Metabolic Engineering of *Streptomyces*

by

Zainab Nosarka

1689766

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Supervisor: Professor Karl Rumbold

Co-supervisor: Dr Sanchia Moodley

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Declaration

I declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

(Signature of candidate)

5th day of September 2023 at Lenasia, Johannesburg South

Abstract

The *Streptomyces* genus demonstrates remarkable potential as a source and host for the production and discovery of industrially relevant secondary metabolites. The genus natively produces approximately 75% of antibiotics and several other compounds encoded by biosynthetic gene clusters (BCGs). However, most BCGs are poorly expressed or dormant under laboratory conditions and thus require metabolic engineering. Several technologies have been developed for this purpose but the pCRISPomyces-2 plasmid system, which employs Cas9 engineering, exhibits the most promising efficacy. This dissertation outlined foundational research to determine the capacity to introduce pCRISPomyces-2 plasmids into *Streptomyces albulus* BCRC11814 that produces high quantities of ε-poly-L-lysine, an antimicrobial and anti-phage compound. In addition, the strain has several other industrially relevant BCGs that have not been studied but possess engineering potential. To achieve the outlined aim, pCRISPomyces-2 plasmids were Sanger sequenced to ensure structural integrity and related functionality. A ClustalW alignment referenced against the plasmid's nucleotide sequence verified a sequence identity > 98%. Subsequently, an intergeneric conjugation system was established by transforming pCRISPomyces-2 plasmids into *Escherichia coli* donor cells with an average transformation efficiency (TE) of 1.49×10^5 cfu/µg. Attempts to optimise TE were hindered by the plasmids' large size and inherent Cas9 toxicity. Thereafter, the transformed donor cells were conjugated with *S. albulus* BCRC11814 and a comparative model strain *Streptomyces coelicolor* A3(2). Successful exconjugants were only obtained with *S. coelicolor* A3(2). The absence of conjugal mating with *S. albulus* BCRC11814, despite optimisation attempts, was hypothesised to result from the presence of a methyl-specific restriction-modification system. This was confirmed by comparative electro-transformation with methylated and non-methylated DNA that demonstrated specificity to *dam* and *dcm* methylated DNA. Furthermore, spontaneous resistance to the selectable marker apramycin was confirmed in both *Streptomyces* strains. Additional efforts are required to effectively introduce pCRISPomyces-2 plasmids into *S. albulus* BCRC11814.

Keywords: Metabolic engineering; *Streptomyces*; Secondary Metabolites; Synthetic Biology; Gene Editing; CRISPR/Cas9; Intergeneric Conjugation

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بِسْمِ اللَّهِ الرَّحْمَـٰنِ الرَّحِيمِ

"In the Name of Allah, the Most Gracious, the Most Merciful"

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List of Abbreviations

AGA- Aminoglycoside antibiotics

Amp- Ampicillin

Apr-Apramycin

attP- Phage-specific attachment sites

AR- Antibiotic resistance

BCG- Biosynthetic gene clusters

CF- Conjugational frequency

Cre/loxP- Cyclization recombinase/ locus of x-over, P1

CRISPR- Clustered regularly interspaced short palindromic repeats

crRNA- CRISPR RNA

CSR- Cluster situated regulators

DAP- Diaminopimelic acid

dH_2O- Distilled water

DMSO-Dimethyl sulfoxide

DNA- Deoxyribonucleic acid

Flp/FRT- Flippase recombinase/ flippase recognition target

HDR- Homology-directed repair

ISP2- International *Streptomyces* Project-2 Medium

kb- kilo base-pairs

LAL- Large AT-binding regulators of the LuxR family

LB- Lysogeny Broth

MIC- Minimum Inhibitory Concentration

Nal- Nalidixic acid

NGG- Nucleotide-guanine-guanine

NHEJ- Non-homologous ending joining

NRPS- Non-ribosomal polypeptide synthase

PAM- Protospacer-adjacent motif

PCR-Polymerase chain reaction

pDNA- Plasmid DNA

PKS- Polyketide synthase

R-M- Restriction-modification

RNA – Ribonucleic acid

SDS- Sodium dodecyl sulphate

SARP- Several ankyrin repeat proteins)

SFM-Soya Flour Mannitol

sgRNA- Single guide RNA

SM- secondary metabolites

TAR- Transformation-associated recombination

TB-Transformation Buffer

T_d- Doubling time

TE- Transformation efficiency

tra- Transfer

tracrRNA- trans-activating CRISPR RNA

XC- Xylanimonas cellulosilytica

YT- Yeast Tryptone

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Chapter 1: Literature review

1.1 *Streptomyces*: A robust organism and bioengineering host

Research strides to acquire secondary metabolites (SMs) and industrially relevant compounds have become of increasing interest across the globe over the past decade. This is accredited to increasing demands due to surges of infectious diseases and the importance of developing more environmentally safe and sustainable industries for the production of chemical, pharmaceutical, fuel and cosmetic products (Holmes et al., 2017; Tack et al., 2019; World Health Organisation, 2020). While several approaches have been researched for this purpose, bioengineering approaches and efforts have dominated with their efficiency and continue to expand steadily.

A sub-field of bioengineering termed metabolic engineering (defined as the modification and optimisation of innately occurring biochemical pathways by genetic manipulation or disruption) has contributed much to the success of sustainable and affordable mass production of new and existing SM compounds (García-Granados et al., 2019). This success is commonly achieved by the exploitation of the bacterial family *Streptomycetaceae. Streptomyces* spp. are well recognised for their versatility as a plentiful source and engineering host for SM production. The applications of *Streptomyces* spp. are relevant in the biofuel, biocatalytic, medical and food industries **(Figure 1)** (Bentley et al., 2002; Ōmura et al., 2001).

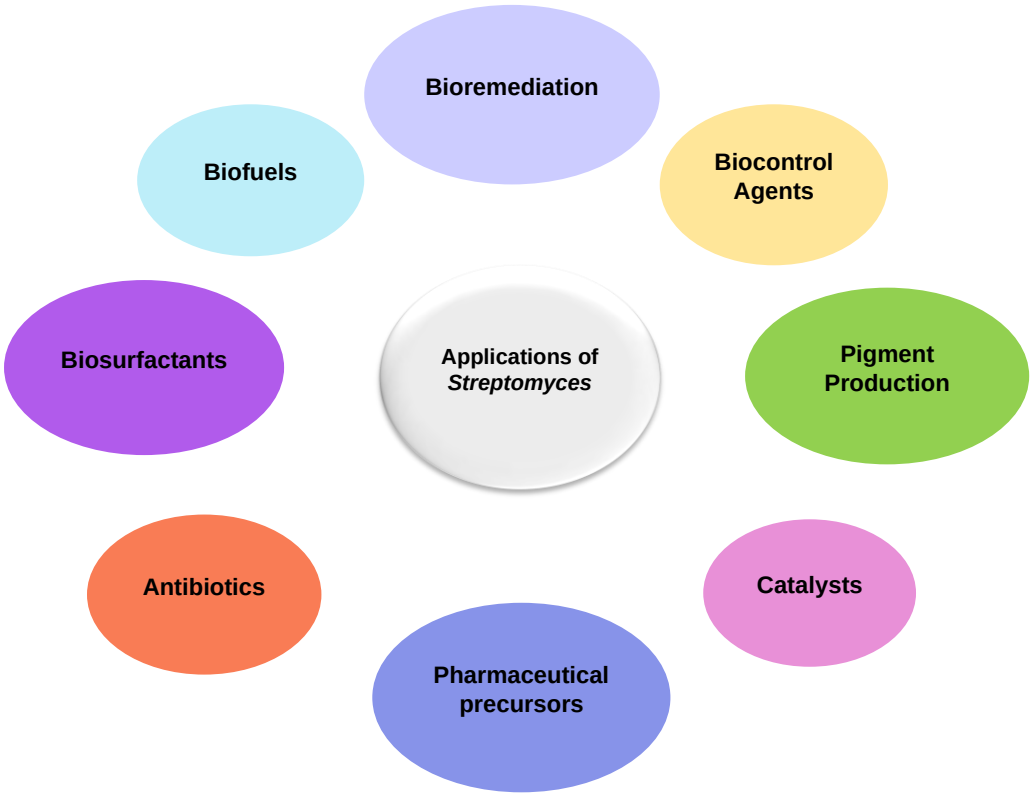

Figure 1: The industrial applications of *Streptomyces* SM compounds. Adapted from Deka et al., (2020)

Streptomyces, belonging to the class of *Actinomycetes*, are gram-positive bacteria that have a distinguishable three-stage growth cycle (**Figure 2)** consisting of spore germination, vegetative mycelia compartmentalisation and aerial mycelia formation and septation (Yagüe et al., 2013). These bacteria commonly inhabit terrestrial habitats due to their saprophytic nature by secretion of various extracellular enzymes, which contributes to their robustness in physically and nutritionally challenging soil habitats (Basilio et al., 2003; Sajid et al., 2011). The overall environmental adaptability of *Streptomyces* is mainly regulated by master pleiotropic transcription factors (PhoP, GlnR, or MtrA) that activate or repress target promoters in response to environmental stimuli cascades (Hwang et al., 2022; Santos et al., 2012; Tschowri et al., 2014).

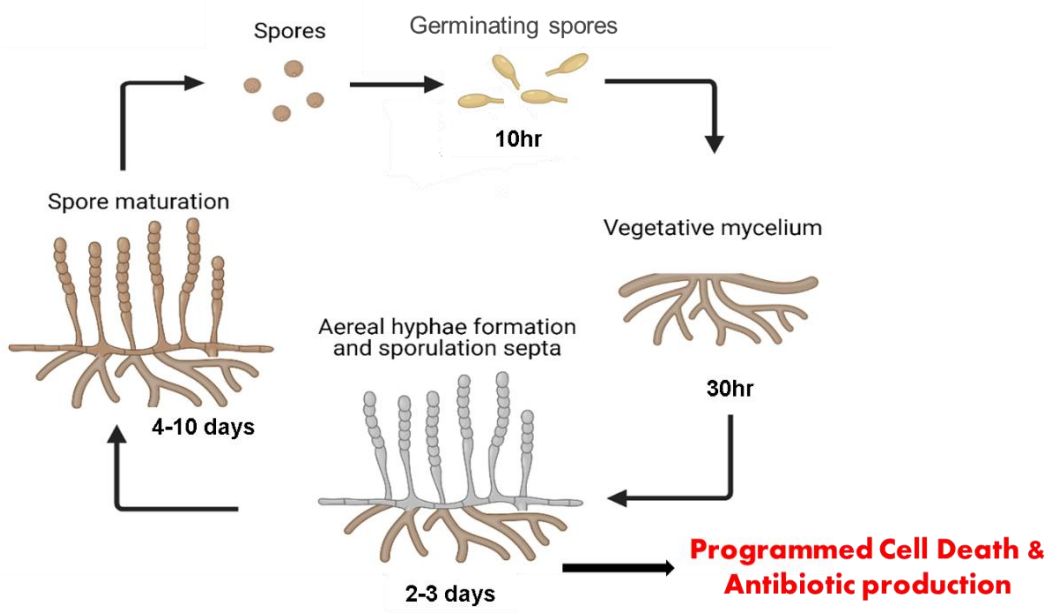

Figure 2: The lifecycle of *Streptomyces.* The microorganisms differentiate into three distinguishable cell types during their lifecycle. The production of SMs is synced with the aerial mycelia phase. Adapted from micro-bites.org with BioRender.

Successively, the ability to regulate and adapt at gene transcriptional level is attributed to the evolution of *Streptomyces* by horizontal gene transfer (Schniete et al., 2018; Treangen & Rocha, 2011).The evolvability of *Streptomyces* can be further observed by their protective spore phase and metabolic flexibility, such as nitrogen suppression and unique glucose repression (Bascaran et al., 1989; Hodgson, 1982). These metabolic processes, amongst others, form part of the primary metabolism of *Streptomyces* which have direct regulation on their secondary metabolism and the type of SMs secreted (Hodgson, 2000). *Streptomyces* simultaneously undergo morphological and physiological differentiation activating the secondary metabolism and allowing for the synthesis of a variety of SMs according to different biosynthetic pathways (Hodgson, 2000; Sola-Landa et al., 2003). Consequently, the metabolic dynamicity of *Streptomyces* and relevant SM products contributes to the interest in using *Streptomyces* as an industrial source for relevant compounds.

