 16,000 x *g* to remove any insoluble material prior to analysis by LC-HRMS. Two microliters of the chemical extract was injected into a Bruker MaXis Impact TOF mass spectrometer equipped with a Dionex Ultimate 3000 HPLC and a Waters Acquity UPLC Peptide CSH C18 column (1.7 µm, 2.1 × 100 mm). The solvents used for elution were solvent A, 0.1% formic acid in water; solvent B, 0.1% formic acid in acetonitrile. The following gradient was used: 0−1.5 min, 1% B; 1.5−3.5 min, 75−95% B; 3.5−4.5 min, 95% B; 4.5− 5 min, 95−1% B; 5−5.5 min, 1% B. The flow rate was 0.7 mL/min.

2.5 Protein expression

2.5.1 Purification trials of SurE

Using standard molecular biology techniques, the coding regions of interest were cloned into pET28a, pET30a or pDB-His-MBP to have an N-terminal 6x His tag, a C-terminal 6x His tag or an N-terminal 6x His- MBP tag, respectively. The expression vectors were then introduced into BL21(DE3) cells and various growth conditions and lysis methods were tested (briefly discussed in chapter 5). Cells were harvested following the expression trial period and the cell pellets weighed. Lysis buffer (100 mM Tris, 500 mM NaCl, 10 mM imidazole, pH 7.9) was added at 10x the volume of the weight

of the cell pellet and the resuspended cell pellets were lysed either by sonication or using the cell disruptor (flow-through option at 20 kpsi or one-shot option at 27 kpsi). The cell debris was removed by centrifugation at 16,000 x *g* for 30 minutes at 4 °C. The soluble fraction was obtained and mixed with 4 mL of prepped Ni-NTA affinity resin (Super Ni-NTA Affinity resin, Generon) and allowed to mix for approximately 20 mins before allowing the mixture to pass through an empty gravity-flow chromatography column (Econo-Pac® Chromatography Columns, Bio-Rad). The flow through was allowed to pass through the column twice to maximise the chances of the protein binding to the resin. The resin was then washed with 6 mL of 20 mM imidazole containing lysis buffer followed by 5 mL of 60 mM imidazole containing lysis buffer and finally by 15 mL of 500 mM imidazole containing lysis buffer. The 500 mM imidazole buffer elution was then passed through a spin concentrator (Vivaspin® 20; Sartorius Stedim) to concentrate the volume to approximately 2.5 mL. The sample was then passed through an equilibrated desalting column (Sephadex G-25 in PD-10 desalting columns, GE Healthcare) before collecting ten 1 mL fractions. Bradford reagent was used to check for the presence of protein. Prior to analysing the samples on the gel, 5 µL of 4 x Laemmlli buffer (Bio-rad) was added to 10 µL of the samples and samples were denatured at 95 °C for 5 mins. The denatured samples were analysed on an SDS-PAGE gel (RunBlue precast gels, expedeon) alongside a blue pre-stained protein standard (#P7706S, New England Biolabs). The gel was run at 200 V for 45 – 60 mins and then stained using InstantBlue (Expedeon). A cell free protein expression kit (PURExpress In Vitro Protein Synthesis Kit, NEB E6800S) was also tried to synthesise SurE.

2.5.2 Purification of SurE

BL21(DE3)(pET28:*surE*) cells were inoculated in autoinduction-LB broth base media including trace elements (Formedium) and allowed to grow for 3 days at 16 °C. Following the incubation period, cells were harvested and resuspended in 10 x volume of the weight in lysis buffer (50 mM HEPES, 200 mM NaCl, pH 7.4). The cells were lysed by sonication (40% power, 2 cycles of 1 m 30 s with pulses every other second). The soluble fraction was collected and mixed with 5 mL of prepped Ni-NTA resin. The sample was allowed to flow through the column slowly before washing the resin with the wash buffer (50 mM HEPES, 200 mM NaCl, 50 mM imidazole, 1 mM EDTA, pH 7.4). The column was washed until there was no more protein detected

in the flow through (analysed using Bradford's reagent). The protein was then eluted from the column using the elution buffer (50 mM HEPES, 200 mM NaCl, 300 mM imidazole, 1 mM EDTA, pH 7.5) as 3 ml fractions and the presence of protein was checked using Bradford's reagent. The resultant fractions were analysed on an SDS-PAGE gel. The purification of SurE was done together with Asif Fazal.

2.6 SNAC assays

Reactions (100 µL) contained 50 mM HEPES (pH 7.5), a peptidyl SNAC-thioester mimic of surugamide A (0.1 mM; SA-SNAC dissolved in methanol) and SurE (18 µg) and were incubated at 30 °C for 5 h along with the control reactions. The reactions were quenched by adding 100 µL of 0.05% trifluoroacetic acid (TFA). The reactions mixtures were then extracted with Phenomenex strata-XL C18 (100 µm, 30 mg, 1 mL) solid-phase extraction column (SPE) and a vacuum manifold. The column was first washed with 1 mL of 100% methanol followed by 1 mL of deionized water. The reaction mixture was then loaded onto the column prior to being washed with 1 mL of deionized water. Metabolites were eluted from the column with 120 µL of 100% methanol. The methanolic extract was centrifuged for 10 mins at 16,000 x *g* to remove any insoluble material before analysis by LC-HR-ESI MSMS on a Bruker MaXis Impact time of flight mass spectrometer equipped with a Dionex UltiMate 3000 high-performance liquid chromatography apparatus with conditions as described previously (Liu et al., 2015).

2.7 Synthesis of Surugamide A

Surugamide A was commercially synthesised through Synpeptide Co., Ltd (now Chinapeptides Co., Ltd). Once synthesised, further QC analyses were carried out at University of Leeds via LC-HR-ESI MSMS on a Bruker MaXis Impact time of flight mass spectrometer equipped with a Dionex UltiMate 3000 high-performance liquid chromatography apparatus with conditions as described previously (Liu et al., 2015).

2.8 Evaluation of antimicrobial activity

2.9.1 *In vitro* antibiotic susceptibility tests

Minimum inhibitory concentrations (MICs) of antibiotic compounds were determined by broth microdilution according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (Cockerill et al., 2012). Antifungal

activity was assessed in the same way, except LB was used instead of Mueller-Hinton broth II (MHBII), and MICs were read after 48 h of incubation at 30 °C. Positive growth controls and solvent controls were included in each experiment.

2.9.2 Checkerboard MICs

Compounds used in checkerboard assays in this study in conjunction with surugamide A were erythromycin (Sigma), cetyltrimethylammonium bromide (CTAB; BDH Laboratory Supplies), nisin (NBS Biologicals) and gramicidin D (Sigma). Stock solutions and serial dilutions of the drugs of interest were prepared to at least their respective MICs. The first antibiotic of the combination was serially diluted along the x-axis, while the second drug was serially diluted along the y-axis of 96 well plates. The indicator organism of choice (*S. aureus* SH100) was diluted in MHBII to OD_{625nm} 0.001 to achieve a cell number of ~ 5 x 10^5 cfu/mL. The diluted culture was then added to the wells to contain a total volume of 100 µL and was incubated shaking at 37 °C for 16 h (personal correspondence with Dr Arya Gupta and Dr Chris Randall). Synergism was calculated using the FIC index. The ΣFIC were calculated as follows: ΣFIC = FIC A + FIC B, where FIC A is the MIC of compound A in the combination/MIC of compound A alone, and FIC B is the MIC of compound B in the combination/MIC of compound B alone. The interaction was considered to be synergistic if the ΣFIC is ≤0.5, indifferent when the ΣFIC is >0.5 to <4 and antagonistic if the ΣFIC is ≥4 (Odds, 2003).

2.9.3 Bioassays with chemical extracts

From an overnight culture, the indicator organism of interest was diluted to OD_{625nm} 0.08125 in LB and was spread on an LB plate with no selection using a rotary platform to allow uniform distribution. Then, small sterilised circular paper discs (6 mm diameter) holding 60 µL of the chemical extract to be tested were placed on the plate and incubated at the appropriate temperature (37 °C for 16 h for *S. aureus* SH1000 and room temperature for 48 h for *C. albicans*) (personal correspondence with Dr Asma Akter).

2.9.4 Whole cell bioassays

Whole cell bioassays with *Streptomyces* strains on solid agar were done by inoculating the centre of an agar plate with 10 µL of spores, which were

allowed to grow for 7 days at 30 °C. The plates were then overlaid with 7 mL of soft nutrient agar (SNA) inoculated with 200 µL of an overnight culture of *S. aureus* SH1000 or *C. albicans.*

2.10 Generation of *S. albus* S4 Δ5

S. albus S4 Δ5 was generated in a multiparty effort with Dr Ryan Seipke and Ellie Harris. *S. albus* S4 Δ5 has mutations in the biosynthetic gene clusters (BGC) encoding the production of antimycin, candicidin, surugamide, albaflavenone and fredericamycin. Dr Seipke made the mutations in the antimycin, candicidin and fredericamycin BGCs. I made the mutations in the surugamide and albaflavenone BGCs. Ellie designed and made all the constructs for the CRISPR/Cas9 based deletions used in the generation of this strain.

Deletion of the antimycin BGC was carried out using Cos213 and the apramycin disruption cassette to delete the gene *antF* following the ReDirect PCR targeted mutagenesis as described previously (Gust et al., 2002) to generate *S. albus* S4 ∆*antF*. Next, *antABCDEFGHIJKLMNO* on Cos213 were replaced with the apramycin resistance cassette as above and then removed by the FLP recombinase, followed by replacement of the *bla* gene with the hygromycin resistance cassette from pIJ10701 as described previously (Gust et al., 2002) to generate Cos213 ∆antFLP B2H (Seipke, Barke, et al., 2011). This mutated cosmid was then mobilised into *S. albus* S4 ∆*antF.* A single transconjugant was chosen and passaged twice in the absence of selection before assessing apramycin and hygromycin sensitivity. An apramycin and hygromycin sensitive isolate was identified and was named *S. albus* S4 ∆1 and was confirmed by PCR using primers RFS236 and RFS237 (Seipke, Barke, et al., 2011). The candicidin BGC was disrupted using the previously constructed *fscC* (*STRS4_02234*) deletion plasmid, pKC1132-UpDn (Seipke, Barke, et al., 2011). The pKC1132-UpDn plasmid was introduced to *S. albus* S4 ∆1 and a single apramycin resistant transconjugant was selected and passaged as above until an apramycin-sensitive isolate was identified, and was named *S. albus* S4 ∆2.

The pCRISPomyces-2 system was used to disrupt the surugamide, fredericamycin and albaflavenone BGCs as described previously (Cobb et al., 2015). Briefly, single-guide RNA protospacers were generated for the

three BGS by annealing oligonucleotides EHS9 and EHS10 (surugamide), EHS3 and EHS4 (albaflavenone) and EHS7 and EHS8 (fredericamycin); the annealed DNA fragments were introduced into the BbsI site of the pCRISPomyces-2 plasmid by Golden Gate Assembly. Secondly, homology directed repair templates were generated by PCR for each of the three BGCs and were cloned into the XbaI site of the pCRISPomyces-2 plasmid using the NEBbuilder HiFi DNA assembly kit (New England Biolabs). The resulting CRISPR/Cas9 editing plasmids, pCRISPomyces-2-sur, pCRISPomyces-2-alb, pCRISPomyces-2-fdm were sequentially mobilised into *Streptomyces*. The temperature sensitive pCRISPomyces-2 plasmids were then cured from the transconjugants by passage in LB at 37 °C (twice) prior to the cultivation of a sporulated lawn on MS agar at 37 °C. Serial dilutions of the resultant spores were replica plated to examine apramycin sensitivity. Apramycin sensitive colonies were identified and were analysed for the respective deletion. The sequential mutations of the surugamide, albaflavenone and fredericamycin BGCs in *S. albus* S4 Δ2 resulted in the generation of the strains: *S. albus* S4 Δ3, *S. albus* S4 Δ4 and the final strain, *S. albus* S4 Δ5.

2.11 Bioinformatic prediction of putative proteins and amino acid sequence alignment

The sequence analysis tools, BLASTP (Altschul et al., 1990; Altschul et al., 1997) and InterPro (Mitchell et al., 2018), were used to predict the proteins encoded by the genes in the surugamide biosynthetic gene cluster by submitting the amino acid sequences of the respective genes to these platforms. Amino acid alignments were done using the Clustal Omega Tool (Sievers et al., 2011).

Chapter 3 Generation of a chromatogram-simplified *Streptomyces albus* S4 heterologous host

Abstract

Secondary metabolites are an important source of medically relevant compounds. Genome sequencing and genome mining of actinomycetes has revealed the presence of many silent biosynthetic gene clusters in their genomes, which are not expressed under standard laboratory conditions. These silent biosynthetic gene clusters have the potential to produce secondary metabolites with important bioactivities. Heterologous expression of biosynthetic gene clusters in an optimised host is a powerful strategy to activate silent gene clusters, to engineer variants and to optimise product yields. The availability of suitable hosts is therefore important in the continued discovery and production of new and useful secondary metabolites. In this study, a chromatogram-simplified *Streptomyces albus* S4 heterologous host was generated. Five gene clusters were targeted and disrupted in *Streptomyces albus* S4 to generate *Streptomyces albus* S4 Δ5. These gene clusters were targeted as they encode the production of known antifungal or antibacterial compounds. The resultant strain does not exhibit antifungal or antibacterial activity. Comparison of chromatograms of *Streptomyces albus* S4 Δ5 and the wild type *Streptomyces albus* S4 revealed that *Streptomyces albus* S4 Δ5 possess a much simpler chromatogram profile, which will ease the detection of compounds that are heterologously produced. Additionally, the cinnamycin encoding biosynthetic gene cluster from *Streptomyces cinnamoneus cinnamoneus* DSM 40005 was successfully expressed in *Streptomyces albus* S4 Δ5 demonstrating its utility. *Streptomyces albus* S4 Δ5 will be a useful tool in the continued discovery and production of new secondary metabolites.

3.1 Introduction

Genome sequencing and genome mining of actinomycetes have revealed the presence of many silent or cryptic biosynthetic gene clusters (BGCs) in their genomes. With that, one of the current challenges in natural product discovery is the development of strategies to activate and study the BGCs of interest (Ziemert et al., 2016; Bekiesch et al., 2016). The strategies to activate the silent BGCs can be divided into two major groups: pleiotropic approaches and cluster-specific approaches (both of which will be explored further in chapter 4) (Zhu et al., 2014; Ziemert et al., 2016). A different way to classify the strategies would be: i) activation of the BGCs in the natural host versus ii) the expression of the BGC heterologously in a surrogate strain (Gomez-Escribano and Bibb, 2011). Heterologous expression is the expression of BGCs from one organism in a surrogate host with a known secondary metabolome (Baltz, 2010; Gomez-Escribano and Bibb, 2011; Rutledge and Challis, 2015). Heterologous hosts are usually faster growers and genetically tractable, which enable genetic manipulation of BGCs even from genetically recalcitrant strains. Heterologous expression can be used to activate a silent BGC, confirm production of a compound by the putative gene cluster, to determine the borders of the BGC and to investigate the biosynthesis of secondary metabolites. It can also be used to yield the compounds, which can then be used for further studies. It is a powerful way of identifying novel compounds and to investigate their properties (Ongley et al., 2013; Rutledge and Challis, 2015; Bekiesch et al., 2016). Additionally, heterologous expression combined with rational engineering and the refactoring of BGCs in a genetically amenable host strain paves the way for combinatorial biosynthesis and molecular engineering to generate natural product variants (Ongley et al., 2013).

3.2 Heterologous expression is a powerful strategy

Many secondary metabolites have evaded discovery due to their biosynthetic pathways not being prevalent in the commonly isolated actinomycetes or because they are expressed poorly or not at all under standard fermentation conditions (Baltz, 2010). Modifications such as growth media variation, precursor feeding and manipulation of cluster-specific regulators can sometimes be used to induce or increase the production of the compound in the native host. However, despite such strategies, many BGCs still only produce the compounds at low yields in the native host (Zhang et al., 2011; Zhu et al., 2014; Baral et al., 2018). Additionally, the

slow growth and sparse sporulation of many strains coupled with the difficulties in their genetic manipulation is often challenging (Baltz, 2010; Bekiesch et al., 2016). Moreover, metagenomic sequences have revealed that out of the approximately 4 million rRNA sequences deposited, less than 1% of these belong to cultured microbes (Konstantinidis and Rosselló-Móra, 2015). Metagenomic sequencing has revealed the presence of many secondary metabolite BGCs in these difficult-to-culture microbes that are capable of producing interesting natural products (Katz et al., 2016). An important approach in overcoming these bottlenecks is to clone the BGC of interest to express them in a heterologous host optimised for production that is genetically amenable (Baltz, 2010; Gomez-Escribano and Bibb, 2011; Zhang et al., 2011; Ongley et al., 2013; Bekiesch et al., 2016; Katz et al., 2016).

To heterologously express a BGC, access to the sequence information and/or the genetic material is required along with tools for its cloning and a well characterised host. Genes encoding secondary metabolites are often clustered together in the genome, which makes cloning these BGCs relatively easier (Bentley et al., 2002; Zhang et al., 2011). Technologies have emerged to capture the pathway of interest to be heterologously expressed (Zhang et al., 2011). There are well established strategies for capturing the BGC of interest for heterologous expression. Target BGCs have classically been captured through the generation and screening of cosmid or bacterial artificial chromosome libraries. The development of direct cloning strategies that bypass library construction such as transformation-associated recombination, φBT1 integrase-mediated direct cloning and RecET mediated recombineering strategies have played a major role in allowing the BGCs to be more accessible for heterologous expression (Fu et al., 2012; Yamanaka et al., 2014; Du et al., 2015). The advances in gaining access to the BGCs have made heterologous expression a powerful tool in the activation and expression of BGCs.

3.3 Heterologous hosts

Successful expression of the BGC and production of the desired product is dependent on many factors and therefore, the choice of host is not trivial. The production of the compound is dependent upon the enzymatic and

metabolic capability of the host to support the biosynthesis of the secondary metabolites (Zhang et al., 2011). Factors such as growth rate, self-resistance if the compound exhibits antibacterial activity, metabolic flux and the availability of precursors play key roles (Zhang et al., 2011; Ongley et al., 2013; Baral et al., 2018). Additionally, the heterologous hosts themselves should be well characterised and genetically tractable (Bekiesch et al., 2016). *Escherichia coli*, *Saccharomyces cerevisiae*, *Bacillus subtilis* and *Pseudomonas* have all been shown to be used as heterologous hosts for the expression of secondary metabolite BGCs from streptomycetes (Zhang et al., 2011; Liu et al., 2016; Baral et al., 2018; Choi et al., 2018). However, it appears that evolutionarily closely linked organisms are most often better suited as hosts for the heterologous expression of BGCs (Ongley et al., 2013; Baral et al., 2018). It is important to consider the precursors required for the production of the target compound and therefore, it is not surprising that streptomycetes are among the most frequently used heterologous host strains, especially for BGCs originating from actinomycetes (Baltz, 2010; Ongley et al., 2013; Bekiesch et al., 2016). Phylogenetically related species will also possess a similar codon usage, which provides a greater translational efficiency compared to distantly related species (Ongley et al., 2013). There are a number of established actinomycete heterologous hosts that are genetically tractable such as *Saccharopolyspora erythraea*, *Salinispora tropica*, *Streptomyces avermitilis, Streptomyces coelicolor, Streptomyces lividans and Streptomyces albus* J1074 that have been used for this purpose (Baltz, 2010; Zhang et al., 2011; Huang et al., 2016; Jia Zhang et al., 2018). A few of the streptomycete heterologous hosts are discussed in this section.

S. avermitilis SUKA5, SUKA17 and SUKA22 are three examples of genome minimised *S. avermitilis* derived heterologous hosts. *S. avermitilis* is the industrial producer of avermectins and therefore, has a good supply of primary metabolic precursors, which is an important factor for a heterologous host (Luo et al., 2015). Several *S. avermitilis* strains have been developed as heterologous hosts with deletions of large DNA segments resulting in reduced genome sizes. These strains are mutants in the production of endogenous secondary metabolites found in the parent strain such as avermectins, filippin, oligomycin, geosmin, neopentalenolactone and a carotenoid, presenting a clean metabolic profile (Komatsu et al., 2010; Komatsu et al., 2013). *S. avermitilis* SUKA5 is deficient in the production of

avermectins, oligomycins and filippins while *S. avermitilis* SUKA17 is also deficient in the production of the terpenoid metabolites geosmin, neopentalenolactone and a carotenoid (Komatsu et al., 2010; Komatsu et al., 2013). With these deletions, approximately 78% of the putative transposase encoding genes were also deleted, which was suggested to further contribute to the stability of the engineered mutants (Komatsu et al., 2010; Komatsu et al., 2013; Ikeda et al., 2014; Luo et al., 2015). These engineered strains have been shown to successfully heterologously express a wide array of secondary metabolite BGCs with the yield of the compounds found to be greater in the engineered host when compared to the parent strain. For example, the heterologous production of chloramphenicol in *S. avermitilis* SUKA22 was ten-fold greater than its production in its native host *Streptomyces venezuelae* (Ikeda et al., 2014).

S. coelicolor M512, M1146, M1152 and M1154 are examples of heterologous host strains derived from the model streptomycete *S. coelicolor* A3(2) (Gomez-Escribano and Bibb, 2011; Bekiesch et al., 2016). *S. coelicolor* is known for its production of the bioactive secondary metabolites actinorhodin (Act), undecyleprodigiosin (Red), calcium-dependent antibiotic (CDA) and coelimycin (CPK). *S. coelicolor* M512 is a mutant in methylenomycin production via the loss of plasmids SCP1 and SCP2 as well as being deficient in the production of Act and Red via the deletion of the pathway specific regulatory genes *redD* and *actII-ORF4* (Floriano and Bibb, 1996). The quadruple deletion strain *S. coelicolor* M1146 is derived from *S. coelicolor* M512 with deletions in the BGCs encoding Act, Red, CDA and CPK possessing an even cleaner metabolic profile (Gomez-Escribano and Bibb, 2011). It has previously been shown that mutations in some genes encoding some of the ribosomal proteins or RNA polymerase can have a positive effect on antibiotic production (the basis for ribosome engineering in switching on silent BGCs) (Shima et al., 1996; Hu et al., 2002; Hosaka et al., 2009). *S. coelicolor* M1152 and M1154 are derived from *S. coelicolor* M1146 with an additional mutation in the RNA polymerase β-subunit encoding gene *rpoB* (M1152) or mutations in both the *rpoB* gene and the ribosomal gene *rpsL* encoding the ribosomal S12 protein (M1154) with the yield of the heterologously expressed compounds usually found to be greater in M1146 even though the growth and sporulation of the strains are slightly reduced (Jones et al., 2013).

S. lividans is very closely related to *S. coelicolor*, differing primarily only in horizontally acquired genes (Rückert et al., 2015). However, they dramatically differ in the expression of the commonly shared secondary metabolite BGCs. While Act, Red and CDA are all produced by *S. coelicolor* normally, the BGCs encoding these products are silent in *S. lividans* (Baltz, 2010). Upon deletion of the polyphosphate kinase gene (*ppk*), whose product (Ppk) is responsible for the formation of polyphosphate (a stock polymer of inorganic phosphate), the production of Act, Red and CDA was induced in *S. lividans* (Chouayekh and Virolle, 2002). Thus, the availability of inorganic phosphate plays a role in the biosynthesis of Act, Red and CDA in *S. lividans*. *S. lividans* TK24 is a plasmid free strain derived from *S. lividans 66*. It is a popular heterologous host especially as it can accept methylated DNA (Rückert et al., 2015). Additionally, its low protease activity has allowed it to be used as a heterologous expression/secretion system for proteins ranging from human cytokines to industrial enzymes such as cellulases (Rückert et al., 2015).

The readily transformable *S. albus* G expresses the restriction endonuclease *Sal*I. *S. albus* J1074 is derived from *S. albus* and is defective in *Sal*I. *S. albus* J1074 is an established heterologous host (Cox and Baltz, 1984; Baltz, 2010). For example, the heterologous expression of the BGC for isomigrastatin from *Streptomyces platensis* NRRL18993 in *S. avermitilis*, *S. coelicolor*, *S. lividans* and *S. albus* revealed that while the production in none of these heterologous hosts matched the production in the parental strain, production of isomigrostatin in *S. albus* J1074 was the closest with 46 mg/L compared to 58 mg/L in the parent strain (Feng et al., 2009). Additionally, *S. albus* J1074 (and *S. lividans*) was also able to heterologously express a BGC from a non-streptomycete marine actinomycete. The thiocoraline BGC from a marine *Micromonospora* strain was successfully expressed in both *S. albus* J1074 and *S. lividans* when the positive regulatory gene *tioA* was transcribed from the *ermEp** promoter (Lombó et al., 2006). As with *S. avermitilis*, reducing the genome size can increase the yield of secondary metabolites produced through heterologous production. The deletion of more than 1.4 Mb resulted in a genome size of approximately 7.62 Mb in *S. avermitilis,* which increased the stability of the strain as well as the yield of the secondary metabolites heterologously produced (Komatsu et al., 2010; Komatsu et al., 2013). *S. albus* J1074 has a naturally minimised genome (6.83 Mb) making it an attractive heterologous host. Additionally, *S. albus*

J1074 is a relatively fast grower and is genetic tractable, which are all important factors when choosing a heterologous host (Baltz, 2010; Zaburannyi et al., 2014).

3.4 *S. albus* S4 as a heterologous host

With the continued discovery of silent, new or interesting BGCs, it will be key to have a suite of *Streptomyces* hosts available to enable rapid identification and high-level expression of specific secondary metabolites (Baltz, 2010; Nah et al., 2017). *S. albus* J1074 has been successful in the heterologous production of a diverse array of secondary metabolites such as fredericamycin, isomigrastatin, napyradiomycin, cyclooctatin, thiocoraline and moenomycin (Wendt-Pienkowski et al., 2005; Lombó et al., 2006; Winter et al., 2007; Feng et al., 2009; Kim et al., 2009; Makitrynskyy et al., 2010). The phylogenetically closely related strain, *S. albus* S4, was originally isolated from the leaf-cutting *Acromyrmex octospinosus* ant colonies by bioprospecting in underexplored niches. (Barke et al., 2010; Seipke, Barke, et al., 2011; Seipke, 2015; Joynt and Seipke, 2018). *S. albus* J1074 and *S. albus* S4 share approximately 80% of the secondary metabolome, however, the genome of *S. albus* S4 is slightly larger (7.61 Mb) and harbours an additional six strain-specific BGCs with their products comprising 21% of its secondary metabolome (Seipke, Barke, et al., 2011; Seipke, Crossman, et al., 2011; Seipke, 2015). Two of the strain-specific BGCs in *S. albus* S4 encodes the production of kendomycin (a type I/III polyketide) and fredericamycin (a type II polyketide) (Seipke, 2015). The fast growth and genetic tractability of *S. albus* S4 coupled with its ability to produce a diverse array of secondary metabolites motivated the initiative to engineer this strain to be a heterologous host.

3.5 Aims and objectives

S. albus S4 produce two antifungals: antimycin and candicidin, but it does not produce an antibacterial compound under standard laboratory conditions (Seipke, Barke, et al., 2011). Submission of its genome sequence to the anitSMASH server (version 2.0) revealed that its genome harbours at least 28 BGCs (Figure 3.1) (Blin et al., 2013; Seipke, 2015). Even though the genome of *S. albus* S4 is predicted to encode secondary metabolite BGCs with the potential to produce metabolites with antibacterial activities, *S. albus* S4 does not exhibit antibacterial activity under standard laboratory conditions (explored further in Chapter 4) (Seipke, 2015). *S. albus* S4 is

predicted to have the potential to produce some known compounds with antibacterial activities such as albaflavenone, fredericamycin and a gramicidin-like compound, which was later identified to be surugamides (surugamides are explored further in Chapters 4 and 5) (Pandey et al., 1981; Gurtler et al., 1994; Takada et al., 2013; Seipke, 2015; Ninomiya et al., 2016). The albaflavenone and surugamide encoding BGCs are conserved in the core metabolome across *S. albus* species while the fredericamycin BGC is unique to *S. albus* S4 (Seipke, 2015).

Antimycin and candicidin are prominent in the chemical extracts prepared from *S. albus S4* (Seipke, Barke, et al., 2011). While no antibacterial activity is seen on the routinely used MS agar, culturing on alternative media conditions may elicit the production of these known antibacterial compounds (as is seen with surugamides, Chapter 4). Therefore, inactivation of these known BGCs is important in the generation of a heterologous host. To generate a chromatogram-simplified *S. albus* S4 heterologous host that is unable to produce any antifungal or antibacterial compounds under routinely used laboratory conditions, this project aims to:

- Generate an *S. albus* S4 strain compromised in production of antimycin, candicidin, albaflavenone, surugamide and fredericamycin (done in a multiparty effort with Dr Ryan Seipke and Ellie Harris)
- Compare the bioactivities of chemical extracts obtained from *S. albus* S4 WT and *S. albus* S4 Δ5 and compare the metabolic profile of the chemical extracts using analytical techniques such as HPLC
- Heterologously express a BGC to demonstrate its exogenous expression capability

Figure 3.1 antiSMASH predicted biosynthetic gene clusters encoded in the genome of *Streptomyces albus* S4. The BGCs targeted to mutate to generate *S. albus* S4 Δ5 are highlighted in red, and they are: antimycin, candicidin, albaflavenone, fredericamycin and surugamide encoding BGCs.

3.6 Results

3.6.1 Construction of *S. albus* S4 Δ5

S. albus S4 Δ5 was constructed by sequentially targeting the BGCs encoding the production of antimycin, candicidin, surugamide, albaflavenone and fredericamycin. The construction of *S. albus* S4 Δ5 is described in greater detail in Chapter 2 (Section 2.10). Dr Ryan Seipke previously made a strain deficient in the production of antimycin and candicidin (Seipke, Barke, et al., 2011), which was renamed *S. albus* S4 Δ2 in this work. An undergraduate student, Ellie Harris, designed and made the constructs for the CRISPR/Cas9 based deletions of surugamide (Δ3), albaflavenone (Δ4) and fredericamycin BGCs. I mutated the surugamide and albaflavenone BGCs and Ryan Seipke mutated the fredericamycin BGC. The overall integrity of the final strain, *S. albus* S4 Δ5 was verified by genome sequencing using Illumia MiSeq (Ryan Seipke, unpublished data).

3.6.2 *S. albus* S4 Δ5 does not exhibit antifungal activity or antibacterial activity

Ethyl acetate extracts were prepared from *S. albus* S4 WT and *S. albus* S4 Δ5 grown in two different liquid media: MS and GYM media (in duplicate). The chemical extracts prepared were checked for antibacterial activity using *Micrococcus luteus* and for antifungal activity using *Candida albicans*. As expected, the chemical extracts prepared from the media only controls of MS and GYM media did not exhibit any bioactivity (Figure 3.2). The chemical extracts prepared from *S. albus* S4 WT obtained from MS or GYM media did not show antibacterial activity against *M. luteus* (Figure 3.2). Only the *S. albus* S4 WT chemical extracts from MS media showed antifungal activity against *C. albicans*. The chemical extracts prepared from *S. albus* S4 Δ5 cultivated in MS or GYM media did not exhibit any antibacterial or antifungal activity (Figure 3.2).

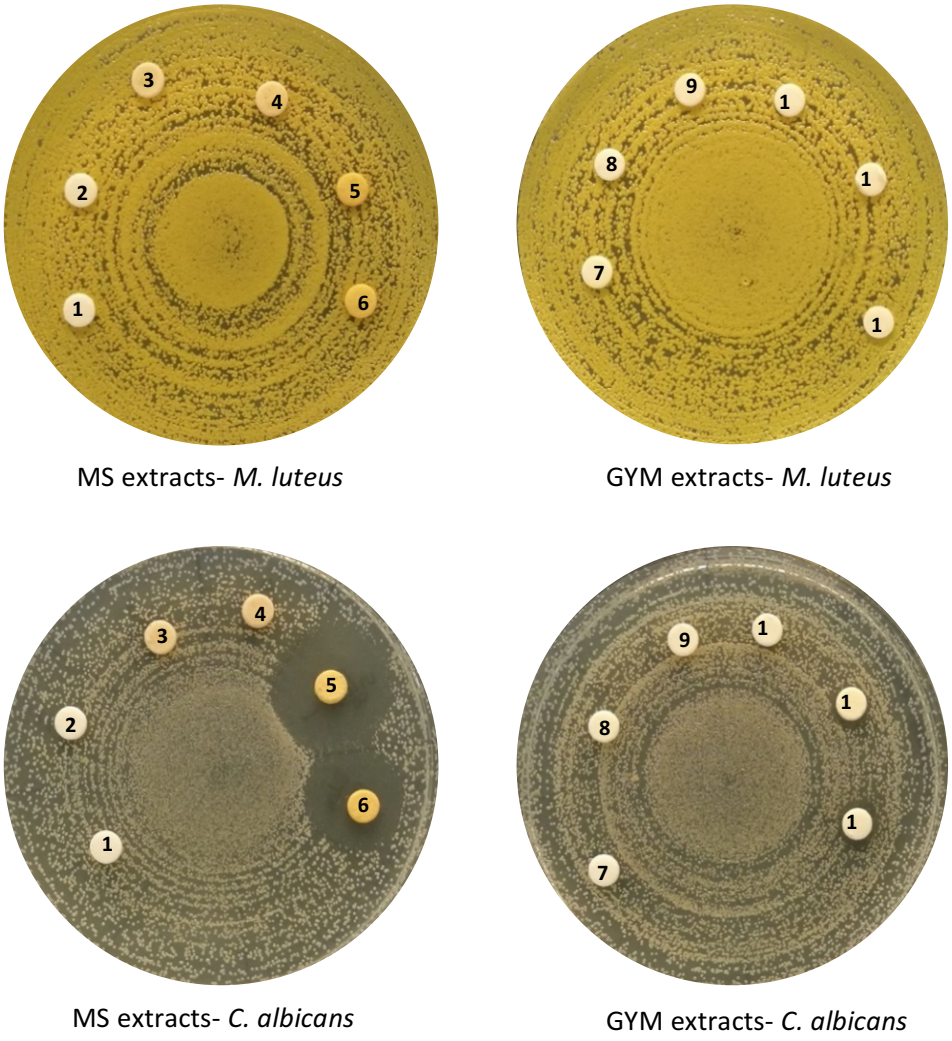


Figure 3.2 Antibacterial and antifungal bioassays of the chemical extracts prepared from *S. albus* S4 WT and *S. albus* S4 Δ5. The strains were cultivated in duplicate in liquid MS and GYM media. Antibacterial activity was assessed against *Micrococcus luteus* and antifungal activity was assessed against *Candida albicans*. The key for the samples are: 1- methanol control; 2- MS media only chemical extract; 3 & 4- MS media *S. albus* S4 Δ5 chemical extracts; 5 & 6: - MS media *S. albus* S4 WT chemical extracts; 7- methanol control, 8- GYM media only chemical extract; 9 & 10- GYM *S. albus* S4 Δ5 chemical extracts; 11 & 12- GYM *S. albus* S4 WT chemical extracts. Only *S. albus* S4 WT grown up in MS media exhibited antifungal activity. None of the other extracts exhibited antibacterial or antifungal activity.

3.6.3 *S. albus* S4 Δ5 has a simplified metabolic profile compared to *S. albus* S4 WT

The chemical extracts prepared from *S. albus* S4 WT and *S. albus* S4 Δ5 cultivated in MS and GYM media were analysed by HPLC to compare the metabolic profiles. The chromatograms of the elutions were monitored at 256 nm. Many of the metabolites produced by *S. albus* S4 WT in the routinely used MS media are absent in *S. albus* S4 Δ5 revealing a significantly less complex chromatogram background (Figure 3.3). Chemical extracts obtained from *S. albus* S4 WT and *S. albus* S4 Δ5 grown in GYM media did not show any obvious differences in the metabolic profile (Figure 3.3).

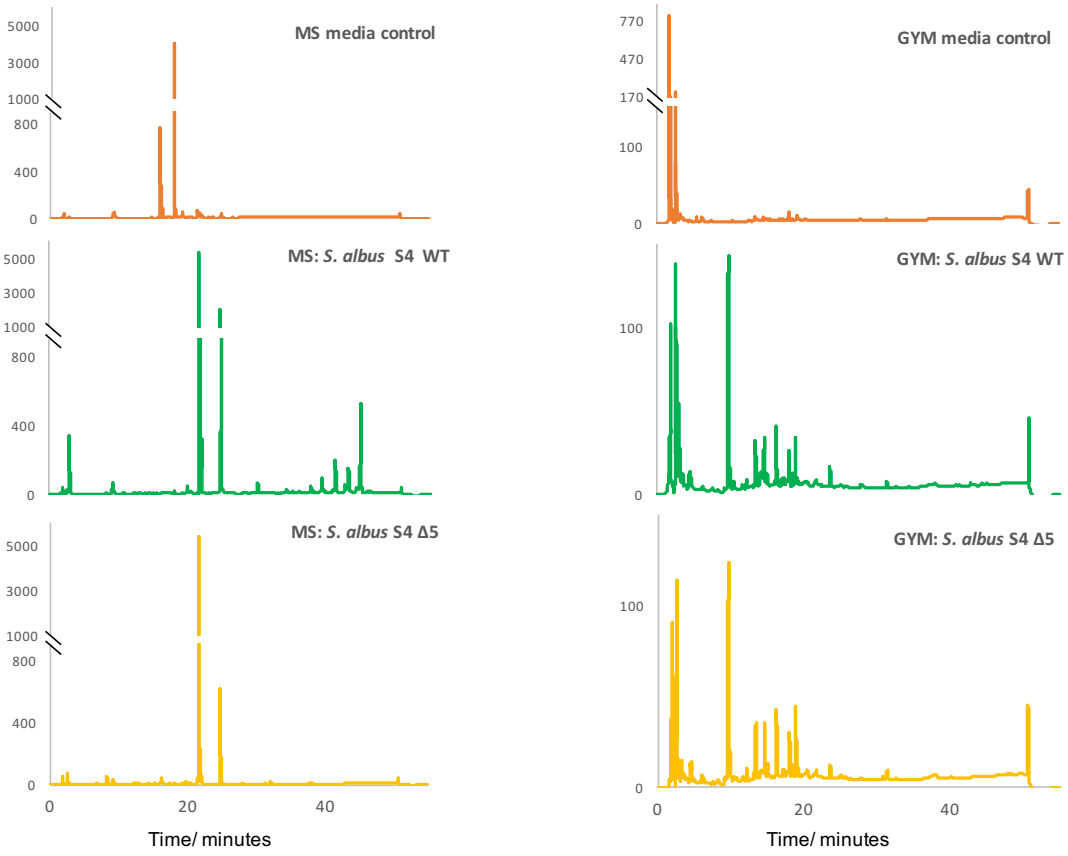


Figure 3.3 Comparison of the HPLC chromatogram profiles of the ethyl acetate extracts from *S. albus* S4 WT and *S. albus* S4 Δ5 cultivated in liquid MS and GYM media. The chromatogram profile of *S. albus* S4 Δ5 cultivated in MS media is cleaner and simpler than that of *S. albus* S4 WT cultivated in MS media. The latter peaks present in the wild type strain cultivated in MS media are absent in the engineered strain. No major differences were observed in the chemical extracts prepared from *S. albus* S4 WT and *S. albus* S4 Δ5 cultivated in GYM media.