The environmental evolution of *Streptomyces* is evident by the rich GC content (more than 70%) conserved in the microorganisms' genome (Šmarda et al., 2014; Wright & Bibb, 1992). The high GC content confers higher-order genomic structure stability and a broader environmental tolerance range in *Streptomyces* compared to other microorganisms with lower genomic G+C content (Ikeda et al., 2012). Furthermore, *Streptomyces* spp. have remarkable genome plasticity that allows for genetic manipulation of non-essential gene arms that flank the centrally conserved genome core region of essential growth genes (approximately 35-45% of the genome) (Zhou et al., 2012). Together, the high GC content and genomic plasticity partially facilitate the capacity of *Streptomyces* as an engineering host.

In tandem, the metabolic features, dynamicity, and genomic structure of *Streptomyces* impart their overall functionality in SM production, as well as act as a model organism for metabolic engineering studies.

1.2 Secondary metabolite production in *Streptomyces* and industrial relevance

The SM metabolism of *Streptomyces* is typically synchronised with aerial mycelia formation (Chater, 1999). The SM production is encoded by genes arranged in clusters, termed biosynthetic cluster genes (BCGs), ranging from several to > 100 kilo base-pairs (kb) (Bentley et al., 2002; Ōmura et al., 2001). Each *Streptomyces* strain is reported to contain between approximately 20-30 BCGs (Ikeda et al.,2003; Nett et al.,2009). The expression of BCGs is regulated by different pathway-specific cluster situated regulator (CSR) families such as SARP (several ankyrin repeat proteins) and LAL (large ATP-binding regulators of the LuxR family) (Sekurova et al., 2004; Wietzorrek & Bibb, 1997). Master transcriptional factors interact with the upstream regions of CSRs to integrate physiological and environmental-specific signals, transduced by phosphorylation cascades, and activate or repress BCG expression (Sola-Landa et al., 2003; van Wezel & McDowall, 2011). Additionally, some CSRs also exhibit pleiotropic regulation on the BCGs (Huang et al., 2005; Makitrynskyy et al., 2020).

The BCGs encode secretory pathways allocated to SM biosynthesis (Takarada et al., 2008). Seven pathways have been reported; namely, the polyketide synthase (PKS) pathway, the hybrid (non-ribosomal polyketide synthetic) pathway, the non-ribosomal polypeptide synthase (NRPS) pathway, the peptide pathway, the carbohydrate pathway, the shikimate pathway and the β-lactam synthetic pathway **(Figure 3)** (Harir et al., 2018; Komatsu et al., 2010). The PKS and NRPS pathways are most reported and studied as they produce most of the industrially relevant SMs (Belknap et al., 2020; van Wezel & McDowall, 2011).

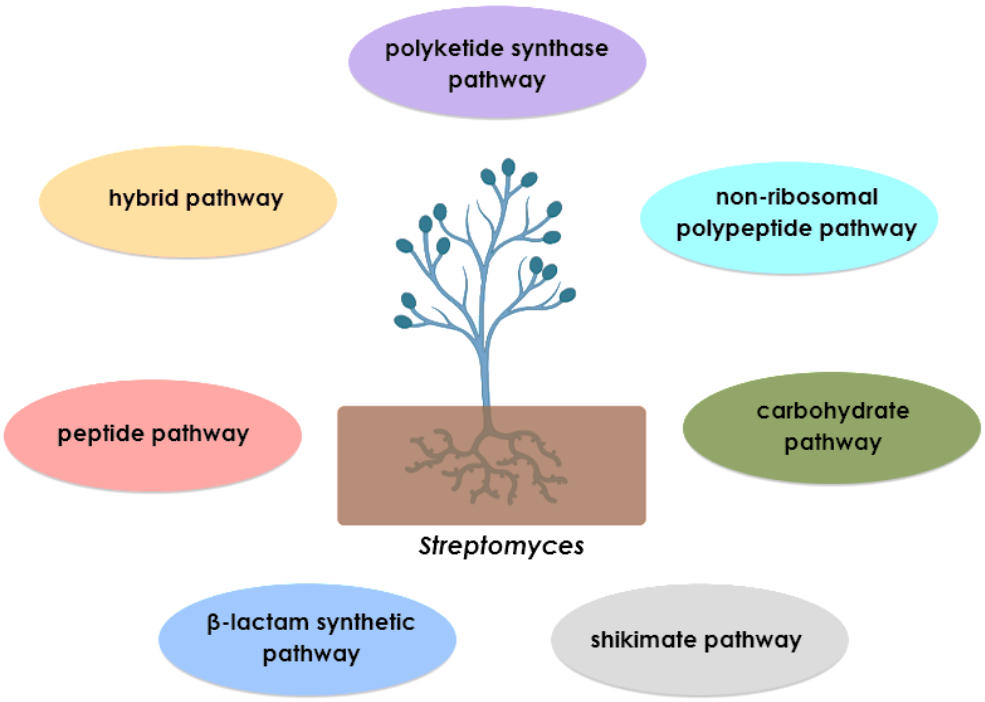

Figure 3: *Streptomyces* biosynthetic secondary metabolic pathways. Each pathway, encoded by BCGs, can produce different SMs that have industrial relevance. Adapted from Harir et al. (2018) with BioRender.

To date, in their innate capacity, more than 80% of naturally derived industrialised antibiotics or their precursors are sourced from *Streptomyces* spp. (Abdel-Razek et al.,2020; Lucas et al.,2013; Luo et al.,2014). Notably, some prevalent antibiotics produced by *Streptomyces* are streptomycin from *Streptomyces griseus*; erythromycin and rifamycin from *Streptomyces avermitilis;* and penicillin G from *Streptomyces griseus* (Baral et al.,2018; Jenke-Kodama et al.,2006). Clinical drugs such as immunosuppressants (cyclosporin and Hygromycin A), antivirals (zanamivir and oseltamivir) and anti-oncogenic compounds (doxorubicin and daunorubicin) are also obtained from *Streptomyces* spp. (Lomovskaya et al.,1999; Otten et al.,1990; Shitara et al.,2000; Uyeda et al.,2001).

Other naturally secreted SMs include biosurfactants used in the cosmetic, pharmaceutical, and mining industries (Steenbergen et al., 2005), vitamins (e.g. vitamin B12) (Hall et al., 1953) and biocatalysts which are used in several industries (e.g. polyhydroxyalkanoate depolymerase and halogenases) (Latham et al., 2016; Martínez et al., 2015; Spasic et al., 2018). In addition to their natural secretion, SM compounds are commonly produced through heterologous expression in *Streptomyces*. Examples of these are biocatalysts, such as xylanase and α-amylase (Belfaquih et al., 2002; Santos et al., 2012); biopeptides such as chitinases (Berini et al., 2019); bioremediation compounds, such as laccases (Berini et al., 2018); and clinical compounds, such as proinsulin (Koller et al., 1989).

These examples only reflect a limited percentage of SM production by *Streptomyces.* To illustrate the richness of *Streptomyces* BCGs, a phylogenetic distribution of BCGs, generated by antiSMASH, from over 1000 genomes is displayed in **Figure 4** (Belknap et al., 2020). Additional SM products and commercialisations of *Streptomyces* can be searched via journal databases and patent/commercialised registers.

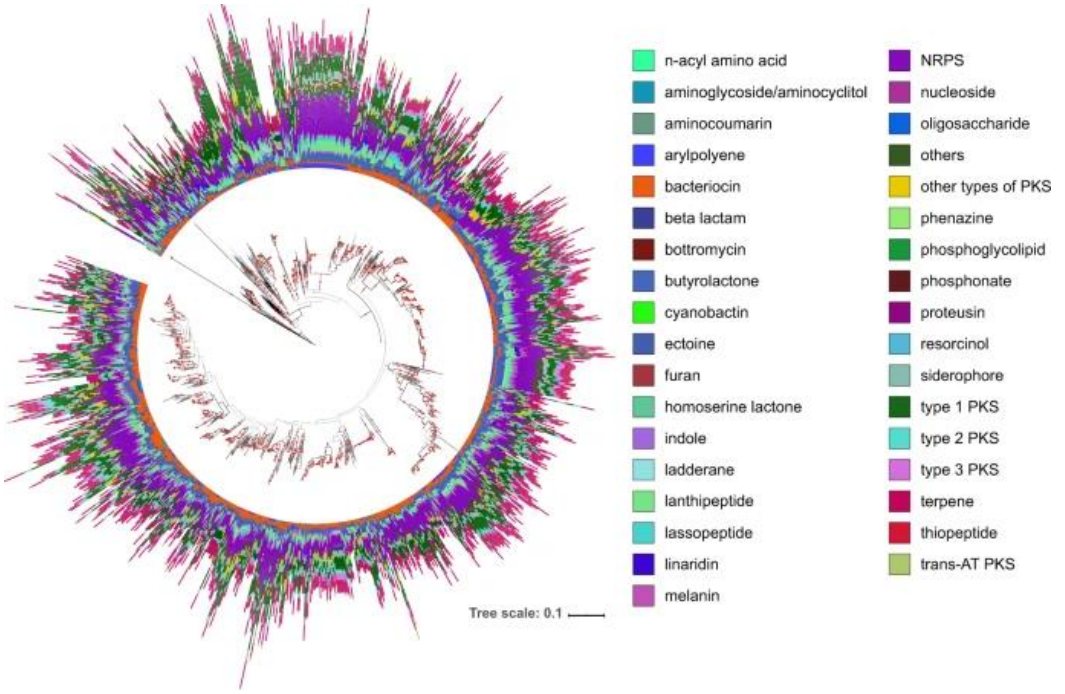

Figure 4: Phylogenetic distribution of BCGs from 1,107 genomes illustrating the SM richness in *Streptomyces.* BGCs were identified using antiSMASH. Extracted from Belknap et al., (2020)

1.3 *Streptomyces albulus*

A particular species with great industrial potential to note is *Streptomyces albulus*. *Streptomyces albulus* has been reported to natively produce high quantities of ε-poly-L-lysine (ε-PL) with different molecular sizes between 25-30 residues (Hamano, Yoshida, et al., 2006). Epsilon-poly-L-lysine is an amino acid homopolymer with an interesting structure that links the ε-amino and α-carboxylic acid functional groups (Shima & Sakai, 1977). This homopolymer exhibits wide-spectrum antimicrobial activity against gram-negative and gram-positive bacteria and anti-bacteriophage activity (Shima et al.,1984; 1982). Furthermore, ε-PLs are biodegradable, water-soluble, and food-safe; thus, they are used for food preservation in several countries (Hamano, 2010; Hirohara et al., 2006);. However, despite its practicality, industrialisation of ε-PL is hindered as research on metabolic

engineering of *S. albulus* strains and the ε-PL encoding BCG is limited (Hamano, 2010).

Streptomyces albulus has several other BCGs that also hold great industrial potential (Baltz, 2016; Fiedler et al., 2005). The draft genome of a novel strain *S. albulus* BCRC11814 has demonstrated several BCGs encoding for other industrially relevant SMs such as terpenes, lanthipeptides, butyrolactone and bacteriocin (Dodd et al., 2013). However, to date, there is limited research on these BCGs or the *S. albulus* genome resulting in a research gap worth exploring.

1.4 Advantages and drawbacks of *Streptomyces* in the bioengineering field

A key reason for the migration to metabolic engineering approaches is cost-effectiveness. *Streptomyces* spp., which are easily adapted to their environments, aid in this feature as they are inexpensive microorganisms to maintain in both lab or industry settings, as they are culturable on simple and low-cost media (Bhave et al., 2013). Previous studies have also reported the ability of some *Streptomyces* spp. to grow on glycerol waste stocks as well as under high salt and heavy-metal conditions (Abbas & Edwards, 1989; Dodd et al., 2018; Vijayakumar et al., 2012). This imparts an appealing feature to ensure sustainability, accessibility and reduce waste.

Specifically, as an engineering host, *Streptomyces* heterogeneously produces several industrial compounds (Komatsu et al., 2010). This feature impressively rivals putative industrialised *Escherichia coli* or yeast species. *Escherichia coli* and yeast tend to exhibit recombinant protein engineering difficulties such as overexpressed recombinant protein aggregation, self-toxicity, inclusion body aggregation, poor post-translational modification (e.g. methylation or glycosylation), improper native folding, lack of secretion and SM retention (Adrio & Demain, 2014; Conde et al., 2004; Cregg et al., 2009; Sevillano et al., 2016; Vieira Gomes et al., 2018). The absence of an outer membrane in *Streptomyces* allows for the direct extracellular secretion of SM products and also eliminates

contamination of endotoxins and pyrogenic lipopolysaccharide (Hamed et al., 2018). Additionally, more efficient extraction is also achieved with minimal requirement for post-purification and processing (Anné et al.,2012; Berini et al.,2020; Pozidis et al.,2001). This brands *Streptomyces* as appealing for desired SM yields with easier downstream processing

Secondary metabolite biosynthesis by other engineered microorganisms mostly occurs during lag or stationary growth stages. Contrastingly, SM biosynthesis by *Streptomyces* spp. can occur in the exponential, stationary, death, and transition (exponential to stationary) stages, thus higher SM yields are procured (Gramajo et al., 1993; Harir et al.,2018; Huang et al.,2001). Gramajo et al., (1993) and Huang et al., (2001) have reported that the highest metabolite production was observed during stage transitions, due to the presence of pleiotropic CSRs. Regarding this, research performed on members of the order *Enterobacterales* has demonstrated successful extension of growth phases by growth media manipulation and modification of gene encoding transcription factors that influence the growth stage (Osuna et al., 1995; Sekar et al., 2018). Therefore, this research could be applied to extend the transition stages of the lifecycle of *Streptomyces*, to enhance SM yield and build on the engineering efficiency.