3.6.4 Heterologous expression of the cinnamycin BGC in *S. albus* S4 Δ5

To demonstrate that *S. albus* S4 Δ5 can heterologously produce secondary metabolites, the lantibiotic cinnamycin encoding BGC from *Streptomyces cinnamoneus cinnamoneus* DSM 40005 was heterologously expressed. The previously described cinnamycin BGC encoding plasmid pIJ10109 was used to generate the recombinant strain *S. albus* S4 Δ5-cin2 (made by Ryan Seipke) (Widdick et al., 2003). Ethyl acetate extracts obtained from the cell pellets of *S. albus* S4 Δ5 and *S. albus* S4 Δ5-cin2 cultivated in liquid TSB were analysed by LC-HRMS. A compound consistent with the chemical formula and mass was only observed in the chemical extract from *S. albus* S4 Δ5-cin2 and as expected; no production was seen in the 'empty' host (Figure 3.4).

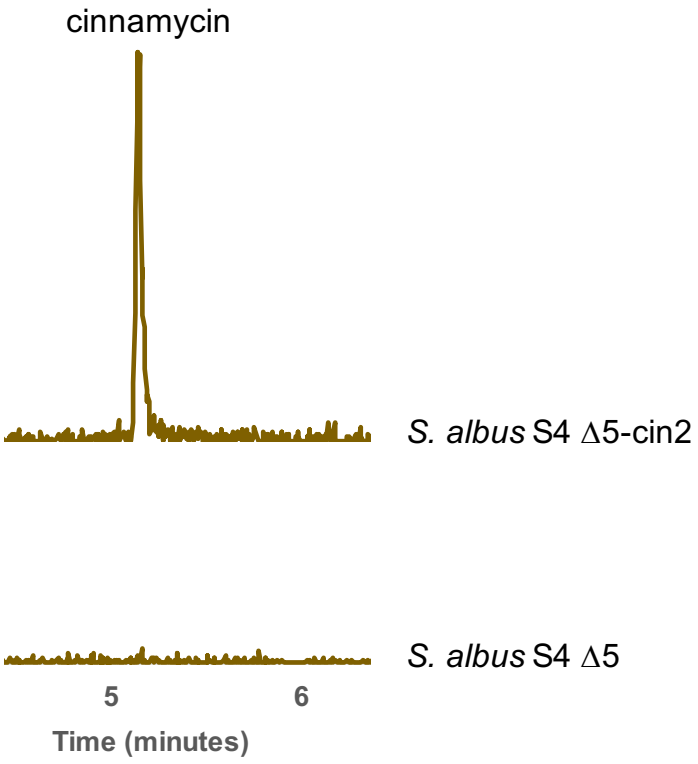


Figure 3.4 Heterologous production of cinnamycin in *S. albus* S4 Δ5. The EICs corresponding to the $[M+2H]^{2+}$ ions are shown for cinnamycin ($C_{89}H_{125}N_{25}O_{25}S_3$). (The y-axis is set to a maximum of 1.8×10^4). Cinnamycin production was observed in the recombinant strain *S. albus* S4 Δ5-cin2 while no production of cinnamycin was observed in the 'empty' heterologous host *S. albus* S4 Δ5

3.7 Discussion

Heterologous expression is a powerful strategy, which plays a key role in the activation of silent BGCs and production of secondary metabolites. Activation of BGCs and improving the yields of secondary metabolites within the native host is often extremely time consuming and laborious (Ongley et al., 2013). Heterologous hosts that are optimised for the production of secondary metabolites can allow faster screening and identification of the compounds with increased yields. It is an especially valuable tool in the expression of BGCs from unculturable or poorly cultivable microbes (Baltz, 2010; Zhang et al., 2011; Ongley et al., 2013). In this study, a chromatogram-simplified *S. albus* S4 ∆5 heterologous host was generated as a tool to aid in the continued discovery of secondary metabolites.

There are many factors to consider in the construction of an optimal host. Deletions in the genome to generate a genome minimised strain and inactivation of native BGCs make way for a cleaner metabolic profile. This is in turn allow the identification and purification of the heterologously produced compound easier and represent a valuable tool in identifying unknown compounds. Furthermore, these deletions increase precursor availability as there will be less competition with the reduced production of endogenous metabolites (Ongley et al., 2013; Bekiesch et al., 2016). Antimycin, candicidin, surugamide, albaflavenone and fredericamycin encoding BGCs in the *S. albus* S4 genome were targeted rationally to make way for a better heterologous host (Figure 3.1). Antimycin and candicidin are prominent in the metabolic profile of *S. albus* S4 grown under standard laboratory conditions (Seipke, Barke, et al., 2011). Therefore, these BGCs were targeted to abolish the production of these compounds. Additionally, both antimycin and candicidin are antifungal compounds (Seipke, Barke, et al., 2011). A heterologous host itself should preferably not exhibit any bioactivity as it can interfere with the screening of exogenous BGCs that may encode the production of bioactive secondary metabolites. The disruption of these BGCs was therefore desired to abolish the existing antifungal activity.

Although, *S. albus* S4 does not exhibit antibacterial activities under standard laboratory conditions, albaflavenone, surugamide and fredericamycin are known compounds with reported bioactivities. Therefore, to reduce the chances of rediscovery when bioactivity is observed as well as to make way

for a better heterologous host that can be used to screen under different conditions, these BGCs were also mutated. Chemical extracts prepared from *S. albus* S4 ∆5 did not exhibit any antifungal or antibacterial activity (Figure 3.2). Additionally, a cleaner metabolic profile is observed with the deletion of the five BGCs when grown in the routinely used MS media, which will make identification and purification of heterologously produced compounds easier. (Figure 3.3). Furthermore, even with the mutations in the five BGCs, *S. albus* S4 ∆5 sporulated and grew equally as well as the *S. albus* S4 WT strain (Ryan Seipke, unpublished data). While this work was in progress, Myronovskyi *et al.,* published a new heterologous host named *S. albus* Del14 (Myronovskyi et al., 2018). Fifteen BGCs in *S. albus* J1074 were mutated to generate *S. albus* Del14 and no secondary metabolites were detected in the chromatogram from the chemical extract prepared from *S. albus* Del14 (Myronovskyi et al., 2018). *S. albus* Del14 adds to the suite of heterologous hosts available and will be a valuable tool in the activation and/or expression of BGCs.

To demonstrate the potential of *S. albus* S4 ∆5 as a heterologous host, the 17 kb lantibiotic cinnamycin BGC from *Streptomyces cinnamoneus cinnamoneus* DSM 40005 was successfully heterologously expressed (Figure 3.4). Current work in the laboratory also aims to heterologously produce different types of secondary metabolites such as polyketides and non-ribosomal peptides along with the use of replicative and integrative vectors to further demonstrate the stability and utility of *S. albus* S4 ∆5 as a heterologous host. Additionally, comparing the production of the heterologously produced compounds in *S. albus* S4 ∆5 with other heterologous hosts can shed further light into the utility of *S. albus* S4 ∆5 as an optimal host. Reducing the genome size has been shown to have a positive impact on the heterologous expression of secondary metabolite BGCs as with *S. avermitilis* (Komatsu et al., 2010; Komatsu et al., 2013). Therefore, future work also aims to target known BGCs to further reduce the genome size along with creating an even more simplified chromatogram profile. As many of the BGCs in the genome in *S. albus* S4 are silent, these mutations can also pave the way to activate these BGCs with the increased precursor availability. Additionally, any cluster-specific approaches that may lead to the activation of these silent BGCs may be identified easier with the newly simplified chromatogram profile.

3.8 Conclusions

Though an ideal ‘super host’ strain is not yet available for the heterologous production of any and every BGC of interest, continued development of heterologous hosts will be of great value in the discovery and production of secondary metabolites. In this study, a new heterologous host*, S. albus* S4 ∆5 was generated. Five BGCs, antimycin, candicidin, albaflavenone, surugamide and fredericamycin BGCs were rationally chosen and mutated to generate *S. albus* S4 ∆5. The final strain has a cleaner metabolic profile than the wild type strain, which will aid in the identification and purification of any heterologously produced compounds. Chemical extracts prepared from *S. albus* S4 ∆5 did not exhibit any antifungal activity with the loss of candicidin and antimycin and no antibacterial was observed either. Furthermore, the utility of *S. albus* S4 ∆5 was demonstrated through the heterologous production of cinnamycin. With the wealth of information available from genome mining efforts about silent BGCs from individual and metagenome sequences, heterologous hosts provide conditions that enable the screening of cloned BGCs under optimal conditions (Ongley et al., 2013). *S. albus* S4 ∆5 was generated as a tool to aid in the continuing discovery and production of new and useful secondary metabolites.

Chapter 4 Identification of surugamides in *Streptomyces albus* S4 and the bioactivity profile of surugamides

Abstract

Genome mining has revealed the presence of many silent biosynthetic gene clusters in the genomes of streptomycetes with the potential to produce new and useful bioactive compounds. Approaches to awaken these silent gene clusters can be classified as pleiotropic approaches and cluster-specific approaches. The microbe of interest in this study, *Streptomyces albus* S4, does not produce any antibacterial compounds under standard laboratory conditions. However, genome mining has revealed the presence of biosynthetic gene clusters in its genome with the potential to produce antibacterial compounds. In this study, minimal medium agar facilitated the production of an antibacterial compound(s) in *S. albus* S4. Submission of the MSMS data prepared from the chemical extracts from *S. albus* S4 grown on minimal media to the GNPS platform identified surugamides within the chemical extract. Surugamide A was previously reported to have antibacterial activity against *Staphylococcus aureus*, however its mode of action is unknown. In this study, surugamide A was identified in the chemical extracts prepared from *S. albus* S4 and gene inactivation studies were used to link the compound to its gene cluster. Surugamide A was commercially synthesised to further investigate its bioactivity profile. Surprisingly, an MIC that is approximately 28 times greater than the reported MIC of surugamide A was observed. As the surugamide biosynthetic gene cluster also encodes the production of a linear peptide, surugamide F, the possibility of surugamide A working synergistically with linear peptide antibiotics was also investigated. In this study, surugamide A demonstrated synergism with gramicidin D.

4.1 Introduction

Genome mining of numerous *Streptomyces* spp. has revealed the presence of many silent biosynthetic gene clusters (BGCs) in their genomes. The published genomes of many actinomycetes have revealed that about 90% of the chemical potential of these microbes still remain to be discovered (Bentley et al., 2002; Wilkinson and Micklefield, 2007). These silent BGCs embody a large reservoir of therapeutic leads (Rutledge and Challis, 2015; Ziemert et al., 2016). Development of strategies to activate these BGCs is crucial in reaching the maximum potential of the genomics-driven endeavour in the discovery of novel antimicrobial compounds (Figure 4.1) (Rutledge and Challis, 2015). The methods developed thus far can be broadly divided into two major categories: pleiotropic approaches and cluster-specific approaches (Figure 4.1). Pleiotropic approaches hardly require any knowledge about specific gene clusters; rather, such approaches work by exerting their influence on more than one gene, triggering global changes and activating BGCs. On the other hand, cluster-specific approaches involve prioritisation of specific silent BGCs and allow more control. Such methods are particularly useful when information regarding the structural novelty of the predicted compound of a specific BGC can be garnered from bioinformatic analyses (Zhu et al., 2014; Ziemert et al., 2016). Though not exhaustively, some of these strategies are discussed in this chapter.

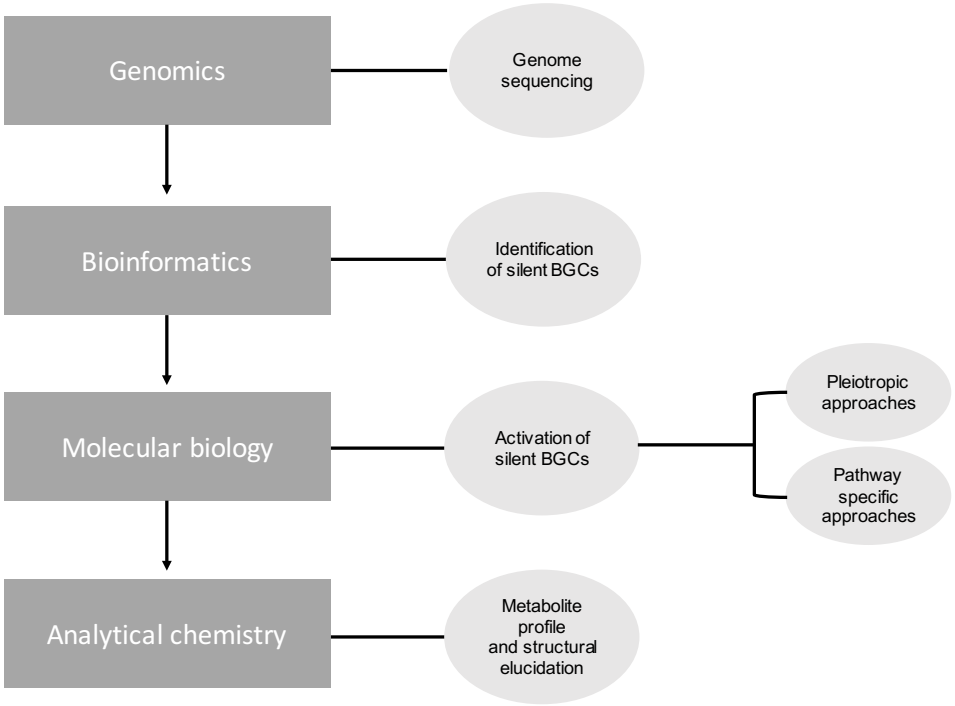


Figure 4.1 Genomics-driven activation of silent gene clusters summary

4.2 Pleiotropic approaches

4.2.1 Ribosome engineering

Due to an altered 30S ribosomal S12 protein responsible for streptomycin resistance, a *Streptomyces lividans* strain was able produce the blue pigmented antibiotic actinorhodin (Act) in ample amounts, which is an otherwise silent BGC in *S. lividans* (Ochi et al., 2004). Thus, it was proposed that modulation of ribosomal proteins or rRNA can lead to the activation of silent BGCs and the concept of 'ribosome engineering' was put forward (Ochi et al., 2004). Guanosine tetraphosphate (ppGpp), synthesised by the ribosomal enzyme ppGpp synthetase (*relA* gene product), is a bacterial alarmone involved in the stringent response (Artsimovitch et al., 2004). During the stringent response, uncharged tRNA, whose levels increase due to amino acid shortage, enter the A-site of the ribosome. When this happens, ppGpp synthetase utilises ATP and GTP to synthesise ppGpp. The alarmone ppGpp then binds directly onto the RNA polymerase and modulates transcription by up/downregulating various promoters (Ochi, 1987; Artsimovitch et al., 2004; Ross et al., 2013). In streptomycetes, morphological differentiation and antibiotic production are interlinked with the ribosomal machinery playing an essential role in the modulation of gene expression during nutrient limitation via ppGpp (Chakraburtty et al., 1996; Hoyt and Jones, 1999).

Therefore, it was postulated that mutations that can make the RNA polymerase to mimic the ppGpp bound conformation may result in the activation of silent BGCs (Ochi et al., 2004). As a result, ribosome engineering utilises sub-lethal concentrations of antibiotics that target the ribosome or RNA polymerase such as rifampicin, streptomycin and gentamycin with the hypothesis that altering the bacterial transcriptional and translational pathways can lead to enhanced levels of gene expression (Ochi et al., 2004; Ochi, 2007; Ochi and Hosaka, 2013). Induction of antibiotic producing silent gene clusters was observed in various actinomycetes by screening and selecting for spontaneous mutants of the genes encoding the RNA polymerase β-subunit (*rpoB*), S12 (*rpsL*) using gentamycin and streptomycin and the RNA polymerase targeting rifampicin, which led to the production and identification of a novel class of antibiotics called piperidamycins from *Streptomyces mauvelicolor* (Hosaka et al., 2009).

4.2.2 Variation in cultivation parameters

Variation in growth conditions is a relatively simple approach in exploiting the secondary metabolism potential of microbes. Changes in the media composition, aeration rate and culturing vessel are all changes that can contribute to the induction of silent BGCs and improvement in production titre (Bode et al., 2002). The term 'OSMAC' (one strain many compounds) was coined by Zeeck and coworkers to describe the ability of strains to produce different secondary metabolites when grown under different fermentation conditions (Bode et al., 2002). Using the OSMAC approach and eight different culture conditions, *Streptomyces* sp. strain C34 was able to produce the newly identified polyketides (PKs) chaxalactins A-C as well as the previously known compounds desferroxamine E, hygromycin A and 5''-dihydrohygromycin A (Rateb et al., 2011).

Using external signals called 'elicitors' is another recognised strategy in activating silent BGCs. The use of both chemical and biological elicitor molecules have proven to be successful in the activation of silent BGCs (Zhu et al., 2014). Chemical elicitation involves the use of compounds that are not of biological origin in the activation process that can lead to changes in the metabolite profile. Some examples of chemical elicitors include rare-earth elements, heavy metal ions and inorganic compounds (Abdelmohsen et al., 2015). Supplementation of culture media with scandium activated the production of Act in *S. lividans* (Kawai et al., 2007). A threshold concentration of ActII-ORF4, the cluster-specific primary transcriptional regulatory protein in Act production, is required for the efficient transcription of its associated genes for Act production. The addition of scandium enhanced the transcription of *actII-ORF4* to activate Act production in *S. lividans* (Kawai et al., 2007). Addition of other rare-earth elements such as lanthanum, yttrium, cerium and europium were also able to upregulate the production of Act in *S. coelicolor* (Kawai et al., 2007). Rare-earth elements are found around the globe, it is presumable that microbes have developed ways to respond to the low level concentrations of these elements to adapt to the conditions they are exposed to (Ochi and Hosaka, 2013).

A large-scale screen of streptomycetes with a compound collection encompassing 30,569 small molecules identified 19 compounds that could remodel the secondary metabolite profile of streptomycetes. Out of the 19

compounds, 4 compounds referred to as the antibiotic remodelling compounds (ARCs), ARC2, ARC3, ARC4 and ARC5 were able to induce the production of Act in *S. coelicolor*, of which ARC2 was the most active (Craney et al., 2012). LCMS (liquid chromatography mass spectrometry) analyses also revealed that the *S. coelicolor* treated with the triclosan like ARC2 showed elevated production levels of the pyranone germicidins A-C and a reduction in the production of prodiginines and the daptomycin-like calcium dependent antibiotic (CDA) (Craney et al., 2012). Addition of ARC2 to cultures of other actinomycetes such as *Kutzneria* sp. 744, *Streptomyces pristinaespiralis* ATCC 25486 and *Streptomyces peucetius* 27952 altered their secondary metabolite profiles also suggesting that ARC2 could be used as an elicitor across various actinomycetes (Craney et al., 2012). ARC2 shares structural similarity with the fatty acid synthesis inhibitor triclosan, which inhibits the enoyl-acyl carrier protein FabI. FabI is responsible for catalysing the final and rate limiting step of fatty acid synthesis. Secondary metabolism such as PK biosynthesis and fatty acid biosynthesis share the common precursors of acetyl- CoA and malonyl-CoA and so, ARC2 may be acting by allowing these precursors to flow to secondary metabolism instead by partially inhibiting fatty acid biosynthesis (Craney et al., 2012).

The addition of organic compounds such as dimethyl sulfoxide (DMSO) and ethanol can induce antibiotic production in some streptomycetes. The production of tetracenomycin C and chloramphenicol increased about 3-fold in *Streptomyces glaucescens* and *Streptomyces venezuelae,* respectively and the production of thiostrepton increased around 2-fold in *Streptomyces azureus* in the presence of 3% DMSO (Chen et al., 2000). Addition of ethanol (6%) dramatically increased the production of jadomycin B in *S. venezuelae* (Doull et al., 1994). Organic compounds such as DMSO and ethanol cause mistranslation, which probably results in the induction of a stress response and the promotion of secondary metabolism (Pettit, 2011). Inhibitors of the zinc containing enzymes and histone deacetylases (HDACs) such as sodium butyrate can activate antibiotic production via epigenetic perturbation (Moore et al., 2012). In eukaryotes, HDACs influence chromatin structure and gene expression by antagonizing histone acetylation (Pazin and Kadonaga, 1997). *S. coelicolor* harbours three HDAC-like genes. Sodium butyrate showed both stimulatory and inhibitory effects on the production of Act in *S. coelicolor* depending on the agar medium used. While addition of sodium butyrate to minimal medium agar activated Act production

in *S. coelicolor*, repression of production was observed on the otherwise permissive R5 medium (Moore et al., 2012). A similar pattern of production was also observed with *N*-acetyl glucosamine (GlcNAc) under poor nutrient and nutrient rich conditions (Rigali et al., 2008).

Components of the cell wall, microbial hormones or molecules involved in signalling carbohydrates or cell derived biopolymers have also been successfully used as elicitors to activate the production of secondary metabolites. GlcNAc, the peptidoglycan cell wall constituent and chitin monomer elicited Act and undecylprodigiosin (Red) production in *S. coelicolor* under poor nutrient (famine) conditions via the de-repression of DasR- a GntR-family regulator. High concentrations of GlcNAc (5-10 mM) activated development and production of antibiotics under famine conditions and blocked antibiotic production under nutrient rich (feast) conditions (Rigali et al., 2008). The DNA binding sites of DasR (*dre*) are located upstream of the genes encoding activators of Act and Red. Electrophoretic mobility shift assays and reverse transcription-PCR revealed that the binding of DasR to the *dre* regions resulted in the down-regulation of antibiotic production (Rigali et al., 2006; Rigali et al., 2008). The PEP-dependent phosphotransferase system PTS is responsible for the import of GlcNAc, which is then deacetylated to form glucosamine-6-phosphate (GlcN-6P). GlcN-6P is a ligand for DasR and its allosteric binding to DasR reduces the affinity of DasR for its binding sites resulting in the de-repression and production of the antibiotics (Rigali et al., 2006; Rigali et al., 2008; Nothaft et al., 2010). Treatment of *Streptomyces clavuligerus*, *S. griseus*, *Streptomyces hygroscopicus*, *S. venezuelae* and *Streptomyces collinus* with GlcNAc also activated antibiotic production under famine conditions (Rigali et al., 2008) Rigali *et al*., found the stimulating effect of GlcNac to be common among various streptomycetes, but not universal (at least not under the conditions studied) and so, elicitation using GlcNAc in streptomycetes is another promising strategy in triggering secondary metabolism (Rigali et al., 2008).

Microbial hormones such as γ-butyrolactones play a major role in regulating morphological differentiation and secondary metabolism in streptomycetes (Hsiao et al., 2009). As with the A-factor in *S. griseus,* many γ-butyrolactones are involved in the regulation and production of secondary metabolites in other streptomycetes such as *Streptomyces virginiae* and

Streptomyces aurefaciens, and the production of virginiamycin and auricin, respectively (Kawachi et al., 2000; Novakova et al., 2011; Zhu et al., 2014). Addition of exogenous γ-butyrolactones can also be used as environmental cues to trigger secondary metabolite production. An example is the production of pimaricin by *Streptomyces natalensis* that is triggered by the addition of *S. griseus* A-factor. In *S. natalensis*, the hydrophilic autoinducer molecule PI factor is normally responsible for the production of pimaricin. *S. natalensis* mutant strain lacking the PI factor is unable to produce pimaricin and production is restored upon addition of the PI factor (Recio et al., 2004). Surprisingly, the production of pimaricin was also restored by the addition of A-factor from *S. griseus,* thus demonstrating the fact that the exogenous addition of these small molecules can also aid in the activation of secondary metabolism (Recio et al., 2004).

Addition of microbial lysates can also elicit antibiotic production in actinomycetes. While pure cultures of *S. coelicolor* only produced low concentrations of Red, the treatment of cultures with live and heat killed *Bacillus subtilis* increased the yield of Red (Luti and Mavituna, 2011; Jaber et al., 2014). On solid medium, the production of Red was observed at the interface between *S. coelicolor* and *B. subtilis* and no production of Red was seen with *S. coelicolor* grown alone (Luti and Mavituna, 2011). Another study, which utilised heat killed *B. subtilis* and *S. aureus* cells, also found upregulated production of Red with a higher yield observed with heat killed *S. aureus* (Luti and Mavituna, 2011). As *S. coelicolor* and *B. subtilis* are both found in the soil, *S. coelicolor* probably has recognition mechanisms in place leading to interspecies interactions (Luti and Mavituna, 2011). The higher production of the antibiotic with the heat killed *S. aureus* may be because *S. aureus* is more foreign to *S. coelicolor* and therefore, simulates a more competitive environment (Abdelmohsen et al., 2015).

4.2.3 Co-cultivation

Another approach in activating silent BGCs is via the co-cultivation of microorganisms. The vast structural diversity observed among the natural products is thought to have evolved through the interactions with other microbes and higher organisms and as a result, being produced only at certain times (Zhu et al., 2014). In nature, many secondary metabolites will only be produced upon detection and receipt of specific signals from their

surroundings or surrounding microbes. Streptomycetes are predominantly soil-dwelling microbes that exist with other microbes. Their interactions with other microbes in the soil therefore has the potential to activate gene clusters to initiate secondary metabolite production (Zhu et al., 2014).

The intricate mechanisms involved in such interactions are not well understood but the activation or repression of metabolite production may be via physical cell-to-cell interactions, via small molecules such as quorum sensing molecules or siderophores, horizontal gene transfer or through the catalytic activation of precursor metabolites (Abdelmohsen et al., 2015). For example, the co-cultivation of *S. lividans* with *Tsukamurella pulmonis*, a mycolic-acid containing actinomycete induced the production of Act and Red in *S. lividans* via cell-to cell interactions (Onaka et al., 2011). Members of the *Corynebacterium* genus along with other closely related strains of *T. pulmonis* were also able to activate the production of the pigment in *S. lividans* (Onaka et al., 2011). In these cell-to-cell interactions, mycolic-acid was identified to be essential. The mycolic-acid mutant of *Corynebacterium glutamicum* was unable to stimulate the production of Red in *S. lividans*. Additionally, the treatment of the co-culture with a mycolic-acid inhibitor also inhibited the production of the red pigment (Onaka et al., 2011).

Co-cultivation of streptomycetes with predator microbes can also induce antibiotic production. Cultivation of *S. coelicolor* with one its predatory microbes, *Myxococcus xanthus*, induced the production of Act (Pérez et al., 2011). The Act producing *S. coelicolor* colonies also repelled the growth of *M. xanthus* around the colonies. Actinorhodin is an antibiotic that shows antibacterial activity against Gram-positive bacteria (Mak and Nodwell, 2017). The inhibition in growth of the Gram-negative *M. xanthus* strain around the Act producing *S. coelicolor* strain suggests that *S. coelicolor* might be able to use Act as a repellent signal against *M. xanthus* (Pérez et al., 2011).

4.3 Cluster-specific approaches

The incredible amount of genome sequence data available has led to the development of sophisticated bioinformatic tools for the characterisation of BGCs. For instance, the computational tool antiSMASH (antibiotics & Secondary Metabolite Analysis Shell) can identify and predict known classes

of secondary metabolite BGCs in the genome sequence. AntiSMASH is also able to provide comprehensive functional annotations of biosynthetic systems such as non-ribosomal peptide synthetase (NRPS) and polyketide synthetase (PKS) systems identified and can predict the possible chemical structures of the compounds predicted to be produced by these megaenzymes (Medema et al., 2011). Advances in bioinformatics have aided researchers in prioritising silent BGCs, which are predicted to encode the production of potentially novel secondary metabolites, thereby lowering the chances of rediscovery (Rutledge and Challis, 2015). Cluster-specific approaches are of great use when a novel structure is predicted via bioinformatics as well as when pleiotropic approaches fail to bear fruit (Zhu et al., 2014; Rutledge and Challis, 2015).

4.3.1 Manipulation of gene cluster specific regulatory genes

Insights into the composite regulation of secondary metabolites in actinomycetes has been invaluable in natural product discovery. Gene cluster specific transcriptional activators or repressors often control the expression of BGCs and the genes encoding these regulators are commonly found within the gene cluster itself. Overexpression of gene cluster specific activator genes or deleting the genes encoding repressors in the BGC is a common and promising strategy in awakening silent BGCs (Baral et al., 2018). Though not exhaustive, some examples where overexpression of activator genes or deletion of repressor genes has led to the activation of silent BGCs are described below.

One of the earliest examples of its use was carried out in *Streptomyces ambofaciens* with the stambomycin BGC. The genome of *S. ambofaciens* harbours a large PKS BGC and transcriptional analysis revealed that this BGC was not expressed under standard laboratory growth conditions. *In silico* analyses predicted that the regulatory gene *samR0484* located within the BGC encodes a putative activator belonging to the LAL (Large ATP binding regulators of the LuxR family) family. Subsequently, the constitutive expression of this activator triggered the production of stambomycins (Laureti et al., 2011). Many such LAL activators have since been identified in the genomes of various actinomycetes (Laureti et al., 2011). Recently, the overexpression of a pathway specific LAL-family activator in *Streptomyces pactum* activated the production of totopotensamides (Chen et al., 2017).

The chattamycin BGC in *Streptomyces chattanoogensis* L10 was successfully activated via the overexpression of a putative pathway specific activator encoded by *chaI,* which led to the discovery of two angucycline antibiotics- chattamycins A and B (Zhou et al., 2015). Thus, the constitutive expression of pathway specific activators is a powerful tool in the activation of silent gene clusters.

Inactivation of repressors is another common strategy used to activate silent BGCs. In *S. ambofaciens*, the kinamycin antibiotics were discovered following the deletion of the gene *alpW,* which encodes a repressor of the otherwise silent type II PKS system (Bunet et al., 2011). Another example is the induced production of gaburedins, a new family of γ-aminobutyrate ureas, in *S. venezuelae* via the deletion of a transcriptional repressor GbnR (Sidda et al., 2014). Another strategy that has the potential to activate silent BGCs is reporter-guided mutant selection. This strategy uses genome scale random mutagenesis to awaken cryptic clusters with a promoter-reporter selection system for successfully identifying the activated mutants based on specific phenotypes. Reporter-guided mutant selection was used to activate a silent gene cluster in *Streptomyces* sp. PGA64, which in turn produced two new anthraquinone aminoglycosides (Guo et al., 2015).

4.3.2 Refactoring

The affordable costs of DNA synthesis in recent years and the emergence of new metabolic engineering strategies has made refactoring BGCs possible. Refactoring of a BGC involves the replacement of the native host promoter of the gene cluster of interest with constitutive or inducible promoters (Baral et al., 2018). For example, a CRISPR-Cas9 knock in strategy, which can introduce heterologous promoters, was used to activate various BGCs in *Streptomyces* species. This strategy was used to activate the production of indigoidine in *Streptomyces albus* with the introduction of the strong promoter *kasO**p upstream of the biosynthetic gene. The CRISPR-Cas9 knock-in strategy was also successful in activating previously uncharacterised silent BGCs in *Streptomyces roseosporus*, *S. venezuelae* and *Streptomyces viridochromogenes* (Zhang et al., 2017). In another study, the insertion and expression of the promoter *ermE**p in a silent gene cluster in *S. albus* J1074 activated the production of two new alteramide derivatives (6-epi-alteramides A and B) (Olano et al., 2014). Refactoring is often used in

heterologous expression where the heterologous host of a minimalised secondary metabolite profile is optimised for production (explored further in chapter 3). Upon successfully cloning and expressing the BGC in an optimised heterologous host, it can help confirm the production of the compounds by the putative cluster, investigate the biosynthesis of natural products and yield the compounds which can then be used for further studies (Gomez-Escribano and Bibb, 2011).

4.3.3 Gene knock out studies or heterologous expression combined with comparative metabolite analyses

For biosynthetic systems where bioinformatic tools are not yet able to predict substrate specificity with confidence, two fruitful strategies that can be utilised are gene knock out/comparative metabolic profiling and heterologous gene expression/comparative metabolic profiling (Corre and Challis, 2007). In gene knock out/comparative metabolic profiling studies, genes from the BGC of interest identified via genome mining that are hypothesized to be required for the metabolite production are first inactivated and the metabolic profile of the culture supernatants of mutant is compared with that of the wild type using analytical techniques such as LCMS. The metabolites that are present in the wild type but not present in the non-producing mutant are likely the products of the gene cluster of interest. These metabolites can then be isolated and characterised structurally (Corre and Challis, 2007; Challis, 2008b). In heterologous expression/comparative metabolic profiling studies, the entire gene cluster is cloned and is expressed in a heterologous host optimised for the production. The metabolic profile of the heterologous host expressing the gene cluster is compared to the same host lacking the gene cluster via analytical techniques. And as with the first approach, the metabolites that are present in the gene cluster containing host but not found in the ‘empty’ host are likely the products of the gene cluster (Corre and Challis, 2007; Challis, 2008b).

4.4 Limitations of pleotropic approaches and cluster-specific approaches

Both pleiotropic and pathway specific approaches have demonstrated the potential of microbes in producing structurally diverse metabolites with interesting bioactivities. Though specific approaches have their benefits,

there are also various limitations to both (Rutledge and Challis, 2015). In comparison to cluster-specific approaches, pleiotropic approaches enable a higher throughput even when very little is known about the regulation of the silent BGC. Pleiotropic approaches can also be applied to a wide variety of strains. However, with such approaches, the results are extremely hard to predict as they are intrinsically empirical. The induction of many BGCs at the same time brings about global changes in the cell and can make identification and isolation of new metabolites extremely difficult (Rutledge and Challis, 2015). On the other hand, gene cluster specific approaches provide more predictability and control. However, these approaches are generally lower throughput compared to pleiotropic approaches as it requires specific strategies for the awakening of each silent BGC and as a result, can be extremely time consuming. Insight is often required about the biosynthetic and regulatory genes of the BGC involved in the production of the metabolite of interest too (Rutledge and Challis, 2015). The regulatory systems are complex, with multiple players involved. Cluster-situated and global regulators can add multiple layers of regulation and can complicate cluster-specific approaches (Liu et al., 2013). Additionally, such approaches can be extremely challenging if the source organism is not genetically amenable (Rutledge and Challis, 2015).

4.5 Silent biosynthetic gene clusters in *Streptomyces albus* S4

Streptomyces albus S4 was originally isolated from the leaf-cutting *Acromyrmex octospinosus* ant colonies (Barke et al., 2010; Seipke, Barke, et al., 2011). The *A. octospinosus* ant species form a mutualistic relationship with the fungus *Leucoagaricus gongylophorus* exchanging food, protection and transport services with the fungal cultivar (Schultz and Brady, 2008). The fungal garden is prone to attack from different microbes such as the necrotrophic fungus genus *Escovopsis*. The ants help defend the fungal garden though grooming, weeding, antifungal secretions and through the application of weed-killers (Schultz and Brady, 2008). The ants also form a symbiotic relationship with actinomycetes such as those belonging to the genera *Pseudonocardia, Amycolatopsis* and *Streptomyces* (Currie et al., 1999; Sen et al., 2009; Haeder et al., 2009). It is thought that the actinomycete bacteria associated with the ants help protect the fungal garden through the production of antimicrobial compounds (Currie et al., 1999; Haeder et al., 2009).

When cultivated using mannitol-soya agar, *S. albus* S4 produces antimycin and candicidin antifungals, but it does not produce an antibacterial compound (Figure 4.2) (Barke et al., 2010; Seipke, Barke, et al., 2011; Seipke, Crossman, et al., 2011). Submission of its genome sequence to the antiSMASH server (version 2.0) revealed that its genome encodes at least 28 BGCs (Figure 4.3) (Blin et al., 2013; Seipke, 2015). Of the 28 predicted BGCs, some are predicted to produce secondary metabolites that may have antibacterial properties (Seipke, 2015). For example, BGC 19 is predicted to encode a terpene biosynthetic system with the putative product predicted to be albaflavenone (Seipke, Barke, et al., 2011; Seipke, 2015). Albaflavenone is a volatile tricyclic sesquiterpene with antibacterial activity (Gurtler et al., 1994; Moody et al., 2012). BGC 22 is an NRPS system whose putative product is predicted to be a lipoglycopeptide- an established antibacterial class against Gram-positive bacteria (Kahne et al., 2005; Seipke, Barke, et al., 2011; Seipke, 2015). Another example is BGC 28, which is an NRPS biosynthetic system that is predicted to produce a gramicidin-like compound (Seipke, Barke, et al., 2011). The albaflavenone and gramicidin-like compound encoding gene clusters (BGCs 19 and 28, respectively) are conserved in the core secondary metabolome across *S. albus* strains, while the lipoglycopeptide BGC is unique to *S. albus* S4 (Seipke, 2015). Therefore, genome mining revealed that the genome of *S. albus* S4 harbours silent gene clusters, which hold the potential to encode the production of secondary metabolites with antibacterial properties.

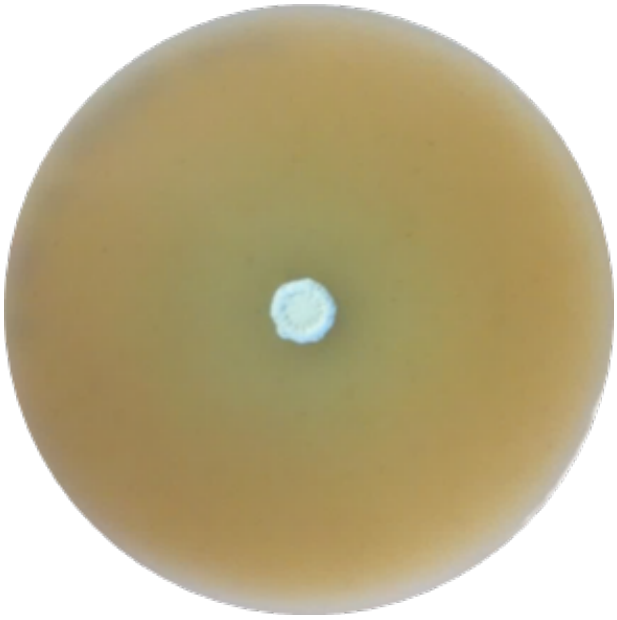


Figure 4.2 *S. albus* S4 does not exhibit antibacterial activity on mannitol-soya flour agar. A whole cell bioassay of *S. albus* S4 challenged with *S. aureus* SH1000.

Figure 4.3 antiSMASH predicted biosynthetic gene clusters harboured in the genome of *S. albus* S4 (antiSMASH version 2.0).

4.6 Aims and objectives

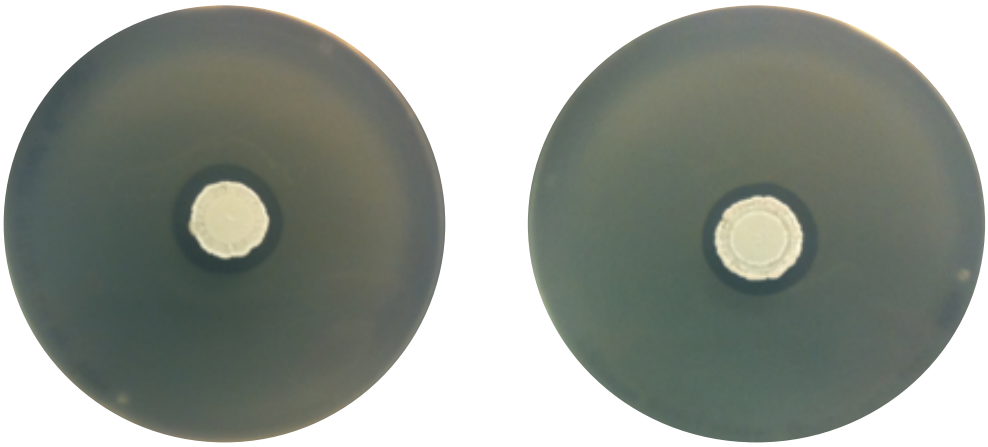
As the genome of *S. albus* S4 encodes silent BGCs with the potential to make secondary metabolites with antibacterial properties, this study aimed to:

- Activate the production of an antibacterial compound(s) using pleiotropic strategies
- Identify and chemically characterise the activated antibacterial compound(s)
- Characterise the bioactivity of the activated antibacterial compound(s)

4.7 Results

4.7.1 Minimal medium facilitates the production of an antibacterial compound in *S. albus* S4

S. albus S4 does not exhibit antibacterial activity on the routinely used MS agar (Figure 4.2). However, mannitol minimal medium (MM, containing 25 mM mannitol) facilitated the production of an antibacterial compound(s). *S. albus* S4 and *S. albus* Δ*ant*Δ*can* (mutant in antimycin and candicidin production and therefore, a cleaner metabolic profile for LC-HRMS analysis) strains both exhibited antibacterial activity against *S. aureus* SH1000 (Figure 4.4). Since the zone of inhibition observed on MM agar although reproducible, was relatively small, elicitor molecules were used to try and enhance the antibacterial activity seen. Following the findings of Rigali *et al.,* the cell-wall component GlcNAc was tested as a chemical elicitor at a final concentration of 10mM under famine (MM) conditions. However, the addition of GlcNAc had no additive effect on the antibacterial activity (Appendix, Figure A2.1). The sizes of the zones of inhibition observed on MM agar remained similar to the sizes observed with the addition of 10 mM GlcNAc. The addition of sodium butyrate to MS agar had no phenotypic effect and no antibacterial activity was observed with *S. albus* S4 (Figure A2.1). Surprisingly, on MM, the addition of sodium butyrate switched off the antibacterial activity (Figure A2.1).



S. albus S4 WT | *S. albus* S4 ΔantΔcan

Figure 4.4 Minimal medium facilitates the production of an antibacterial compound in *S. albus* S4 WT. *S. albus* S4 WT and *S. albus* S4 Δ*ant*Δ*can* (mutant in antimycin and candicidin production) both exhibit antibacterial activity against *S. aureus* SH1000 on MM.

4.7.2 Surugamides identified in the *S. albus* S4 chemical extracts prepared from MM

Ethyl acetate extracts prepared from *S. albus* S4 grown on MM agar retained antibacterial activity (Figure A2.2) and were analysed by LC-HRMSMS. The MSMS data obtained was submitted to the GNPS (Global Natural Products Social molecular networking) infrastructure (Mohimani et al., 2016; Wang et al., 2016). One of the tools the GNPS platform offers is DEREPLICATOR. DEREPLICATOR compares the fragmentation patterns of peptidic natural products within the experimental MSMS input data against a database of theoretical MSMS spectra of a chemical library. The dereplication algorithms identify peptide spectrum matches and statistically significant matches based on similarities are reported (Mohimani et al., 2016). In this study, the GNPS platform identified antimycin (antifungal), homosalate (hormone disruptor), cyclopentyladenosine (A1 adenosine receptor agonist), benzalkonium chloride (BAC, a quaternary ammonium compound) and the GNPS DEREPLICATOR tool identified surugamide A (SA) and D within the chemical extract (Figure A2.3). Of the compounds detected by GNPS, two are reported to have antibacterial activity. BAC is a cationic surfactant often used in disinfectants owed to its biocidal properties (Kim, Hatt, et al., 2018; Kim, Weigand, et al., 2018). Additionally, SA has

been reported to have antibacterial activity (Wang et al., 2014). While BAC is a broad-spectrum biocide, SA is a non-ribosomal peptide (NRP) with specific bioactivity and an unknown mode of action (Wang et al., 2014; Kim, Hatt, et al., 2018). Therefore, SA was chosen to be investigated further.

Surugamides are a group of NRPs that were first identified from the marine *Streptomyces* sp. JAMM992 (Takada et al., 2013). The *sur* BGC encodes the production of five cyclic octapeptides surugamides A-E (SA-SE) and a linear decapepetide named surugamide F (SF) (Takada et al., 2013; Ninomiya et al., 2016). A schematic diagram of the surugamide (*sur*) BGC from *S. albus* S4 is presented in Figure 4.5. SA was previously reported to have antibacterial activity against *S. aureus.* The surugamide BGC is further explored in chapter 5. Here, the fragmentation pattern of SA from the MSMS data from *S. albus* S4 chemical extract matched the fragmentation pattern of SA in the GNPS database (Figure A2.4). Further LC-HRMS analyses confirmed the presence of SA in the chemical extract prepared from *S. albus* S4 Δ*ant*Δ*can* (Figure 4.5). In order to corroborate these data, a CRISPR/Cas9 mutational strategy was used to mutagenize the *sur* BGC, which resulted in the generation of the strain Δ*ant*Δ*can*Δ*surA* (Figure A2.5; the generation of *S. albus* S4 Δ*ant*Δ*can*Δ*surA* was done together with Ellie Harris). Ethyl acetate extracts prepared from *S. albus* S4 Δ*ant*Δ*can*Δ*surA* were analysed by LC-HRMS to check for the presence/absence of SA. Deletion of *surA* abolished the production of SA, linking the compound to the BGC (Figure 4.5).

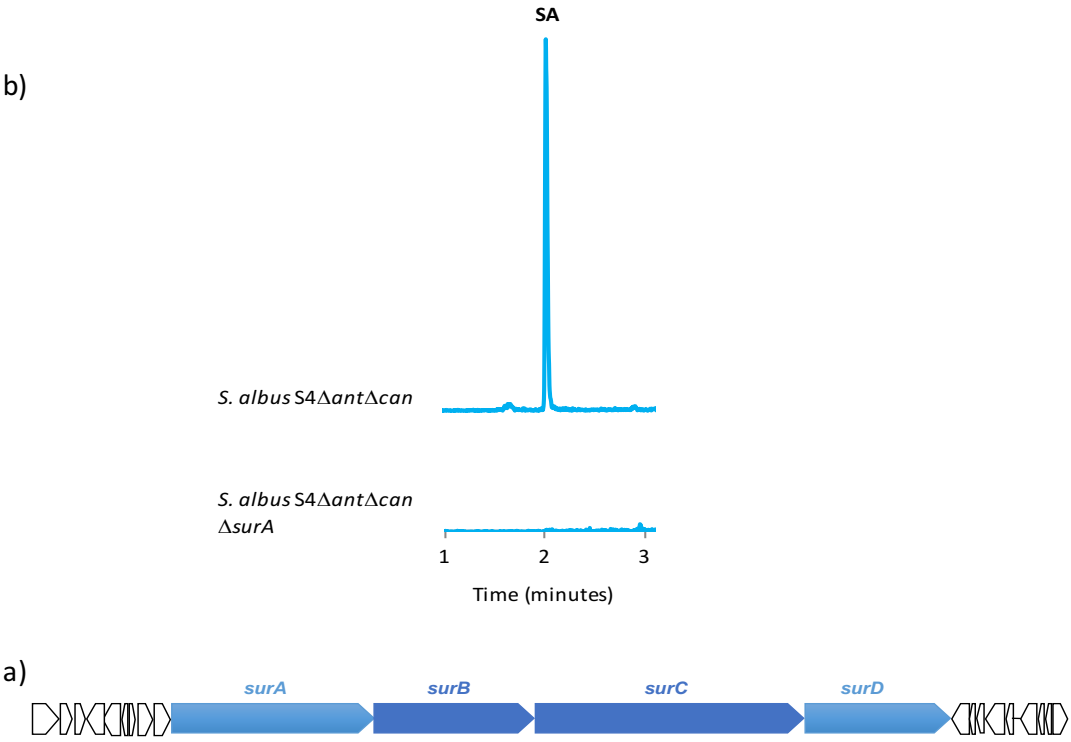
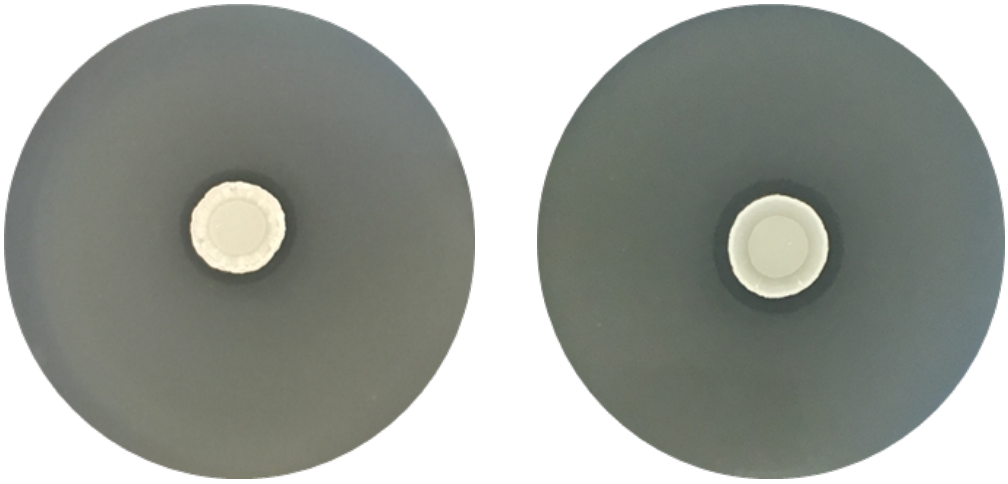


Figure 4.5 *S. albus* S4 encodes the production of surugamides. a) A schematic of the surugamide biosynthetic gene cluster in *S. albus* S4. The BGC comprises of four non-ribosomal peptide synthetases (*surABCD*) of which *surAC* encodes the production of surugamide A (SA) and *surBC* encodes the production of surugamide F. b) The EICs corresponding to the $[M+H]^+$ ions are shown for SA ($C_{48}H_{81}N_9O_8$). While SA is present in the chemical extracts from *S. albus* S4 Δ*ant*Δ*can*, the production is abolished in *S. albus* S4 Δ*ant*Δ*can*Δ*surA.*

4.7.3 *S. albus* S4 Δ*ant*Δ*can*Δ*surA* retained antibacterial activity

To check whether the activity seen on MM agar was attributable to SA, whole cell bioassays against *S. aureus* SH1000 were carried out with *S. albus* S4 Δ*ant*Δ*can* and *S. albus* S4 Δ*ant*Δ*can*Δ*surA*. However, *S. albus* S4 Δ*ant*Δ*can*Δ*surA* still retained antibacterial activity on MM (Figure 4.6).



S. albus S4 Δ*ant*Δ*can* *S. albus* S4 Δ*ant*Δ*can*Δ*surA*

Figure 4.6 Whole cell bioassays of *S. albus* S4 Δ*ant*Δ*can* and *S. albus* S4 Δ*ant*Δ*can*Δ*surA* on MM against *S. aureus* SH1000. The surugamide A mutant *S. albus* S4 Δ*ant*Δ*can*Δ*surA* retained antibacterial activity on MM.

4.7.4 Commercially synthesised SA

SA is reported to have antibacterial activity against *S. aureus,* however, its mode of action is unknown (Wang et al., 2014). Therefore, to probe further into its bioactivity profile *in vitro*, SA was commercially synthesised. Detailed LC-HRMS was performed to verify that the synthesised compound was in fact SA. The synthetic SA and biologically produced SA both possess the same retention time and their respective MS2 fragmentation patterns are identical (Figure 4.7).

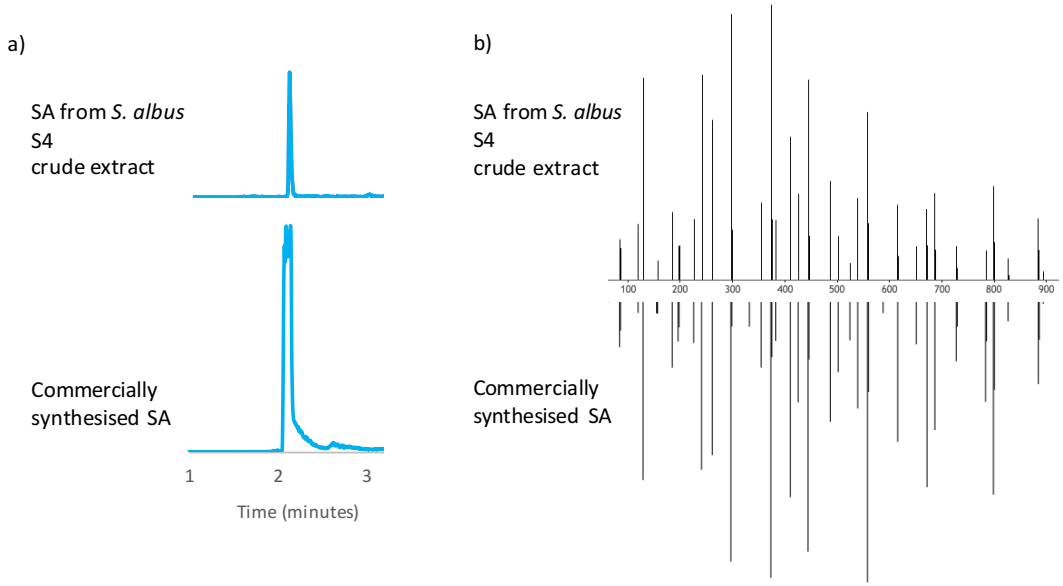


Figure 4.7 Comparison of commercially synthesised surugamide A (SA) with SA from the chemical extracts of *S. albus* S4. a) The EICs corresponding to the $[M+H]^+$ ions are shown for SA ($C_{48}H_{81}N_9O_8$) from the chemical extract from *S. albus* S4 and commercially synthesised SA. Both possess the same retention time. b) The fragmentation pattern of SA from the MSMS data from the chemical extract of *S. albus* S4 compared to that of the commercially synthesised SA. The fragmentation patterns were obtained from the GNPS platform.

4.7.5 Antimicrobial activity of SA

To probe into the bioactivity profile of SA, minimum inhibitory concentration (MIC) assays were performed with SA against *Escherichia coli* BW25113, *Candida albicans* and *S. aureus* SH1000. SA did not exhibit any antibacterial activity against *E. coli* and no antifungal activity was observed against *C. albicans* (Figure 4.8). Surprisingly, in this study, SA had an MIC of 256 μg/mL against *S. aureus* SH1000, which is approximately 28 times greater than the reported MIC of SA (Figure 4.8).

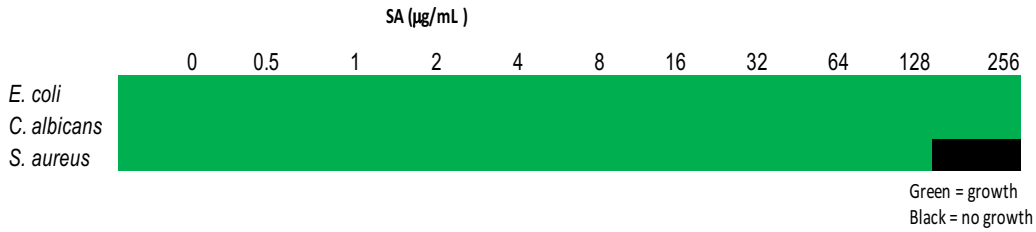


Figure 4.8 Bioactivity profile of surugamide A against *E. coli* BW25113, *C. albicans* and *S. aureus* SH1000. Bioactivity was only seen against *S. aureus* and the MIC was 256 μg/mL

4.7.6 SA shows synergism with other linear membrane damaging antibiotics

In lieu of the lack of antibacterial activity with SA, we hypothesised that SA and SF may work synergistically. The organisation of the biosynthetic genes (*surABCD*) of the *sur* BGC means that SA and SF are both presumably co-produced at the same time (Figure 4.5). To explore the possibility of SA and SF working together as a compound antibiotic to exert antibacterial activity, checkerboard MIC assays were used to investigate whether SA demonstrated synergism with the linear peptide antibacterial compound gramicidin D. Gramicidin D is a linear polypeptide antibiotic complex comprising of a heterogeneous mix of gramicidin A, B and C; of which gramicidin A is the most abundant (80%) (Kelkar and Chattopadhyay, 2007). Looking at the chemical structures of SF and gramicidin A, both compounds appear to share structural similarity (Figure 4.9). Checkerboard MIC assays of SA were also performed with the membrane damaging detergent cetyltrimethylammonium bromide (CTAB) and the cyclic, non-membrane damaging antibiotic erythromycin. The chemical structures of SA, SF, gramicidin A, CTAB and erythromycin are presented in Figure 4.9.

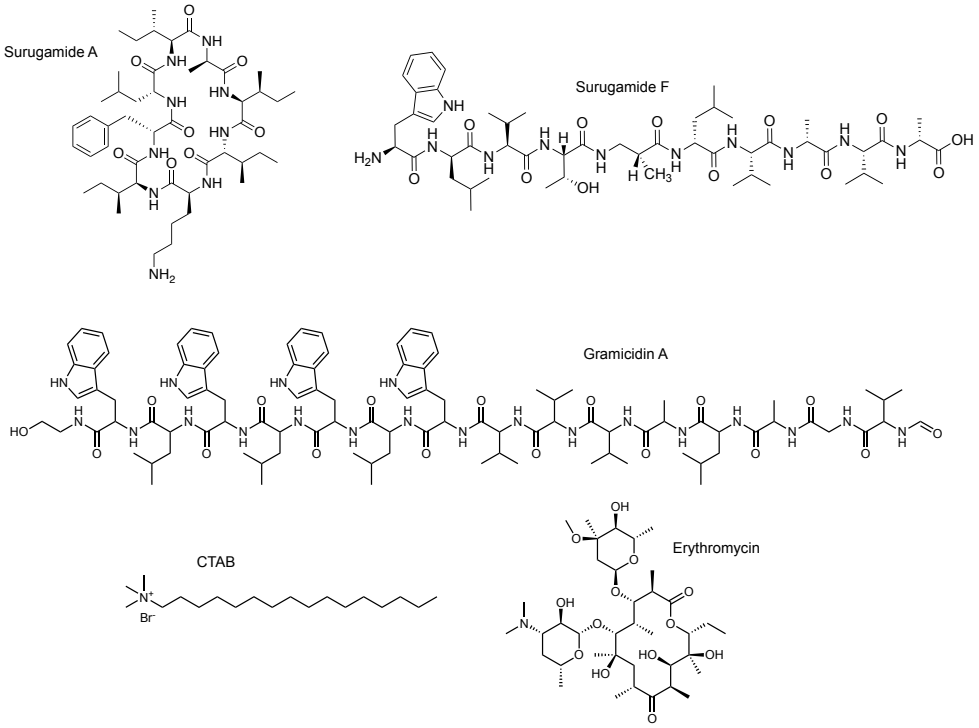


Figure 4.9 Chemical structures of surugamide A, surugamide F, gramicidin A, CTAB and erythromycin

The MICs of the compounds against *S. aureus* SH1000 were first determined before carrying out the checkerboard assays. The MICs were as follows: 0.5 μg/mL for gramicidin D, 2 μg/mL for CTAB and 0.25 μg/mL for erythromycin. In the checkerboard assays, SA and gramicidin D demonstrated synergism (Figure 4.10) with an FIC (fractional inhibitory concentration) of 0.281. Neither CTAB nor erythromycin showed any effect, with inhibition in growth only seen at the respective MICs of the compounds (Figure 4.10).

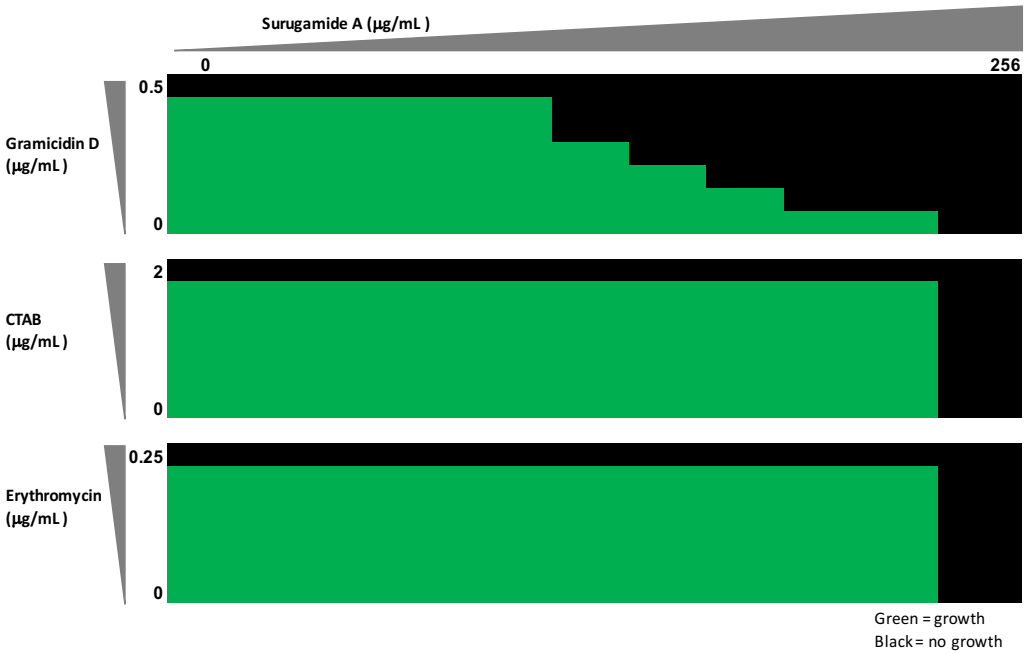


Figure 4.10 Checkerboard assays of surugamide A with gramicidin D, CTAB and erythromycin. Synergy was only observed with SA and gramicidin D with an FIC of 0.281, which is consistent with synergism. SA did not show any interaction in terms of antibacterial activity with CTAB or erythromycin, with inhibition in the growth of *S. aureus* only observed at the respective MICs of the compounds.

4.7.7 The titre of SF is much lower than SA *in vivo*

On MM, though the production of SA is readily detectable, hardly any SF is detected (Figure 4.11). In the first ever report of SF, liquid ISP2 media was used as the growth media for *Streptomyces* sp.JAMM992 (Ninomiya et al., 2016). When *S. albus* S4 is cultivated using ISP2, the yield of SF increases, but it is still dramatically lower than that of SA (Figure 4.12, Figure A2.6). Comparing the peak area of SA and SF from the ISP2 culture chemical extracts, the titre of SF is 98.9% lower than that of SA. Additionally, a bioassay of the chemical extract obtained from *S. albus* S4 grown in liquid ISP2 media did not exhibit any antibacterial activity against *S. aureus* SH1000 (Figure 4.12). The relatively low titre of SF produced by *S. albus* S4 may explain the lack of bioactivity of crude extracts.

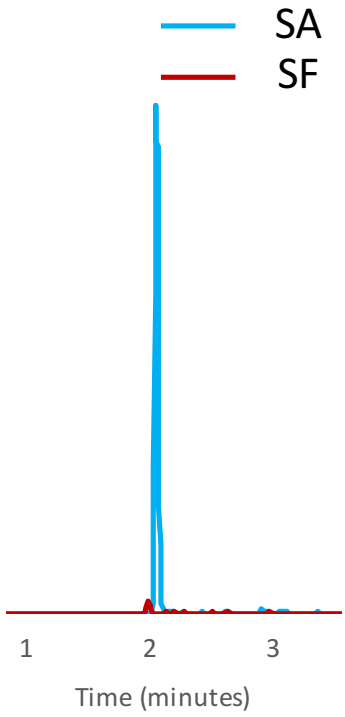


Figure 4.11 Production of surugamide A (SA) and surugamide F (SF) in MM. The EICs corresponding to the $[M+H]^+$ ions are shown for SA ($C_{48}H_{81}N_9O_8$) and SF ($C_{52}H_{85}N_{11}O_{12}$). While SA is easily detectable in the chemical extracts of *S. albus* S4 grown on MM agar, the yield of SF is drastically lower. The y-axis intensity scale is set to a max of 1.2 x 10^5.

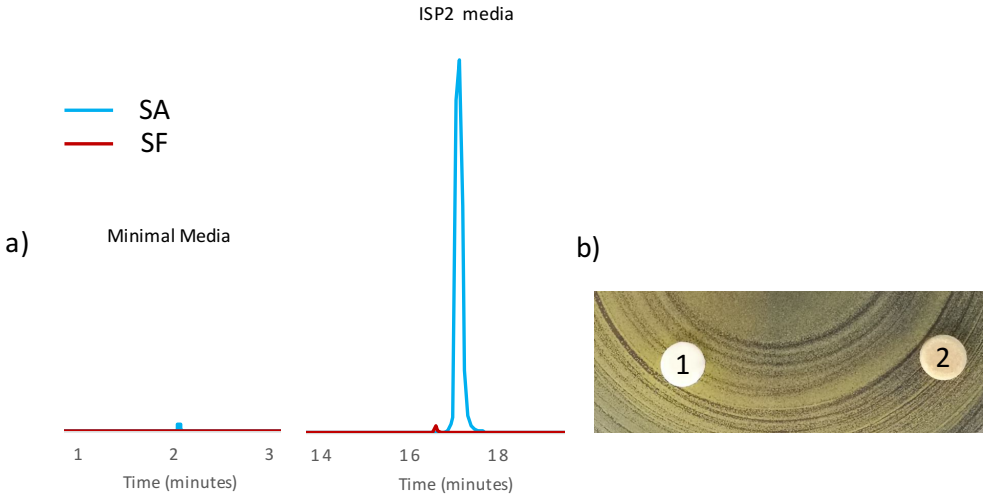


Figure 4.12 Comparison of the production of surugamide A (SA) and surugamide F (SF) in MM to ISP2 liquid cultures. a) The EICs corresponding to the $[M+H]^+$ ions are shown for SA ($C_{48}H_{81}N_9O_8$) and SF ($C_{52}H_{85}N_{11}O_{12}$). The y-axis scale is set to a max of 6 x 10^6. Due to technical issues at the time, the extracts were run on two different columns. The production of SA and SF by *S. albus* S4 is greater in ISP2 than on MM agar. However, the yield of SF is still drastically lower. b) Antibacterial assays of the chemical extract prepared from *S. albus* S4 grown in ISP2. 1- methanol, 2- chemical extract prepared from *S. albus* S4. No antibacterial activity against *S. aureus* was observed with the *S. albus* S4 chemical extract.

4.8 Discussion

Pleiotropic approaches hold great potential in the activation of silent BGCs (Zhu et al., 2014; Baral et al., 2018). In this study, the use of MM agar facilitated the production of an antibacterial compound(s) in *S. albus* S4 (Figure 4.4) and led to the identification of a group of compounds called surugamides from *S. albus* S4 (Figure A2.3, Figure A2.4, Figure 4.5). Although such approaches are generally high throughput, they bring about global changes in the metabolome making identification of the compound(s) responsible for the observed bioactivity challenging (Baral et al., 2018). Even though SA is reported to have antibacterial activity in the literature, it was not responsible (at least not solely) for the antibacterial activity observed on MM as the SA mutant still retained antibacterial activity (Figure 4.6). The antibacterial activity observed could be attributable to BAC or another compound(s). Additionally, pleiotropic approaches also do not make use of the information available from genome sequencing and genome mining to its full potential as such approaches are not able to target the most interesting gene clusters. As a result, the use of this approach also suffer from rediscovery issues (Baral et al., 2018). Therefore, the use of data driven platforms such as GNPS is highly valuable as they enable dereplication to identify previously known compounds (Wang et al., 2016; Mohimani et al., 2016). Recently, the putative gramicidin-like compound that *S. albus* S4 shares with *S. albus* J1074 was in fact identified to be surugamides in *S. albus* J1074 using the GNPS platform and was confirmed through experimental work (Mohimani et al., 2016). In this study, the GNPS platform identified surugamides from *S. albus* S4 MM agar extracts (Figure A2.3) and experimental work with the SA mutant *S. albus* S4 Δ*ant*Δ*can*Δ*surA* matched the compound to the BGC as the production of SA was abolished in the mutant strain (Figure 4.5).

Surugamides are a known group of compounds with SA reported to have antibacterial activity (Takada et al., 2013; Wang et al., 2014; Ninomiya et al., 2016). However, their mechanism of action is unknown, which fuelled the initiative to further explore the bioactivity profile of SA. SA did not exhibit antifungal activity against *C. albicans* or antibacterial activity against *E. coli* (Figure 4.8). Previously, SA was reported to have antibacterial activity against *S. aureus* with an MIC of 10 μM (= 9 μg/mL) (Wang et al., 2014). However, in this study, SA had an MIC of 256 μg/mL, which is approximately 28 times greater than the reported MIC (Figure 4.8). In the report by Wang *et*

al., SA was purified from a *Streptomyces* isolate from an underground coal mine fire site, *Streptomyces* sp. RM-27-46. The isolated compound was identified to be SA via correlation of ^{1}H-NMR and ^{13}C-NMR analyses data and HRMS data to what is reported in the literature, which was then used in the MIC assay (Wang et al., 2014). In contrast, in this study, commercially synthesised SA was used (Figure 4.7) as purification of secondary metabolites can often be extremely challenging (Rutledge and Challis, 2015). However, it was surprising that the commercially synthesised SA showed a much higher MIC than the reported MIC of SA. The isolation and purification of secondary metabolites is challenging and laborious and as a result, the compound can sometimes be contaminated with other molecules from the crude extract (Bucar et al., 2013; Xiao et al., 2013). This possibly could explain the lower MIC of SA that was previously reported, where a low but potent concentration of a contaminant might be present, which is likely the source of the bioactivity observed.

The higher MIC of SA led us to hypothesize that SA and SF may function synergistically. Many examples of antibiotics that work synergistically have been reported (Acar, 2000). For example, streptomycin and penicillin G acts synergistically with an increased uptake of streptomycin into enterococci in the presence of penicillin, which acts on the cell wall. The combination therapy of penicillin G and streptomycin in turn reduced the frequency of relapse in entercoccal endocarditis treatment (Reynolds and Rowley, 1953; Moellering and Weinberg, 1971). Quinupristin-dalfopristin is a combination of two semi-synthetic streptogramin antibiotics, which exhibit synergistic antibacterial activity against a wide array of Gram-positive bacteria by stabilising the binding of each other to the ribosome (Finch, 1996; Speciale et al., 1999).

As purified/synthesised SF was not available, gramicidin D was used to initially test this hypothesis in a preliminary test. Gramicidin A, which makes up 80% of gramicidin D is a linear peptide antibiotic and shares structural similarity with SF (Figure 4.9). Linear gramicidins are ionophores that work by forming well-defined cation specific ion channels with a pore size of ~4 Å. The ion channels formed are permissive to the passage of monovalent cations (Kelkar and Chattopadhyay, 2007). In the checkerboard assays conducted, SA displayed synergism with gramicidin D. Synergism was determined using the FIC index with a value of ≤ 0.5 interpreted as a

synergistic interaction (Schwalbe and Steele-Moore, L (Ed.) Goodwin, 2007). The ΣFIC of SA and gramicidin D was calculated to be 0.281, which is consistent with synergism. SA did not exhibit any synergism with the control compounds CTAB or erythromycin (Figure 4.10). CTAB is a cationic surfactant, which works by rupturing the cell membrane resulting in cell lysis (Simões et al., 2005). SA did not demonstrate any interaction with the detergent CTAB in terms of antibacterial activity with growth inhibition only seen at the respective MICs of SA (256 μg/mL) and CTAB (2 μg/mL), suggesting that the interaction of SA and the peptide antibiotic gramicidin D is specific (Figure 4.10). Additionally, as with CTAB, SA did not show any changes in the antibacterial activity in the presence of the macrolide antibiotic erythromycin with inhibition in growth only seen at their respective MICs (Figure 4.10). To speculate, it maybe that SF plays a role in helping SA gain access to its target site, or it may be that SA and SF interact to work together as a compound antibiotic.

As the genes for the production of SA and SF are arranged in an operon in the surugamide BGC, both compounds are most likely produced at the same time (Figure 4.5). However, the yield of SF is drastically lower compared to that of SA. While on MM SF was barely detectable (Figure 4.11), chemical extracts prepared from *S. albus* S4 grown in ISP2 cultures showed that SF was produced at a detectable yield (Figure A2.6). However, the titre of SF was ~98.9% lower than that of SA, which may explain why no antibacterial activity was observed with the *S. albus* S4 ISP2 crude extract (Figure 4.12). It may be that a sufficient quantity of SF is not available for SA and SF to exhibit synergism. Future work with purified/synthesised SF on its own and with SA can shed light on whether they do in fact act synergistically. If they are in fact synergistic, to my knowledge, two compounds that are produced from the same biosynthetic locus has previously not been reported to exhibit such an interaction. The synergistic interaction of SA with gramicidin D is an interesting phenomenon. Use of other linear membrane damaging antibiotics as well as structurally different membrane damaging antibiotics (such as nisin and gramicidin S) can also provide further insight (Guilhelmelli et al., 2013).

4.9 Conclusions

In this study, the use of MM facilitated the production of an antibacterial compound(s) in *S. albus* S4, which does not otherwise exhibit antibacterial activity under standard laboratory conditions. The GNPS platform identified a group of compounds called surugamides from the chemical extracts of *S. albus* S4 grown on MM. The production of SA was experimentally verified, linking the compound to its BGC. In this study, the MIC of SA against *S. aureus* was 28 times greater than the reported MIC of SA. Additionally, SA demonstrated synergism with the ionophore gramicidin D, which may suggest that SA demonstrates a similar synergistic interaction with SF to exert its antibacterial activity. However, this hypothesis still needs to be experimentally verified. With the advances in genome sequencing and genome mining, this study used platforms such as antiSMASH and GNPS to predict and then identify surugamides in *S. albus* S4 demonstrating the utility of such tools. The identification of the compound enabled an informative decision to be taken to pursue the compound further. In this genomics era, the growing field of genomics-driven natural product discovery hold great potential in the activation of silent gene clusters and in the identification and discovery of new and/or useful compounds (Baral et al., 2018).

Chapter 5 Assembly line release of surugamides

Abstract

Non-ribosomal peptides (NRPs) are a large family of structurally complex and diverse natural products, often with remarkable biologically and therapeutically relevant activities. However, the structural complexity of these peptides can pose significant challenges in their chemical synthesis and engineering. NRPs are synthesised by large multifunctional mega enzymes called non-ribosomal peptide synthetases (NRPSs) with a modular, assembly line-like synthetic logic. In a canonical NRPS system, a terminally located *cis*-acting thioesterase domain is responsible for the hydrolytic release or the macrocyclisation of the mature peptide. The surugamide (*sur*) biosynthetic gene cluster includes four NRPS genes (*surABCD*). SurAD specify the biosynthesis of a cyclic octapeptide named surugamide A and SurBC specify the biosynthesis of a linear decapeptide named surugamide F. Unlike canonical NRPS systems, the terminal NPRSs within the Sur pathway (SurC and SurD) lack C-terminal *cis*-acting TE domains**.** In this study, the assembly-line release of surugamide A and F in *Streptomyces albus* S4 are explored. Understanding alternative release mechanisms is important expanding the synthetic biology toolbox available for engineering the biosynthesis of NRPs. The roles of a putative α/β hydrolase (SurF) and a putative β-lactamase (SurE) in the synthesis of surugamide A and F were characterised *in vivo*. Gene inactivation studies of *surF* and *surE* established that SurF was not required for the production of surugamide A and F. Reduced production titres of surugamide A and F were observed in the absence of SurF. However, SurE was essential for the synthesis of surugamide A and F, with the production of both compounds abolished in the SurE production mutant. *In vitro* assays with recombinant SurE and an *N*-acetyl cysteamine thioester mimic of surugamide A further confirmed the *trans*-acting thioesterase function of SurE.

5.1 Introduction

Non-ribosomal peptides (NRPs) are a diverse family of structurally complex secondary metabolites, often with a wide range of therapeutically relevant activities. Some examples of NRPs include vancomycin (antibacterial), cyclosporine (immunosuppressant), bleomycin (antitumor agent) and myxothiazol (antifungal) (Figure 5.1). Bacteria and fungi are the most prolific producers of NRPs. Genome sequencing and bioinformatics analyses have revealed great insight into the distribution of NRPs and their pathways. Actinobacteria such as *Streptomyces* are prolific producers of these secondary metabolites (Süssmuth and Mainz, 2017). NRPs can be linear, cyclic or branched cyclic in their structure and can further structural complexity by modifications such as glycosylation, oxidative cross-linking or heterocyclisation (Sieber and Marahiel, 2003; Martínez-Núñez and López, 2016; Komaki et al., 2018).

NRPs are synthesised by large, multifunctional enzymes called non-ribosomal peptide synthetases (NRPSs) that have an assembly line like synthetic logic (Martínez-Núñez and López, 2016). They are not limited to the 20 canonical amino acids, instead they are capable of using different monomers such as non-proteinogenic amino acids, fatty acids and α-hydroxy acids and are able to use both L- and D- versions of the 20 amino acids. This in turn, contributes to their structural versatility and most likely contributes to their bioactive properties also (Miller and Gulick, 2016; Süssmuth and Mainz, 2017). NRPSs are large multimodular enzymes and bacterial NRPSs often have their modules spread over many discrete NRPSs (type II NRPS) (Hur et al., 2012).

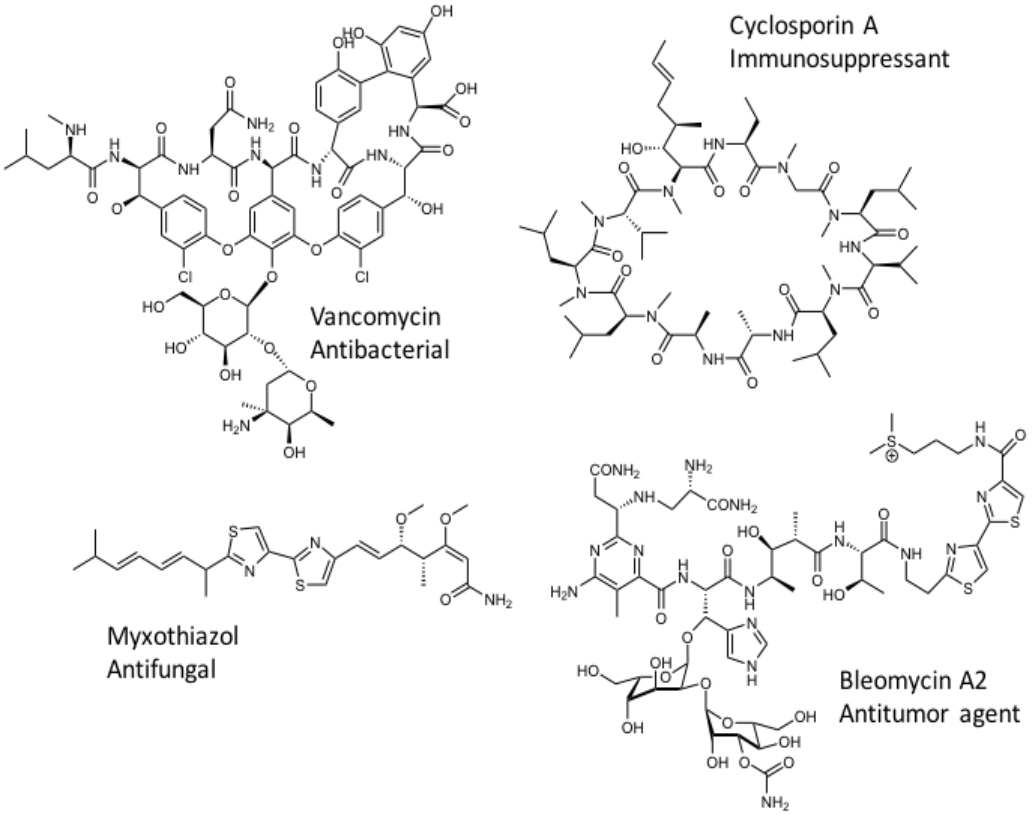


Figure 5.1 Examples of non-ribosomal peptides

5.2 Modular assembly line like synthetic logic of NRPSs

Each module in the NRPS is responsible for the incorporation of a specific amino acid into the growing peptide chain (Figure 5.2). NRPSs can encode many domains, which include (but is not constrained to) adenylation (A), condensation (C), peptidyl carrier protein (PCP) or thiolation (T) and thioesterase (TE) domains (Sieber and Marahiel, 2003; Süssmuth and Mainz, 2017). The initiation module is the first module of the NRPS and is comprised of an A domain and a T domain (Figure 5.2). The initiation module is followed by elongation modules which contain C domains as well as A and T domains. Elongation continues until it reaches the termination module where the final domain of the module is a TE domain (Figure 5.2). A, C and T domains are required in each module but a single TE domain can terminate peptide synthesis. The order of the modules collinearly corresponds to the primary amino acid sequence of the peptide. The typical modular organisation of an NRPS system is: A-T-(C-A-T)$_n$-TE with some of the modules containing some tailoring domains (Sieber and Marahiel, 2003; Süssmuth and Mainz, 2017).