Despite, the extensive SM repertoire and dynamic engineering efficacy of *Streptomyces* spp., metabolic engineering and commercial SM production strategies with this host are hampered by several drawbacks.

The foremost obstacle is caused by BCGs that encode for SM production. Recent studies have reported that nearly 922 BCGs have been discovered but most tend to be innately “silent” or express poorly under laboratory conditions (Lee et al., 2020; Thanapipatsiri et al., 2016; Zhang et al., 2017). This is largely attributed to the lack of adequate environmental stimuli to transduce CSRs. Typically, this obstacle can be averted with homologous activation of native CSRs in the *Streptomyces* genome, but this is commonly achieved with poor engineering efficiency(Zhang et al., 2016). Higher engineering efficiencies are achieved with heterologous expression

approaches to active transcription by constitutive or inducible promoters but necessitates preliminary genetic engineering as large portions of the genome must be cloned and precisely regulated to procure high SM yields (Chen et al.,2010; Kang et al.,2016; Zhang et al.,2017).

Alternatively, engineering by crosstalk-exhibiting Sec-protein translocation and twin-arginine translocation pathways has shown not only to activate the BCGs but also increase SM yields; however, this requires genetic recombination procedures with *Streptomyces* to introduce the respective systems (Goosens et al.,2014; Schaerlaekens et al.,2004). Gene transfer in *Streptomyces* poses a second obstacle due to the presence of a thick cell wall barrier and protective restriction-modification (R-M) systems that are prevalent in most *Streptomyces* species(MacNeil, 1988; Sànchez et al., 1985). Approximately 90% of *Streptomyces* genomes possess one of four reported R-M systems (type I, II, III and IV), but only two R-M systems (type II and IV) have been well studied (Mruk et al., 2019; Roberts et al., 2007).

Type II is responsible for cleaving exogenous DNA selectively at specific sites, but not endogenous methylated DNA and Type IV is the methyl-specific restriction system that cleaves heterologous methylated DNA (Bair & Black, 2007; Suzuki, 2011). To date, the mechanisms of *Streptomyces* R-M systems are unclear due to the variety in diversity, distribution, and specificity of R-M systems (Roberts et al., 2003; Rodic et al., 2017). However, the type IV methyl-specific restriction systems is the most implicated system in limiting genetic engineering of *Streptomyces* (Kwak et al., 2002; MacNeil, 1988). Therefore, heterologous expression with alternative microorganisms such as *E. coli* or yeasts are industrially employed instead but consequently, lower SM yields are procured. Moreover, the reduced capacity to engineer *Streptomyces* hinders the ability to uncover the new BCGs that still exist, thus reducing potential research efforts (Cobb et al., 2015; Donald et al., 2022).

Streptomyces are an economic option, but their feasibility is limited in industrial applications. The vegetative mycelial phase of *Streptomyces* is characterised by dense coiled mycelia formation that poses a third obstacle. The dense mycelia result in fermentation difficulties as viscous and heteromorphic cultures are produced (Sevillano et al., 2016). This negatively affects economic feasibility and functionality as it results in fermenter stirring, gas and nutrient, and heat transfer limitations (Sevillano et al., 2016). This limitation can be resolved by mechanical, or protein-overexpression-induced mycelia fragmentation (Celler et al., 2012). However, mechanical approaches can subsequently result in mycelial structural damage and impact SM secretion (van Dissel et al., 2015). Thus, protein overexpression approaches are preferred but require effective metabolic engineering knowledge.

The limitations mentioned above not only pose as single obstacles but as accumulated limitations that severely impact the metabolic engineering of *Streptomyces*. However, to date, *Streptomyces* spp. continues to be the first source choice for SMs and with advancing research and techniques the practicability and advantages of engineering *Streptomyces* may outweigh its drawbacks.

1.5 The *Streptomyces* genetic engineering toolbox

Gene transfer and recombinant techniques for genetic engineering have been well-studied in *Streptomyces*. This is achieved by vectors such as integrative vectors, autonomous or conditional replicative vectors, or non-replicative vectors with selectable antibiotic markers (Fayed et al., 2014; Muth et al., 1989; Wehmeier, 1995). They can be either low or high copy-number vectors that are designed with specific elements such as promoters, temperature-sensitive and gene inserts to target specific sites or genomic elements of *Streptomyces*. Subsequently, these vectors are transferred to *Streptomyces* by transformation (direct or protoplast-assisted) or conjugation for homologous or heterologous expression of BCGs (**Figure 5**) (Musiol-Kroll et al., 2019).

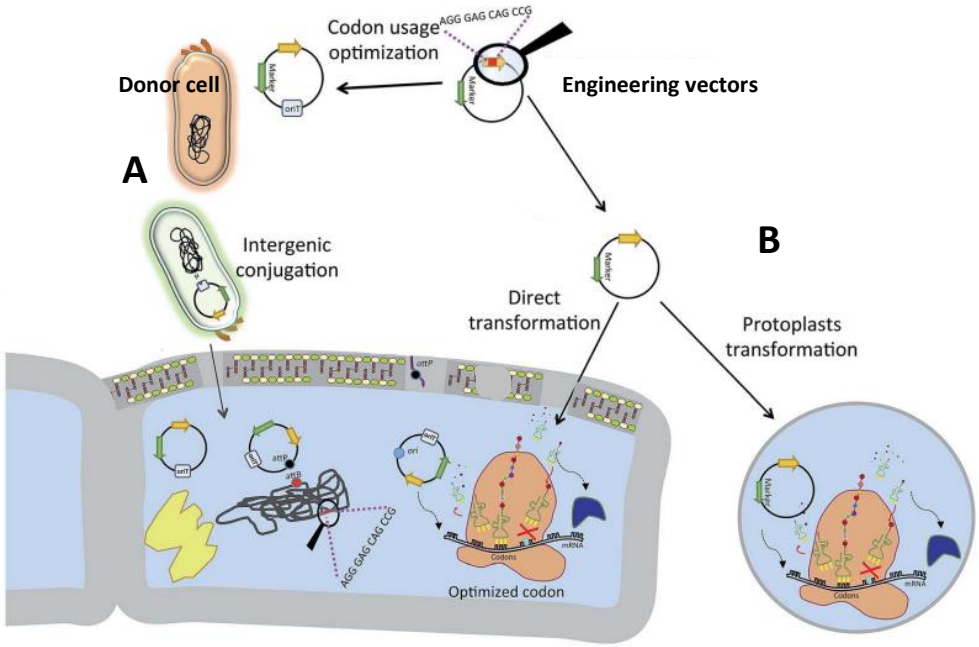

Figure 5: Illustration of gene transfer techniques for genetic manipulation of *Streptomyces* BCGs. Gene transfer of vectors to engineer BCGs is achieved by two common techniques A) intergeneric conjugation and B) direct or protoplast-assisted transformation. This illustration was adapted with permission from Musiol-Kroll et al.,(2019).

Conjugation, especially intergeneric conjugation, is preferred as it enables the successful transfer of small and large-sized plasmids as well as cosmids (Griffiths et al., 2000; Kieser et al., 2000). This is due to conjugation circumventing limitations posed by transformation such as the preliminary formation of protoplasts (removal of the gram-positive cell wall), *Streptomyces* inherent R-M systems interference with DNA transfer, and overall low transformation frequency (Baltz, 1998; Kwak et al., 2002; Paranthaman & Dharmalingam, 2003).

Conjugation, which exploits the mechanism of horizontal gene transfer, is described as the unidirectional transfer of genetic material between the direct but temporary cell-to-cell union of bacteria by pilus formation (Kurosawa et al.,2008; Soucy et al.,2015). The transfer system consists of a conjugal donor that can donate DNA

and a conjugal recipient that inherits the DNA (Griffiths et al., 2000). The transfer is not an actual exchange of DNA but is more correctly described as a leaking of substances, thus physical contact between cells is vital (Griffiths et al., 2000). Overall, conjugational transfer techniques are superior to transformation; however, it is worth noting that conjugation in *Streptomyces* requires specific methylation-deficient donor strains; is time-consuming; and is easily prone to contamination(Fouces et al., 2000; Kieser et al., 2000).

1.6 The *Streptomyces* metabolic engineering toolbox

1.6.1 Homologous vs heterologous expression approaches

Broadly, the metabolic engineering of *Streptomyces* is achieved by homologous or heterologous expression. Homologous expression commonly includes native promoter replacement, competitive pathway and negative regulatory gene knockout, positive regulation gene overexpression by specific CSR targeting, extracellular signal CSR activation, and pathway reconstruction ribosomal manipulation (Chen et al., 2010; Laureti et al., 2011; Lu et al., 1999; Smanski et al., 2009; Wang et al., 2019). However, homologous expression approaches have poor efficiency (Galm & Shen, 2006; Sianidis et al., 2006). Few studies have reported superior SM yield in native strains (Dubé et al., 2008; Miao et al., 2005). This is due to the tendency of *Streptomyces* to be recalcitrant to laboratory culturing due to inactive CSRs and their pleiotropic modulation, thus producing exceedingly low or no yields of SMs (Li et al., 2009; Tanaka et al., 2013).

The expression by cloning of target BCG genes in heterologous hosts takes precedence. Heterologous expression is manipulated by the use of strong promoters, overexpression of global or pathway-specific regulators, and metabolic flux engineering(Huo et al., 2012; Lyu et al., 2020; Viollier et al., 2001). The transfer of BCGs into heterologous hosts is achieved by traditional or advanced BCG construct assembly. However, due to the large size of BCGs and poor efficiency achieved with traditional strategies, such as bacterial artificial

chromosome library construction; advanced strategies are more commonly employed (Bentley et al., 2002; Nett et al., 2009; Zhang et al., 2016). Advanced strategies include phage recombination, transformation-associated recombination (TAR), or clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system (Cobb et al.,2015; Du et al.,2015; Li et al.,2018; Lu et al.,2010; Yamanaka et al.,2014).

1.6.2 Heterologous construct assembly strategies

1.6.2.1 The phage recombination approach

Site-specific genetic engineering of *Streptomyces* can be achieved by tyrosine and serine recombinase families derived from phages. Tyrosine recombinases include Cre/loxP (cyclization recombinase/ locus of x-over, P1), Flp/FRT (flippase recombinase/ flippase recognition target) and λ phage recombination systems (Gust et al., 2003; Komatsu et al., 2010). Alternatively, serine recombinases included *Streptomyces* integrases from phages ϕC31, ϕBT1 and VWB that catalyse at phage-specific attachment sites (*attP*) (Thorpe et al., 2000; Van Mellaert et al.,2007). These recombinase systems allow for entire gene cluster manipulation and avert polar effects such as transcriptional gene inactivation at the integration site that results in cell differentiation defects (Van Mellaert et al., 2007). Successful genome editing has been observed with Cre/loxP and ϕBT1 systems (Herrmann et al., 2012; Wu et al., 2012).

A prominent issue with serine phage recombination is that it depends on integrative vectors for gene transfer (Musiol-Kroll et al., 2019; Wu et al., 2012). These vectors are derived from *Streptomyces* and thus share high sequence identity with the target genome. This could potentially cause large deletions and genome rearrangements when homologous recombination occurs (Musiol-Kroll et al., 2019). Furthermore, recombinase systems are multistep techniques that are laborious as they require cosmid library generation and create FRT/*IoxP* site scars, thus causing low genetic engineering efficiencies (Gust et al., 2003; Liu et al., 2009; Zhao et al., 2020).