NRP synthesis is initiated with the post-translational modification of a conserved serine residue in the T domain where a 4'-phosphopantetheinyl transferase (PPTase) adds a flexible 4'-phosphopantetheine (PPT) group converting the protein from its *apo*- state to the *holo*- state (Miller and Gulick, 2016; Winn et al., 2016). The A domain confers the specificity of the amino acid to be incorporated and is responsible for the recognition and activation of the amino acid. Following the covalent modification of the T domain, the A domain of the initiation module activates the amino acid through adenylation using ATP to generate an aminoacyl adenylate intermediate (Sieber and Marahiel, 2003; Miller and Gulick, 2016; Süssmuth and Mainz, 2017). The T domain then covalently links the activated amino acid to the 4'- PPT arm via a thioester bond generating an aminoacyl-*S*-T intermediate. The A domain of the second module similarly activates its cognate amino acid and a T domain tethered amino-acyl thioester intermediate is generated. The adjacent C domain then catalyses the formation of a peptide bond between the two aminoacyl-*S*-Ts thereby allowing the translocation of the growing peptide onto the T domain of the second module (Figure 5.2) (Miller and Gulick, 2016; Winn et al., 2016). Additional modification domains such as epimerisation (E) domains may also be present. Epimerisation domains can change the stereochemistry of amino acids from L- to L- and D- by deprotonating and reprotonating the α-carbon of the amino acid when it is tethered to a T domain. Elongation continues in an N-terminal to C-terminal fashion until it reaches the C-terminally located TE domain (Figure 5.2). The peptide is cleaved from the assembly line either by an intramolecular reaction with an internal nucleophile, which gives rise to a cyclic peptide with or without oligomerisation or by hydrolysis, which results in a linear peptide (Sieber and Marahiel, 2003; Miller and Gulick, 2016; Süssmuth and Mainz, 2017).

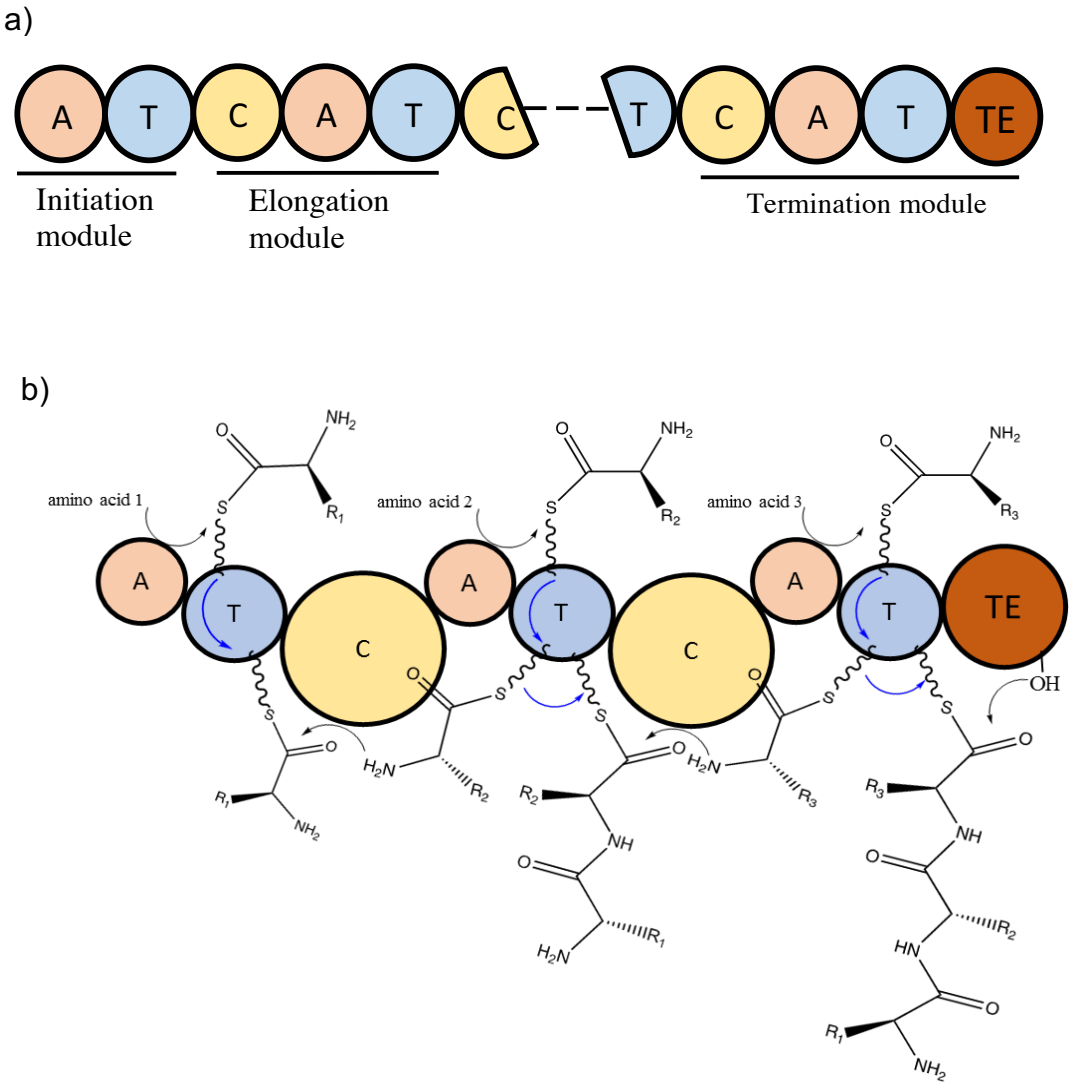


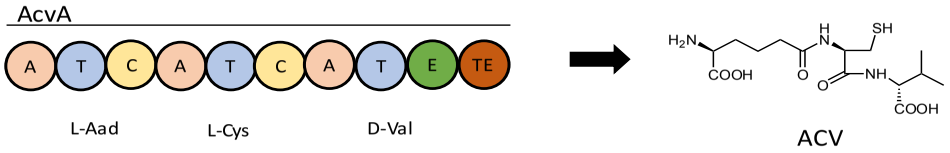
Figure 5.2 Typical modular organisation of NRPSs (a) and the modular assembly line like synthetic logic in NRP biosynthesis (b). T domains within NRPSs are post-translationally modified where the 4'- PPT group of cofactor CoA is transferred onto a conserved serine residue of the T domain. This flexible PPT arm allows the shuttle of the tethered intermediates down the assembly line. A domain then activates the amino acid, which is then covalently linked to the 4' PPT arm of the primed T domain to form the aminoacyl-*S*-T intermediate. The adjacent C domain then catalyses the formation of a peptide bond between two cognate aminoacyl-*S*-Ts. The peptide elongation carries on down the assembly line until a terminally located thioesterase domain is reached**.** The mature peptide chain must be released once it reaches the end of the assembly line in order to reactivate the megasynthetase for the next cycle of synthesis (Winn et al., 2016) (b- adapted from (Winn et al., 2016)).

5.3 Different biosynthetic strategies of NRPs

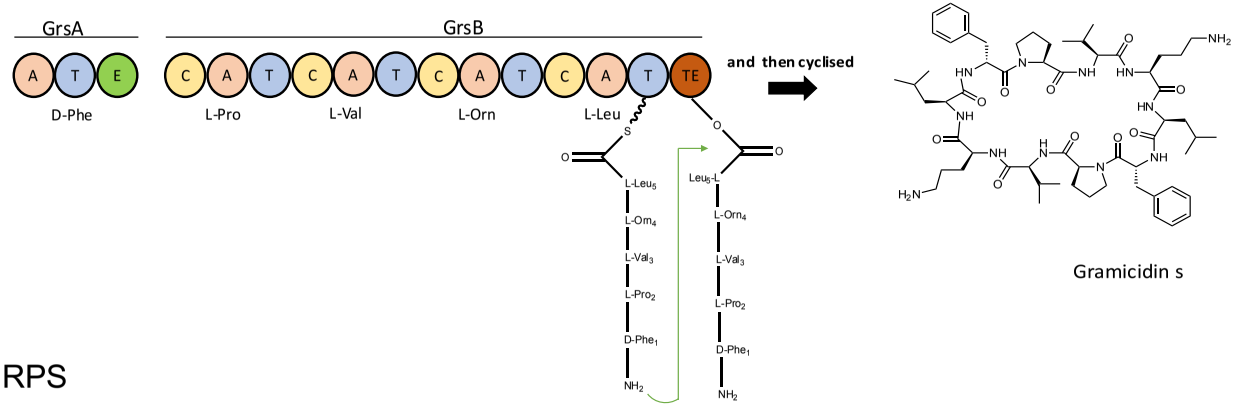
The vast majority of NRPSs follow the collinearity rule to catalyse stepwise peptide condensation; however, there are systems that deviate from this typical biosynthetic strategy (Sieber and Marahiel, 2003; Süssmuth and Mainz, 2017). NRPSs can be divided into three major classes: type A, B and C (Figure 5.3) (Mootz et al., 2002; Felnagle et al., 2008). Type A NRPSs such as the β-lactam precursor L-δ-(α-aminoadipoyl)-L-cysteinyl-D-valine (ACV) biosynthetic system strictly follow the collinearity rule where the amino acid sequence is determined by the order and number of modules of the NRPS (Figure 5.3) (Weber et al., 1994; Mootz and Marahiel, 1997; Byford et al., 1997; Felnagle et al., 2008). Each domain is only used once. Peptide biosynthesis is initiated at the first module, sometimes referred to as the loading module, with elongation occurring down the assembly line until the peptide is released from the terminal module. This biosynthetic strategy is the simplest and the best characterised (Mootz et al., 2002; Felnagle et al., 2008).

In type B NRPS systems, the modules and domains are used more than once to generate the NRP. In such systems, after the first cycle of synthesis is completed, the product remains tethered to the terminal domain while another cycle ensues (Figure 5.3) (Mootz et al., 2002; Felnagle et al., 2008). Each of the peptide chain remains tethered to the terminal domain until the total biosynthesis is complete at which point the final product is released from the assembly line (Mootz et al., 2002; Felnagle et al., 2008). An example of such a system is the biosynthetic machinery for gramicidin S (Figure 5.3) (Krätzschmar et al., 1989; Hoyer et al., 2007). Lastly, type C or non-linear NRPSs differ from the typical C-A-T modular arrangement along with certain domains functioning more than once in the biosynthesis of the peptide such as in the biosynthesis of vibriobactin (Figure 5.3) (Keating et al., 2000a; Keating et al., 2000b; Mootz et al., 2002; Felnagle et al., 2008).

Type A NRPS



Type B NRPS



Type C NRPS

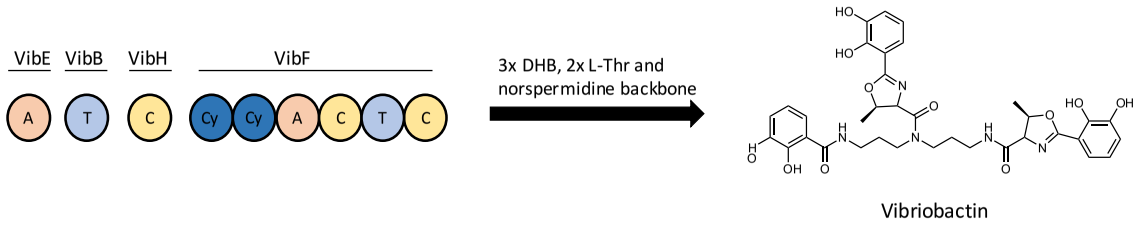


Figure 5.3 The three different kinds of NRPS assembly strategies

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5.4 Canonical assembly line release of NRPs

Usually located at the C-terminus of the NRPS, the TE domain is the last domain to play a role in NRP synthesis, where it can either act as a hydrolase or a cyclase and is responsible for the release of the mature peptide from the megasynthetase (Figure 5.4) (Miller and Gulick, 2016). TE domains are approximately 30 kDa and are homologous to those found in fatty acid synthases and polyketide synthases (PKSs) (Challis and Naismith, 2004; Du and Lou, 2010; Bloudoff and Schmeing, 2017). They belong to the α/β hydrolase superfamily, which includes proteases, lipases and esterases. They have a defined active site with the catalytic triad Ser, His and Asp along with a variable 'lid' region that is thought to play a role in their substrate specificity (Du and Lou, 2010; Miller and Gulick, 2016).

TE domains catalyse the enzymatic release of the peptide in a two-step reaction. First, the His and Asp residues activate the Ser residue as a nucleophile through hydrogen bonding (Figure 5.4) (Weissman, 2015; Miller and Gulick, 2016). The nucleophilic hydroxyl oxygen group of the active site Ser then attacks the carbonyl thioester of the peptidyl-*S*-TE intermediate to form a peptidyl-*O*-TE intermediate. Thus, the nascent peptide chain is transferred from the last T domain onto the hydroxyl group of the active site serine to form a peptidyl-*O*-TE intermediate (Du and Lou, 2010). In the second step, this intermediate is released by the cleavage of the ester carbonyl through the attack of a nucleophile. The nucleophilic attack can occur through water by hydrolysis or through the attack by an intramolecular nucleophile (Figure 5.4) (Challis and Naismith, 2004; Du and Lou, 2010). TE-catalysed hydrolytic peptide release of the NRP leads to a linear product as in the biosynthesis of ACV (Byford et al., 1997; Du and Lou, 2010). The other major product release route and the most commonly observed mechanism in NRP release is via intramolecular cyclisation, where the hydroxyl or amino group of the nascent peptide chain acts as an internal nucleophile to result in a cyclic product such as daptomycin (Kopp et al., 2006; Du and Lou, 2010). The structural restrictions of the cyclic peptide plays a role in the enhanced bioactivity observed in NRPs as well as in providing resistance to proteolytic degradation (Du and Lou, 2010; Weissman, 2015).

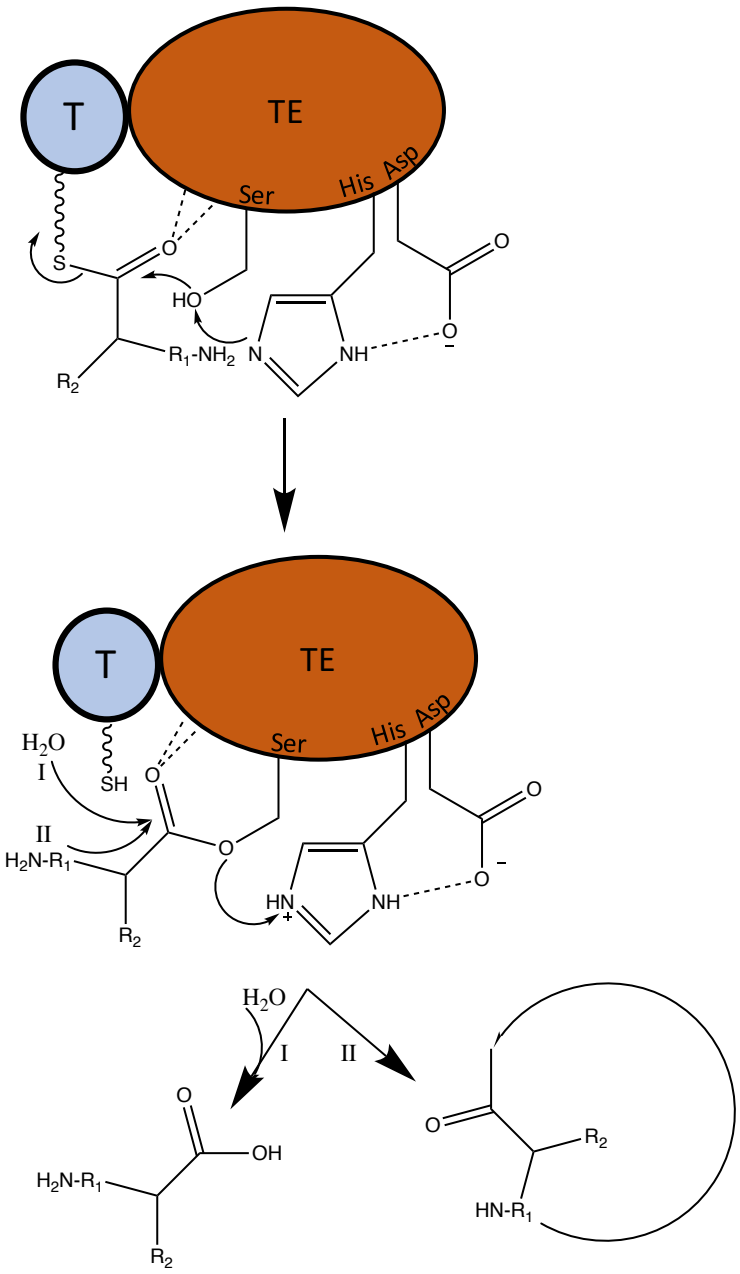

Figure 5.4 TE domain mediated release of a peptide through hydrolysis(I) or cyclisation (II)

Insights into the structural information of a TE domain was first obtained from the crystal structure of the surfactin (Srf) TE domain excised from the NRPS SrfC from *Bacillus subtilis* (Tseng et al., 2002). Sequence similarity shared by Srf-TE with TE domains from other NRPSs suggested it to be a prototype for TE domains (Bruner et al., 2002). Srf-TE is a 28 kDa globular protein and belongs to the α/β hydrolase superfamily with its characteristic fold and the catalytic triad defined by the amino acid residues Ser80, His207 and Asp107. The lid region of Srf-TE is composed of three α-helices that

reach over the active site and adopt two conformations called open and closed (Bruner et al., 2002). The open state allows unobstructed access to the active site whereas in the closed state, the lid occludes the active site. The closed state was proposed to be the ground state of the enzyme (Bruner et al., 2002). Srf-TE also has a distinctive bowl-shaped hydrophobic aromatic active site cavity with two cationic amino acid residues (Lys111 and Arg120) playing a role in directing the peptide cyclisation (Tseng et al., 2002; Bruner et al., 2002). Often, water can act as a competing nucleophile to release the peptide through hydrolysis. The mutational change of both Lys111 and Arg120 residues to Ala, resulted in an increase in the hydrolytic release of the peptide (Tseng et al., 2002). According to the Srf-TE model, the terminal T domain bound surfactin peptidyl substrate is directed into the active site region where it is transferred onto Ser80, which is activated by the His207 and Asp107 residues. The peptidyl-O-TE intermediate accommodated in the bowl region is then cyclised to enable the macrocyclising chain terminating activity through the intramolecular nucleophilic attack by the β-hydroxyl group of the fatty acid on the peptidyl-*O*-TE acyl enzyme ester bond, which results in the final lactone product (Tseng et al., 2002; Bruner et al., 2002).

Structural information from the TE domain excised from the fengycin synthetase (FenB) shed further light on how these domains work (Samel et al., 2006). Fen-TE is a 27.5 kDa, globular protein responsible for the regio- and stereospecific macrocyclisation and assembly line release of fengycin in *B. subtilis.* It contains a distinctive 6-Å deep, 25-Å long open canyon-like active site with the catalytic Ser84 residue found at the bottom of the central part of the crevice (Samel et al., 2006). The lid-region of Fen-TE is much shorter than that of Srf-TE suggesting access to the catalytic triad is unrestricted (Samel et al., 2006). Furthermore, a closed state was not observed, at least not under the crystallisation conditions tested, suggesting it is unlikely that the lid region plays a crucial role in the recognition of the substrate (Samel et al., 2006). It has been proposed that some TE domains utilise the open/closed conformations of the lid to control peptide cyclisation, perhaps dictated by the presence of more than one suitable nucleophile within the substrate (Miller and Gulick, 2016).

5.5 *N*-acetyl cysteamine thioesters (SNACs)

Given the importance of the compounds produced by NRPS systems, biochemical and structural characterisation of these biosynthetic systems are areas of great interest (Süssmuth and Mainz, 2017). However, studying these systems *in vitro* is challenging owed to the complexity of these assembly lines and the requirement of covalent attachment of the intermediates to the T domains before they can be recognised by the catalytic domains as substrates (Kittilä and Cryle, 2018). CoA or rather, its substructure PPT are important components of NRPS assembly lines (as well as PKSs and FASs). The use of CoA in such studies is extremely challenging owed to its complex structure which is made up of five stereogenic centres and its inherent instability arising from its many phosphate linkages (Franke and Hertweck, 2016). Therefore, chemical tools to study these systems *in vitro* are greatly valuable. The truncated *N*-acetyl cysteamine analogue has been found to be a good substitute for CoA and related thioesters in such studies. The SNAC moiety is identical to the terminal part of 4'-PPT arm and is therefore a good mimic of the natural T domain bound peptidyl substrate (Figure 5.5) (Ehmann et al., 2000; Kohli et al., 2002; Sieber and Marahiel, 2003). In regards to studying release mechanisms, linear peptides synthesised with a SNAC moiety attached at the C-terminus can be used to mimic the natural peptidyl-S-T substrate (Seieber and Marahiel, 2003).

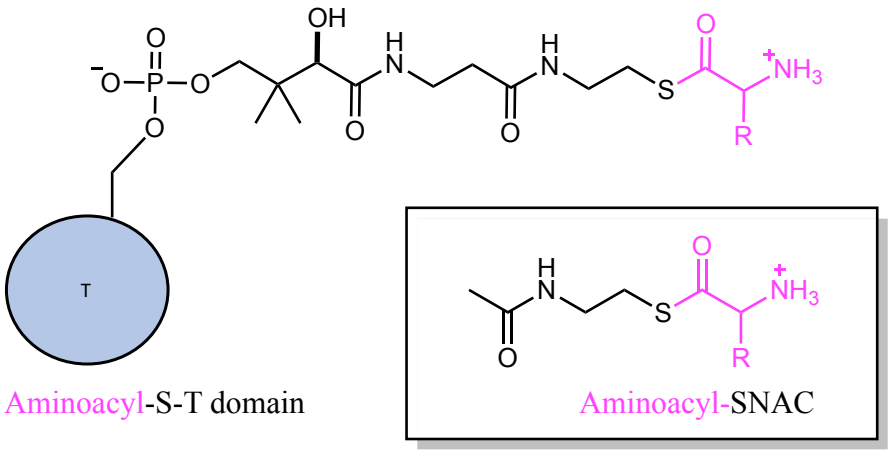

Figure 5.5 *N*-acetyl cysteamine thioesters (SNACs) mimic the terminal part of the 4' PPT arm. SNACs function as good biomimetics and represent an important chemical tool set in studying specific biosynthetic steps in greater detail (Fig adapted from (Ehmann et al., 2000)).

5.6 Chemoenzymatic cyclisation

TE domain mediated macrocyclisation of NRPs is the most understood and is considered to be the canonical assembly line release mechanism (Marahiel et al., 1997). However, the chemical synthesis of NRPs is often associated with relatively low yields, is expensive and rather challenging (Sieber and Marahiel, 2003). For example, even though cyclisation is entropically favoured, the steric repulsion of the residues in the ring structure along with the use of protecting groups to achieve proper regiochemistry has proven synthetic cyclisation to be incredibly difficult (Kohli et al., 2002; Sieber and Marahiel, 2003). As truncated TE domains remain active *in vitro*, there is considerable interest in exploiting them as biocatalysts to generate medicinally important cyclic NRPs and to generate new chemical scaffolds for testing (Trauger et al., 2000; Sieber and Marahiel, 2003; Grünewald et al., 2004). The chemical synthesis of a linear peptide by solid-phase synthesis chemistry followed by TE mediate cyclisation that bypasses the use of protecting groups and formation of unwanted by-products would be highly desirable (Kohli et al., 2002; Sieber and Marahiel, 2003).

Trauger *et al.,* first demonstrated the *in vitro* cyclisation activity of the excised TE from tyrocidine A synthetase (TycC) using a SNAC thioester mimic. Incubation of the excised TycC-TE with the tyrocidine decapeptide-SNAC revealed efficient cyclisation to tyrocidine A with only a minor flux to hydrolysis (cyclisation/hydrolysis ratio of 6:1) (Trauger et al., 2000). Further experiments with a wide array of peptidyl-SNAC substrates with various compositions of amino acids, lengths and stereochemistry revealed that the TycC-TE only recognised the C- and N- terminal residues of the substrate, depending on its identity and stereochemistry (Trauger et al., 2000). This in turn allowed room for the alteration of residues within the peptide backbone as well as the investigation of substrates of various lengths. The tyrocidine analogue library that was thus created was screened for compounds with better or altered activity. Analogues that showed better broad spectrum activity towards Gram-positive and Gram-negative bacteria as well as reduced human erythrocyte lysis compared to that of tyrocidine A were identified demonstrating the potential of TE domains as biocatalysts (Trauger et al., 2000; Kohli et al., 2002). A few more examples that foreshadow the utility of excised TE domains are described below.

Tyc-TE also exhibited activity *in vitro* towards the SNAC mimic of gramicidin S (Trauger et al., 2000). Gramicidin S is a cyclic decapeptide synthesised by a type B NRPS. It is formed as result of the dimerization between two identical pentapeptides followed by its subsequent cyclisation to form the final decapeptide (Figure 5.3) (Krätzschmar et al., 1989; Hoyer et al., 2007). Comparison of gramicidin S and tyrocidine A precursors revealed that both shared the same two N-terminal amino acid residues (D-Phe-Pro) and three C-terminal residues (Val-Orn-Leu) (Trauger et al., 2000). As the Tyc-TE only required the conserved C- and N- terminal residues for substrate recognition, it was reasoned that the Tyc-TE should be able to both dimerise and cyclise a gramicidin S pentapeptide-SNAC. Upon incubation of the SNAC substrate with the excised Tyc-TE, production of gramicidin S was observed (Trauger et al., 2000). Grünewald *et al*., showed that the excised TE domain from the calcium dependent antibiotic (CDA) NRPS from *S. coelicolor* could be used to make daptomycin derivatives (Grünewald et al., 2004). Daptomycin and CDA belong to the same family of acidic lipopeptides and both possess decapeptide lactones that share 5 amino acids at the same positions in the ring. The CDA-TE was shown to have remarkable tolerance towards amino acid substitutions in the CDA peptide backbone and was able to synthesise daptomycin derivatives that are otherwise not accessible via chemical modification of the parent compound (Grünewald et al., 2004).

The excised TE domains present an attractive means for the development of new or better NRPs. However, even with the use of the excised TEs, the yield observed is often much lower than with the natural substrate. Additionally, it is hard to draw general conclusions about the substrate selectivity of TE domains with substrate specificities ranging from the largely permissive TycC-TE to the very restricted specificity of SrfC-TE. Improvement of TE domains with increased promiscuity and efficiency is therefore a highly attractive area of research (Tseng et al., 2002; Bruner et al., 2002; Sieber and Marahiel, 2003; Du and Lou, 2010). Such insights play a major role in the rational engineering of biosynthetic pathways to generate natural products with desired characteristics to explore and develop new drug leads.

5.7 Type I and II thioesterases

There are two types of TEs that have been found to be associated with NRPSs: type I and type II. Type I TEs are located at the C-terminal end of the termination module of the multimodular NRPS and are thus integrated in the NRPS machinery. Srf-TE, Tyc-TE and Fen-TE are all examples of type I TEs (Tseng et al., 2002; Samel et al., 2006; Miller and Gulick, 2016). Type II TEs on the other hand, are stand-alone discrete hydrolytic enzymes that are not covalently liked to the megasynthetase. Unlike type I TEs, type II TEs do not appear to have an essential role in the NRP synthesis, rather they are involved in the housekeeping functions (Schwarzer et al., 2002; Du and Lou, 2010). Disruption of type I TEs results in the loss of production, whereas disruption of type II TEs decreases yield by ~30–95%, but do not abolish the synthesis of the product (Challis and Naismith, 2004; Yeh et al., 2004; Claxton et al., 2009).

The *trans*-acting type II TEs are present in several NRPS gene clusters and is exemplified by the type II TE in the surfactin biosynthetic gene cluster (BGC). Type II TEs act as proofreading enzymes and clear misloaded T domains and aberrant intermediates (Schwarzer et al., 2002). These mistakes can arise from PPTase accidentally post-translationally installing an acyl-CoA instead of CoA or when an incorrect amino acid is adenylated and loaded onto the T domain (Schwarzer et al., 2002). The type II TEs are able to hydrolyse the incorrectly loaded acyl or peptidyl groups and restore the activity of the NRPS (Schneider and Marahiel, 1998; Schwarzer et al., 2002). These enzymes must be capable of recognising all the T-domains that are misprimed and hydrolyse any incorrect addition while having a low specificity for the correctly growing peptide chain. In studies conducted by Claxton et al., (2009) on RifR, a type II TE from the hybrid NRPS/PKS rifamycin BGC, it was revealed that RifR possessed a broad substrate specificity with a greater affinity for carrier proteins that were misacylated over the natural rifamycin building blocks (Claxton et al., 2009).

Structural studies of type II TEs have revealed them to adopt an α/β hydrolase fold similar to type I TEs (Linne et al., 2004). Comparison of the two types of TEs in the surfactin BGC revealed that the type II TE had a catalytic triad similar to that of the type I TE consisting of Ser86, Asp190 and His216 (Linne et al., 2004). The Asp163 residue was found to be critical for

the structural stability of the protein, as the mutant was structurally unstable even though it was active (Linne et al., 2004). The Srf type II TE was also observed to have a shorter lid, which only partially obstructed the active site cavity. It also had a shallower active site cavity that is more easily accessible than the type I TE and can only tolerate small acyl substituents on the 4'-PPT arm (Koglin et al., 2008).

Although TE domain mediated peptide release is the most common release mechanism, there are examples of NRPS systems that lack a terminally located TE domain especially in fungal NRPS biosynthetic systems. In such cases, there are examples of other terminally located domains such as a reductase (R) domain or a condensation like (C_T) domain, which have been shown to perform the same function (Weissman, 2015).

5.8 Other termination domains

5.8.1 Reductase domains

R domains are sometimes found in the terminal NRPS modules in place of a TE domain especially in fungi and are responsible for the NRP release from the assembly line (Du and Lou, 2010). These R domains share sequence similarity with the short chain dehydrogenase/reductase (SDR) superfamily and adopts the Rossmann-fold structure (Kavanagh et al., 2008; Manavalan et al., 2010). R domains typically require NAD(P)H as a cofactor for the reductive cleavage of the thioester bond between the nascent peptide chain and the T domain (Manavalan et al., 2010). A stable macrocyclic imine is then formed by the attack of either a side chain or the N-terminal amino group by the C-terminal alcohol or aldehyde group of the freed peptide chain (Ehmann et al., 1999; Keating et al., 2001; Manavalan et al., 2010).

The best studied reductive release mechanism of an NRPS system is of lysine in *Saccharomyces cerevisiae* with the Lys2 protein which has an A-T-R domain arrangement (Ehmann et al., 1999). The terminally located R domain is responsible for the release of the T domain bound α-aminoadipate by the regiospecific reduction of its C6 carboxylate to its aminoadipate semialdehyde, which is in turn reductively transaminated to lysine. The reductive release of lysine is carried out by the protein pair Lys2/Lys5 (Ehmann et al., 1999). The PPTase Lys5 is responsible for the conversion of the T domain from its *apo*- state to the *holo*- state. The *holo*-Lys2 is then

able to activate the aminoadipate and generate the aminoadipoyl-*S*-T-acyl intermediate just like a canonical A-T domain pair (Ehmann et al., 1999). The R domain then utilises NADPH to reduce the thioester bond of the aminoadipoyl-*S*-T-peptidyl intermediate to a hemithioaminal linkage, which spontaneously decomposes to form the C6 aldehyde product. The semialdehyde then condenses with glutamate to form saccharopine, which is in turn hydrolysed to form the final products lysine and a-ketoglutarate (Ehmann et al., 1999; Keating et al., 2001; Du and Lou, 2010).

An example of reductive release of a bacterial NRP is in the biosynthesis of the linear pentadecapeptide gramicidin A in *Bacillus brevis*. Two reductases, an integrated R domain and a discrete reductase are required for the formation of the final product (Kessler et al., 2004; Schracke et al., 2005). The BGC for gramicidin A synthesis comprises of four NRPSs, LgrA, LgrB, LgrC and LgrD with an R domain found at the C-terminus of LgrD. The LgrD R domain is responsible for the release of the peptidyl thioester from the NRPS in an NAD(P)H dependent reduction step generating an aldehyde intermediate (Kessler et al., 2004). This intermediate is then further reduced by a discrete NADPH dependent aldoreductase (LgrE) to generate the final product gramicidin A (Kessler et al., 2004; Schracke et al., 2005).

While TE domain mediated release mechanism utilises oxoester chemistry, the R domain mediated release mechanisms uses thioester chemistry (Du and Lou, 2010) In TE mediated release, the thioester chemistry is switched to oxoester chemistry when the peptidyl chain is transferred from the 4'-PPT of the T domain to the hydroxyl group of the active site serine for the product release by hydrolysis or cyclisation. Although not completely understood, in R-domain mediated release, it is most probable that the peptidyl chain is not transferred from the T domain to R domain, rather the R domain yields a thiohemiacetal that is still bound to the 4'-PPT via the transfer of a hydride from NAD(P)H to the thioester. The 4'-PPT of the T domain is then regenerated following the release of the aldehyde (Du and Lou, 2010).

5.8.2 Condensation-like domains

In addition to R domains, some NRPS systems, especially those that release cyclic NRPs in fungi, utilise condensation like domains (C_T) for the assembly line release (Gao et al., 2015). For example, the *in vitro* re-constitution and

biochemical characterisation of the 450 kDa NRPS tryptoquialanine synthetase (TqaA) involved in the biosynthesis of the fumiquinazoline F in *Penicillium aethiopicum* revealed that its terminal C_T domain was responsible for the formation of 10-membered macrocycle, which then underwent a spontaneous intramolecular cyclisation to form the final product (Gao et al., 2015; Zhang et al., 2016). Crystal studies of the TqaA-C_T domains revealed that as with typical C domains, TqaA-C_T also adopted a pseudo-dimeric open sandwich (V-shaped) structure (Zhang et al., 2016). However, the C_T domain possessed a distinctive α1 helix and a shorter α2 helix atypical to that of canonical C domains. This in turn results in the obstruction of the acceptor site and solvent channel, which promotes cyclisation by averting the nucleophilic attack by the solvent (Zhang et al., 2016).

C_T and TE domains possess distinctly different folds and use different mechanisms to release the final products (Gao et al., 2015; Zhang et al., 2016). In the two-step TE mediated release, the peptidyl chain is transferred to the active site serine on the TE, which is followed by an intramolecular nucleophilic attack to cleave the ester carbonyl to result in a cyclic product. However, as with typical C domains, no covalent adduct with the enzyme is expected to be formed during the C_T domain mediated cyclisation (Fischbach and Walsh, 2006; Gao et al., 2015). The catalytic histidine is instead responsible for the deprotonation of the amine nucleophile enabling its attack on the thioester carbonyl which enables cyclisation and release in one step (Gao et al., 2015). Additionally, while TE domains can use both peptidyl-SNACs and peptidyl-T domains as substrates, studies with the TqaA-C_T domain, revealed that it required the natural T domain partner for the cyclization reaction (Ehmann et al., 2000; Kohli et al., 2002; Gao et al., 2015). Only the T domain-tethered peptide substrates were recognised by the C_T domain while the peptidyl-SNAC or peptidyl-CoA substrates were not. This contrasts with most TE domains studied, which could accept the mimics of the peptidyl-thioesters (Sieber et al., 2003; Bloudoff and Schmeing, 2017). Fengycin TE is an exception, which required interaction with the T domain for the cyclisation (Sieber et al., 2003; Sieber et al., 2004).

Another example of C domain catalysed chain release is observed in the biosynthesis of the enediyene-C1027 chromophore in *Streptomyces globisporus* (Lin et al., 2009). The chromophore comprises of an enediyne

core, a deoxy aminosugar, a benzoxazolinate moiety and a β-amino acid. The BGC encoding the enediyne antitumor antibiotic C-1027 has an NRPS mechanism for the incorporation of the (*S*)-3-chloro-5-hydroxy-*β*-tyrosine moiety (Van Lanen and Shen, 2008). The A domain (SgcC1), T domain (SgcC2) and the C domain (SgcC5) of the minimal NRPS system act as free standing proteins (Lin et al., 2009). SgcC5 was able to catalyse the condensation reaction to catalyse an ester bond formation between a mimic of the enediyne core ((*R*)-1-phenyl-1,2-ethanediol) and the SgcC2 bound (*S*)-3-chloro-5-hydroxy-*β*-tyrosine leading to a C-domain mediated release (Lin et al., 2009). SgcC5 required the T domain-tethered molecule as its substrate. Additionally, SgcC5 was also able to catalyse an amide bond formation between the SgcC2-tethered *β* amino acid and an enediyne core mimic with an amine as a nucleophile at the C-2 position (*R*)-2-amino-1-phenyl-1-ethanol). SgcC2 was the first C domain shown to be able to catalyse both amide and ester bonds (Lin et al., 2009; Chang et al., 2018).

5.9 Assembly line release of surugamides

Surugamides are a group of NRPs that were first identified from the marine *Streptomyces* sp. JAMM992 (Takada et al., 2013). The surugamide (*sur)* BGC consists of four successive NRPS genes *surA, surB, surC* and *surD* (Figure 5.6**).** Surugamides A-E (SA-SE) are cyclic octapeptides encoded by *surA* and *surD* (Figure 5.6). SA is the major product with SB-SE thought to be formed as a result of the permissive substrate specificity of the A domains (Takada et al., 2013). SA contains four isoleucine residues, whereas one of the isoleucine residues is replaced by a valine at different positions in SB-SE (Takada et al., 2013). Genes *surB* and *surC* encode the production of an unrelated linear decapeptide named surugamide F (SF) (Figure 5.6) (Ninomiya et al., 2016). The surugamides were recently also identified in *S. albus* J1074 (Mohimani et al., 2016; Xu et al., 2017). Through work carried out in this study, the surugamide BGC was identified in *S. albus* S4 with the production of SA and SF verified using LC-HRMS (see Chapter 4). Since the initial discovery of surugamides, the corresponding BGC has been found to be widespread among streptomycete strains closely related to *S. albus* J1074 (Ryan Seipke, unpublished work).