1.6.2.2 Transformation-associated Recombination approach

The transformation-associated recombination (TAR) approach enables the direct cloning of large BCGs without the need to construct clone libraries (Yamanaka et al., 2014). This approach appertains to the *in vivo* homologous recombination of *Saccharomyces cerevisiae* (Kouprina and Larionov 2016; Orr-Weaver et al.,1981). A target BCG is directly inserted via homologous recombination into the integrative TAR vector pCAP01, hosted in yeast cells to avoid multiple recombination events (Yamanaka et al., 2014). Thereafter, the target BCG is manipulated in the yeast cell or shuttled to *E. coli* for manipulation by systems like λ phage recombinases or CRISPR/Cas9 (Mitousis et al.,2020; Yamanaka et al.,2014). The manipulated BCG can be conjugated into a heterologous host as the vector pCAP01 contains an origin of DNA transfer gene and *Streptomyces*-derived φC31 integrase gene and its targetcas9cas9 *attP* site (Yamanaka et al., 2014).

Notably, the TAR system can also be used for the homologous expression of BCGs (Wu et al., 2017). However, the efficiency of obtaining clones with the correct BCG construct is < 5% with the standard protocol; thus, this system must be used in combination with other systems or tools to increase efficiency (Lee et al.,2015; Mitousis et al.,2020).

1.6.2.3. The CRISPR/Cas9 approach

The introduction of CRISPR/Cas9 technologies has revolutionised genetic engineering and currently dominates metabolic engineering approaches. Specifically in *Streptomyces*, advancements with recalcitrant strain manipulation, BGC discovery and SM yield have been achieved (Kang et al., 2016; Yu et al., 2018; Zhang et al., 2017). The technology is based on the natural response of a bacterial defence system that operates with precise targeting to repair chromosomal disruptions (Barrangou et al., 2007). The native function of CRISPR/Cas9 as a defence system includes a crRNA (CRISPR ribonucleic acid), encoded as 20-30bp spacers with complementary targeting specificity; and a tracrRNA (trans-activating

CRISPR ribonucleic acid), to form a crRNA-tracrRNA duplex (Jinek et al., 2012). The crRNA-tracrRNA duplex then recruits the Cas9 endonuclease and cleaves the target site on the chromosome to introduce double-strand breaks (Jinek et al., 2012).

The double-strand breaks are repaired by non-homologous end joining (NHEJ) or homology-directed repair (HDR) (Jinek et al., 2012). This system has been repurposed for engineering microorganisms by modifying the crRNA-tracrRNA duplex into a single synthetic guide RNA (sgRNA) (Cobb et al.,2015). Additionally, guide RNA sequences are inserted into the editing system to cleave at target sites for site-specific engineering of genomes (Cobb et al.,2015). The mechanism of the editing system is initiated with the specific sgRNA recognising the NGG (nucleotide-guanine-guanine) protospacer-adjacent motif (PAM) on the chromosomes (Barrangou et al., 2007). Subsequently, the Cas9 is recruited and cleaves to form double-strand breaks on the targeted site of the chromosome according to the sgRNA (Barrangou et al., 2007). If a donor DNA insert is coupled with the editing system, it is placed at the target site.

Initial engineering studies of the CRISPR/Cas9 system in *Streptomyces* were achieved with phages, thus termed multiplexed site-specific genome engineering (MSGE). The MSGE approach was based on the "one integrase-multiple *attB* site" and facilitated the integration of multiple stable BGCs into the native and artificial *attB* sites of the host genome in a single step. While this system has reported remarkable efficiency it required the pre-addition of artificial *attB* sites into the host genome by CRISPR/Cas9 (Huang et al., 2015). Additionally, the insertion of the artificial *attB* sites into genetically elusive industrial *Actinomycetes* is laborious (Li et al., 2019).

Recently, to advance Cas9 technologies for *Streptomyces*, several Cas9-based engineering vectors have been reported such as the pCRISPomyces plasmid, the one-step pKCcas9dO editing plasmid, the CRISPR/Cas9 vector, the CRISPR/dCas9-mediated multiplex gene repression vector and the CRISPR/Cas base editing system vector (Cobb et al.,2015; Huang et al.,2015; Tong et al., 2019;

Zhao et al.,2018). All these vectors were designed with similar elements such as a thermosensitive pSG5 replication region. Furthermore, several optimised derivatives have been designed from these vectors (Alberti & Corre, 2019; Zhang & Voigt, 2018). However, the engineering efficiency of these vectors varies considerably due to the presence of cas9 toxicity, expressed under strong promoters, in host strains (Zhang & Voigt, 2018). Furthermore, the toxic effect may be amplified by the non-specific binding to NGG-PAM sequences within the *Streptomyces* genome, particularly when sgRNA is not provided (Zhang & Voigt, 2018).

The highest efficiencies were reported for the pCRISPomyces plasmids and its derivatives, which have been studied in several *Streptomyces* strains (Alberti & Corre, 2019; Cobb et al., 2015; Yeo et al., 2019; Zhao et al., 2020). The mechanism of the pCRISPomyces expression system is based on the type II CRISPR/Cas9 system, discovered in S*treptococcus pyogenes*, which has demonstrated significant success and efficacy (Cobb et al.,2015). Two plasmid systems were developed, pCRISPomyces-1 (includes the *cas9* and both the tracrRNA and crRNA cassette) and pCRISPomyces-2 (includes *cas9* and a sgRNA cassette) **(Figure 6)** (Cobb et al.,2015). The pCRISPomyces plasmids allowed for the flexible custom insertion of spacers (approximately 20bp-30kbp) (Cobb et al.,2015).

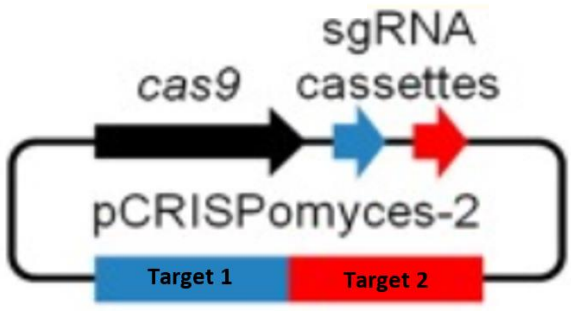

Figure 6: Diagram of pCRISPomyces-2 gene-editing system for multitarget gene editing with sgRNA construct. Adapted from Cobb et al., (2015).

Considering the two systems, a distinguishable engineering efficiency of 70-100% was achieved with the pCRISPomyces-2 plasmid (Cobb et al.,2015; Zhao et al.,2020). This system was also designed with a second sgRNA construct to achieve multiplex genome editing (Cobb et al.,2015). Additionally, the pCRISPomyces-2 plasmid and derivatives are compatible with heterologous and homologous expression engineering and can be used in combination with other available technologies such as the TAR system (Alberti & Corre, 2019). In combination, the efficiency of a flexible gene-editing system such as pCRISPomyces holds promise as an effective engineering tool with wide application. However, *Streptomyces* strains respond differently to *cas9* expression and engineering. Therefore, it would be necessary to determine the baseline effects of the pCRISPomyces plasmids in a specific strain engineering.

1.7 Dissertation statement

Several engineering tools have been developed to harness the engineering potential of *Streptomyces*. However, many of these tools do not possess adequate engineering efficiency to be effectively employed in industrial settings. To resolve this limitation, many engineering tools are used in combination to achieve a specific output but this requires preliminary research of the target *Streptomyces* strain. This dissertation outlines baseline research conducted on *S. albulus* BCRC11814 for future metabolic engineering. *Streptomyces albulus* BCRC11814 produces SMs, such as ε-PLs, and its genome carries several unstudied BCGs. However, there is very limited research on the metabolic engineering of this particular strain and its engineering limitations regarding the presence of R-M systems. Therefore, a crucial research gap exists with regard to knowledge of *S. albulus* BCRC11814 and necessitates research to uncover its potential as an industrial strain or source for SMs. Engineering tools such as pCRISPomyces plasmids, which have exhibited good engineering efficiency, could aid in achieving this long-term aim but the response of *S. albulus* BCRC11814 to the introduction to pCRISPomyce-2 plasmids is unknown and must be established before achieving future metabolic engineering.

1.8 Aims and cbjectives

This study aimed to introduce pCRISPomyces-2 plasmids in *Streptomyces* strains, for prospective multiplex genome editing and metabolic engineering in *S. albulus* BCRC11814. It was hypothesised that the successful introduction of pCRISPomyces-2 plasmids in *S. albulus* BCRC11814 could be achieved.

Aims were set to be achieved by the following objectives:

1. To verify the pCRISPomyces-2 plasmids by Sanger sequencing.

2. To transform pCRISPomyces-2 plasmids into a conjugal donor strain *E. coli* WM6026

3. To establish a successful intergeneric conjugational system for the transfer of pCRISPomyces-2 plasmids in *S. albulus* BCRC11814.

4. To determine the presence of methyl-specific restriction-modification systems in *S. albulus* BCRC11814 by electro-transformation.

5. To verify the susceptibility of *Streptomyces* strains to the aminoglycosidic antibiotic apramycin with minimum inhibitory concentration assays.

Chapter 2: pCRISPomyces-2 plasmid sequence verification and transformation into conjugative *Escherichia coli* WM6026

2.1 Introduction

In a study by Cobb et al. (2015), two replicative pCRISPomyces were designed for the genetic engineering of *Streptomyces*; however, the second version the pCRISPomyces-2 plasmid has garnered attention for its efficient design and promising engineering efficacy. The original study reported a 70-90% engineering efficiency with the pCRISPomyces-2 plasmid in several different *Streptomyces* strains through intergeneric conjugation. Catering to the Cas9 functionality of pCRISPomyces-2 plasmid, the plasmid has been constructed with a sgRNA expression cassette and a Cas9 protein that is expressed under a strong promoter, *rpsLp (XC)* derived from *Xylanimonas cellulosilytica* (Cobb et al., 2015; Ye et al., 2020). The sgRNA expression cassette contains a *Bbs*I-flanked *lacZ* cassette for the assembly of target-specific spacer sequences. Furthermore, the plasmid has an *Xba*I restriction site for the insertion of additional editing templates via Gibson assembly or traditional ligation (Cobb et al., 2015). A vector map is displayed in **Figure 7**.

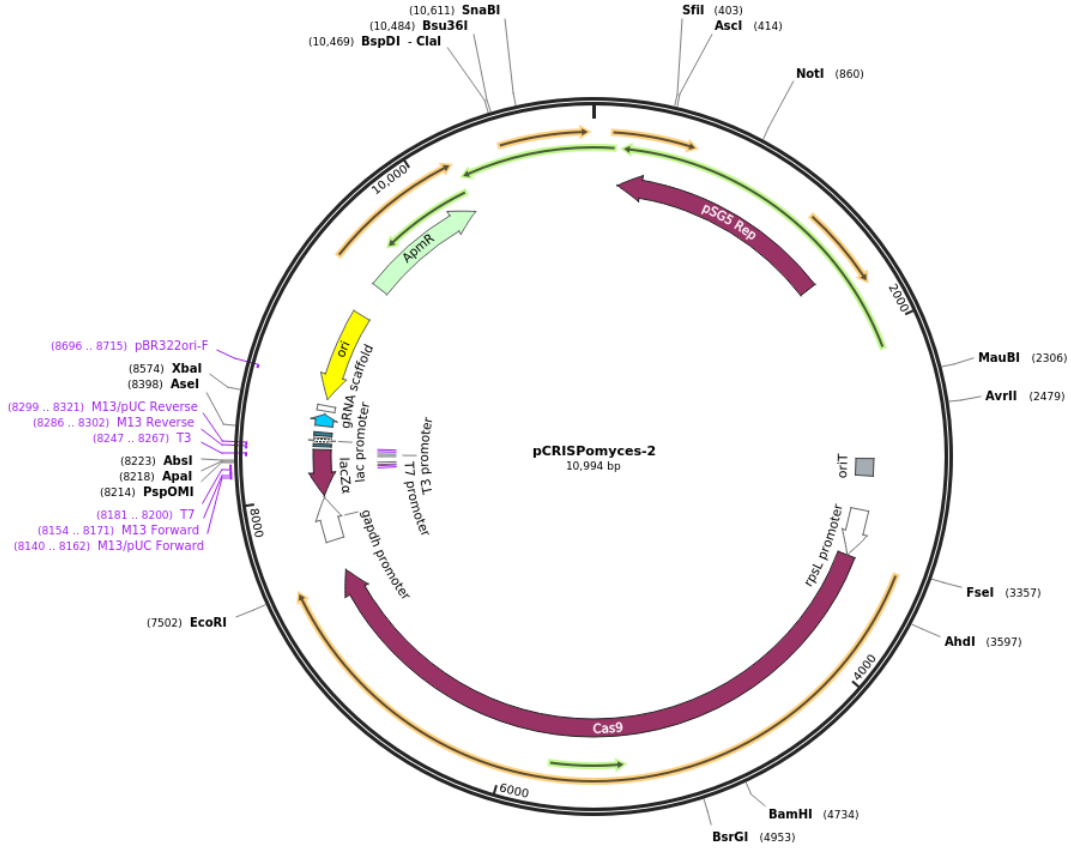

Figure 7: A plasmid map of pCRISPomyces-2 displaying its functional elements specific to the plasmid's CRISPR/Cas9. This vector map was extracted from Addgene.org (plasmid ID: 61737)

Additionally, the plasmid's features include a colicin E1 origin, that enables its replicative ability in donor *E. coli* strains; an RP4 origin of transfer and transfer (tra) genes, derived from IncP plasmids that enables intergeneric conjugation with target *Streptomyces* recipient strains; and an apramycin (Apr) resistance gene (*aac(3) IV*), as a selectable marker (Cobb et al., 2015; Davies & O'Connor, 1978; Lyras & Rood, 1998; Sabik et al., 1983). Furthermore, the pCRISPomyces-2 plasmid has a conditional suicide feature through the presence of a pSG5 temperature-sensitive *rep* region that enables plasmid clearance post-engineering (Cobb et al., 2015; Muth et al., 1989). Together these elements and the specific CRISPR/Cas9 elements make up the pCRISPomyces-2 plasmid and confer its efficiency.

This chapter outlines the preliminary work performed to achieve the subsequent objectives of this dissertation. The work aimed to extract and verify the pCRISPomyces-2 plasmid DNA (pDNA) and then subsequently transform the plasmid into *E. coli* WM6026. The initial quality verification control step is generally performed by sequencing analysis or enzyme restriction (Gallegos et al., 2020). However, sequence analysis is preferred as it provides observable data of the plasmid's sequence to detect single point mutations that occur from DNA synthesis or structural mutations that may have occurred during plasmid assembly (Czar et al., 2009; Marchand & Peccoud, 2012). This avoids potential bottlenecks caused by the plasmid's structural integrity or related functionality and ensures viable introduction of the plasmid into *E. coli* WM6026 and *S. albulus* BCRC11814.

Escherichia coli strains, such as *E. coli* WM6026, have been engineered as conjugative donor cells for *Streptomyces* intergeneric conjugation studies (Blodgett et al., 2007). Conjugative *E. coli donor* strains differ functionally from typical cloning strains such as *E. coli* DH5-alpha and act as a donor means to deliver the cloned DNA to target microorganisms (Thoma & Schobert, 2009). These donor strains, of which several exist, are strategically engineered with methylation defective genes to bypass strict methyl-specific R-M systems, especially present in *Streptomyces* (Flett et al., 1997; Purdy et al.,2002; MacNeil, 1988). These methylation genes generally encode methyltransferases that cleave exogenous DNA at specific sites as part of the R-M systems in *E. coli*, particularly lab strains (Marinus & Løbner-Olesen, 2014). However, careful genetic engineering of the donor strains can circumvent this problem.

The donor strains are also genetically manipulated to grow with exogenously supplied auxotrophic factors which consequently result in a slower doubling time. This allows for growth synchronisation during the conjugal mating process (Allard et al., 2015; Clarkson & Meadow, 1971). However, it is interesting to note that the manipulation of the auxotrophic genes does not bear any negative effects on the transformation efficiency of donor cells when cloning the subject DNA (Collens et

al., 2008; Prías-Blanco et al., 2022). In addition, several other features may also be engineered into the donor strain according to the specific design of the conjugation system and the recipient strain (Blodgett et al., 2007).

In particular, *E. coli* WM6026 has been engineered with a defective *hsd* gene encoding the EcoK adenine methyltransferase and is diaminopimelic acid (DAP) deficient through deletion of the *dapA* gene (Blodgett et al., 2007). Importantly, DAP is vital for the cross-linkage of peptidoglycan in the cell wall and is also required for lysine synthesis in *E. coli* strains (Born and Blanchard 1999). Therefore, DAP must be exogenously provided in the growth media of *E. coli* WM6026 to enable growth. Furthermore, DAP deficiency in *E. coli* WM6026 confers its donor cell quality as it results in slower doubling times which enables growth synchronisation during downstream conjugal mating (Allard et al., 2015). However, given the presence of Cas9 protein in the pCRISPomyces-2 plasmids, it was hypothesised to negatively affect transformational efficiency.

2.2 Methods

2.2.1 pCRISPomyces-2 plasmid DNA verification

2.2.1.1 pCRISPomyces-2 plasmid DNA extraction

The pCRISPomyces-2 pDNA, maintained in *Escherichia coli* DH5-alpha, was donated by Professor Huimin Zhao (Addgene plasmid ID: 61737) and extracted for downstream experiments. A glycerol stock of *E. coli* DH5-alpha/pCRISPomyces-2 was streaked on lysogeny broth (LB; 10 g tryptone, 5 g NaCl, 5 g Yeast extract, 1 L dh_2O, pH 7.0) agar, prepared with 10g agar and supplemented with 50 µg/ml Apr selectable maker. The agar plates were then incubated at 30°C for 16-18h. A single colony from the streak agar plate was inoculated into 100 ml Apr-supplemented LB broth and incubated overnight at 30°C with shaking at 220 rpm. Thereafter, the inoculum was sub-cultured, as per the aforementioned conditions, in 50 ml Apr-supplemented LB broth until an OD_{600} (Optical Density) of 0.3 was measured with

a S-200 VIS spectrophotometer (BOECO, Hamburg, Germany). Subsequently, pCRISPomyces-2 pDNA was extracted in triplicate from the inoculum with a ZR Plasmid Miniprep kit (Zymo Research, Irvine, CA, USA), as per the manufacturer's instructions. The extracted pDNA was quantified and quality was assessed with a Nanodrop One (Thermo Fisher Scientific, Massachusetts, USA) and stored at -20°C for subsequent use.

2.2.1.2 ClustalW alignment of pCRISPomyces-2 plasmid sequencing outputs

A partial sequence of the pCRISPomyces-2 plasmid was verified by Sanger sequencing as a quality control step to detect structural mutations in the plasmid backbone or CRISPR/Cas9 functional elements. The extracted pDNA from **Chapter 2, section 2.2.1.1** was subjected to Sanger sequencing by Inqaba Biotech (Pretoria, South Africa). Forward primers 5'-GACGGCCAGTGAGCGCGCG-3' (position 8162- 8180) and 5'- CCGCTTACCGGATACCTGTC-3' (position 8755-8774) were designed to obtain amplicon sizes of 598bp and 687bp, respectively. BioEdit Sequence Alignment Editor© version 7.2.5 (Hall, 1999) was used to align the sequencing outputs and obtain a total amplicon size of 1280bp. ClustalW alignment was then used to verify the sequence identity against the full *Streptomyces* expression codon-optimised Cas9 nucleotide sequence (plasmid ID: 61737) obtained from Addgene.org as a GenBank file.

2.2.2 Transformation of pCRISPomyces-2 plasmid into *Escherichia coli* WM6026

2.2.2.1 Culturing of Escherichia coli WM6026

Escherichia coli WM6026 donated by Professor Karl Rumbold (IMBL, Molecular and Cell Biology, University of Witwatersrand, South Africa) was streak-plated from glycerol stocks on LB agar supplemented with 19 µg/ml DAP auxotrophic marker and incubated at 37°C overnight. A single colony of *E. coli* WM6026 was inoculated into 50 ml DAP-supplemented LB broth and incubated at 37°C with shaking at 220 rpm for 16-18 h. The inoculum was sub-cultured, as per the

aforementioned conditions, by adding 2 ml inoculum into 250 ml DAP-supplemented LB broth and growing the subculture until an OD_{600} of 0.3-0.4 (mid-exponential phase) was reached. Thereafter, the inoculum was immediately chilled in an ice/dH_2O slurry and subsequently used to prepare electro- or chemical-competent cells.

2.2.2.2 Transformation by electroporation

Electroporation of *E. coli* WM6026 with the pCRISPomyces-2 plasmid was performed according to the methodology by Banta et al.,(2020), with minor modification.

a. Preparation of electrocompetent cells

The electrocompetent cells were prepared by pelleting 50 ml chilled inoculum of *E. coli* WM6026 in a chilled (4°C) centrifuge (Beckman Coulter, Allegra X-30) at 4000 x g for 10 min. The supernatant was removed, and the pellet was washed twice in an equal volume of 15% cold glycerol. The pellet was resuspended in 5 ml of 15% cold glycerol and washed again. Finally, the pellet was resuspended in 2 ml of 15% glycerol and 50 µl volumes were aliquoted into single-use cryovials. The aliquoted cells were immediately used or stored at -80°C until electroporation.

b. Electroporation

A 50 µl aliquot of electrocompetent cells was thawed on ice and mixed with 1 µl of purified pCRISPomyces-2 pDNA (10 ng/µl). The mixture was carefully transferred to a chilled sterile 0.2cm gap electroporation cuvette. The mixture was electroporated with a Bio-Rad Micropulser™ (Johannesburg, South Africa) as per the manufacturer's instructions, using the "EC2" pre-set for a single pulse delivery at 2.5 kV voltage. Post-electroporation, the transformed mixture was immediately resuspended in 500 µl of SOC (Super Optimal broth with Catabolite repression; 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM

$MgSO_4$,20 mM glucose in 1 L H_2O with pH 7.0), supplemented with 19µg/ml DAP. Thereafter, the transformed suspension was transferred into a 1.5 ml microcentrifuge tube and incubated at 37°C with shaking at 250 rpm for 1 h without any antibiotic selection. Post-incubation, the transformed suspension was spread-plated in volumes of 50,100 and 200 µl on prewarmed LB broth, supplemented with 19 µg/ml DAP and 50 µg/ml Apr, and incubated at 30°C for 16-18 h. The transformants were then manually counted. The experiment included a negative control that lacked the addition of pCRISPomyces-2 pDNA and a positive pUC19 control selected with 100 µg/ml Amp (ampicillin).

2.2.2.3 Transformation by heat Shock

Heat shock transformation was performed as described by Tu et al.,(2005) with chemical-competent cells that were prepared according to Inoue et al.,(1990).

a. Preparation of chemical-competent cells

The chemical-competent cells were prepared by pelleting 50 ml chilled inoculum of *E. coli* WM6026 at 4000 x g for 10 min, 4°C. The pellet was resuspended in 25ml chilled sterile TB (Transformation Buffer; refer to **Appendix A** for preparation) and incubated on ice for 25 min. The cell suspension was pelleted again, as per the aforementioned conditions, and finally resuspended in 2 ml cold TB. The competent cell suspensions were mixed with 100% glycerol to obtain a final concentration of 20% glycerol then aliquoted in 100 µl volumes into single-use cryovials and stored at -80°C.

b. Heat-shock transformation

An aliquot of chemical-competent cells was thawed on ice and mixed with 3.0 µl of pCRISPomyces-2 pDNA (10 ng/µl) and 1 µl 40% DMSO (Dimethyl sulfoxide) before the mixture was incubated on ice for 30 min. Thereafter, the mixture was heat-shocked at 42°C for 90 s and immediately incubated on ice for 2 min. The

transformed mixture was resuspended in 500µl prewarmed SOC media, supplemented with 19 µg/ml DAP, and incubated at 37°C with shaking at 250 rpm without antibiotic selection for 1h. Post-incubation, similarly as per **Chapter 2, section 2.2.2.2 b**, the transformed suspension was spread-plated with a pre-plating 10x dilution for each plating volume and incubated until transformants appeared and were counted. The experiment was also performed with a negative control lacking the addition of pCRISPomyces-2 pDNA and a positive pUC19 control selected with 100 µg/ml Amp.

2.2.3 Screening of transformants by colony PCR

The transformed *E. coli* WM6026 were screened for the uptake of the pCRISPomyces-2 plasmids by colony PCR as described by Kirby & Patel, (2021) and Thakkar & Saraf, (2017), with minor modification. Random colonies of *E. coli* WM6026/pCRISPomyces-2 from each transformation method were streaked on selective LB agar, supplemented with 19 µg/ml DAP and 50 µg/ml Apr, then incubated at 30°C for 16-18 h. Three random single colonies from different plates were isolated and resuspended in 20 µl nuclease-free water and then subjected to colony PCR using M13/pUC primers (forward: 5'-GTAAAACGACGGCCAGT-3' and reverse: 5'-GGAAACAGCTATGACCATG-3'). The colony PCR reaction mix in a total volume of 50 µl contained 2 µl cell suspension, 5 µl x10 ThermoFisher DreamTaq buffer, 1 µl 10 mM DNTP mix, 0.5 µl ThermoFisher DreamTaq Polymerase (5 U/µL), 2.5 µl each of 10 mM M13/pUC forward and reverse primer, and 36.5 µl nuclease-free water. The reaction mix was amplified with a 2720 thermal cycler (Applied Biosystems, Massachusetts, USA) at the following cycling conditions: initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 90 s, and final extension at 72°C for 10min. Gel electrophoresis was used to analyse 10 µl of the colony PCR product with 50 bp Thermo Scientific GeneRuler DNA ladder on a 1.2% agarose gel. The agarose gel included a negative control of untransformed *E. coli* WM6026 and a positive control of “pure” extracted pCRISPomyces-2 pDNA.