In the surugamide BGC, genes *surABCD* are organised in an operon with the four genes presumably transcribed at the same time and SA and SF both co-produced (Figure 5.6). Genes *surA/surD* are responsible for the

biosynthesis of the cyclic compound SA and *surB/surC* are responsible for the biosynthesis of SF (Figure 5.6) (Ninomiya et al., 2016). Following the canonical NRPS assembly line logic, a TE domain is expected to be found at the C-terminal end of both SurD and SurC for the release of SA and SF, respectively. However, intriguingly, both SurD and SurC lack terminally located TE domains (Figure 5.6). Neither do they end in an alternate termination domain such as an R domain or a C domain (Figure 5.6). Bioinformatic tools such as antiSMASH, NRPS-PKS and Pfam are consistent with both SurD and SurC ending in an E domain at the C-terminus end (Figure 5.6, Appendix Figure A3.1) (Ansari et al., 2004; Medema et al., 2011).

To investigate the possibility of a *trans*-acting release factor(s), other genes predicted to be encoded within the antiSMASH defined boundaries of the *sur* BGC were considered (Figure A3.2). While there are additional hypothetical proteins predicted to be encoded by genes within the BGC, BLAST and InterPro analyses predicted proteins are listed in Table 5.1 (and Figure A3.2). Examination of the bioinformatically predicted proteins revealed most of them to be regulators (e.g. Sur4, Sur12), transporters (e.g. Sur10, Sur11) or proteins involved in the folate pathway (e.g. Sur18, Sur19). Two genes of particular interest were *surF* and *surE.* Gene *surF* is predicted to encode a protein with an α/β hydrolase fold (InterPro Family IPR029058). As TEs also typically contain an α/β hydrolase fold, *surF* was chosen to be investigated further for a potential role in the release of either or both SA and SF.

Gene *surE* is predicted to encode a protein belonging to the β-lactamase superfamily (InterPro Family IPR012338). SurE is very different to and much larger than canonical TE domains such as Srf-TE and Fen-TE and lack the catalytic triad found in typical TEs (A3.3). While β-lactamases are not known to act as *trans*-acting release factors in NRPS systems, stand-alone metallo-β-lactamases have been shown to be responsible for the release of some fungal PKs. The absence of a TE domain in the PK asperthecin biosynthetic pathway in *Aspergillus nidulans* and the presence of an essential gene encoding a β-lactamase (*aptB*) in the asperthecin BGC led to the proposal that the β-lactamase may function as a *trans*-acting release factor in fungal PKS systems (Szewczyk et al., 2008; Li et al., 2011).

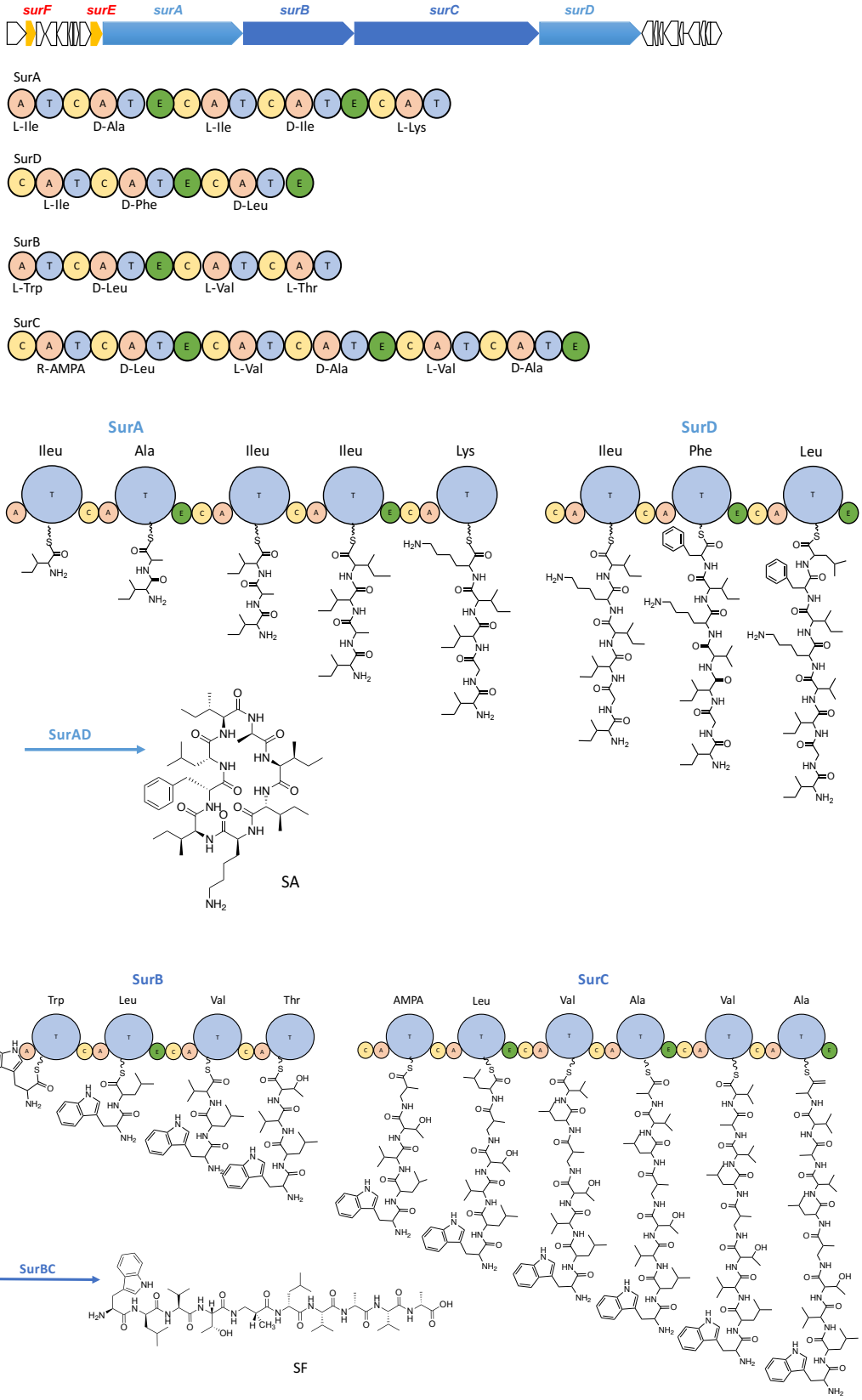

Figure 5.6. Schematic representation of the surugamide BGC, the modular organisation of the NRPSs and their respective assembly line logic

Biochemical evidence for this hypothesis was provided from work on the *Aspergillus terreus* PKS atrochrysone carboxylic acid synthase (ACAS) responsible for the biosynthesis of atrochrysone. This biosynthetic system also lacks a terminal TE domain or an alternate termination domain. The assembly line release mechanism in turn was shown to be via the metallo-β-lactamase ACTE (Awakawa et al., 2009; Li et al., 2011; Chooi and Tang, 2012). While PKs and NRPs have distinct structures, their biosynthetic strategies are very similar. Both NRPSs and PKSs follow an assembly line like synthetic logic, use carrier proteins (acyl carrier protein or ACP instead of T domains in PKSs) and both systems use the 4'-PPT moiety for precursor activation. A TE domain located in the terminal module is responsible for release of the PK in a canonical PKS system as well (Du and Lou, 2010; Weissman, 2015). Though, the β-lactamases reported with TE activity are from PKS systems and in fact, in all cases thus far, from fungi, it may be possible for such a system to exist in bacteria too. Additionally, in the BGC encoding the cyclic lipoglycopeptide mannopeptimycin, the NRPS system lacks a terminal TE domain and it was suggested that MppK, an essential protein in the synthesis of mannopeptimycin, which belongs to the β-lactamase superfamily may function as the termination/cyclisation protein (Magarvey et al., 2006). SurE shares 39% homology with MppK.

Table 5.1. Putative proteins encoded by the surugamide biosynthetic gene cluster

Protein	Deduced function
SurA	NRPS
SurB	NRPS
SurC	NRPS
SurD	NRPS
SurE	β-lactamase
SurF	α/β hydrolase fold containing protein
Sur1	NADH-quinone oxidoreductase subunit D/H family protein
Sur2	Predicted membrane protein
Sur3a/3b	Two-component system
Sur4	ArsR family transcriptional regulator
Sur5	Aldehyde dehydrogenase family protein

Sur6	MbtH-like protein
Sur7	ABC transporter permease
Sur8	ABC transporter ATP binding protein
Sur9	GntR family transcriptional regulator
Sur10	MFS transporter
Sur11	Major facilitator transporter
Sur12	TetR family transcriptional regulator
Sur13	MFS transporter
Sur14	ABC transporter substrate binding protein
Sur15	ABC transporter permease
Sur16	ABC transporter permease
Sur17	ABC transporter ATP binding protein
Sur18	Dihydropteroate synthase
Sur19	Dihydroneopterin aldolase
Sur20	2-amino-4-hydroxy-6-hydroxymethyldihydropteridine diphosphokinase
Sur21	GTP cyclohydrolase 1 Fol E
Sur22	FtsH family protein
Sur23	Hypoxanthine phosphoribosyl transferase family protein
Sur24	tRNA (Ile)- lysidine synthetase
Sur25	Zinicin-like metallopeptidase type 2

5.10 Aims and Objectives

In the absence of an obvious cis-acting release mechanism for SA and SF, two gene candidates (*surE* and *surF*) were chosen to be investigated as potential *trans*-acting release factors. To investigate the potential roles of SurE and SurF in the synthesis of SA and SF, this study aimed to:

- Use gene inactivation and complementation studies coupled with LC-HRMS to investigate the production titre of SA and SF in *S. albus* S4 ∆*surE and* ∆*surF* null mutants
- Carry out an *in vitro* investigation using purified protein and a SNAC thioester mimic of SA

5.11 Results

5.11.1 Deletion of *surF in S. albus* S4 lowers the titre of SA and SF

The Redirect recombineering protocol was used to generate *S. albus* S4 Δ*surF* (Figure 5.7) in order to examine the role of the α/β hydrolase fold containing SurF in the synthesis of SA and SF. Chemical extracts prepared from *S. albus* S4 wild type (WT) and *S. albus* S4 Δ*surF* were analysed by LC-HRMS to check for the presence of SA and SF (Figure 5.8). Deletion of *surF* did not abolish the production of SA or SF. However, the titre of SA and SF were both reduced compared to the WT levels. A 62% reduction in the titre of SA and 17% reduction in the titre of SF was observed (Figure 5.8). To examine whether the production level would return to WT levels with a functional copy of the gene, the complementation strain S4 Δ*surF attB* φBT1::pIJ10257-*surF* was constructed (Figure A3.4) and ethyl acetate extracts were prepared for chemical analysis. Upon complementation, production of both SA and SF were restored, with production of SA to near WT levels and SF titre exceeding that of the WT strain levels (Figure 5.8). Compared to the 62% loss in the titre of SA to the WT titre, upon complementation, the loss was only 16% in comparison to the WT titre of SA. With SF, interestingly, the titre upon complementation was even greater than that of WT with a 57% increase compared to the titre of SF in the WT strain (Figure 5.8).

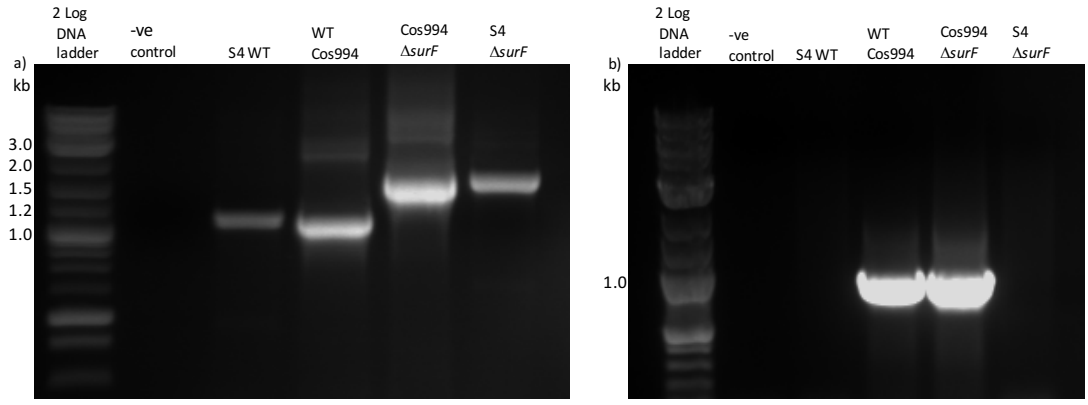

Figure 5.7 PCR verification of *S. albus* S4 Δ*surF*. a) Verification using primer set DT171 and DT172. If Apra cassette has replaced *surF*, then EPL: 1649 bp; if not, 1084 bp. b) Verification using primer set RFS96 and RFS97 to check for loss of kanamycin cassette. A double crossover event to generate S4 Δ*surF* would result in the loss of the kanamycin cassette from the cosmid backbone.

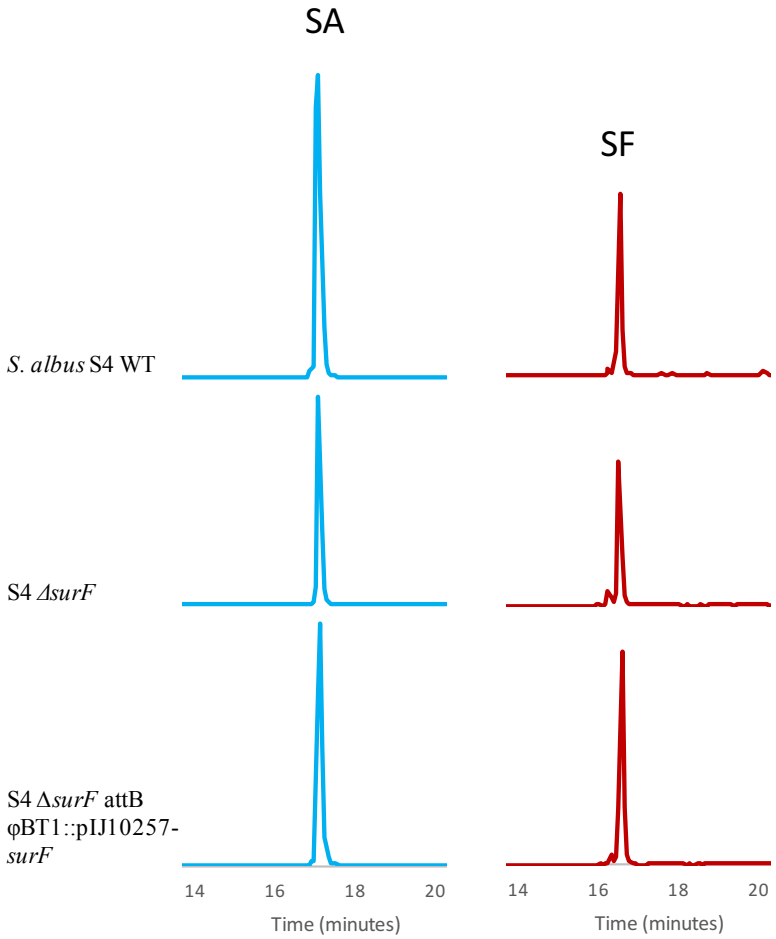

Figure 5.8 Deletion of *surF* results in a lower titre of surugamide A (SA) and surugamide F (SF). The EICs corresponding to the $[M+H]^+$ ions are shown for SA ($C_{48}H_{81}N_9O_8$) and SF ($C_{52}H_{85}N_{11}O_{12}$). The y-axis intensity scale for SA is set to a max 6 x 10^6 and the scale for SF is set to 2 x 10^5. The peak area of SA and SF in the WT are 57254844 and 628891, respectively and in S4 Δ*surF*, the peak area for both SA and SF were reduced to 21537268 and 524318 demonstrating a 62% and 17% decrease respectively. Upon complementation, in S4 Δ*surF attB* φBT1::pIJ10257-*surF,* the production of SA and SF returned to almost/greater than WT levels. The peak area of SA in the complemented strain was 48279612 and SF was 987327, which corresponds to only a 16% loss of SA compared to that of WT SA titre and an increase in SF titre by 57% compared to that of the WT SF titre.

5.11.2 Deletion of *surE in S. albus* S4 abolishes the production of SA and SF

To investigate the role of the predicted β-lactamase family protein encoded by *surE*, the mutant strain *S. albus* S4 Δ*surE* was generated following the Redirect recombineering protocol (Figure 5.9). As with *S. albus* S4 Δ*surF,* chemical extracts prepared from *S. albus* S4 WT and *S. albus* S4 Δ*surE* were analysed by LC-HRMS to examine the effect of the deletion of *surE* on the production of SA and SF**.** The deletion of *surE* completely abolished the production of both SA and SF (Figure 5.10). Upon complementation of the mutant with a functional copy of the gene, the strain *S. albus* S4 Δ*surF attB* φBT1::pIJ10257-*surF* was generated (Figure A3.5). Chemical analyses confirmed that production of both SA and SF was restored in the complemented strain confirming that *surE* is essential in the biosynthesis of both SA and SF **(**Figure 5.10).

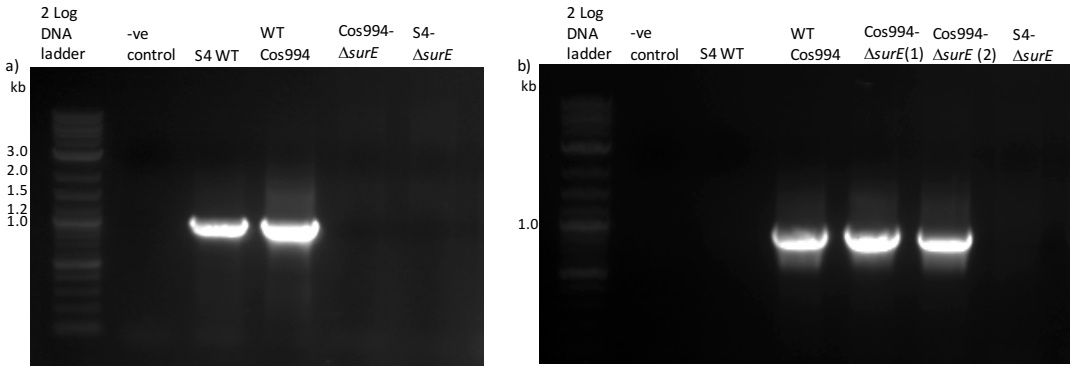

Figure 5.9 PCR verification of *S. albus* S4 Δ*surE*. a) Verification using primer set DT163a and DT64a checking for an internal fragment of *surE*. If *surE* is present, EPL: 953 bp. b) Verification using primer set RFS96 and RFS97 to check for loss of kanamycin cassette. A double crossover event to generate S4 Δ*surE* would result in the loss of the kanamycin cassette from the cosmid backbone.

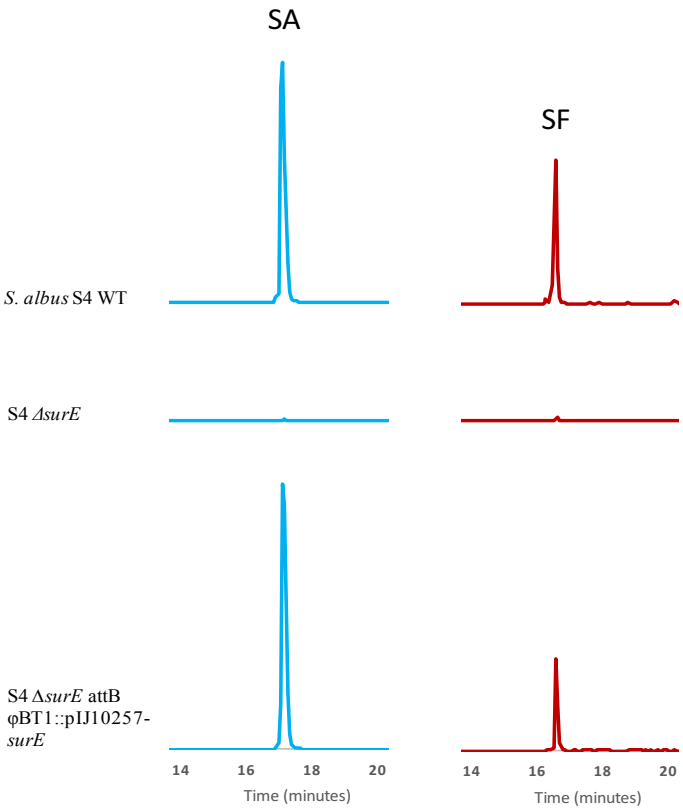

Figure 5.10 SurE is essential for the production of surugamide A (SA) and surugamide F (SF). The EICs corresponding to the $[M+H]^+$ ions are shown for SA ($C_{48}H_{81}N_9O_8$) and SF ($C_{52}H_{85}N_{11}O_{12}$). The y axis scale for SA is set to a max 6 x 10^6 and the scale for SF is set to 2 x 10^5. Upon deletion of *surE*, production of both SA and SF were abolished. Upon complementation, in S4 Δ*surE attB* φBT1::pIJ10257-*surE,* production of both SA and SF were restored.

5.11.3 Purification trials of SurE

As the *in vivo* results showed that SurE was essential for the production of SA and SF, SurE was desired to be purified to reconstitute the results *in vitro*. The *in vitro* reconstitution of the results would confirm that SurE is solely able to release the peptides and is responsible for the effect observed. However, the purification of SurE proved to be rather challenging. It was after many failed attempts that SurE was successfully purified (section 5.11.4). In the initial expression trial, C- and N- terminal 6x His tagged SurE were trialled. However, hardly any production of recombinant SurE was seen (Figure A3.6). Expression trials revealed SurE to be in the insoluble fraction under most conditions tested. The conditions tested are described briefly in the following paragraphs.

In the initial trial, BL21(DE3)-pET28a-*surE*/pET30a-*surE* cells were cultivated using LB at 37 °C until OD_{600} ~0.6 at which point, the cells were induced with IPTG (final concentration 1 mM) and grown overnight at 16 °C. Following the incubation period, cells were pelleted and resuspended in 10 times the volume of the weight of the pellet in the lysis buffer. The pellets were then lysed by sonication and the soluble fraction was collected and samples were analysed on an SDS-PAGE gel. However, hardly any production of recombinant SurE was seen (Figure A3.6). Quick induction trials were attempted with 0.5 mM and 1 mM IPTG concentrations by growing the cells at 37 °C, inducing the cells at OD_{600} ~0.6 and a further 4 h incubation at 37 °C before harvesting the cells and collecting the soluble fraction. However, SurE was still found in the insoluble fraction (Figure A3.7). An expression trial at 16 °C overnight in autoinduction media also yielded SurE in the insoluble fraction (Figure A3.7). In an effort to make SurE more soluble, *surE* was cloned into the vector pDB-His-MBP. However, a quick induction trial in LB revealed His_6-MBP-SurE to still be in the insoluble fraction (Figure A3.8). To consider alternate lysis methods, lysis via a cell disruptor was also explored. Cell disruption with the flow through option at 20 kpsi still yielded His_6-MBP-SurE in the insoluble fraction (Figure A3.9). BL21(DE3)-pDB-His-MBP-*surE* cells grown in autoinduction media (Terrific broth) for 2 days at 16 °C and cells from a quick induction trial with BL21(DE3)-pET28a-*surE* were harvested and lysed with the cell disruptor using the OneShot option at 27 kpsi and the samples were analysed on a gel. However SurE was still found in the insoluble fraction (Figure A3.10).

Whilst this work was being carried out, another group successfully showed that SurE was responsible for the offloading of SB *in vitro,* and in the process had purified the *Streptomyces albidoflavus* NBRC12854 SurE homologue (Kuranaga et al., 2018). However, the methodology reported in their study (Kuranaga et al., 2018) did not yield soluble SurE in my hands (Figure A3.11). To explore the possibility of using a SurE homologue, *S. albus* J1074 *surE* was cloned into pDB-His-MBP. However, quick expression trials revealed His_6-MBP-J1074 SurE to be in the insoluble fraction still **(**Figure A3.12). The possibility of denaturing the protein to solubilise SurE (to then refold it later was investigated) with *surE* expressed from pET28a (with Dr Justin Clarke). Following harvesting after a quick induction trial, addition of 7 M urea containing lysis buffer followed by sonication yielded SurE in the soluble fraction (Figure A3.13). The soluble fraction was collected and

loaded on to the AKTA equipped with an HisTrap FF Crude column (1 mL column). The AKTA was used to purify SurE by refolding it using a gradient of washes (100% 7 M urea, 0% imidazole to 0% urea 100% 500 mM imidazole containing buffers). However, almost all of the SurE was lost in the flow through (Figure A3.14). A cell free protein expression kit was also tried to produce SurE. However, SurE failed to be synthesised *in vitro* and only the positive control protein was successfully synthesised.

5.11.4 Purification of SurE and protein identification

SurE was successfully produced from BL21(DE3)-pET28a-*surE* cells grown in autoinduction media in the conditions described in Chapter 2 (2.5.2.) His_6-SurE is predicted to be 49764.13 Da and a band consistent with this was seen on the gel (Figure 5.11). The purification of SurE was done together with Asif Fazal.

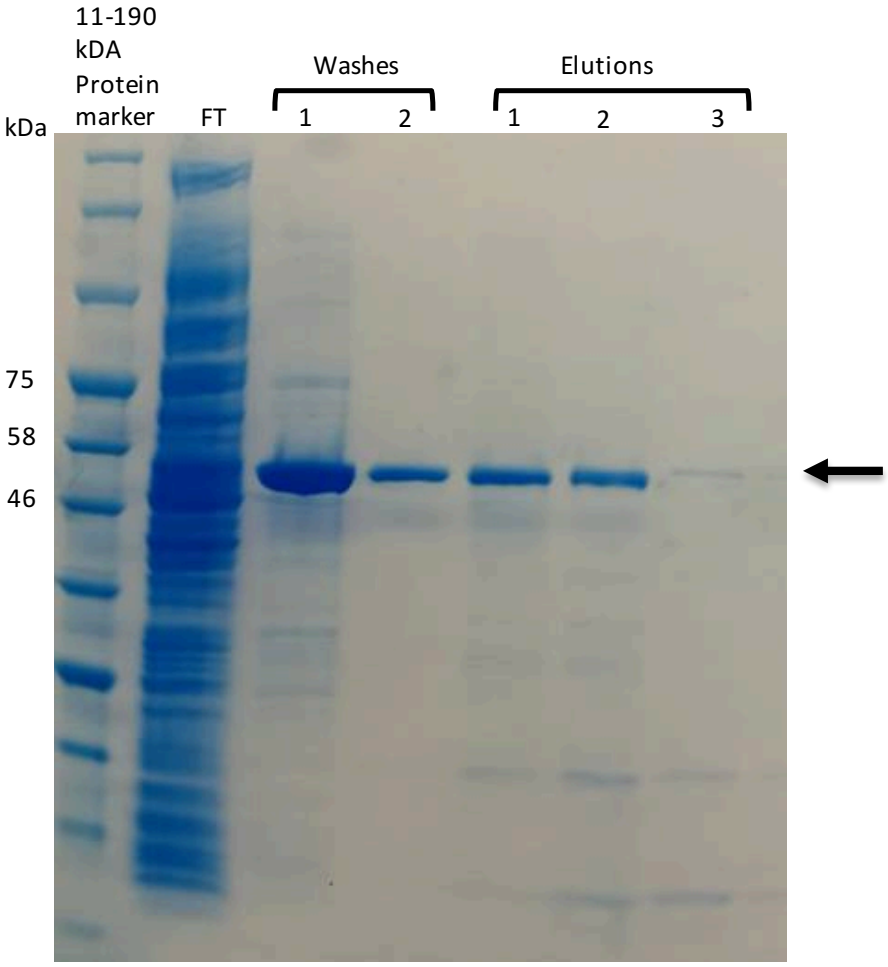

Figure 5.11 Purification of His_6-SurE. His_6-SurE is expected to be 49764.13 Da and a band consistent with this was observed (denoted by the black arrow)

The protein elution samples were concentrated and desalted for further analysis. To verify that the band seen on the gel was in fact His_6-SurE, the protein sample was analysed by the University of Leeds mass spectrometry facility (Figure A3.15). N-terminal methionine excision is a very common post translational modification in proteins and is often critical for the function and stability of the protein. Mass shifts of -131 corresponds to the loss of a methionine (Giglione et al., 2004; Bonissone et al., 2013). His_6-SurE is predicted to be 49764.13 Da and His_6-SurE without the N-terminal methionine is predicted to 49632.93 Da. The mass observed in the protein mass spectrum data for His_6-SurE is 49630.52 $\pm$ 4.08. The mass of His_6-SurE minus the N-terminal methionine falls into the error range of the observed mass further confirming that the protein purified is indeed His_6-SurE.

5.11.5 Analysis of the *in vitro* activity of SurE on SA-SNAC

The purified His_6-SurE was used in *in vitro* assays with a SNAC thioester mimic to recapitulate the *in vivo* results and to verify the chain releasing activity of SurE. The SNAC used in this study was kindly made by Dr Daniel Francis. Although Dr Francis arduously attempted to make both SA and SF SNACs, he was only successful in making the SA-SNAC with adequate purity. Synthesis of SF-SNAC was particularly challenging due to its hydrophobic nature and the presence of the non-proteinogenic amino acid R-AMPA (3-amino-2-methylpropionic acid). Therefore, the assay was only performed with SA-SNAC. If SurE is active *in vitro* and is responsible for the release of SA by its macrolactamisation, then the enzyme should be able to produce SA from the SA-SNAC.

The assay revealed that His_6-SurE was indeed active and that it was able to act on the SA-SNAC to generate the final cyclic product SA (Figure 5.12). As expected there was no SA or SA-SNAC present in the buffer or the His_6-SurE reaction controls (Figure 5.12). In the SA-SNAC only control, a very low amount of SNAC less SA was present (probably left over from the synthesis and purification of SA-SNAC). To account for this small amount of SA, the area under the peak was calculated for SA in the SA-SNAC only control and this was subtracted from the peak area of SA in the actual reaction (Figure 5.12). In the presence of His_6-SurE and SA-SNAC, conversion of the SA-SNAC to SA was seen, however the SA-SNAC was

not completely converted to SA even after prolonged incubation. Comparing the area underneath the curve, 63.8% of the SA-SNAC was converted to SA and in regard to the total amount of material, 92.6% of the SA-SNAC was recovered between the SA-SNAC and SA in the reaction.

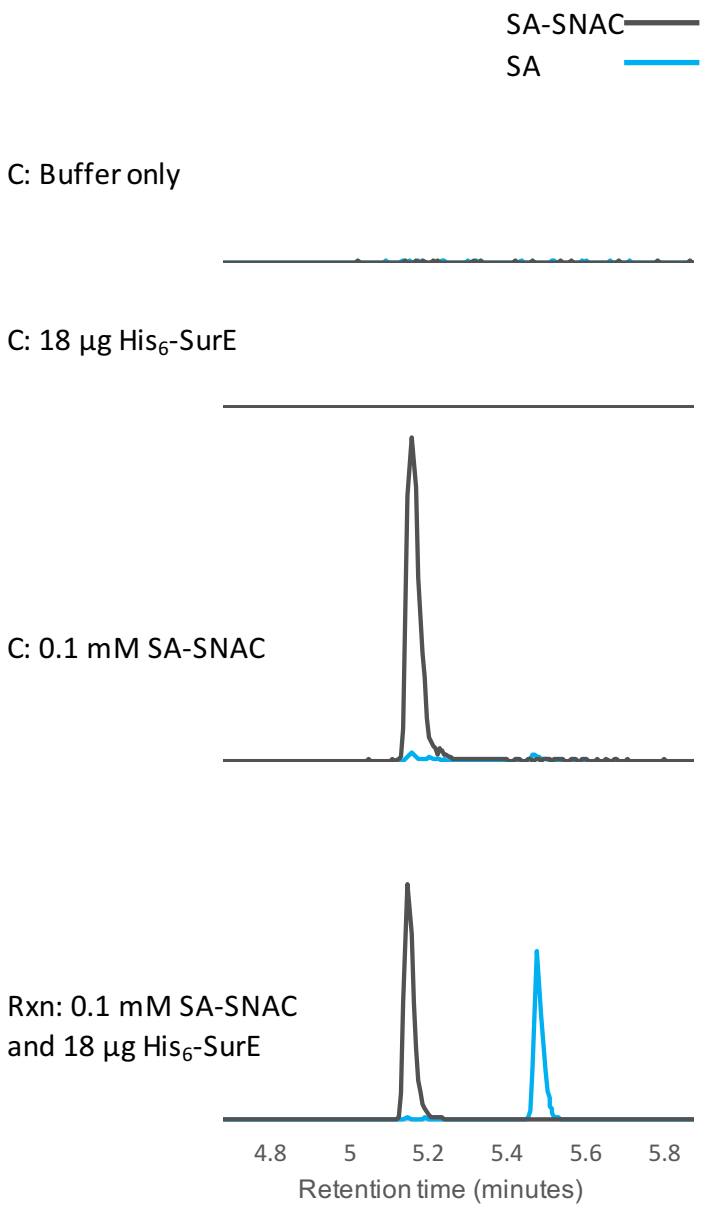

Figure 5.12 SurE is able to convert the surugamide A-SNAC to surugamide A. The EICs corresponding to the $[M+H]^+$ ions are shown for SA-SNAC ($C_{52}H_{90}N_{10}O_9S$) and SA ($C_{48}H_{81}N_9O_8$). Neither SA-SNAC nor SA are seen in control reactions. In the presence of SurE and SA-SNAC, SurE is able to convert the SA-SNAC to SA

5.12 Discussion

As observed usually with type II TEs associated with NRPSs, the deletion of *surF* resulted in a reduction in the titre of the final products SA and SF (Figure 5.8). Sequence alignment of SurE with the type II TEs SrfTEII and RifR revealed the conserved catalytic triad of Ser, His and Asp, typical of the α/β hydrolase fold superfamily (A3.16). Bioinformatically, SurF is predicted to belong to IPR029058, while type II TEs usually belong to IPR012223. Additionally, type II TEs normally have the hydrolase motif GHSMG (A3.16) while SurF possess the GXSXG motif (A3.16). However, the observed reduction in the titre of SA and SF is consistent with SurF being a type II TE. Though, the tire of both SA and SF were reduced, titre of SA (62%) was reduced more than that of SF (16%) (Figure 5.8). The genes encoding the production of SA and SF are co-transcribed, however, the titre of SA in the WT strain is greater than that of SF under the growth conditions tested (titre of SA is ~91 times greater than that of SF in the WT). To speculate, it maybe that because the rate of synthesis of SA is greater than that of SF, SurF has a greater observable impact on the titre of SA. It may also be that SurF is one of the rate limiting factors in the production of SF, where the available SurF may work competitively on SA synthesis. SF also requires the non-proteinogenic amino acid R-AMPA. The availability of AMPA could be another limiting factor in SF synthesis.

Surprisingly, in the complementation strain, while the titre of SA was almost back to WT levels, the titre of SF was greater than that is seen in the WT by 57%. In the complemented strain *surF* is overexpressed from the *ermE** promoter. It may be that with a stronger expression of *surF* compared to that of the native promoter, the increase in the availability of SurF has a directly positive impact on the synthesis SF. In the picromycin/methymcyin PKS biosynthetic system, while the deletion of its associated type II TE had no effect on the PK production levels, its coexpression in the heterologous host *Streptomyces lividans* increased the production of the PK up to sevenfold (Tang et al., 1999; Chen et al., 2001). It is unclear why only certain NRPS and PKS systems possess type II TES and why their deletion is only fully detrimental to some. However, the role they play in efficiency of these megasynthetases is important (Kotowska and Pawlik, 2014). The results obtained in this study suggest that SurF has a similar role to that of type II TEs.

SurE, however, is essential for the synthesis of both the cyclic SA and linear SF (Figure 5.10). Deletion of *surE* abolished the production of both SA and SF (Figure 5.10) and the production was restored in the complementation strain (Figure 5.10). Additionally, the purified SurE was active *in vitro* and was able to utilise the SA-SNAC thioester mimic to produce the final compound SA (Figure 5.12). These results support the hypothesis that SurE is a *trans*-acting release factor. It is especially interesting that SurE seems to be responsible for the release of both SA and SF, a cyclic and a linear product, respectively. SA is probably released by the off-loading of the linear peptidyl-*S*-thioester intermediate followed by a head to tail cyclisation whereas SF is probably released via a hydrolytic route with the release of the peptidyl-*S*-thioester intermediate.

β-lactamases can be classified into Classes A-D depending on their amino acid sequence similarity (Bush and Jacoby, 2010). Classes A, C and D function via serine ester hydrolysis mechanisms while class B comprises of metallo β-lactamases (Bush and Jacoby, 2010; Öztürk et al., 2015). Metallo-β-lactamases can be further subdivided into B1, B2 and B3 subclasses (Bush and Jacoby, 2010). SurE, lacks the conserved metal binding residues found in canonical metallo-β-lactamases and therefore is unlikely to be a metallo-β-lactamase (A3.17 and A3.18). Instead, SurE shares the active site serine (SXXK) with Class A, C and D β-lactamases (A3.19). β-lactamases are believed to have evolved from the bacterial cell wall biosynthetic enzymes penicillin binding proteins (PBPs) and most members of both families possess a catalytic serine in their active site (Massova and Mobashery, 1998). PBPs are divided into three major groups: Class A, B and C. Classes A and B comprise of high molecular weight (HMW) PBPs and Class C consists of low molecular weight (LMW) PBPs. Class A HMW PBPs are bifunctional proteins that are capable of transglycosylase and transpeptidase activities while Class B PBPs are mono-functional proteins with transpeptidase activity. Class C LMW PBPs comprises of D, D carboxypeptidases and/or D, D endopeptidases (Massova and Mobashery, 1998). Sequence alignment of SurE with Class A, B and C PBPs (A3.20) revealed that SurE had the catalytic residues arranged in a similar fashion to that of LMW Class C PBPs (A3.20.3). The Class C LMW PBPs can be further subdivided into subclasses namely A, B and C based on their sequence similarity. The subclass B LMW PBPs have a similar active site composition to Class C β-lactamases. Sequence alignment of SurE with the

subclass B LMW PBP and Class C β-lactamases (A3.21), revealed that SurE behaves the same way and shares most catalytic sites with the Class C β-lactamases (the conserved H residue is replaced by a K residue in Class C β-lactamases). These analyses show that SurE shares all the catalytic residues characteristic of subclass B LMW PBPs that share similarities with Class C-β-lactamases.

Whilst this study was being carried out, Kuranaga *et al.,* demonstrated that SurE was responsible for the release of SB using a SNAC thioester mimic of SB (Kuranaga et al., 2018). The study showed that the recombinant SurE was able to use the linear SB-SNAC to produce the mature SB. They chose to work on SB instead of SA (or SC-E) as the synthesis of SB does not require the rare amino acid D-Ileu. The total chemical synthesis of SB in their study revealed additional insight into the synthesis of these cyclic peptides (Kuranaga et al., 2018). SurE was unable to cyclise the peptidyl SNAC of SB with the C-terminal D-Leu replaced with an L-Leu. The data collected suggested that SurE has specificity for D-amino acids at the C-terminus of its substrate (Kuranaga et al., 2018). The last of A domain of SurD encodes a D-Leu and of SurC encodes a D-Ala. PBPs or DD peptidases also generally recognise D-amino acids (D-Ala-D-Ala in most cases), SurE being similar to the PBPs likely also cyclises SA by recognising the terminal D amino acid of the SA intermediate (Massova and Mobashery, 1998; Öztürk et al., 2015; Kuranaga et al., 2018).