2.2.4 Comparison of growth curves for untransformed and transformed *E. coli* WM6026

Growth curves were modelled to determine if the pCRISPomyces-2 plasmid had any metabolic effects on the *E. coli* WM6026 post-transformation. Growth curves of untransformed and transformed *E. coli* WM6026 were produced simultaneously according to the modified methodology by Cornu et al., (1999). A single colony of untransformed and transformed *E. coli* WM6026 were each inoculated in 200ml of LB broth, supplemented with 19 µg/ml DAP. Additionally, 50 µg/ml Apr was added for the transformed *E. coli* WM6026 to select for the pCRISPomyces-2 plasmid. Both inoculums were incubated at 30°C, with shaking at 250rpm, and OD_{600} readings were obtained hourly with a 200 VIS spectrophotometer (BOECO, Hamburg, Germany) over 24 hours. An exponential curve ($y = Ae^{Bx}$) was produced with the obtained data to determine the difference in doubling time between untransformed and transformed *E. coli* WM6026 (refer to **Appendix B** for calculations).

2.2.5 Statistical Analysis

The transformation efficiency (TE; cfu/µg) was determined by the following equation: number of transformants/ µg of pDNA x final recovery volume (ml)/ volume played (ml). The statistical significance comparing TEs of transformation experiments was determined by an unpaired t-test or one-way ANOVA with α=0.05 conducted on the average data result obtained from three independent experiments.

2.3 Results and Discussion

2.3.1 pCRISPomyces-2 plasmid DNA extraction and verification

The pCRISPomyces-2 pDNA was extracted for quality control and downstream applications such as genetic recombination. The quality of pCRISPomyces-2 pDNA obtained from initial extractions displayed A_{260}/A_{230} values > 3.0 indicating organic compound contamination. Despite the A_{260}/A_{230} ratio as a debatable secondary measure of pDNA quality, because of its sensitivity to the make-up of elution buffers, the abnormally high values observed were suspected to cause potential downstream problems such as pDNA degradation or sequencing errors (Glassing et al., 2016; Lucena-Aguilar et al., 2016). In order to avoid such problems, re-extraction was performed with smaller inoculum inputs and alteration of the extraction process by extended centrifuge times or repetition of extraction steps. However, the consequent yield of pCRISPomyces-2 pDNA (<20 ng/µl) was significantly compromised as quality improved.

Therefore, extractions with higher yields but relatively lower contamination were selected. The pCRISPomyces-2 pDNA yields from three selected extractions ranged between 64.1-115.1 ng/µl with A_{260}/A_{280} and A_{260}/A_{230} values above 1.8 and 2.0 respectively (**Table 1**). However, to avoid potential bottlenecks in downstream experiments, the pCRISPomyces-2 pDNA extractions 1 and 3 (A_{260}/A_{230} values > 2.2) were reserved for recombination and cloning applications as they did not classify as “pure”. Previous studies have reported that contaminated DNA has an insignificant effect on cloning experiments thus purification was not required (Zimmermann & Neil, 1996). However, the thawing cycles for these extractions were minimised to reduce the acceleration of pDNA degradation. Alternatively, the pCRISPomyces-2 pDNA extraction 2 displaying the best “pure” quality was used for sequencing or polymerase chain reaction (PCR) control applications.

Table 1: pCRISPomyces-2 pDNA quantification and quality assessment.

Extraction	pDNA concentration (ng/µl)	A_{260}/A_{280}	A_{260}/A_{230}
1	64.1	1.88	2.24
2	115.1	1.80	2.17
3	79.9	1.85	2.30

Consequently, the pCRISPomyces-2 sequencing outputs were obtained from the pDNA extraction by Sanger sequencing. An annotated ClustalW alignment of the aligned pCRISPomyces-2 sequencing outputs and an edited reference *Streptomyces* expression codon-optimised Cas9 nucleotide sequence, displaying the selected sequenced region, was presented below as **Figure 8**. The ClustalW alignment that included the multiple cloning sites, sgRNA scaffold cassette, the origin of replication, and *aac (3) IV* sequences indicated > 98% identity. The presence of single point mutations observed was likely to have occurred from sequencing errors caused by pDNA degradation. However, the overall structural and hence functional integrity of the pCRISPomyces-2 plasmid sequenced region was maintained.

Notably, the sequenced regions which included the multiple cloning site (position 8215-8575) and sgRNA scaffold (position 8444-8519) displayed >90% identity. Thus, their structural integrity and relational functionality were maintained in the pCRISPomyces-2 plasmid. The multiple cloning sites contain a *lacZ* cassette and the *Xba*I restriction site for the assembly of target-specific spacer sequences and additional editing templates, respectively. Cooperatively, these elements guide the Cas9 protein and consequently enable the CRISPR/Cas9 expression of the pCRISPOmyces-2 plasmid (Cobb et al., 2015). Therefore, it was crucial to verify these specific sequenced regions for future experimental procedures. In hindsight, this study could have been improved by sequencing and verifying the fragment

containing the Cas9 protein as well as to increase the accuracy of the pCRISPOmyces-2 plasmid verification.

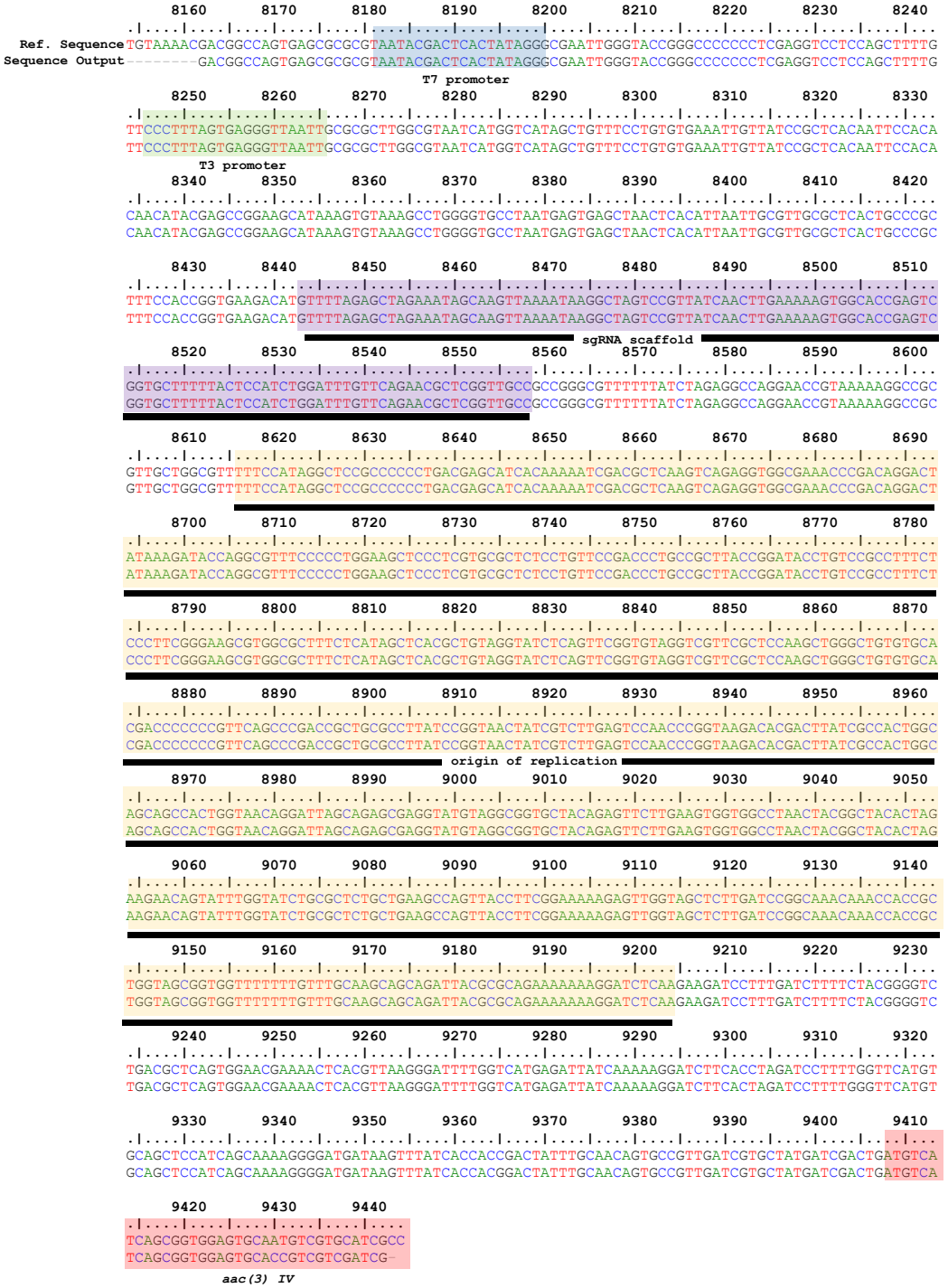

Figure 8: Annotated ClustalW alignment output generated with BioEdit Sequence Alignment Editor© version 7.2.5. A partial sequence of the

pCRISPomyces-2 plasmid (labelled as Sequence Output) was sequenced with a forward T7 promoter primer and ClustalW aligned with the selected region of the reference *Streptomyces* expression codon-optimised Cas9 nucleotide sequence (plasmid ID: 61737; labelled as Ref. sequence).

2.3.2 Transformation of pCRISPomyces-2 plasmid into *Escherichia coli* WM6026

Transformation by electroporation and heat-shock was performed with methylation-defective *E. coli* WM6026 for the uptake of high-copy pCRISPomyces-2 plasmids. The transformation methods were compared by their efficiency of plasmid uptake.

2.3.2.1 Transformation by electroporation

Electroporation is the standard method for bacterial transformation due to its efficiency and efficacy in producing high TEs. These features were appealing for the introduction of pCRISPomyces-2 plasmids in *E. coli* WM6026. However, in comparison to previously reported TEs of 10^9 cfu/µg (Dower et al., 1988), a significantly lower average TE of 3.42×10^4 cfu/µg was initially obtained. Three influencing factors were considered for the poor TE: the quality of competent cells given the low TE initially observed with the control experiment using pUC19 plasmids, the size of the pCRISPomyces-2 plasmids and the concentration of pCRISPomyces-2 pDNA at 30 ng/µl.

Consequently, the competent cells were prepared again, and the final resuspension volume was reduced from 4 to 2 ml to obtain a higher competent cell concentration (cfu/ml). The use of a higher competent cell concentration was based on the rationale of transformation frequency such that TE would improve as the number of competent cells increased and more competent cells would be available for pDNA uptake (Dower et al., 1988). A minor improvement in TE was observed with the re-preparation confirming the poor quality of previously used competent cells.

However, the average TE remained $<10^5$ cfu/µg but could be elucidated by the other potential influencing factors.

A study by Szostková & Horáková (1998) reported that the electroporation of *E. coli* strains with increasing plasmid sizes from 4.4kb resulted in decreased TE. The pCRISPomyces-2 plasmids used for electroporation in this study have a plasmid size of 10 995 bp, which was substantially larger than those used in the previously mentioned study. Furthermore, only the pCRISPomyces-2 plasmid construct without sgRNA inserts was used in this study. This confirmed the pCRISPomyces-2 plasmids' larger size as an influencing factor for poor TE but also foreshadowed a further decline in TE when target sgRNA sequences are inserted into the plasmids further increasing the plasmids' size which could impact downstream experiments.

In tandem, the concentration of pCRISPomyces-2 pDNA as a third potential influencing factor was considered. It is known that pDNA concentration affects electroporation efficiency (Szostková & Horáková,1998). The presence of a direct relationship between the pDNA concentration and the metabolic burden imposed by the pCRISPomyces-2 plasmids Cas9 protein was assumed to impact on TE. To determine if optimisation of pDNA concentration would improve TE, electroporation was performed with pCRISPomyces-2 pDNA concentrations at 10, 20 and 40 ng/µl in comparison to the initial concentration of 30 ng/µl **(Figure 9)**. The results indicated a 10-fold improvement in TE from the initial 2.07×10^4 cfu/µg to 1.07×10^5 cfu/µg as pDNA concentration decreased from 40 ng/µl to 10 ng/µl. The trend in TE indicated that lower pDNA concentrations yielded higher TE as a significant decrease (p-value: $9.23\times10^{-9} < \alpha$) in TE was observed as pDNA concentrations increased (**Figure 9**).

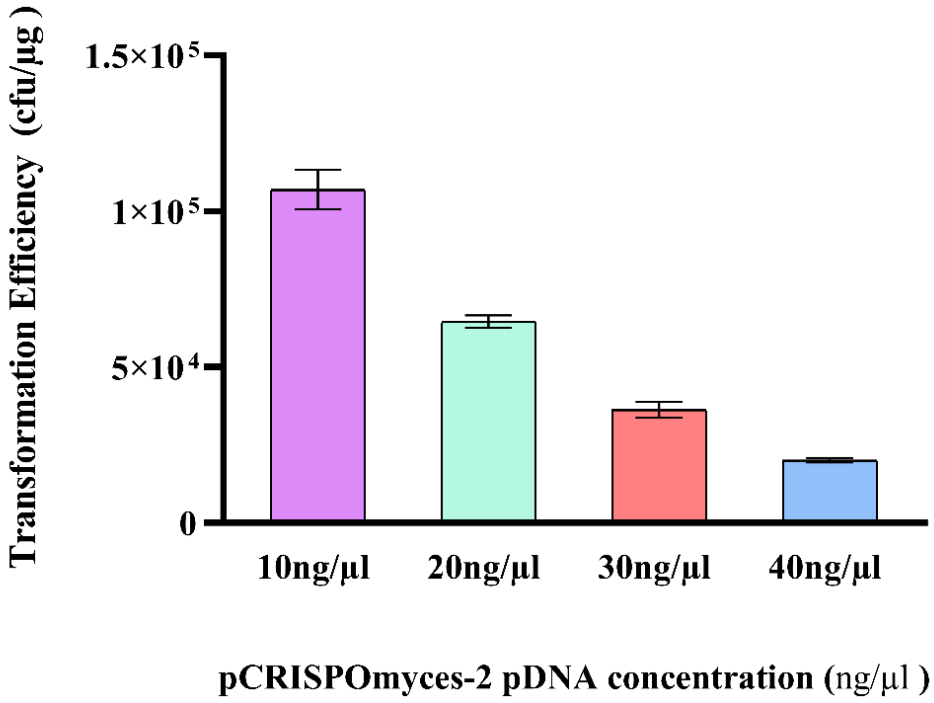

Figure 9: Transformation efficiency of *E. coli* WM6026 with increasing concentrations of pCRISPomyces-2 pDNA. Average TE was obtained from triplicate electroporation experiments. A significant decrease (p-value: $9.23\times10^{-9} < \alpha$) in transformation efficiency was observed as pCRISPomyces-2 pDNA increased indicating toxicity of Cas9 protein expression.

These results could be elucidated by a study conducted by (Szostková & Horáková, 1998) which reported a decrease in TE when pDNA concentration was increased beyond the saturation concentration and when larger plasmids were introduced. The decrease was likely due to an aggregative effect of decreased viability in certain microorganisms resulting from the compromised cell wall porosity and the metabolic burden of the exogenous plasmid (Szostková & Horáková, 1998). The microorganism would need to recover its natural cell wall porosity while simultaneously adjusting to the metabolic burden imposed by the exogenous plasmid (Szostková & Horáková, 1998). This metabolic burden would be inflated if the pDNA uptake was increased beyond the competence of the microorganism, particularly with the presence of a larger plasmid with a Cas9 protein such as the pCRISPomyces-2 plasmid.

The varied size transformants, including reduced-sized colonies, were observed when a pCRISPomyces-2 pDNA concentration of 40 ng/µl was used. Taking cognizance of the Cas9 protein in pCRISPomyces-2 plasmids, small colonies and poor TE was not uncommon as hypothesised. The extensive expression of Cas9 proteins has been reported in several microorganisms (Misra et al., 2019; Racharaks et al., 2021). The primary cause of the toxicity in this study was attributed to the control of Cas9 expression under a strong constitutive promoter *rpsLp(XC)*, which has been reported to drive Cas9 protein toxicity (Ye et al., 2020). Moreover, given that this study focused on determining the baseline effects of the pCRISPomyces-2 plasmids, sgRNA was not included. This likely further influenced factor the low TE as non-specific binding of NGG-PAM sequences within the *E. coli* genome may have occurred (Zhang & Voigt, 2018).

Further attempts to improve TE did not result in any significant change and it was suspected that excessive reduction in pCRISPomyces-2 pDNA concentration would revert the improved TE. A final TE of 1.07×10^5 cfu/µg for electroporation was concluded.

2.3.2.2 Transformation by heat-shock

To determine if TE could be improved by a change of transformation method, heat-shock transformation was performed. Heat-shock, although typically more effective with smaller plasmids, would enable change in the duration at which the competent cells' increased porosity was maintained, enabling entry of larger plasmids. This change could be achieved with less detrimental effects on the viability of transformed cells (Pope & Kent, 1996). A heat-shock method developed by Tu et al., (2005) reported high efficiencies similar to those expected with electroporation. Thus, this heat-shock transformation method was used. Taking into consideration the effects of pDNA concentration observed from electroporation experiments, heat-shock was performed at pCRISPomyces-2 pDNA concentrations of 10 and 30 ng/µl. Transformation efficiencies of 1.49×10^5 and 5.10×10^4 cfu/µg were obtained for 10 and 30 ng/µl, respectively.

The heat-shock TEs obtained at different pCRISPomyces-2 pDNA concentrations displayed results analogous to those obtained with electroporation. A reduction in pDNA concentration yielded higher TEs for heat-shock. In comparison to electroporation, the average TE for heat-shock with 10 ng/µl was significantly higher at 1.49×10^5 cfu/µg however the TE remained $< 10^6$ cfu/µg which is the suggested standard minimum for cloning applications. Statistical analysis displayed a p-value of $7.0 \times 10^{-4} < \alpha$ when comparing transformation methods **(Figure 10)**. However, as previously mentioned, the size of the pCRISPomyces-2 plasmid will increase when sgRNA sequences are inserted which might further reduce TE. Given the limited effectiveness of heat-shock for larger plasmids, heat shock may no longer be a viable transformation method once target sequences are inserted into pCRISPomyces-2.

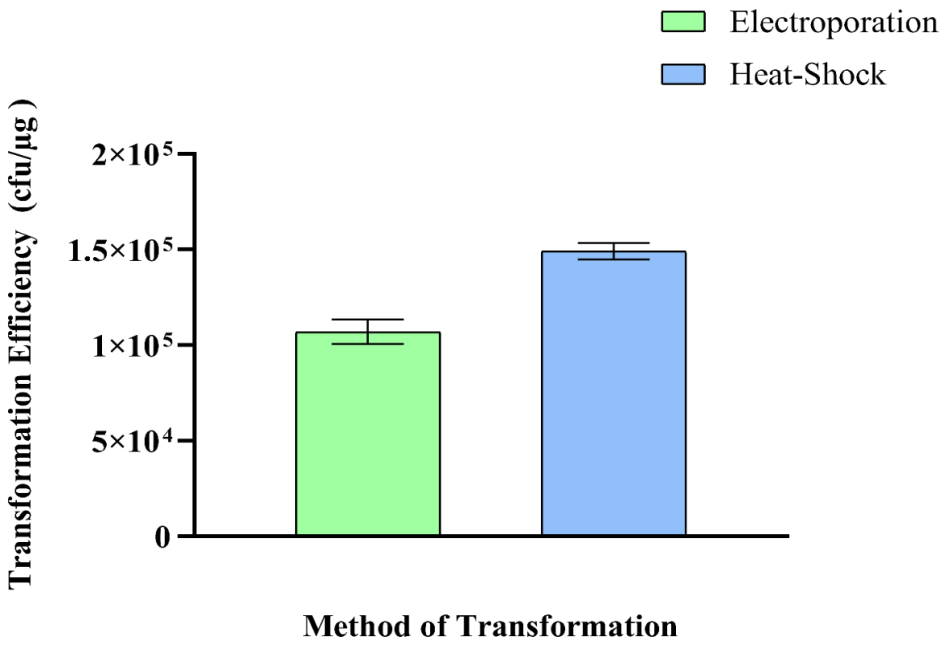

Figure 10: A comparison of transformation efficiency achieved by transformation methods with pCRISPomyces-2 pDNA. Electroporation and heat-shock were performed with *E. coli* WM6026 for the uptake of pCRISPomyces-2 plasmids. A significant increase (p-value: $7.0 \times 10^{-4} < \alpha$) in transformation efficiency was observed with heat shock at 1.49×10^5 cfu/µg.

The reduced stability of the pCRISPomyces-2 plasmids in *E. coli* WM6026 resulting in low TE could be attributed to the presence of R-M systems. *Escherichia coli* WM6026 has been engineered with a defective *hsd* gene but two other methyltransferases are still functional in this strain. DNA adenine methyltransferase, encoded by the *dam* gene, and DNA cytosine methyltransferase, encoded by the *dcm* gene (Marinus & Løbner-Olesen, 2014; Zhou et al., 2012). It is well-reported that R-M systems limit TE and reduce plasmid stability (Guss et al., 2012; Yasui et al., 2009). Therefore, methylation by *dam* and *dcm* encoded methyltransferases was likely to decrease pCRISPomyces-2 plasmid stability as several corresponding restriction sites are contained in the plasmid.

In consideration of the comparative results and TE influencing factors, the method of transformation did not have an overall significance on TE but rather it confirmed that a cumulative effect of influencing factors drove the overall poor TEs observed. However, an interesting paradoxical relationship may be observed with the pCRISPomyces-2 Cas9 protein toxicity and application of the transformed *E. coli* WM6026 as donor cells for intergeneric conjugation. Transformation efficiency is a partial indicator of a transformed cell population's fitness. Through uptake, plasmids impart genetic elements or induce metabolic changes to which the cells must adapt (Kassen & Bataillon, 2006). Thus, influencing the transformed cells' fitness which in turn modulates the conjugative transfer of plasmids (Millan et al., 2014). If the plasmids have a favourable influence on the transformed cells, the plasmids could be retained in the cells by epigenetic inactivation of conjugative *tra* genes in plasmids (Koraimann & Wagner, 2014; Pölzleitner et al., 1997). Inversely, an unfavourable influence from the plasmids such as Cas9 protein toxicity would decrease the cells' fitness and increase its donor ability (Dimitriu et al., 2016; Svara & Rankin, 2011).

Given this dynamic, the inverse was observed as the low TE obtained from both transformation methods indicated a reduction in the transformed cell fitness. A reduction in fitness was further observed, by a slower doubling time of T_d=1.49 h for transformed donor *E. coli* WM6026/ pCRISPomyces-2 compared to the

doubling time of T_d=1.14 h for untransformed *E. coli* WM6026 in **Figure 11** (Moradigaravand & Engelstädter, 2014; Rathmann et al., 2023). Therefore, the low TE indirectly indicated the probability of downstream conjugation such that it was partially favourable for its application as donor cells because donor ability would have increased to reduce the metabolic burden imposed by the Cas9 protein toxicity(Rathmann et al., 2023). Moreover, the success of downstream intergeneric conjugation necessitated a minor level of Cas9 protein toxicity to enhance the donor cells' ability to donate the pCRISPomyces-2.