5.13 Conclusions

From this study, SurF was found to function as a type II thioesterase and together with the results presented by Kuranaga *et al.,* this is the first report of a PBP homologue, which has been shown to responsible for an NRPS assembly line release (Kuranaga et al., 2018). SurE is especially interesting in that it is able to release the cyclic peptide via macrolactamisation and the presumably the linear peptide most likely through a hydrolytic route. This work contributes to the understanding of how NRPs are off loaded from the megasynthetases. With the wealth of knowledge now available through genome sequencing and genome mining, this study contributes to expand the understanding of how NRPs may be off loaded in some existing and ‘to be discovered’ NRPS systems. With the pharmaceutical potential that many NRPs possess, industrially producing NRPs, to synthesise derivatives or to chemically synthesise NRPs are longstanding goals of the NRPS field.

Understanding alternative release mechanisms will expand the synthetic biology toolbox available for engineering the biosynthesis of NRPs and will provide an opportunity to increase the chemical space of important NRP therapeutics (Martínez-Núñez and López, 2016).

Chapter 6 General discussion and conclusions

Since their discovery, antibiotics have revolutionised human medicine. The discovery of penicillin in 1928, followed by the use of sulphonamides and later the development of streptomycin, tetracycline and chloramphenicol ushered in the Golden Age of antibiotic discovery (Clardy et al., 2009). As a result, huge progress was made in managing many highly infectious diseases (Renwick et al., 2015). Without the treatments that we have in place through antibiotics, many of the medical achievements that we take for granted today would not be possible (Renwick et al., 2015; Katz and Baltz, 2016). However, the rise in antibiotic resistance combined with the decline in antibiotic discovery has put antibiotic therapy in great jeopardy. Diseases and the microbial agents responsible, which were previously thought to be controlled by antibiotics are returning with the emergence of multi-drug resistance. Consequently, this has placed a significant burden on healthcare globally (Wright, 2010; Livermore, 2011; Silver, 2011). Therefore, continued discovery and development of new antibiotics is crucial.

Most antibiotics used in the clinic are derived from natural products or secondary metabolites obtained predominantly from actinomycetes (Barka et al., 2016). Within the *Actinobacteria* phylum, the soil-dwelling streptomycetes are responsible for two-thirds of all the antibiotics used in the clinic (Clardy et al., 2006; Barka et al., 2016). Owed to their biochemical diversity, potency and selectivity, secondary metabolites remain the lead molecules for the development of antibiotics. Even though it was suggested that soil might be an exhausted source of antibiotics, genome sequencing and genome mining has revealed the untapped wealth of secondary metabolites encoded in the genomes of actinomycetes (Challis, 2008b; Wright, 2010; Ziemert et al., 2016). Activation of these silent BGCs along with understanding the biosynthesis of these compounds will be key in tapping into their chemical potential (Wilkinson and Micklefield, 2007).

Chapter 3 describes the generation of a new heterologous host, *Streptomyces albus* S4 Δ5. Heterologous expression is a cluster-specific approach where the BGC of interest is expressed in a genetically amenable surrogate host (Zhang et al., 2011; Ongley et al., 2013). It can be a powerful tool in isolating the secondary metabolite that is predicted to be encoded by

a BGC identified through genome mining. Heterologous expression also provides a means to characterise the biosynthetic pathway in a genetically tractable host as well as a means to refactor the pathway to generate new variants of the compound (Zhang et al., 2011; Ongley et al., 2013). It can also aid in optimising the production of a desired compound (Ongley et al., 2013). Therefore, heterologous hosts represent a valuable tool in antibiotic discovery and development efforts.

Five BGCs were rationally targeted and mutated in *S. albus* S4 to generate *S. albus* S4 ∆5. *S. albus* S4 is a relatively fast growing, genetically tractable strain with the capability to produce a diverse array of secondary metabolites. The BGCs that were mutated encoded the production of two antifungal compounds (antimycin and candicidin; (Seipke, Barke, et al., 2011)) and three previously known antibacterial compounds, which are not produced by *S. albus* S4 under standard laboratory conditions (surugamide, albaflavenone and fredericamycin (Seipke, 2015)). The resultant strain, *S. albus* S4 ∆5 did not exhibit any antifungal or antibacterial activity. The abolishment of the production of these compounds will aid in minimising rediscovery when bioactivity is observed. With the deletion of these BGCs, a significantly less complex chromatographic profile was obtained from the chemical extracts, which will ease detection of heterologously produced compounds in *S. albus* S4 ∆5. The cinnamycin BGC was successfully heterologously expressed in *S. albus* ∆5, demonstrating its potential as a heterologous host. Therefore, in this study (in a multiparty effort with Dr Ryan Seipke and Ellie Harris), a new heterologous host was generated, which is hoped to be used as a tool that will aid in the discovery and development of new and useful chemical entities.

Chapter 4 describes the activation of a silent BGC, which led to the identification of surugamides in *S. albus* S4. The pleiotropic approach of varying growth conditions resulted in the production of an antibacterial compound(s) in *S. albus* S4 when grown on mannitol minimal medium (MM) agar. Submission of the MSMS data from obtained from the ethyl acetate extracts prepared from *S. albus* S4 grown on MM to the GNPS (Global Natural Products Social molecular networking; (Mohimani et al., 2016; Wang et al., 2016)) platform revealed the presence of the surugamide A (SA) and D within the chemical extract. Surugamides are a group of non-ribosomal peptides (NRPs) that were originally isolated from a marine *Streptomyces*

sp. (Takada et al., 2013). The *sur* BGC encodes the production of the cyclic octapeptides surugamides A-E as well as a linear decapeptide named surugamide F (SF) (Takada et al., 2013; Ninomiya et al., 2016). SA was previously reported to have antibacterial activity against *Staphylococcus aureus* (Wang et al., 2014). In this study, gene inactivation studies were used to successfully match surugamides to its BGC in *S. albus* S4. The antibacterial activity seen on MM agar still remained even in the SA production mutant. Pleiotropic approaches bring about global changes in the cell and as a result, can activate more than one BGC. While pleiotropic strategies are high throughput, they offer less control making identification of compounds challenging (Rutledge and Challis, 2015).

However, as the bioactivity profile and mechanism of action of SA was unknown, it was chosen to be further explored and was therefore commercially synthesised. SA did not exhibit any bioactivity against *Candida albicans* or *Escherichia coli.* The MIC (minimum inhibitory concentration) of SA against *S. aureus* was previously reported to be 9.1223 μg/mL. However, in this study the MIC of SA against *S. aureus* was approximately 28 times greater (256 μg/mL) than the reported MIC. In the previous report, SA was purified from the microbe itself. The purification process of natural products is a challenging and laborious process. Natural products exist as complex matrices and therefore, isolation of specific compounds can be extremely challenging (Xiao et al., 2013). As a result, it can sometimes be contaminated with other compounds from within the crude chemical extract. It is possible that such a contaminant might be responsible for the lower MIC of SA that was previously observed. The *sur* BGC also encodes the production of the linear peptide, SF. We therefore hypothesised that SA and SF may work synergistically to exert their antibacterial activity. As synthesised or purified SF was not available, this hypothesis was tested with the structurally similar linear peptide antibiotic gramicidin D. SA and the membrane damaging antibiotic gramicidin D did in fact exhibit synergism while SA did not demonstrate synergism with either of control compounds (CTAB and erythromycin). These results suggest that SA may work synergistically with SF. However, this hypothesis still needs to be verified with SA and SF themselves. If they do in fact exhibit synergism, to my knowledge, this will be the first report of two compounds that are produced from the same biosynthetic locus exhibiting such a phenomenon to exert their bioactivity.

Chapter 5 explores the biosynthetic assembly line release of SA and SF. In a canonical non-ribosomal peptide synthetase (NRPS) system, a terminally located thioesterase (TE) domain is responsible for the release of the mature peptide from the assembly line (Du and Lou, 2010; Süssmuth and Mainz, 2017). In the absence of a TE domain, a reductase (R) domain or a condensation (C) domain has also sometimes been shown to be able to release the peptides (Du and Lou, 2010). In the *sur* BGC, *surAD* encodes the production of the cyclic octapeptides SA-E and *surBC* encodes the production of the linear decapeptide SF (Ninomiya et al., 2016). However, both SurD and SurC lack a C-terminally located TE, C or R domains (Ninomiya et al., 2016). In this study, two proteins, SurE and SurF were investigated for their roles in releasing SA and SF from the biosynthetic assembly line.

The gene *surE* encodes the production of a putative β-lactamase and SurF encodes the production of a putative α/β fold containing protein. In this study, it was found that SurE was required for the production of both SA and SF and that recombinant SurE was able to produce SA from the SA-SNAC further confirming its *trans*-acting assembly line release activity. This study also supports the findings of Kuranaga *et. al.,* where SurE was shown to be responsible for the release of SB using its SNAC thioester mimic (Kuranaga et al., 2018). SurE shares the catalytic residues with penicillin binding proteins (PBPs). Together with the findings of Kuranaga *et al.,* this is this the first report of a PBP homologue acting as *trans*-acting release factor in an NRPS system. Additionally, in this study, SurF acted similarly to a Type II TE, where the production titres of SA and SF were reduced in the absence of SurF. NRPs constitute a major class of natural products with diverse activities ranging from antibacterials to immunosuppressants (Du and Lou, 2010; Butler et al., 2013). In the chemical synthesis of NRPs, the cyclisation of these peptides pose various challenges. A key bottleneck and one that is poorly understood is the substrate fidelity of the C-terminal *cis*-acting thioesterase domains that release the mature peptide from the assembly line. Therefore, understanding alternate release mechanisms will provide valuable insights for the industrial production of NRPs as well as to chemically synthesise derivatives (Sieber and Marahiel, 2003). This study is hoped to contribute to the expanding understanding of assembly line release mechanisms of NRPs.

Actinomycetes, particularly streptomycetes, have long been known to be prolific producers of complex secondary metabolites. The genomics era has further revealed their chemical potential with the wealth of secondary metabolite chemistry that remain to be mined (Nett et al., 2009). With the rise in antimicrobial resistance combined with the decline in the antibiotic discovery pipeline, there is an urgent need for new antibiotics. The work in this thesis is hoped to be a stepping stone in the discovery efforts for new antibiotics and to further the understanding of the biosynthesis of some of these compounds.

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Appendices

Appendix A1 - Primers

Table A1 List of primers used in this study

Primer name	Sequence (5'- 3')	Description
EHP39	caccaggacttcttcacg	Verification of S4 Δ*ant*Δ*can*Δ*surA*
EHP40	gagggagaagaagttgtcgtgg	Verification of S4 Δ*ant*Δ*can*Δ*surA*
EHP35	gtcgtgaatctcctgatcg	Verification of S4 Δ*ant*Δ*can*Δ*surA*Δ*alb*
EHP36	tacggctacctctacatcgacc	Verification of S4 Δ*ant*Δ*can*Δ*surA*Δ*alb*
EHS9	acgc cacctcacgcggcaccggga	Protospacer sequence for pCRISPomyces-2-*sur*
EHS10	aaac tcccggtgccgcgtgaggtg	Protospacer sequence for pCRISPomyces-2-*sur*
EHS4	aaac tttctcgcggcggcccggca	Protospacer sequence for pCRISPomyces-2-*alb*
EHS5	acgc ggagatggcctcggtcggga	Protospacer sequence for pCRISPomyces-2-*alb*
EHS7	acgc gtacgcctgctccatggaga	Protospacer sequence for pCRISPomyces-2-*fdm*
EHS8	aaac tctccatggagcaggcgtac	Protospacer sequence for pCRISPomyces-2-*fdm*
EHP19	tgccgccgggcgtttttttatgtacgtcatgtccacctcc	Upstream HDR arms for pCRISPomyces-2-*sur*
EHP31	cgtaagcctgggtgttgtcctacagctcgctcagttcg	Upstream HDR arms for pCRISPomyces-2-*sur*
EHP27	cgcgaactgagcgagctgtaggacaacacccaggcttacg	Downstream HDR arms for pCRISPomyces-2-*sur*

EHP28	cggccttttacggttcctggcctctccttcaccgacttcagc	Downstream HDR arms for pCRISPomyces-2-*sur*
EHP7	tgccgccgggcgttttttatgtgtactggttccgctc	Upstream HDR arms for pCRISPomyces-2-*alb*
EHP8	tttgttcgtgcctgctttcccgaatccaccaccgaac	Upstream HDR arms for pCRISPomyces-2-*alb*
EHP9	ggcgttcggtggtggattcgggaaagcaggcacgaac	Downstream HDR arms for pCRISPomyces-2-*alb*
EHP10	cggccttttacggttcctggcctggttggatgcgaagagg	Downstream HDR arms for pCRISPomyces-2-*alb*
EHP15	tgccgccgggcgttttttatcgctcttcctgctgttgttgg	Upstream HDR arms for pCRISPomyces-2-*fdm*
EHP16	ggtcatgtggtagccgttggttggcccttcacgatcatctcc	Upstream HDR arms for pCRISPomyces-2-*fdm*
EHP17	agatgatcgtgaagggccaaccaacggctaccacatgacc	Downstream HDR arms for pCRISPomyces-2-*fdm*
EHP18	cggccttttacggttcctggcctatggtcttgaggtcgttgaagc	Downstream HDR arms for pCRISPomyces-2-*fdm*
DT159	aacgtgtttcacctgggctc	*surF* cosmid screen
DT160	acggccgaaaagaagggcaa	*surF* cosmid screen
RFS184	ccattattatcatgacattaa	Cos994 insert-end sequencing
RFS185	gtccgtggaatgaacaatgg	Cos994 insert-end sequencing
DT161	agggtccggtgggttgcagtgggcgcaggggacgcagtg**attccggggatccgtcgacc**	REDIRECT primers for deleting *surE*
DT162	gcgtcgcggggcgcccggccggccgagtggggcgggtca**tgtaggctggagctgcttc**	REDIRECT primers for deleting *surE*
DT163a	agctggtcatgcagttcgtct	Verification of S4 Δ*surE*
DT164a	atcgtgggtcaccatcagctc	Verification of S4 Δ*surE*
DT167	cggatcacgcgggacggccgaaaagaagggcaagttgtg**attccggggatccgtcgacc**	REDIRECT primer for deleting *surF*
DT168	acgccgcgcgaggcgtctgccggccccctgccggttca**tgtaggctggagctgcttc**	REDIRECT primer for deleting *surF*
DT169	tcgtcatggtgatgggctcc	Verification of S4 Δ*surF*
DT170	gtacagcggggtcagcacg	Verification of S4 Δ*surF*

DT171	acttcctcaaccacaccacg	Verification of S4 Δ*surF*
DT172	cggttcaacgtgtttcacct	Verification of S4 Δ*surF*
RFS115	cattcttcgcatcccgcct	Apramycin resistance cassette
RFS116	ctccttccgtagcgtcc	Apramycin resistance cassette
RFS96	atatctagaggatccgaagccctgcaaagtaaactggatg	Kanamycin resistance cassette
RFS97	atatctagaggatcctcagaagaactcgtcaagaaggcga	Kanamycin resistance cassette
DT208	atcacatatggtgccgatcgaacgcatcaa	construction of pIJ10257-*surF*
DT209	atctaagctttcaggcgcgctgcgcgaaga	construction of pIJ10257-*surF*
DT210	atcacatatggtgggtgccgaggggcgga	construction of pIJ10257-*surE*
DT211	atctaagctttcagagccggtgcatggccc	construction of pIJ10257-*surE*
DT045	tgaccgggaacaccgtgctcac	Hygromycin resistance cassette
DT046	cggaaggcgttgagatgcagtt	Hygromycin resistance cassette
RFS582	agcccgacccgagcacg	pIJ10257 insert sequencing primer
DT206	atctcatatggtgggtgccgaggggcgga	Construction of pet28a-*surE*, pDB-His-MBP-*surE* and pet30a-*surE*
DT207	atcactcgaggagccggtgcatggccc	Construction of pet30a-*surE*
DT213	atcactcgagtcagagccggtgcatggccc	Construction of pet28a-*surE* and pDB-His-MBP-*surE*

Restriction endonuclease sites are underlined and Redirect cassette sequences are bolded. Sequences in red represent the overlap sequences for the HDR arms for generating the pCRISPomyces-2 constructs

Appendix A2

Figure A2.1

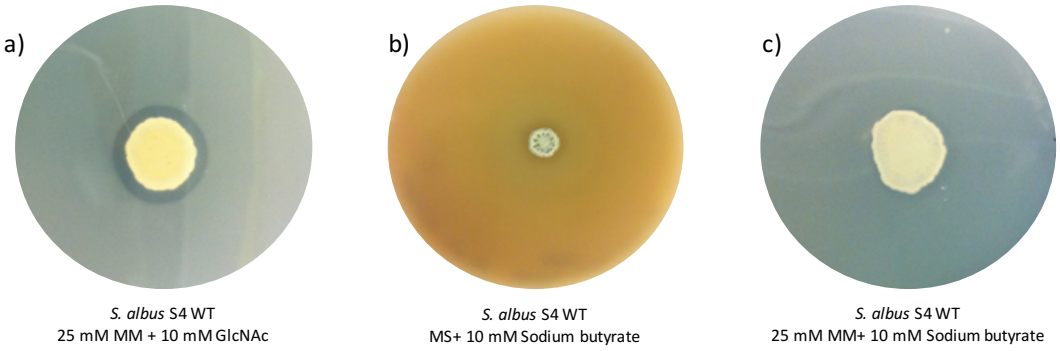


Figure A2.1 Whole cell bioassays of *S. albus* S4 against *S. aureus* SH1000 under different cultivation parameters. a) *S. albus* S4 grown on MM agar containing 10 mM N-acetylglucosamine. b) *S. albus* S4 grown on MS agar containing 10 mM sodium butyrate. c) *S. albus* S4 grown on MM agar containing 10 mM sodium butyrate. Antibacterial activity is seen on MM agar + 10 mM N-acetyl glucosamine while the addition of 10 mM sodium butyrate to the MM media suppresses the production of the antibacterial compound(s). On MS agar containing 10 mM sodium butyrate, no antibacterial activity is seen.

Figure A2.2

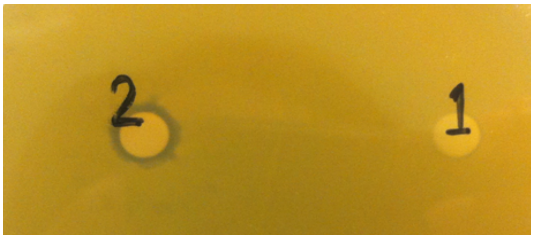


Figure A2.2 Chemical extracts prepared from *S. albus* S4 grown on MM retained antibacterial activity against *S. aureus* SH1000. 1: Methanol control; 2: Chemical extract prepared from *S. albus* S4 MM agar plates.

Figure A2.3

Filter	ViewLib	Compound_Name
1	ViewLib	Antimycin A1
2	ViewLib	Antimycin A2
3	ViewLib	Antimycin A3
4	ViewLib	Benzalkonium chloride (C12)
5	ViewLib	Dereplicator Identification - E'Surugamide_A'
6	ViewLib	Dereplicator Identification - E'Surugamide_D'
7	ViewLib	Homosalate
8	ViewLib	MLS002153196-01 N6-CYCLOPENTYLADENOSINE

Figure A2.3 Surugamides A and D were identified by the GNPS server in the MSMS data from the chemical extracts of *S. albus* S4 grown on MM agar

Figure A2.4

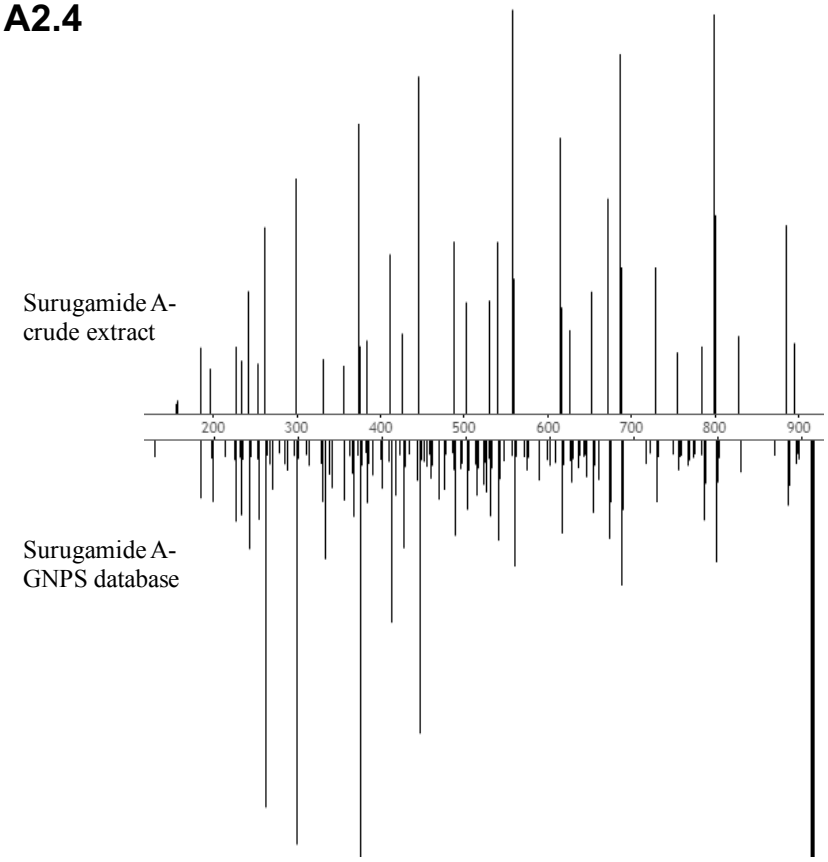


Figure A2.4 The alignment of the fragmentation pattern of surugamide A from the MSMS data of *S. albus* S4 WT chemical extract and SA in the GNPS database

Figure A2.5

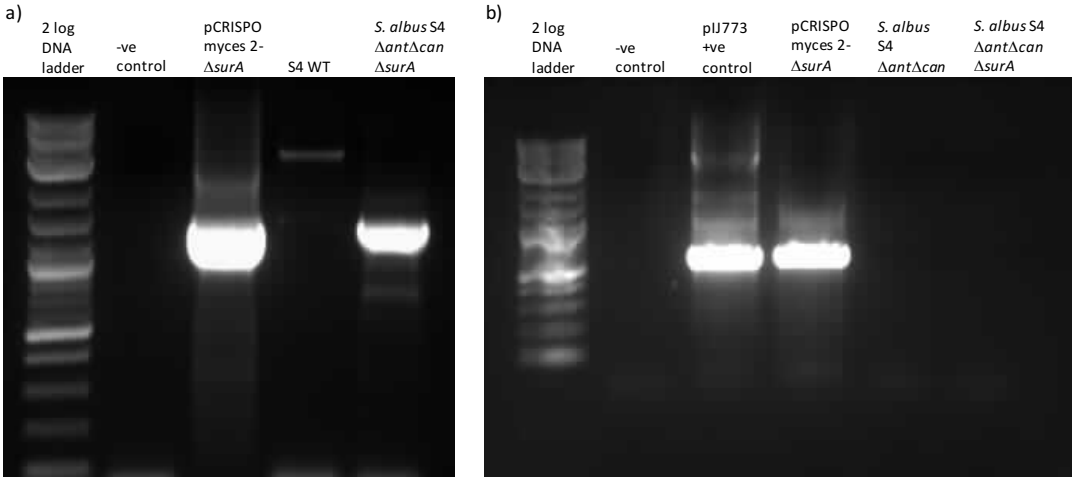


Figure A2.5 PCR verification of *S. albus* S4 Δ*ant*Δ*can*Δ*surA*. a) Verification using primer set EH_P39 and EH_P40. If the region of interest is successfully deleted, EPL: 1302 bp, if WT, EPL: 3161 bp. b) Checking for the loss of the pCRISPOmyces plasmid by checking for the absence apramycin resistance encoding assette. If the plasmid is successfully lost, then no product should be seen. If the cassette is present, EPL: 663 bp (using primer set RFS115 and RFS116)

Figure A2.6

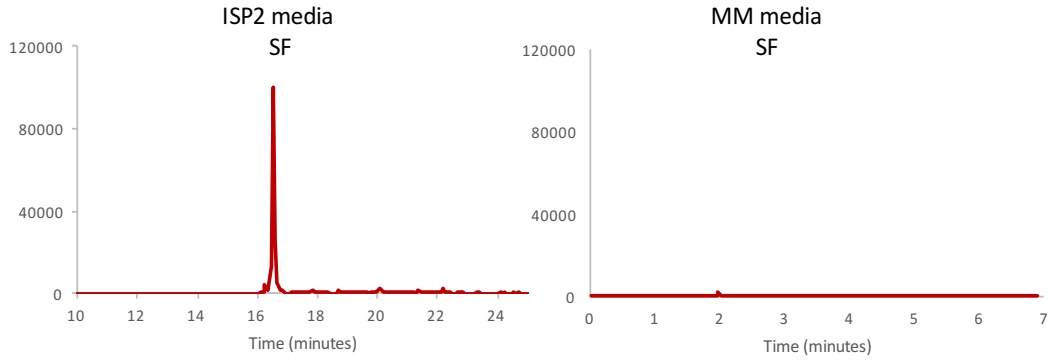


Figure A2.6 Production of surugamide F in ISP2 media compared to MM media agar. Production of SF by *S. albus* S4 is greater in ISP2 liquid cultures than on MM agar.

Appendix A3

Figure A3.1

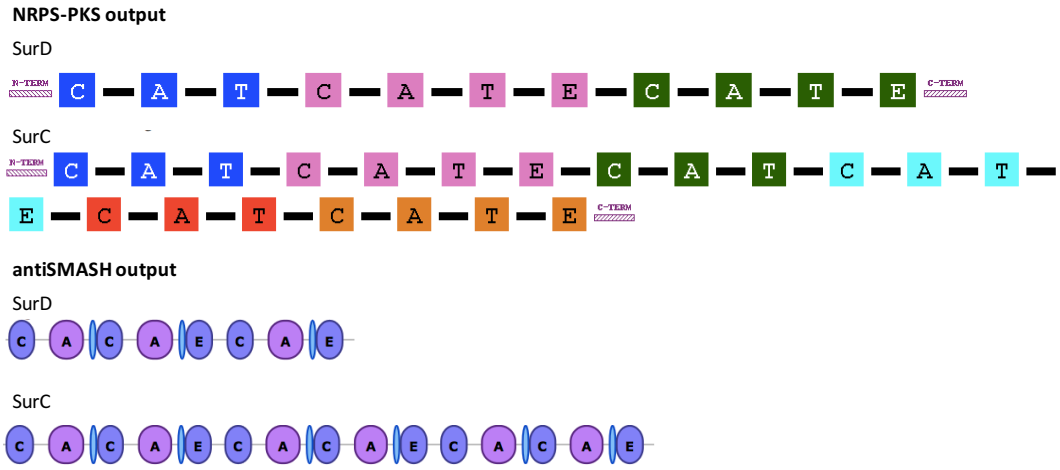


Figure A3.1 NRPS-PKS and antiSMASH outputs for *surD* and *surC*

Figure A3.2

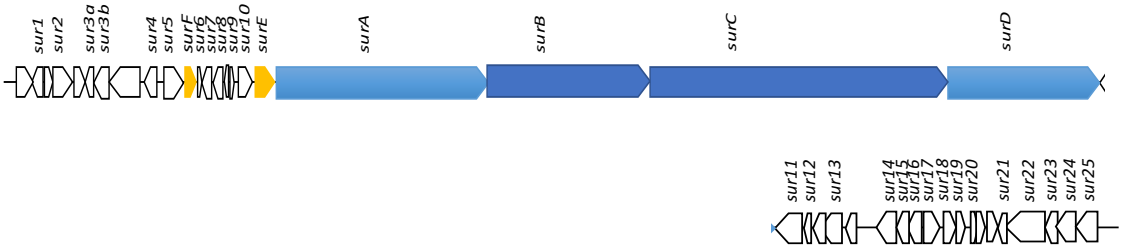


Figure A3.2 A schematic arrangement of the surugamide biosynthetic gene cluster in *S. albus* S4. (Genes encoding hypothetical proteins are not named)

A3.3

A3.3 Sequence alignment of SurE with FenTE and SurfTE

```
SurE       VGAEGAERDAVGALFEELVREHRVTGAQLAVYRDGALSEYATGLASVRTGEPVTPRTGFP      60
SrfTE      ------------------------------------------------------------      0
FenTE      ------------------------------------------------------------      0

SurE       FGSVTKFLTAELVMQFVCDGDLDLDDPLAGLLPDLGRAADPALGTATVRQLLSHTAGVVD     120
SrfTE      ----------------------------------------GG----------SDGLQD       8
FenTE      -----------------------------------AR---SQLS----------AAGEQH      12
                                                 .         : *  .

SurE       SIEYDEMRGPSYRRFASACARQPALFPPGLAFSYSNTGY--------CLLG-AVIEAASG     171
SrfTE      VTIMNQDQ----------EQIIFAFPPVLGYGLMYQNLSSRLPS-YKLCAFDFIEEEDR      56
FenTE      VIQLNQQG----------GKNLFCFPPISGFGIYFKDLALQLNHKAAVYGFHFIEEDSR      61
               ::             :    ***  .:.    .          : .  .**  .

SurE       MDWWTAMDSCLLRPLGIEPAFLHDPRPGQGGATRPVAEGHALRAGGERAERVDHMASLSL     231
SrfTE      LDR-------------------------------------------------------       59
FenTE      IEQ-------------------------------------------------------       64
           ::

SurE       AAAGGLVGSATDLVTAARPHLADRKTFAQHDLLPEDAVLAMRTCVPDAEPFGLADGWGLG     291
SrfTE      -------------------------------------------------------       59
FenTE      -------------------------------------------------------       64

SurE       LMRHGTGDGAWYGHDGAVGGASCNLRIHPDRSLALALTANSTAGPKLWEALVARLPEAGL     351
SrfTE      -------------------------------------------------------       59
FenTE      -------------------------------------------------------       64

SurE       DVGHYALPVPDSAPLAPDAGHLGTYANGDLELMVTHDAAGDLFLTRESYSDYRLSLHEDD     411
SrfTE      -------------------------------------------------------       59
FenTE      -------------------------------------------------------       64

SurE       LFVARSGEPGALPITGRFVREHPAGPVALLQYGGRAMHRL--------------------     451
SrfTE      -------------YADLIQKLQPEGPLTLFGYSAGCSLAFEAAKKLEEQGRIVQRIIMVD     106
FenTE      -------------YVSRITEIQPEGPYVLLGYSAGGNLAFEVVQAMEQKGLEVSDFIIVD     111
                       .. : . :* ** .*: *..     :

SurE       -------------------------------------------------------       451
SrfTE      SYKKQGVSDLDGRTVESDVEALMNVNRDNEALNSEAVKHGLKQKTHAFYSYYVNLISTGQ     166
FenTE      AYKKDQSITADTE------------NDDSAAYLPEAVRETVMQKKRCYQEYWAQLINEGR     159

SurE       -------------------------------------------------------       451
SrfTE      VKADIDLLTSGADFDM--PEWLASWEEATTGVYRVKRGFGTHAEMLQGETLDRNAEILLE     224
FenTE      IKSNIHFIEAGIQTETSGAMVLQKWQDAAEEGYAEYTGYGAHKDMLEGEFAEKNANIILN     219

SurE       ------  451
SrfTE      FLNTQT  230
FenTE      ILDKI-  224
```

Figure A3.4

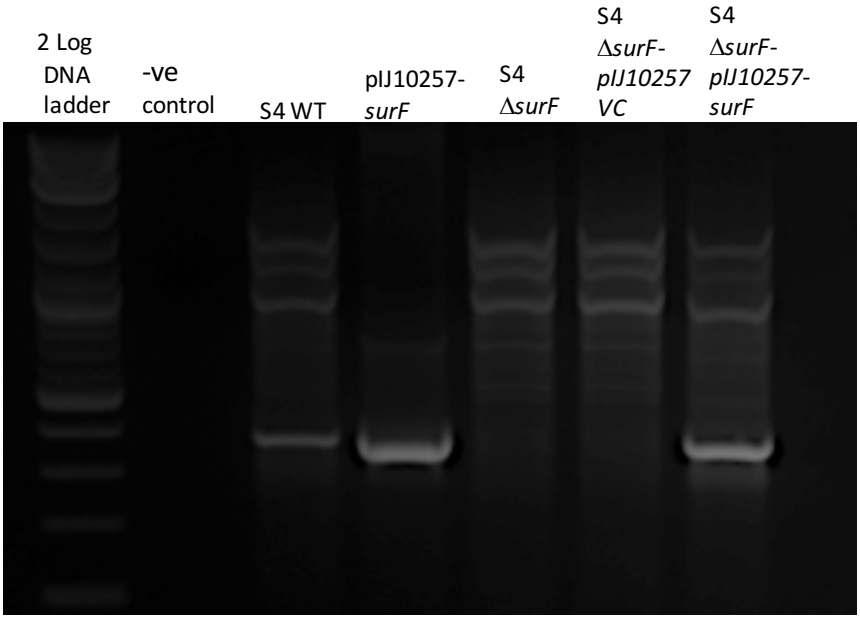


Figure A3.4 PCR verification of S4 Δ*surF*-pIJ10257-*surF*. a) Verification using primer set DT169 and 170 checking for an internal fragment of *surF*. If *surF* present, EPL: 383 bp

Figure A3.5

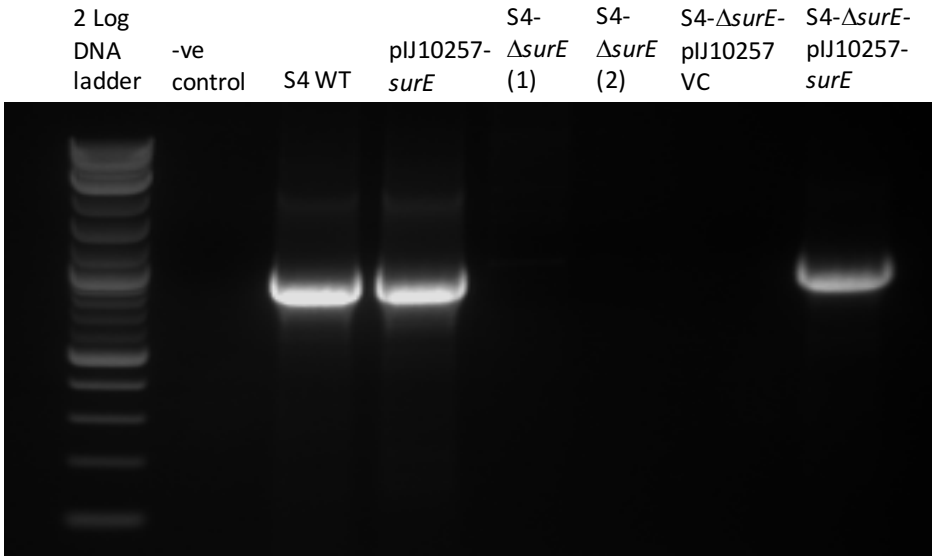


Figure A3.5 PCR verification of S4 Δ*surE*-pIJ10257-*surE*. a) Verification using primer set DT163a and 164a checking for an internal fragment of *surE*. If *surE* present, EPL: 953 bp

Figure A3.6

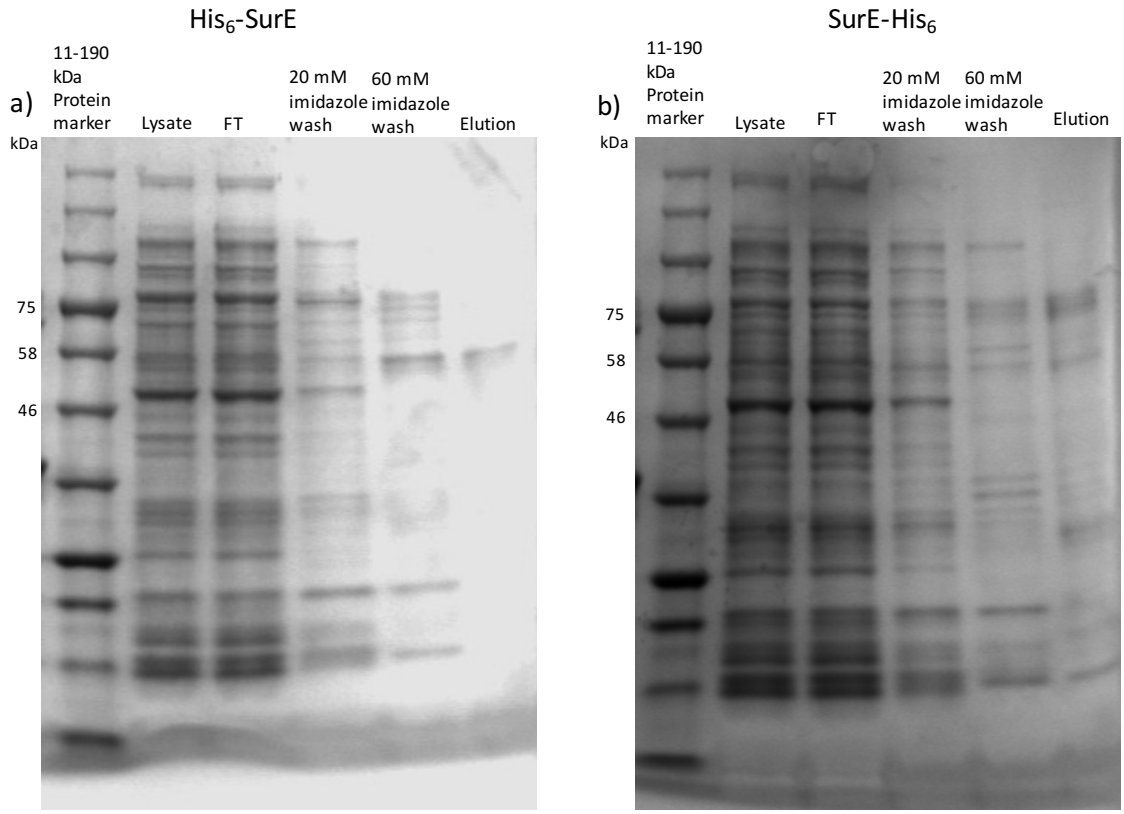


Figure A3.6. Initial expression trial of SurE from pET28a and pET30a vectors. SurE expressed from pET28a (a) is predicted to 49764.13 Da and SurE expressed from pET30a (b) is expected to be 48665.80 Da

Figure A3.7

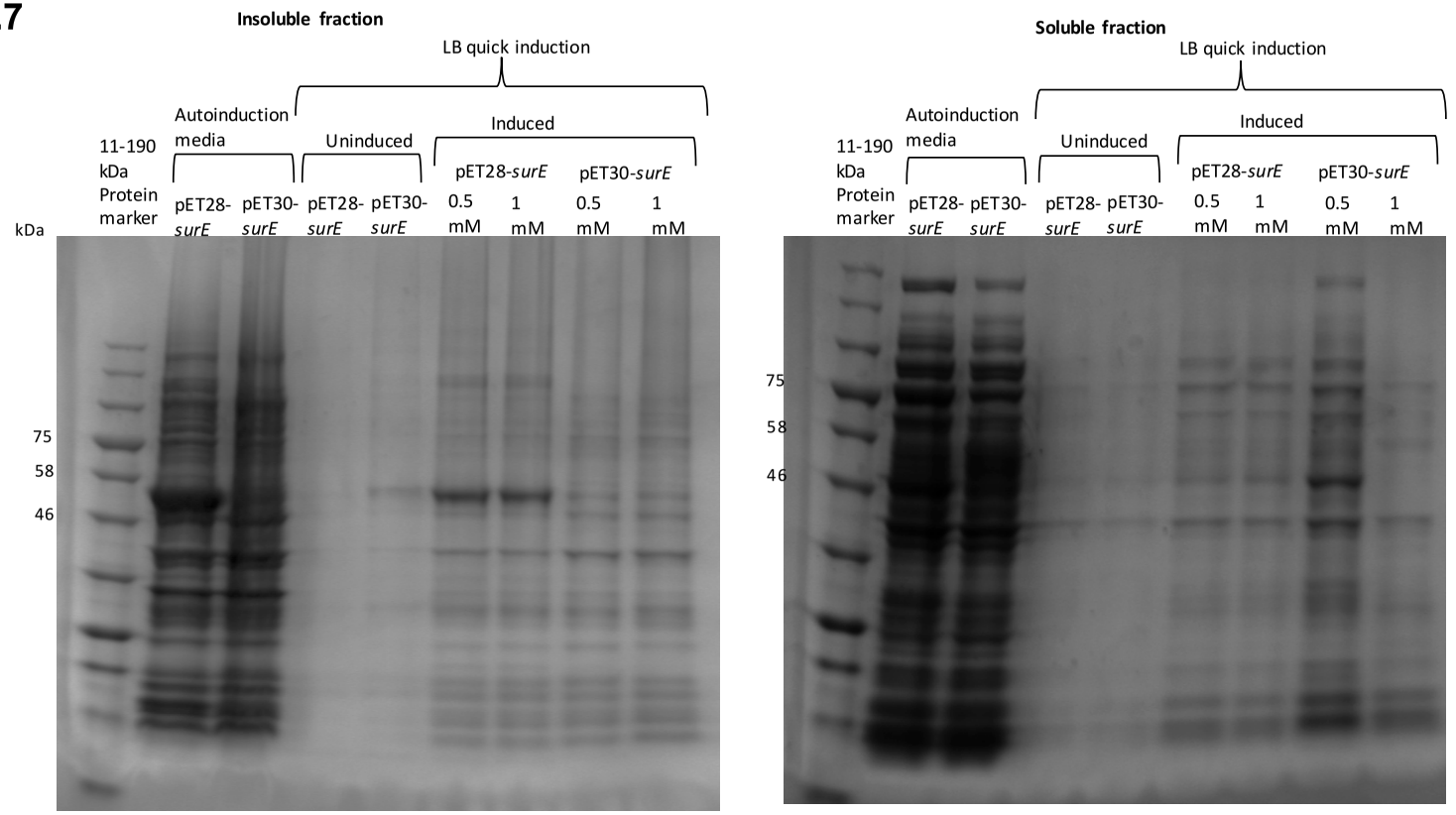


Figure A3.7. Expression trial of SurE from pET28a and pET30a in autoinduction media and in LB quick induction trial conditions. SurE expressed from pET28a is predicted to be 49764.13 Da and SurE expressed from pET30a is expected to be 48665.80 Da. LB induction trials were carried out with 0.5 mM and 1 mM IPTG. SurE expressed from pET28a in autoinduction media and LB quick indication trials were found in the insoluble fraction. SurE was not well expressed from pET30a. The 0.5 mM soluble fraction sample of SurE from pET30a appears to be darker owed to an error with more sample being loaded on relative to the other wells on the same gel.

Figure A3.8

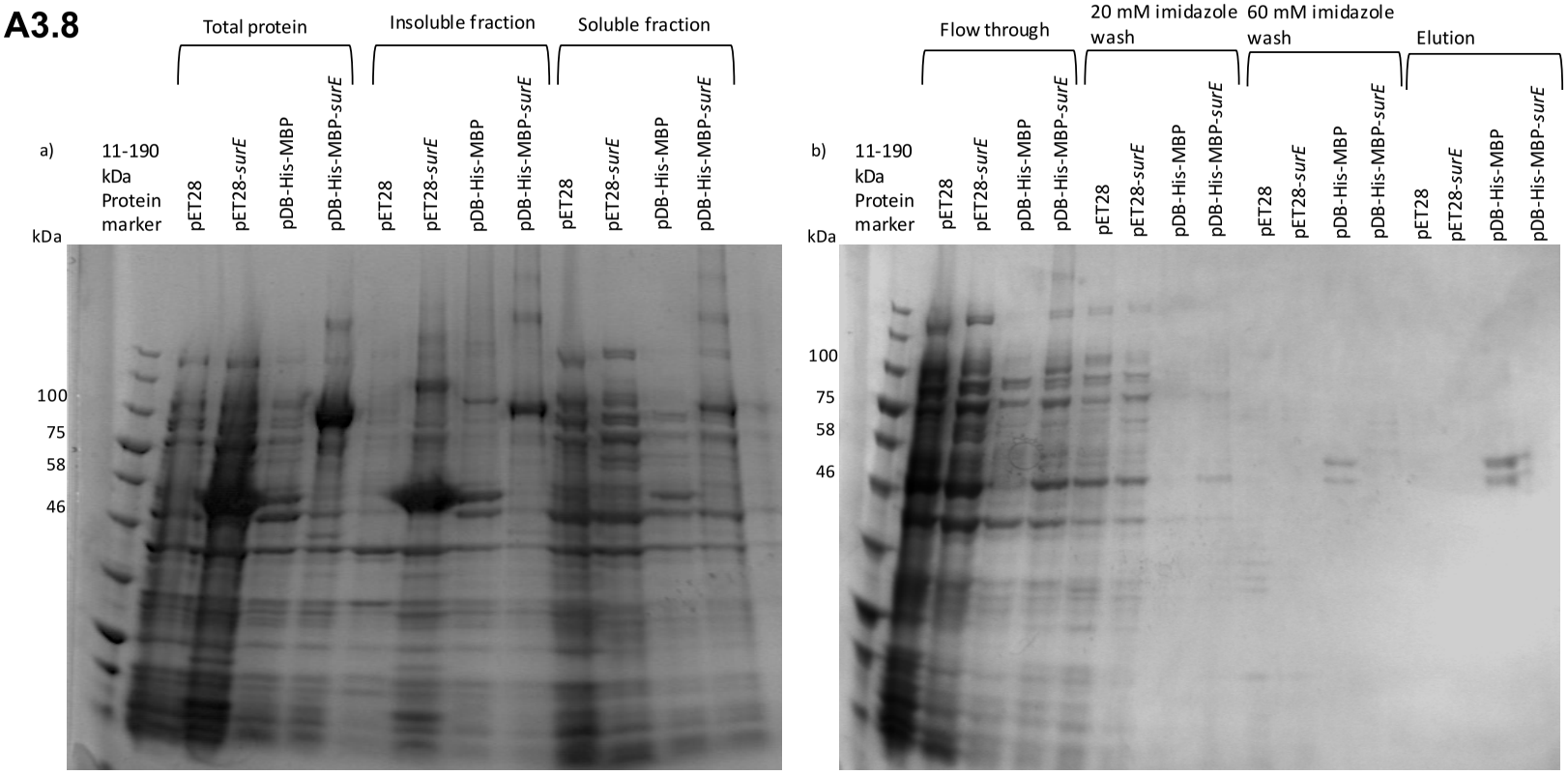


Figure A3.8. Expression of SurE from pET28a and pDB-His-MBP vectors in LB quick induction trial conditions. SurE expressed from pET28a is expected to be 49764.13 Da, SurE expressed from pDB-His-MBP is expected to be 91075.76 Da and His_6-MBP is expected to be 47550.77 Da. Expression of SurE from pET28a revealed His_6-SurE to be in the insoluble fraction and there was no His_6-SurE in the elutions, only His_6-MBP control was found in the elutions.

Figure A3.9

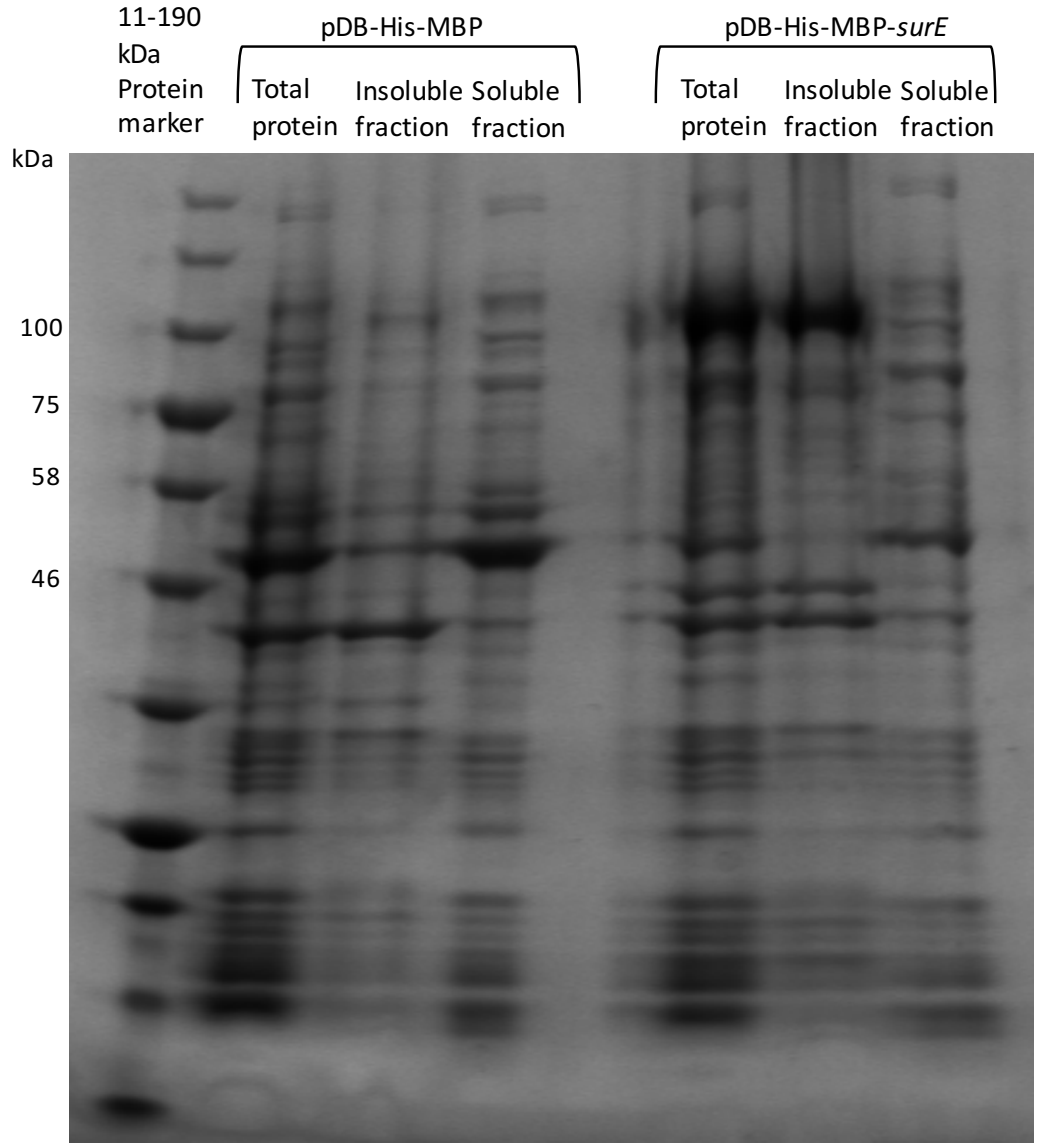


Figure A3.9 Lysis using cell disruptor with the flow through option at 20 kpsi. His_6-MBP-SurE was still present in the insoluble fraction, only His_6-MBP was found in the soluble fraction. SurE expressed from pET28a is expected to be 49764.13 Da, SurE expressed from pDB-His-MBP is expected to be 91075.76 Da and His_6-MBP is expected to be 47550.77 Da.

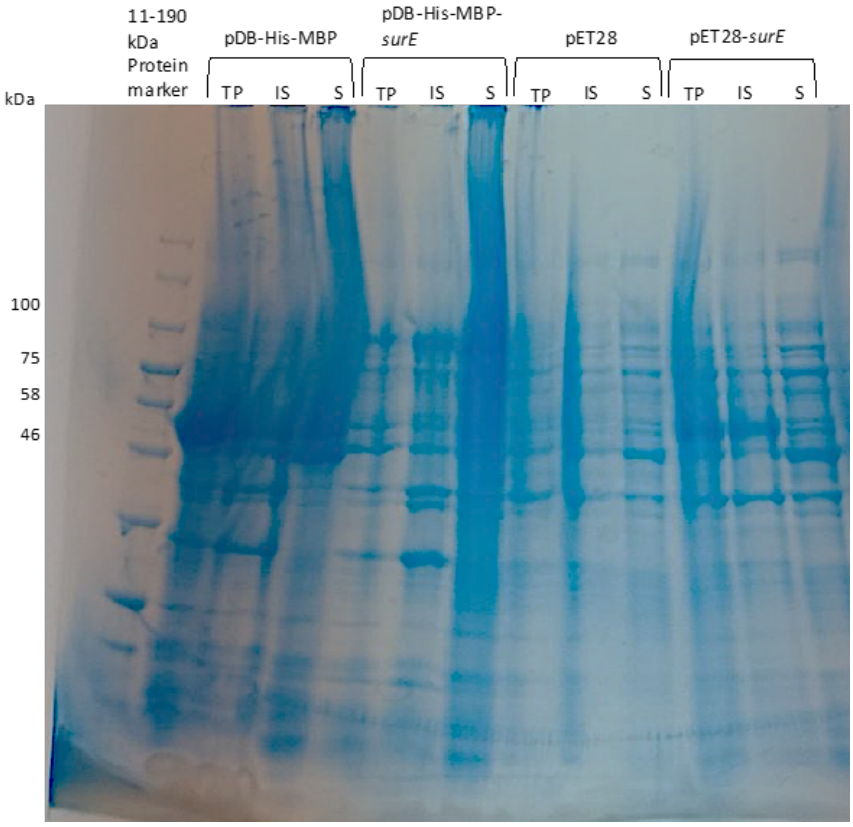


Figure A3.10 Lysis using cell disruptor with the OneShot option at 27 kpsi. (TP- total protein, IS- insoluble fraction, S- soluble fraction). SurE expressed from pET28a is expected to be 49764.13 Da, His_6-MBP-SurE is expected to be 91075.76 Da and His_6-MBP is expected to be 47550.77 Da. His_6-MBP-SurE is quite hard to see, but a band of the approximate size can be seen in the insoluble fraction. His_6-SurE was still present in the insoluble fraction, only His_6-MBP was found in the soluble fraction.

Figure A3.11

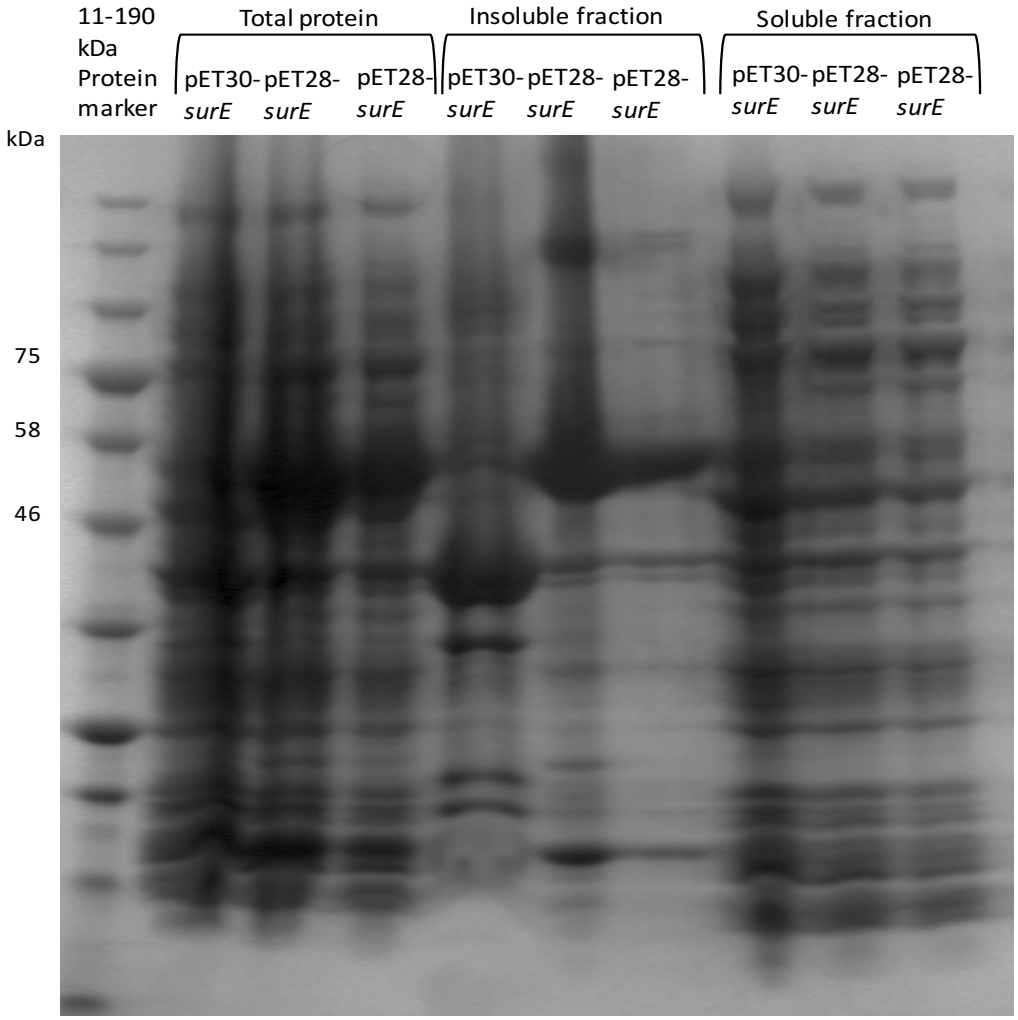


Figure A3.11 Expression trial of SurE from pET28a and pET30a vectors in 2xYT media. SurE expressed from pET28a is expected to be 49764.13 Da and SurE expressed from pET30a is expected to be 48665.80 Da. SurE expressed from pET28a was found in the insoluble fraction. SurE was not expressed well from pET30a.

Figure A3.12

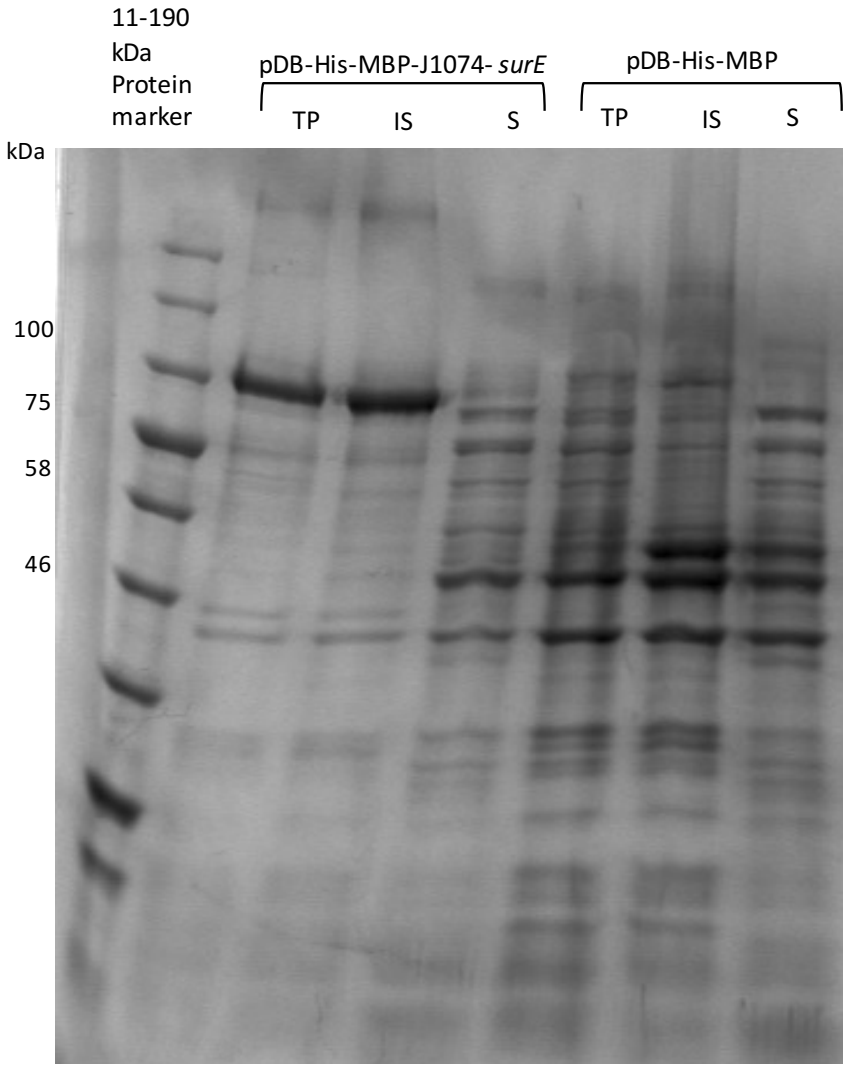


Figure A3.12. Expression trial of *S. albus* J1074 SurE homologue from pDB-His-MBP from LB quick induction media. (TP: total protein, IS- insoluble fraction, S- soluble fraction). His_6-MBP-J1074-SurE is expected to be 91029.78. His_6-MBP-SurE was still found in the insoluble fraction.

Figure A3.13

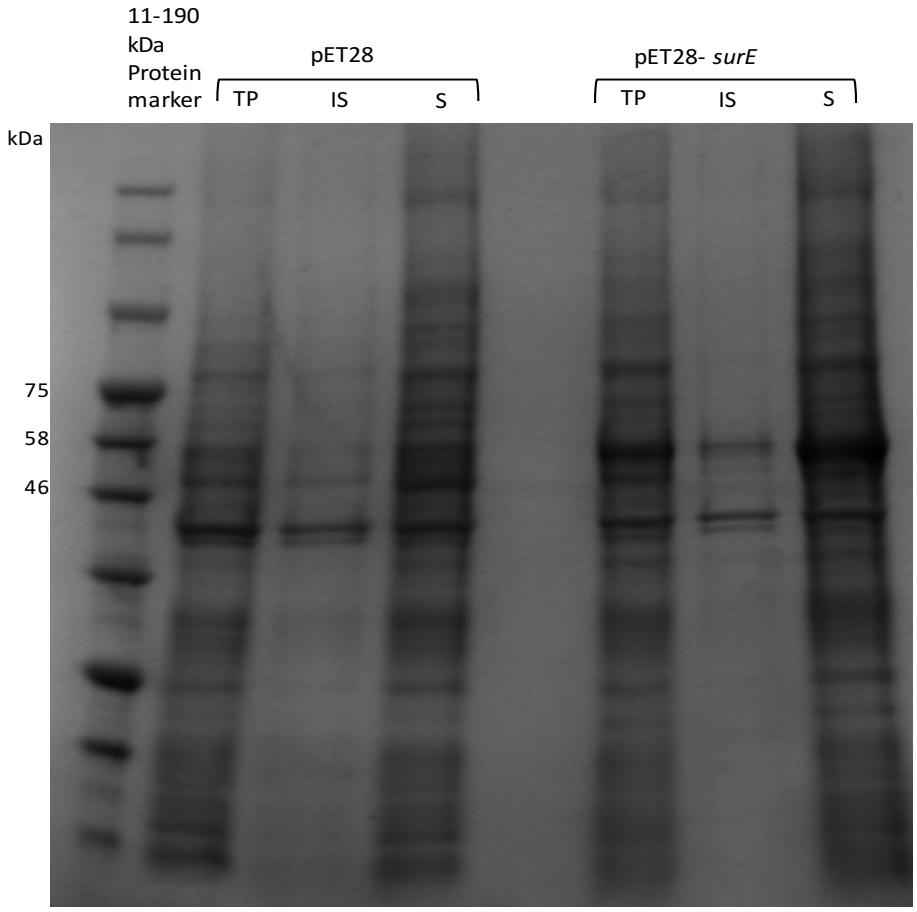


Figure A3.13 7M urea containing lysis buffer yields SurE in the soluble fraction. (TP- total protein, IS- insoluble fraction, S- soluble fraction). Recombinant His_6-SurE is expected to be 49764.13 Da.

Figure A3.14

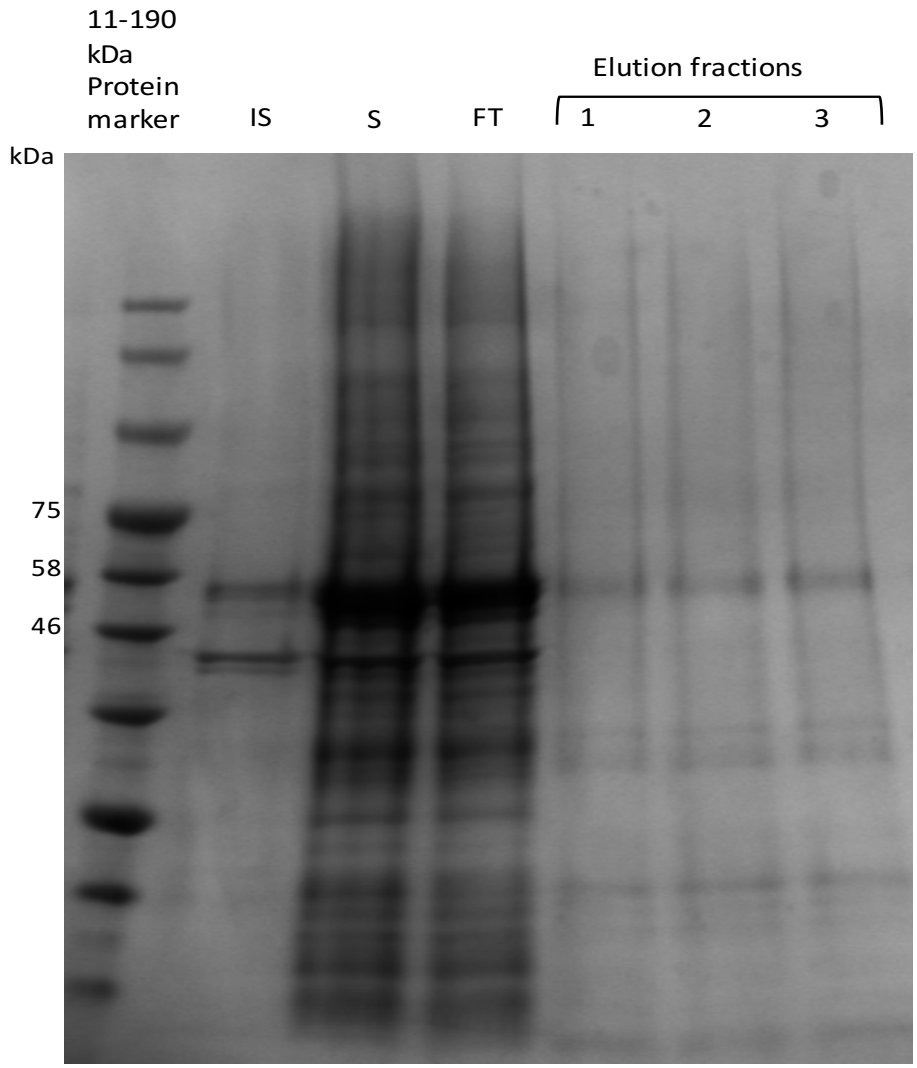
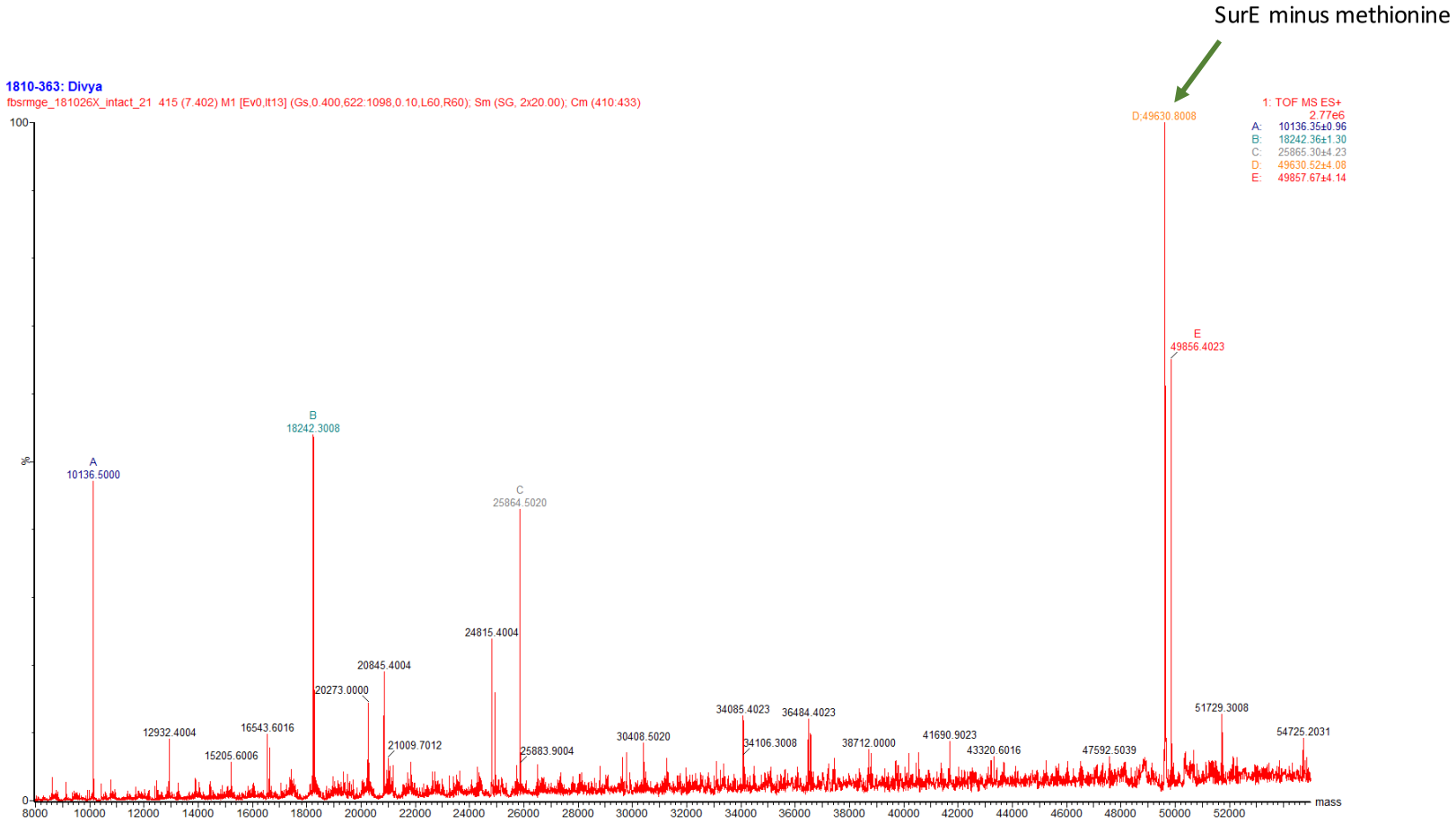


Figure A3.14. Purification trial of His_6-SurE yielded from the 7M urea lysis by refolding on the AKTA. Most of the recombinant SurE was lost in the FT. His_6-SurE is expected to be 49764.13 Da (IS: insoluble fraction, S: soluble fraction, FT: flow through).

Figure A3.15


SurE minus methionine
1810-363: Divya
fbsrmge_181026X_intact_21 415 (7.402) M1 [Ev0,It13] (Gs,0.400,622:1098,0.10,L60,R60); Sm (SG, 2x20.00); Cm (410:433)
1: TOF MS ES+
2.77e6
A: 10136.35±0.96
B: 18242.36±1.30
C: 25865.30±4.23
D: 49630.52±4.08
E: 49857.67±4.14
D:49630.8008
E
49856.4023
B
18242.3008
A
10136.5000
C
25864.5020
24815.4004
20845.4004
20273.0000
12932.4004
16543.6016
15205.6006
21009.7012
25883.9004
30408.5020
34085.4023
36484.4023
34106.3008
38712.0000
41690.9023
43320.6016
47592.5039
51729.3008
54725.2031
%
mass

A3.16

A3.16. Sequence alignment of SurF with SrfTEII (PDB: 2RON) and RifR (GenBank: AAG5299) The catalytic residues of SrfTEII and RifR are bolded in red and is found to be conserved in SurF. The hydrolase signature sequence is highlighted.

```
SurF         VPIERINGIRLHYEESGSGEPVVMVMGSGAGGRSWHLHQVPALRAAGYRVVTFDNRGIPP    60
RifR         MHRPEAEKWLRRFERAPDAR-ARLVCLPHAGGSASFFFPLAKALAPAVEVLAVQYPGRQ-    58
SrfTEII      -----MSQLFKSFD--ASEK-TQLICFPFAGGYSASFRPLHAFLQGECEMLAAEPPGHG-    51
                   .     ::   . . . ::    *** :  :  :        .::: :  *

SurF         TDACAEGFTVDDMVADT---AGLIEHLGLGPCRLVGTSLGAHVAQELCLARPE--LVSQV    115
RifR         --DRRHEPPVDSIGGLTNRLLEVLRPFGDRPLALFGHSMGAIIGYELALRMPEAGLPAPV    116
SrfTEII      --TNQ-TSAIEDLEELTDLYKQELNLRPDRPFVLFGHSMGGMITFRLAQKLEREGIFPQA    108
                       ::.:   *      :.     *  *.* *:*. :  .*.    .  :   .

SurF         ALLATRG------RDDAMRRA-QSRAEREFHDAGGVLTPLYGATVRALQNLSPATLRSEK    168
RifR         HLFASGRRAPSRYRDDDVRGASDERLVAELRKLGGSD----------------AAMLADP    160
SrfTEII      VIIS--AIQPPHIQRKKVSHLPDDQFLDHIIQLGGMP----------------AELVENK    150
              :::         : . :    :.:   .: . **                  * :  :

SurF         EIQDWLDIFEMAPLSGAPGHRAQLGIDMTADRLPAYRRITTPCLVVGFADDLILPAHLGR    228
RifR         ELLAMV----------LPAIRSDYR-AVETYRHEPGRRVDCPVTVFTGDHDPRVSVGEAR    209
SrfTEII      EVMSFF----------LPSFRSDYR-ALEQFELYDLAQIQSPVHVFNGLDDKK-CIRDAE    198
             *:   .           *. *::    :   .     ::  *  *.   .*       ..

SurF         EVADAIPGARYVEVADAGHYGYLERPDQVNNALLDFFAQRA---------    269
RifR         AWEEHTTGPADLRVLPGGHFFLVDQAAPMIATMTEKLAGPALTGSTGGNS    259
SrfTEII      GWKKWAKD-ITFHQFDGGHMFLLSQTEEVAERIFAILNQHPIIQP-----    242
                .   .   ..   .**   :.:   :   :   :
```

A3.17

A3.17. Sequence alignment of SurE with ACTE (active site residues of ACTE are bolded in red)

```
SurE         GAEGAERDAVGALFEELVREHRVTGAQLAVYRDGALSEYATGLASVRTGEPVTPRTGFPF      60
ACTE         ----------------------------------------------------MKRGGYR-      7
                                                                   * *:

SurE         GSVTKFLTAELVMQFVCDGDLDLDDPLAGLLPDLGRAADPALGTATVRQLLSHTAGVVDS      120
ACTE         -QINKALNISA---F--ENYLDIQH---DHLPKLNDVEQ--L-SPRVLRVLGQNAG----      51
              .:.* *. .    *  :. **::.   . **.*. . :  * :  * ::*.:.**

SurE         IEYDEMRGPSYRRFASACARQPALFPPGLAFSYSNTGYC-----LLGAVIEAASGMDWWT      175
ACTE         ----------------------------KFTLQGTNTYIVGTGRERLIIDTGQGIPEWT      82
                                        *: ..*.          :*::..*:  **

SurE         AMDSCLLRPLGIEPA-----------------------------FLHDPRPGQGGATRPV      206
ACTE         DLISSTLRDSAITLSHVLLTHWHGDHTGGVPDLIRLYPHLSNSIFKHSSSN----GQQPI      138
              : *. **  .*  :                            * *.       . :*:

SurE         AEGHALRAGGERAE-------RVDHMASLSLAAAGGLVGSATDLVTAARPHLADRKTFAQ      259
ACTE         IDGQVFHVEGATVRAMHSPGHSHDHMCFILEE---------------------------E      171
              :*:.::. *  ..         ***. :                              :

SurE         HDLLPEDAVLAMRTCVPDAEPFGLADGWGLGLMRHGTGDGAWYGHDGAVGGASCNLRIHP      319
ACTE         NAMFTGDNVLGHGT-----SAVEL--------------LGIWMASLRLMQSSGCRVGYPA      212
             : ::  * **.  *     . . *              * * .    : .:.*.:

SurE         DRSLALALTAN-----------------------------STAGPKLWEALVARLPEAG      349
ACTE         HGAVIADLLAKIAGELDQKARREARVVRTLARNKREEQSKGRSKGSMTVQELVTAMHGKG      272
```

```
          . ::   * *:                              : *    : **: :   *

SurE      LDVGHYALPVPDSAPLAPDAGHLGTYANGDLELMVTHDAAGDLFLTRESYSDYRLSLHED      409
ACTE      LDDQVRTMA------LE-------PFINEVL-----GKLAGD------GCVAFEVRRGEK      308
          **    ::       *         : *  *      . ***      .   :.:   *.

SurE      DLFVARSGEPGALPITGRFVREHPAGPVALLQYGGRAMHRL*     450
ACTE      RWFIVNDV---------------TSSP---------------     320
            *:...                :.*
```

A3.18 Sequence alignment of SurE with metallo-β-lactamases. (Bolded in black are the histidine metal binding site and in red are the cysteine metal binding sites).

A3.18.1 Sequence alignment of SurE with BcII (a B1 class of metallo-β-lactamase, PDB ID: 2BFZ)

```
SurE      VGAEGAERDAVGALFEELVREHRVTGAQLAVYRDGALSEYATGLASVRTGEPVTPRTGFP      60
BcII      ------------------------------------------------------------      0


SurE      FGSVTKFLTAELVMQFVCDGDLDLDDPLAGLLPDLGRAADPALGTATVRQLLSHTAGVVD      120
BcII      ------MKKNTLLKVGLCVGLLGTIQFVST----IS---SVQASQKVEKTVIKNETGTIS      47
                : .  *:   :* * *.  : ::     :.   .   .  . : ::.: :*.:.

SurE      SIEYDEMRGPSYRRFASACARQPALFPPGLAFSYSNTGYCLLGAVIEAASGMDWWTAMDS      180
BcII      ---ISQLNKNVWVHTELGSFNGEAVPSNGLVLN-TSKGLVLV--------DSSWDDKLTK      95
              .::.   : :   .. .  *:   **.:. :..*  *:        . .*   : .

SurE      CLLRPLGIEPAFLHDPRPGQGGATRPVAEGHALRAGGERAERVDHMAS------LSLAAA      234
BcII      ELIEMV--EKKFQK------RVTDVIITHAHADRIGGIKTLKERGIKAHSTALTAELAKK      147
           *:. :  *  * :        :   ::..** * ** :: :   : :       .**

SurE      GGLVGSATDLVTAARPHLADRKTFAQHDLLPEDAVLAMRTCVPDAEPFGLADGWGLGLMR      294
BcII      NGYEEPLGDLQTVTNLKFGNM----------------------KVETFYPGKG------H      179
          .*      ** *.:. ::.:                        ..* *  ..*      :

SurE      HGTGDGAWYGHDGAVGGASCNLRIHPDRSLALALTANSTAGPKLWEALVARLPEAGLDVG      354
BcII      TEDNIVVWLPQYNILVG-GCLVKSTSAKDLGN----VADAYVNEWSTSIENVLKR-----      229
             .  .*  : . : * .* ::    :.*.      : *  : *.: : .: :

SurE      HYALPVPDSAPLAPDAGHLGTYANGDLELMVTHDAAGDLFLTRESYSDYRLSLHEDDLFV      414
BcII      -----YRNINAVVPGHGEVGD--------------------------KGLLLHTLDLLK      257
                 :   :.*. *.:*                              * **  **:

SurE      ARSGEPGALPITGRFVREHPAGPVALLQYGGRAMHRL 451
BcII      ------------------------------------ 257
```

A3.18.2 Sequence alignment of SurE with CphA (a B2 class of metallo-β-lactamase, PDB ID: 1X8G)

```
SurE      VGAEGAERDAVGALFEELVREHRVTGAQLAVYRDGALSEYATGLASVRTGEPVTPRTGFP      60
CphA      -----------------MM-------------------------------------------      2
                           ::

SurE      FGSVTKFLTAELVMQFVCDGDLDLDDPLAGLLPDLGRAADPALGTATVRQLLSHTAGVV-      119
CphA      ----KGWMKCGL------AGAVVL-------------------------MASFWGGSVR      26
              . ::.. *       * : *                         : *. .* *
```

```
SurE      -DSIEYDEMRGPSYRRFASACARQPALFPPGLAFSYSNTGYCLLGAVIEAASGMDWWTAM      178
CphA      AAGMSLTQVSGPVYVVEDNYY--------------VQENSMVYFGAKGVTVVGATWTPDT      72
            .:.  :: ** *    .                 .:..   :**   :. *  *

SurE      DSCLL---RPLGIEPAFLHDPRPGQGGATRPVAEGHALRAGGERAERVDHMASLSLAAAG      235
CphA      ARELHKLIKRVSRK----------------PVLEV-------------------------      91
             *    : :. :                ** *

SurE      GLVGSATDLVTAARPHLADRKTFAQHDLLPEDAVLAMRTCVPDAEPFGLADGWGLGLMRH      295
CphA      ------------------------------------------------------------      91


SurE      GTGDGAWYGHDGAVGGASCNLRIHPDRSLALALTANSTAGPKLWEALVARLPEAGLD---      352
CphA      ----------------------------INTNYHTDRAGGNAYWKSIGAKVVSTRQTRDL      123
                                      :     :: :.*   *::: *:: .:

SurE      -V---------GHYALP-VPDSAPLAPDAGHLGTYANG--DLELMV---THDAAGDLFLT      396
CphA      MKSDWAEIVAFTRKGLPEYPDLPLVLPNVVHDGDFTLQEGKVRAFYAGPAHTPDGIFVYF      183
                    : .**  **   : *:. * * ::    .:. :    :*   * :.

SurE      RE---SYSDYRLSLHEDDL-FVARSGEPG--------ALPITGRFVR--EHPAGPVALLQ      442
CphA      PDEQVLYGNCILKEKLGNLSFADVKAYPQTLERLKAMKLPIKTVIGGHDSPLHGPELIDH      243
           :    *.:  *. : .:* *.  .. *          ***.  :    .   **  : :

SurE      YGGRAMHRL--        451
CphA      YEALIKAAPQS        254
```

A3.18.3 Sequence alignment of SurE with FEZ1 (a B3 class of metallo-β-lactamase, PDB ID: 1K07)

```
SurE      VGAEGAERDAVGALFEELVREHRVTGAQLAVYRDGALSEYATGLASVRTGEPVTPRTGFP      60
FEZ1      ------------------------------------------------------------      0


SurE      FGSVTKFLTAELVMQFVCDGDLDLDDPLAGLLPDLGRAADP-ALGTATVRQLLSHTAGVV      119
FEZ1      ---MKKVLSLTALMMVLNHS--SFAYPMPNPFPPFRIAGNLYYVGTDDLASYLIVT----      51
             :.*.*:   :* .: ..  .:  *: . :* :  *.:   :**  : . *  *

SurE      DSIEYDEMRGPSYRRFASA-CARQPAL--FPPGLAFSYSNTGYCLLGA-VIEAASGMDWW      175
FEZ1      -------PRGNIL--INSDLEANVPMIKASIKKLGFKFSDTKILLISHAHFDHAAGSE--      100
                 **     : *   *. * :      *.*.:*:*   *:.   :: *:* :

SurE      TAMDSCLLRPLGIEPAFLHDPRPGQGGATRPVAEGHALRAGGERAERVDHMASLSLAAAG      235
FEZ1      ------LI--------------------------------KQQTKAKYMVMDEDVSVILSG      123
                *:                                    :*: :    .:*:  :*

SurE      GLVGSATDLVTAARPHLADRKTF-AQH---DLLPEDAVLAMRTCV-P---DAEPFGLADG      287
FEZ1      G----KSDFHYAN-----DSSTYFTQSTVDKVLHDGERVELGGTVLTAHLTPGHTRGCTT      174
          *     :*:  *      * .*: :*    .:* :.  : :   *            .

SurE      WGLGLMRHGTGDGAWYGHDGAVGGASCNLRIHPDRSLALALTANSTAGPKLWEALVARLP      347
FEZ1      WTMKLKDHGKQYQAVI-----IGS----IGVNPGYKLVDNI-----TYPKIAE-------      213
          * : *  **.   *       :*.    : ::*. .*.  :     : **: *

SurE      EAGLDVGHYALPVPDSAPLA-PDAGHLGTYANGDLELMVTHDAAGDLFLTRESYSDYRLS      406
FEZ1      ----DYKHS-IKVLESMRCDIFLGSHAGMFDLKNKYV-LLQKGQNNPFVDPTGCKNYIE-      266
              *  *  : * :*       ..* * :   :  : : :.. .: *:   . .:*

SurE      LHEDDLFVARSGEPGALPITGRFVREHPAGPVALLQYGGRAMHRL  451
FEZ1      QKANDFYTELKKQETA----------------------------  282
           : :*::.  . :  *
```

A3.19

A3.19 Sequence alignment of SurE with Class A (TEM-I and PSE4), Class C (AmpC and CMY2) and Class D (OXA2 and OXA10) β-lactamases. The catalytic residues SXXK are highlighted in red. (PDB IDs: TEM-I- 1BTL, PSE4- 4ID4, AmpC- 1KE4, CMY2- 1ZC2, OXA2- 1K38, OXA10- 1E3U).

```
OXA2       -----------MAIRIFAILFSIFSLATFAHAQEGTLERSDWRKFFS----EFQAKGTIV     45
OXA10      -------------MKTFAAYVIIACLSS-TALAGSITENTSWNKEFS----AEAVNGVFV     42
TEMI       MSIQHFRVALIPFFAAFCLP-------VFAHPETL----VKVKDAEDQLG---ARVGYIE     46
PSE4       ----------MKFLLAFSLLIPS---VVFASSSKFQQVEQDVKAIEVSLS---ARIGVSV     44
SurE       ---------------------------------GAEGAERDAVGALFEELVREHRVTGAQL     28
AmpC       -----------MFKTTLCALLITASCSTFAA---PQQINDIVHRTITPLIEQQKIPGMAV     46
CMY2       ----------MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAV     50
                                                                   *

OXA2       VADERQAD--------RAMLVFDPVRSKKRYSPASTFKIPH---TLFALDAGAVRDEFQI     94
OXA10      LCKSSS----------KSCATNDLARASKEYLPASTFKIPN---AIIGLETGVIKNEHQV     89
TEMI       L-DLNSGKI------------LESFRPEERFPMMSTFKVLLCGAVLSRVDAGQEQLGR--     91
PSE4       L-DTQNGEY------------W-DYNGNQRFPLTSTFKTIACAKLLYDAEQGKVNPNS--     88
SurE       A-VYRDGALSEYATGLASVRTGEPVTPRTGFPFGSVTKFLT--------------------     68
AmpC       A-VIYQGKPYYFTWGYADIAKKQPVTQQTLFELGSVSKTFT--------------------     86
CMY2       A-VIYQGKPYYFTWGKADIANNHPVTQQTLFELGSVSKTFN--------------------     90
                .                         :   *. *

OXA2       FRWDGVNRGFAGHNQDQDLRSAMRNSTVWVYELFAKEIGDDKARRYLKKIDYGNADPSTS     154
OXA10      FKWDGKPRAMKQWERDLTLRGAIQVSAVPVFQQIAREVGEVRMQKYLKKFSYGNQNISGG     149
TEMI       ---------------RIHYSQNDLVEYSPV---TEKHLTDGMT---VRELCSAAITMSDN     130
PSE4       ---------------TVEIKKADLVTYSPV---IEKQVGQAIT---LDDACFATMTTSDN     127
SurE       ----------------------------------------AELV---MQFVCDGDLDLDDP     86
AmpC       ---------------------------------------GVLG---GDAIARGEIKLSDP     104
CMY2       ---------------------------------------GVLG---GDAIARGEIKLSDP     108
                                                               .    .

OXA2       NGDYWIEGSLAISAQ-----------------------------EQIAFLRKL------Y     179
OXA10      IDKFWLEGQLRISAV-----------------------------NQVEFLESL------Y     174
TEMI       TAANL-------------------------------LLTTIGGPKELTAFLHNMGDHVTRL     160
PSE4       TAANI-------------------------------ILSAVGGPKGVTDFLRQIGDKETRL     157
SurE       LAGLLPDLGRAADPALGTATVRQLLSHTAGVVDSIEYDEMRGP-S---YRRFAS----AC     138
AmpC       TTKYWPELTAKQWNGIT---LLHLATYTAGGLPLQVPDEVKSSSD---LLRF-------Y     151
CMY2       VTKYWPELTGKQWQGIR---LLHLATYTAGGLPLQIPDDVRDKAA---LLHF-------Y     155
                                                          .

OXA2       RNELP-FRVEHQRLVKDLMIVEAGRNWILRAKTGWEG-------RMGWWVGWVEW-PTGS     230
OXA10      LNKLS-ASKENQLIVKEALVTEAAPEYLVHSKTGFSGVGTESNPGVAWWVGWVEK-ETEV     232
TEMI       DRWEPELNEAIPNDERDTTMPA---AMATTLRKLLTGELLTLASRQQLIDWMEADKVAGP     217
PSE4       DRIEPDLNEGKLGDLRDTTTPK---AIASTLNKFLFGSALSEMNQKKLESWMVNNQVTGN     214
SurE       ARQPALFPPGLAFSY-------------SNTGYCLLGAVIEAASGMDWWTAMDS-----C     180
AmpC       QNWQPAWAPGTQRLY-------------ANSSIGLFGALAVKPSGLSFEQAMQT-----R     193
CMY2       QNWQPQWTPGAKRLY-------------ANSSIGLFGALAVKPSGMSYEEAMTR-----R     197
            .                                *

OXA2       VFFAL------NIDTPNRMDDL-----------------------------------------     246
OXA10      YFFAF------NMDIDNESKLP-----------------------------------------     248
TEMI       LLRS-ALPAGWFIADKSGAGER--GSRGIIAALGPDGKPSRIV---VIYTT---------     262
PSE4       LLRS-VLPAGWNIADRSGAGGF--GARSITAVVWSEHQAPIIV---SIYLA---------     259
SurE       LLRPLGIEPAFLHDPRPGQGGATRPVAEGHALRAGGERAERVDHMASLSLAAAGGLVGSA     240
AmpC       VFQPLKLNHTWINVPPAEEKNYAWGYREGKAVHVSPGALD----------AEAYGVKSTI     243
CMY2       VLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSPGQLD----------AEAYGVKSSV     247
            :

OXA2       -----------------------------------------------------------     246
OXA10      -----------------------------------------------------------     248
TEMI       ---------------GSQATM---------------------------------------     268
PSE4       ----------------QTQASM---------------------------------------     265
SurE       TDLVTAARPHLADRKTFAQHDLLPEDAVLAMRTCVPDAEPFGLADGWGLGLMRHGTG---     297
AmpC       ED----------MARWVQSNLKPLDIN---EKTLQQGIQLAQSRYWQTGDMYQGLGWEM     289
CMY2       ID----------MARWVQANMDASHVQ---EKTLQQGIALAQSRYWRIGDMYQGLGWEM     293
```

```
OXA2       ----FKRE--------AIVRAILRSIEALPPNPAVNSDAAR------------------     275
OXA10      ----LRK---------SIPTKIMESEGIIGG----------------------------     266
TEMI       ------------------------------------------------------DERN     272
PSE4       ------------------------------------------------------EERN     269
SurE       ----------------------------------DGAWYGHDGAVGGASCNLRIHPDRS     322
AmpC       LDWPVNPDSIINGSDNKIALAARPVKAITPPTPAVRASWVHKTGATGGFGSYVAFIPEKE     349
CMY2       LNWPLKADSIINGSDSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKN     353

OXA2       ------------------------------------------------------------     275
OXA10      ------------------------------------------------------------     266
TEMI       RQIAEIGASLI-------KHW--------------------------------------     286
PSE4       DAIVKIGHSIF-------DVYTSQSR-----------------------------------     288
SurE       LALALTANSTAG-----PKLWEALVARLPEAGLDVGHYALPVPDSAPLAPDAGHLGTYAN     377
AmpC       LGIVMLANKNYPNPARVDAAWQILNALQ---------------------------------     377
CMY2       LGIVMLANKSYPNPVRVEAAWRILEKLQ---------------------------------     381

OXA2       ------------------------------------------------------------     275
OXA10      ------------------------------------------------------------     266
TEMI       ------------------------------------------------------------     286
PSE4       ------------------------------------------------------------     288
SurE       GDLELMVTHDAAGDLFLTRESYSDYRLSLHEDDLFVARSGEPGALPITGRFVREHPAGPV     437
AmpC       ------------------------------------------------------------     377
CMY2       ------------------------------------------------------------     381

OXA2       ------------     275
OXA10      ------------     266
TEMI       ------------     286
PSE4       ------------     288
SurE       ALLQYGGRAMHRL    450
AmpC       ------------     377
CMY2       ------------     381
```

A3.20 Sequence alignment of SurE with Class A and B high molecular weight PBPS and class C low molecular weight PBPs

A3.20.1 Sequence alignment of SurE with HMW Class A PBPs: **PBP2 and PBP1b** (PBP2- *S. aureus*, GenBank X62288, PBP1b- PDB ID 3FWL)

```
SurE       ------------------------------------------------------------     0
PBP1b      MAGNDREPIGRKGKPTRPVKQKVSRRRYEDDDDYDDYDDYEDEEPMPRK-GKGKG--KGR     57
PBP2       ------------------------------------MTEN-KGSSQPKKNGNNGGKSNSK     23

SurE       ------------------------------------------------------------     0
PBP1b      KPRGKRGWLWLLLKLAIVFAVLIAIYGVYLDQKIRSRIDGKVWQLPAAVYGRMVNLEPDM     117
PBP2       KNRNVKRTIIKIIGFMIIAFFVVLLLGILL-------FAYYAWKAPAFTEAKLQDPIPA-     75

SurE       ------------------------------------------------------------     0
PBP1b      TISKNEMVKLLEATQYRQVSKMTRPGEFTVQANSIEMIRRPFDFPDSKEGQVRARLTFDG     177
PBP2       ------------------------------------------------------------     75

SurE       ------------------------------------------------------------     0
PBP1b      DHLATIVNMENNRQFGFFRLDPRLITMISSPNGEQRLFVPRSGFPDLLVDTLLATEDRHF     237
PBP2       ---------------KIYDKNGELV--KTLDNGQRHEHVNLKDVPKSMKDAVLATEDNRF     118
```

```
SurE       ----------------------GAEGAE---RDAVGALFEELVREHRVTG-----AQLA     29
PBP1b      YEHDGISLYSIGRAVLANLTAGRTVQGASTLTQQLVKNLFL---SSERSYWRKANEAYMA     294
PBP2       YEHGALDYKRLFGAIGKNLTGGFGSEGASTLTQQVVKDAFL---SQHKSIGRKAQEAYLS     175
                                 :**.   :: *   *     ..:        * ::

SurE       V-----Y-RDGALSEYATGLASVRTGEPVTPRTGFPFGSVTKFLTAELVMQFVCDGDLDL     83
PBP1b      LIMDARYSKDRILELYMNEVYLGQSG--DNEIRGFPLASLYYFGRP------VEELSLDQ     346
PBP2       YRLEQEYSKDDIFQVYLNKIYYSD------GVTGIKAAAKYYFNKD------LKDLNLAE     223
                 * :*  :. * . :             *:  .:   *         : : .*

SurE       DDPLAGLLPDLG---RAADPA--LGTATVRQLLSHTAGVVDSIEYDEMRG----------     128
PBP1b      QALLVGMVKGASIYNPWRNPKLALERRNLVLRLLQQQQIIDQELYDMLSARPLGVQ----     402
PBP2       EAYLAGLPQVPNNYNIYDHPKAAEDRKNTVLYLMHYHKRITDKQWEDAKKIDLKANLVNR     283
           :  *.*:    .      .*       .    * :    : .  ::

SurE       ----------------PSYRRFASACARQPALFPPGLAFSYSNTGYCLLGAVIEAASGMD     172
PBP1b      ---PRGGV----ISPQPAFMQLVRQE------LQAKLGDKV-----------KDLSGVK     437
PBP2       TAEERQNIDTNQDSEYNSYVNFVKSE------LMNNKAFKDENLG-------NVLQSGIK     330
                       :: .:.         :    . .                **:.

SurE       WWTAMDSCLLRPLGIEP---AF-L------HDPRPG------QGGATRPVAEGHALRAGG     216
PBP1b      IFTTFDSVAQDAAEKAAVEGIPALKKQRKLSDLETAIVVVDRFSGEVRAMVGGSEPQFAG     497
PBP2       IYTNMDKDVQKTLQNDVDNGSFYK-----NKDQQVGATILDSKTGGLVAISGGRDFKDVV     385
            :* :*.                       * . .        *    :  *   :

SurE       ERAERVDHMASLSLAAAGGLVGSATDLVTAARPHLADRKTFAQHDLLPEDAVLAMR-TCV     275
PBP1b      YN------RAMQARRSIGSLAKPATYLTALSQPKIYRLNTWIADAP------IALR---Q     542
PBP2       NR------NQATDPHPTGSSLKPFLAY----GP-AIENMKWATNHA------IQDESSYQ     428
            .              *.             *      .:  .       :  .

SurE       PDAEPFGLADGWGLGLMRHGTGDGAWYGHDGAVGGASCNLR--IHPDR--SLALALTANS     331
PBP1b      PNGQVWSP------------QNDDRRYSESGRVMLVDALTRSMNVPTVNLGMALGLP---     587
PBP2       VDGSTF---------------RNYDTKSHGTVSIYDALRQSFNIPALKAWQSVKQNAGN     472
            :.. :                 .      * *   ..  :    *      ::

SurE       TAGPKLWEALVARLP-----EAGLDVGHYALPVPDSAPLAPDAGHLGTYANGD-LELMVT     385
PBP1b      -AVTETWIKLGVPK-DQLHPVPAMLLGALNLTPIEVAQAFQTIASGGNRAPLSALRSVIA     645
PBP2       DAPKKFAAKLGLNYEGDIGPSEVLGGSASEFSPTQLASAFAAIANGGTYNNAHSIQKVVT     532
            *  :    *             :  .   :   : *      .  *.      :. :::

SurE       HDA-------------------------------------------------AGDLFLT     395
PBP1b      EDGKVLYQSFPQAERAVPAQAAYLTLWTMQQVVQRGTGRQLGAKYPNLHLAGKTG-----     700
PBP2       RDGETIEYDHTSH-KAMSDYTAYMLAE-MLKGTFKPYGSAYGHGVSGVNMGAKTGTGTYG     590
           .*.                                                  :*

SurE       RESYSDYRLSLHED-DLFV-A--------------------RSGEPGALP----ITGRFV     429
PBP1b      ---------TTNNNVDTWFAGIDGSTVTITWVGRDNNQPTKLYGASGAMS----IYQRYL     747
PBP2       AETYSQYNLPDNAAKDVWINGFTPQYTMSVWMGFSKVK---QYGENSFVGHSQQEYPQFL     647
                      :   * :. .                        *  . :        :::

SurE       REHPAGPVALLQ-----------------------------------------YGGRAMHRL     450
PBP1b      ANQTPTPLNLVPPEDIADMGVDYDGNFVCSGGMRILPVWTSDPQSLCQQ-----SEMQQQ     802
PBP2       YENVMSKIS-------SRDGEDFKRPSSVSGSIPSINVSGSQDNNTTNRSTHGGSDTSAN     700
            ::    :

SurE       ----------------------------------------    450
PBP1b      PSGNPFDQSSQPQQQPQQQPAQQEQKDSDGVAGWIKDMFGSN    844
PBP2       SSGTAQSNNN--------TRSQQS------------------    716
```

A3.20.2 Sequence alignment of SurE with HMW Class B PBPs: PBP2 and PBP2b (PBP2- *N. meningitidis*, X59628 GenBank, PBP2B- UniProtKB Q07868)

```
SurE       ------------------------------------------------------------      0
PBP2B      -MI-------QMPKKNKF---M--NRGAAILSICFALFFFVILGRMAYIQITGKANGEVL      47
PBP2       MLIKSEYKPRMLPKEEQVKKPMTSNGRISFVLMAIAVLFAGLIARGLYLQTVT---YNFL      57


SurE       ------------------------------------------------------------      0
PBP2B      ATKATEQHEKKRTIEASRGSILDRKGKVIAEDTATYKLIAILDKKMTTDVKHPQHVVNKE      107
PBP2       KEQGDNRIVRTQTLPATRGTVSDRNGAVLALSAPTESLFAVPKEMKEM---------PSA      108
```

```
SurE      ------------------------------------------------------------      0
PBP2B     KTAEALSKVINLDKADILDILNKDAKQVEFGSAGRDITYSQKQKIEKMKLPGISFLRDTK      167
PBP2      AQLERLSELVDVPVDVLRNKLEQKG--KSFIWIKRQLDPKVAEEVKALGLENFVFEKELK      166


SurE      ----------------GAEGAERDAVGALFEELVREHRVTGAQLAVYRDGALSEYATGLA      44
PBP2B     RYYPNGVFASNLIGYAEVDEETNEISGAMGLEKVLDKYLKER------DGYVTYESDK-S      220
PBP2      RHYPMGNLFAHVIGFTNIDGKGQ-----EGLELSREDSLRGE------DGAKVVLRDN-K      214
                         :   .        *   :. :         **


SurE      SVRTGEPVTPRTGFPFGSVTKFLTAELVMQFVCDGDL--------------DLDDPLAGL      90
PBP2B     GW-ELPNSKNKITAPKNGDNVYLTIDQKIQTFLEDSMTKVAQKYNPKKIMAAVVDPKTGK      279
PBP2      GNIVDSLDSPRNSVPKNGQDMILSLDQRIQTLAYDELNKAVAYHKAKAGAVVVLDAQTGE      274
          .       . :   * ..    *: :  :* .  ..:              : *  :*


SurE      LPDLGRAADPALGTATVRQLLSHTAGVVDSIEYDEMRGPSYRRFASACARQ---------      141
PBP2B     VLAMGQR--PSFDPNKR-DVTN---YYNDLISYAYEPGSTMKIFTLAAAMQENVFNANEK      333
PBP2      ILALVNS--PAYDPNQP-GQANSEQRRNRAVTDMIEPGSAMKPFTIAKALDSGKVDATDT      331
          :  : .   *: .        .        :      * : : *: * * :


SurE      ----------------------PALFPPGLAFSYSNTGYCLLGAV--------------      164
PBP2B     YKSGTFEVGGAPVKDHNNGVGWGPTTYHDGVL-RSSNVAFAKLAKEKLGYDRLNQYLHKF      392
PBP2      FNTLPYKIGPATVQDTHVY----PTLDVRGIMQKSSNVGTSKLSA-------MFTPKEMY      380
                            *:    *:    **.. . *.


SurE      -----------------IEAASGMDWWTA---MDSCLLRPLGIEPAFLHDPRPGQGGATR      204
PBP2B     NFYQKTGI------DLPGEVSSKINFKYE---FDKAST-------------AYGQASAVT      430
PBP2      DFYHDLGVGVRMHSGFPGETAGLLRSWRRWQKIEQATM-------------SFGYGLQLS      427
                         *.:. :        ::..                * .


SurE      PVAEGHALRAGGERAERVDHMASLSLAAAGGLVGSATDLVTAARPHLADRKTFAQHDLLP      264
PBP2B     PIQQIQAA---------------TAIANDGKMM----------------KPYVIDHIVDP      459
PBP2      LLQLARAY---------------TVLTHDGELL----------------PVSFEKQAVVP      456
           :   :*                  ::  * ::                  . .: : *


SurE      EDA-------------VLA------MR----TCVPD---AEPFGLADGWGLGLMRHGTGD      298
PBP2B     DKDKTIYQNKPESAGTPISASTAKKVRDILGEVVTSKIGTGQAYKIEGFDVAGK---TGT      516
PBP2      K------------GKRVIKASTAKKVRELMVS-VTEAGGTGTAGAVDGFDVGAK---TGT      500
          .                :       :*      * .   :      :*:.:.     **


SurE      GAWYGHDGAVGGASCNLRIHPDRSLALALTANSTAGPKLWEALVARLPEAGLDVGHYALP      358
PBP2B     AQIAGKGGYLDGTDN--------YIFSFMGMAPKDDPELLIYVAVQQPQL---KAGQSSS      565
PBP2      ARKLVNGRYVDNK----------HVATFIGFAPAKNPRVIVAVTIDEPTA---NGYY-GG      546
          .    :.  :..            :   :      .*.:   :.   *      .


SurE      VPDSAPLAPDAGHLGTYANGDLELMV--------------------------------TH      386
PBP2B     DPVSEIFNPTMKNSLHYLNIEPTEKSDSDKEETKAQTMPDLTDQTVAAAQKKAKEENLTP      625
PBP2      VVTGPPFKKIMGGSLNILGVSPTKPLTAAAVKTPS-------------------------      581
             .  :           . .


SurE      DAAGDLFLTRESYSDYRL-SLHEDDLFVARSGEPGALPITGRFVREHPAGPVALLQYGGR      445
PBP2B     IVIGSDVAVKEQYPKADEEVLTNQKVFLKTGGKIKMPDMTGWS-------RREVLQYGEL      678
PBP2      ------------------------------------------------------------      581


SurE      AMHRL---------------------------------          450
PBP2B     AGIHIEVSGQGYAVSQSVKKDKEIKDKTVIKVKFKNPD          716
PBP2      ------------------------------------            581
```

A3.20.3. Sequence alignment of SurE with Class C LMW PBPs: PBP4*, R61 and ADP (the catalytic residues are bolded in red; PDB IDs: PBP4*- 1TVF , R61 D-alanyl-D-alanine carboxypeptidase- 1IKI, ADP- 4Y7P)

```
SurE      ---------------------------------------------VGAEGAERDAVGALF      15
PBP4*     ---------------------------------------------MKQNKRKHLQTLF      13
R61       --------------MVSGTVGRGTALGAVLLALLAVPAQAGTAAAADLPAPDDTGLQAVL      46
ADP       MKTRSQITCASLALLIAGS---SLLYTTQTLIVKAEPTQSVSSSVQTSTQRDRNSVKQAV      57
                                                              .   :   .
```

```
SurE        EELVREHRVTGAQLAVYRDGALSEYATGLASVRTGEPVTPRTGFPFGSVTKFLTAELVMQ     75
PBP4*       ETLGEKHQFNGTVLAAEGGDILYHHSFGYAEMTEKRPLKTNSLFELASLSKPFTALGIIL     73
R61         HT-ALSQGAPGAMVRVDDNGTIHQLSEGVADRATGRAITTTDRFRVGSVTKSFSAVVLLQ     105
ADP         RD-TLQLGFPGILAKTSEGGKTWSYAAGVANLSSKKPMKTDFRFRIGSVTKTFTATVVLQ     116
            .    .    *    .  ..     : * *.    . :.    * ..*::* ::*  ::

SurE        FVCDGDLDLDDPLAGLLPDLGRAADPALGTATVRQLLSHTAGVVDSIEYDEMR-------     128
PBP4*       LEEKGILGYEDKVDRWLPGFP------YQGVTIRHLLNHTSGLPDYMGWFFANWDS----     123
R61         LVDEGKLDLDASVNTYLPGLLP-----DDRITVRQVMSHRSGLYDYTNDMFAQTVPGFES     160
ADP         LAEENRLNLDDSIEKWLPGVIQGNGYDDKQITIRQLLNHTSGIAEYTRSKSFDLMD----     172
            :  .. *. :  :   **..           *:*:::.* :*: :

SurE        --GP--SYRRFASAC--ARQPALFPPGLAFSYSNTGYCLLGAVIEAASGMDWWTAMDSCL     182
PBP4*       --HKIAVNQDIVDMLMNEGLSGYFEPNEGWMYSNTGYVLLAVIIEKASGMSYADFIKTSI     181
R61         VRNKVFSYQDLITLSLK--HGVTNAPGAAYSYSNTNFVVAGMLIEKLTGHSVATEYQNRI     218
ADP         -TKKSYRAEELVKMGIS--MPPDFAPGKSWSYSNTGYVLLGILIETVTGNSYAEEIENRI     229
                   . :              *. .: ****.: : . :**  :* .     .. :

SurE        LRPLGIEPAFLHDPRPGQGGATRPV---------AEGHALRAGGERAERVDHMASLSLA-     232
PBP4*       FLPAGMNETRVYNRR-LSPERIDHYAYGYVYDVHSETYVLPDELEETNY-VVY--LDGIQ     237
R61         FTPLNLTDTFYVHPDTVIPG--TH----------ANGYLTPDEAGGALVDSTEQTVSWAQ     266
ADP         IEPLELSNTFLPGNSSVIPG--TK---------HARGYIQLDGASE-PKDVTYYNPSMGS     277
            : *  :  :                         :. :                 .

SurE        AAGGLVGSATDLVTAARPHLADRKTFAQHDLLPEDAVLAMRTCVPDAEPFGLADGWGLGL     292
PBP4*       GDGTVNSVTSDLFRFDQ-------ALYQDDFISKASKESAFSPVRLN--NGETIDYGFGW     288
R61         SAGAVISSTQDLDTFFS-------ALMSGQLMSAAQLAQMQQWTT----VNSTQGYGLGL     315
ADP         SAGDMISTADDLNKFFS-------YLLGGKLLKEQQLKQMLTTVPTG--EAALGRYGLGI     328
            . * : . : **             :   .::           .           :*:*

SurE        MRHGT-GDGAWYGHDGAVGGASCNLRIHPDRSLALALTANSTAGPKLWEALVARLPEAGL     351
PBP4*       VLQNSPEKGRIVSHSGGWPGYSTMMIRYIDHRKTLIYLSNKEEDTEYEQAI-LKAAEHIL     347
R61         RRRDLSCGISVYGHTGTVQGYYTYAFASKDGKRSVTALANTSNNVNVLNTM-ARTLESAF     374
ADP         YETKLPNGVSIWGHGGSIPGFVTFAGGTLGGKHTLAVNLNSLNA-ESPDPF-KNILLAEF     386
                        .* *   *         .   ::    *.    :  : : .     :

SurE        DVGHYALPVPDSAPLAPDAGH------------LGTY------------ANGDLELMVTH     387
PBP4*       FG------QPYDVPERPADKKKKAIDTAIYSRYVGSYLLQDGTAAQVTTENERLYLEIAG     401
R61         CG------KPTTAKLRSATSSATTVE----------------------RHEDIAPGIAR     405
ADP         SK------------------------------------------------------------     388


SurE        DAAGDLFLTRESYSDYRLSLHEDDLFVARSGEPGALPITGRFVREHPAGPVALLQYGGRA     447
PBP4*       QLRLELFPSSE----TR-------FFLRA------LSVEVEFTLGEDAAKSFILYEDGSE     444
R61         D------------------------------------------------------------     406
ADP         ------------------------------------------------------------     388


SurE        MHRL--- 451
PBP4*       EEAVRTK 451
R61         ------- 406
ADP         ------- 388
```

A3.21

A3.21 Sequence alignment of SurE with Class C (subclass B) LMW PBPs and Class B β- lactamases. (LMW PBP subclass B: PBP4*, R61; Class B β- lactamases: AmpC, CMY2, conserved catalytic residues are bolded in red (the conserved H residue is replaced by a K residue in Class C β- lactamases); (PDB IDs: PBP4*- 1TVF , R61 D-alanyl-D-alanine carboxypeptidase- 1IKI, AmpC- 1KE4, CMY2- 1ZC2)

```
PBP4*      ---------------------------------MKQNKRKHLQTLFETLGEKHQFNGTVL     27
SurE       ---------------------------------GAEGAERDAVGALFEELVREHRVTGAQL     28
R61        MVSGTVGRGTALGAVLLALLAVPAQAGTAAAADLPAP-DDTGLQAVLHTALSQGAPGAMV     59
AmpC       -----------MFKTTLCALLITASCSTFAA---PQQINDIVHRTITPLIEQQKIPGMAV     46
CMY2       ----------MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAV     50
                                                .     .     .:   *  :

PBP4*      AAEGGDILYHHSFGYAEMTEKRPLKTNSLFELASLSKPFTALGIILLEEKGILGYEDKVD     87
SurE       AVYRDGALSEYATGLASVRTGEPVTPRTGFPFGSVTKFLTAELVMQFVCDGDLDLDDPLA     88
R61        RVDDNGTIHQLSEGVADRATGRAITTTDRFRVGSVTKSFSAVVLLQLVDEGKLDLDASVN     119
AmpC       AVIYQGKPYYFTWGYADIAKKQPVTQQTLFELGSVSKTFTGVLGGDAIARGEIKLSDPTT     106
CMY2       AVIYQGKPYYFTWGKADIANNHPVTQQTLFELGSVSKTFNGVLGGDAIARGEIKLSDPVT     110
            .   .     : * *.    . :.    * ..*::* :..         * :  .

PBP4*      RWLPGFPYQ------GVTIRHLLNHTSGLPDYMGWFFAN----WDS--HKIAVNQDIVDM     135
SurE       GLLPDLGRAADPALGTATVRQLLSHTAGVVDSIE---------YDE--MRGPSYRRFASA     137
R61        TYLPGLLPDD-----RITVRQVMSHRSGLYDYTNDMFAQTVPGFESVRNKVFSYQDLITL     174
AmpC       KYWPELTAKQ-----WNGITLLH-----LATYTAGGLPLQVP------DEVKSSSDLLRF     150
CMY2       KYWPELTGKQ-----WQGIRLLH-----LATYTAGGLPLQIP------DDVRDKAALLHF     154
              * :            :  :      :                            :

PBP4*      LMNEGLSGYFEPNEGWMYSNTGYVLLAVIIEKASGMSYADFIKTSIFLPAGMNETRVYNR     195
SurE       C--ARQPALFPPGLAFSYSNTGYCLLGAVIEAASGMDWWTAMDSCLLRPLGIEPAFLHDP     195
R61        S--LKHGVTNAPGAAYSYSNTNFVVAGMLIEKLTGHSVATEYQNRIFTPLNLTDTFYVHP     232
AmpC       Y--QNWQPAWAPGTQRLYANSSIGLFGALAVKPSGLSFEQAMQTRVFQPLKLNHTWINVP     208
CMY2       Y--QNWQPQWTPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQPLKLAHTWITVP     212
                      *.    *:*:.  : . :    :* .        :: *  :  :

PBP4*      RLSPERIDHY----------AYGYVYDVHSETYVLPDELEETNYVVY------LDGIQGD     239
SurE       RPG--QGGATRPVAEGHALRAGGERAE--RVDHMASLSL-AAAGGLVGSATDLVTAARPH     250
R61        DTV--IPGTH----------ANGYLTP--DE----------AGGALVDSTEQTVSWAQSA     268
AmpC       PAE--EKN-Y----------AWGYREG--KAVHVSPGALDAEAYGVKSTIEDMARWVQSN     253
CMY2       QNE--QKD-Y----------AWGYREG--KPVHVSPGQLDAEAYGVKSSVIDMARWVQAN     257
                  .           * *                    :          :

PBP4*      GTVNSVTSDLFRFDQALYQDDFISKASKESAFSPVRLNNGETIDYGFGWVLQNSPEK---     296
SurE       LADR------KTFAQ---HDLLPEDAVL-AMRTCVPDAEPFGLADGWGLGLMRHGT----     296
R61        GAVISSTQDLDTFFSALMSGQLMSAAQLAQMQQW----TTVNSTQGYGLGLRRRDLSCGI     324
AmpC       LKPL-----------DINEKTLQQGIQLAQSRYW----QTGDMYQGLGWEMLDWPVNPDS     298
CMY2       MDAS-----------HVQEKTLQQGIALAQSRYW----RIGDMYQGLGWEMLNWPLKADS     302
                          : .                    * *  :

PBP4*      ---------------------------GRIVSHSGGWPGYSTMMIRYIDHRKTLIYLSNK     329
SurE       -------------------------GDGAWYGHDGAVGGASCNLRIHPDRSLALALTANS     331
R61        SVYG----------------------------HTGTVQGYYTYAFASKDGKRSVTALANT     356
AmpC       IINGSDNKIALAARPVKAITPPTPAVRASWVHKTGATGGFGSYVAFIPEKELGIVMLANK     358
CMY2       IINGSDSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVMLANK     362
                                        : *   *         :    :   :*.

PBP4*      EEDTEYEQ-AILKAAEHILFGQPYDVPERPADK----KKKAIDTAIYSRYVGSYLLQDGT     384
SurE       TAGPK-----LWEALVARLPEA----GLDVGH-YALPVPDSAPLAPDAGHLGTYA-----     376
R61        SNNVNV-LNTMARTLESAFCGKPTTAKLRSATSSATTVERHEDIAPGI------------     403
AmpC       NYPNPARVDAAWQILNA----------LQ-------------------------------     377
CMY2       SYPNPVRVEAAWRILEK----------LQ-------------------------------     381
                    .

PBP4*      AAQVTTENERLYLEIAGQLRLELFPSSETRFFLR-ALSVEVEFTLGEDAAKSFILYEDGS     443
SurE       ---------------NGDLELMVTHDAAGDLFLTRESYSDYRLSLHED--DLFVA-RSG-     417
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R61         ----------------------ARD----------------------------------      406
AmpC        ---------------------------------------------------------------      377
CMY2        ---------------------------------------------------------------      381


PBP4*       EEEAV-------RTK------------------    451
SurE        EPGALPITGRFVREHPAGPVALLQYGGRAMHRL    450
R61         ---------------------------------    406
AmpC        ---------------------------------    377
CMY2        ---------------------------------    381
```