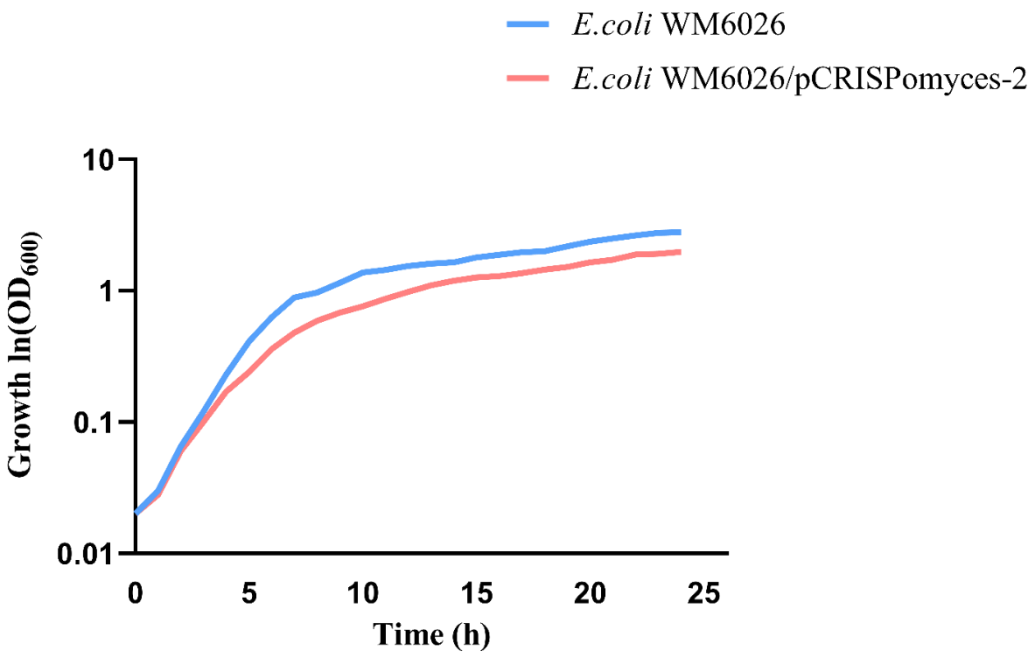

Figure 11: Growth curves of untransformed *E. coli* WM6026 and transformed *E. coli* WM6026/ pCRISPomyces-2 cells. A reduction in doubling time was observed with transformed *E. coli* WM6026 cells (T_d=1.49h) compared to untransformed *E. coli* WM6026 cells (T_d=1.14h). The reduction was attributed to the introduction of pCRISPomyces-2.

2.3.3 Screening of transformants by colony PCR

Post-transformation, *E. coli* WM6026 transformants were screened for the uptake of pCRISPomyces-2 plasmids using M13/pUC primers. A gel electrophoresed product displaying the colony PCR products of three random colonies each from electroporation and heat-shock transformations is shown in **Figure 12**. Colony PCR of transformants from electroporation experiments displayed positive bands in lanes 4-6. Alternatively, colony PCR of transformants from heat-shock experiments were verified by positive bands in lanes 7-9, respectively. A correct amplicon size of 181 bp which included the multiple cloning sites of the pCRISPomyces-2 plasmids was observed for all respective transformants, thus successful transformations for both methods were verified. However, larger bands of approximately 700 bp appearing in lanes 4-9, displaying positive bands, are likely to have occurred due to non-specific primer binding with the gDNA, as commonly observed when performing colony PCR.

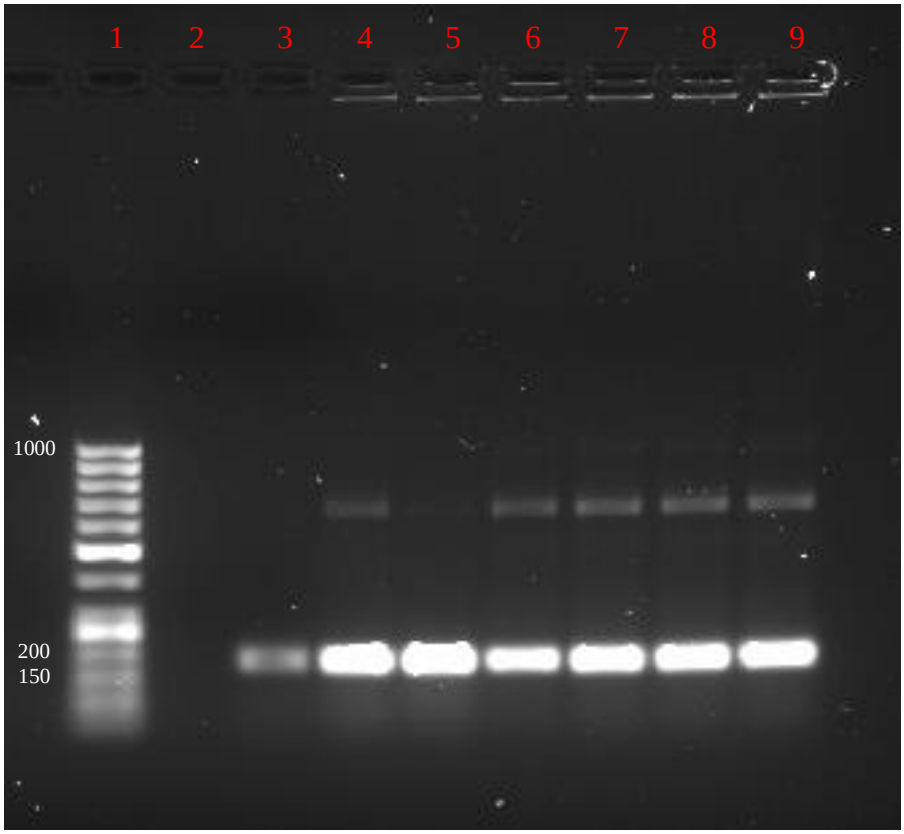

Figure 12: Colony PCR screening of *E. coli* WM6026 transformants with pCRISPomyces-2 pDNA. Lanes 4-9 displayed positive bands of 181 bp which verified the uptake of pCRISPomyces-2 pDNA by electroporation and heat-shock transformation. Lanes 1, 2 and 3 displayed the 50 bp molecular weight marker, negative, and positive controls respectively.

2.4 Conclusion

The quality of pDNA extracted from pCRISPomyces-2 plasmids was important for the verification of the plasmid integrity to reduce downstream bottlenecks. A Sanger sequencing output was conducted on extracted pCRISPomyces-2 pDNA and aligned with ClustalW against the reference *Streptomyces* expression codon-optimised Cas9 nucleotide sequence. The alignment confirmed an identity >98%. This provided sufficient verification of the structural and related functional integrity of the pCRISPomyces-2 pDNA backbone and its CRISPR/Cas9 elements. Subsequently, transformation of *E. coli* WM6026 with pCRISPomyces-2 pDNA

was achieved and verified by colony PCR. A comparatively low average TE of 1.06 $\times 10^5$ cfu/µg was obtained for electroporation transformation. This was despite the optimisation of pDNA concentrations to minimise the metabolic burden of pCRISPomyces-2 plasmids. As a result, heat-shock transformation was performed to optimise TE by increasing the time at which pDNA uptake was enabled for the large pCRISPomyces-2 plasmids. A significant improvement was obtained with an average TE of 1.49 $\times 10^5$ cfu/µg but TE remained $<10^6$ cfu/µg. The overall low average TE for both transformation methods indicated that the pDNA concentration, large plasmid size and inherent toxicity associated with the Cas9 protein expression may have contributed to the low TE. However, low TE could be observed as a favourable indication for the application of the transformed *E. coli* WM6026 as donor cells for intergeneric conjugation. Such that the low TE indicates a reduction in donor cells' fitness, which enhances its donor ability in conjugation applications.

Chapter 3: Intergeneric conjugation with *Streptomyces* strains and *E. coli* WM6026/pCRISPomyces-2

3.1 Introduction

The harnessing of the unique metabolic diversity of *Streptomyces* as promising industrial potential is limited by several factors as discussed in **Chapter 1**. Given the different metabolic and genetic engineering techniques that exist for *Streptomyces* to overcome these limitations, intergeneric conjugation with *E. coli* donor cells has been reported to be most efficient and effective (Baltz, 1998; Mazodier et al., 1989; Paranthaman & Dharmalingam, 2003). The technique effectively introduces exogenous DNA into *Streptomyces* strains, bypassing the methyl-specific R-M systems (Bierman et al., 1992; MacNeil, 1988). However, this largely depends on the strictness of the R-M system in a specific *Streptomyces* strain and the extent of methylated modification which occurs in the donor cells (Peng et al., 2018; Wang et al., 2011).

This chapter aimed to establish an intergeneric conjugation system with *S. albulus* BCRC11814 and *hsd* methylation defective *E. coli* WM6026 strains harbouring pCRISPomyces-2 plasmids. The relevance of *S. albulus* BCRC11814 was previously outlined in **Chapter 1.** In addition, intergeneric conjugation was also performed with a model strain *Streptomyces coelicolor* A3(2) as a comparative experiment. *Streptomyces coelicolor* A3(2) is a well-reported strain that has been subjected to several genetic engineering techniques to develop *Streptomyces* 'super-hosts' for heterologous expression (Chater, 1999; Gomez-Escribano & Bibb, 2011). The strain has a linear chromosome of 8.6 million bp which includes approximately 20 reported BCGs (Bentley et al., 2002). This particular *Streptomyces* strain makes an excellent model as it is well-known for its strict methyl-specific R-M systems and has metabolic genes that are highly conserved within the genus (Borodina et al., 2005; Chater, 1999). Moreover, *S. coelicolor* A3(2) has been previously engineered with pCRISPomyces-2 plasmids (Ye et al., 2020).

The development of an intergeneric conjugation system is typically predicated upon several influencing factors. These factors included the ratio of donor-to-recipient, the conjugation media composition, temperature, pH, mating time, and the growth phases of donor and recipient cells (Fernandez-Astorga et al., 1992; Simonsen et al., 1990; Wang & Jin, 2014). These factors were used to develop an intergeneric conjugation system in this study as well. To briefly overview the intergeneric conjugation system, conjugal mating was expected to occur if the influencing factors provided favourable conjugation conditions such that the pCRISPomyces-2 *tra* genes were expressed and an F-pilus, associated with the type IV secretion system, is assembled for 'mating' (**Figure 13**)(Grahn et al., 2000; Lawley et al., 2003; Mazodier et al., 1989). Hence, donor *E. coli* WM6026 cells transformed with pCRISPomyces-2 plasmids (outlined in **Chapter 2)** were expected to 'mate' with recipient *S. albulus* BCRC11814 or *S. coelicolor* A3(2) cells and donate the pCRISPomyces-2 plasmids.

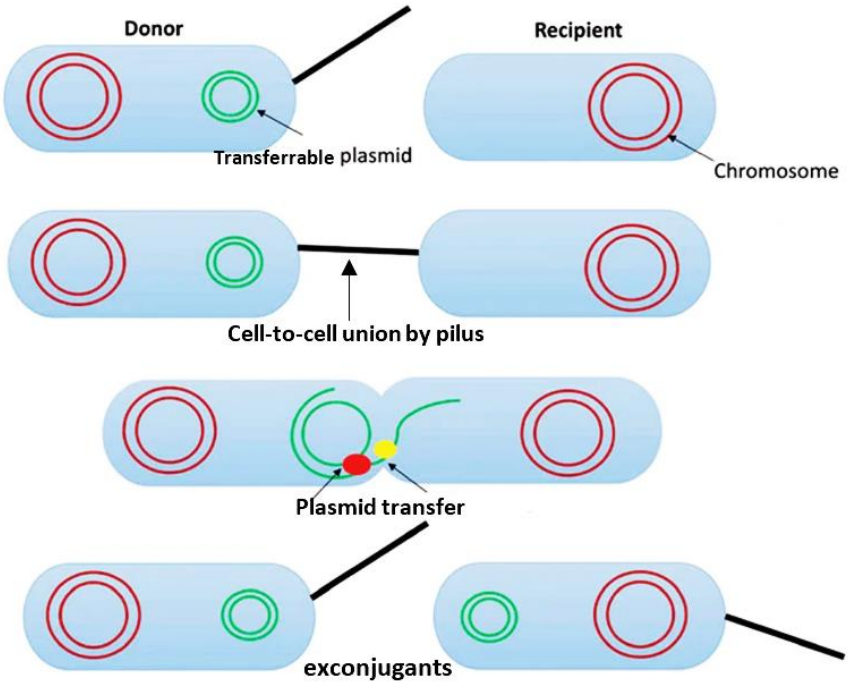

Figure 13: Diagram illustrating conjugational transfer as occurring *Streptomyces*. Adapted from Deng et al., (2017)

The system included three markers with a functional and verification role. Apramycin would be the plasmid selectable marker to verify the uptake of pCRISPomyces-2 plasmids (Cobb et al., 2015). Nalidixic acid (Nal) would be the counter-selective marker as it aids in halting conjugal mating and clearing of the *E. coli* donor cells by disruption of DNA synthesis in the donor cells (Hane, 1971). Lastly, the auxotrophic marker DAP would act as a second counter-selective marker by enabling donor cell growth during conjugal mating and also ensuring the absence of the donor cells during the exconjugant purification process (Allard et al., 2015).

Overall, a seamless intergeneric system has been reported in previous studies (Flett et al., 1997; Paranthaman & Dharmalingam, 2003). However, it should be noted that the work completed in this Chapter did not include the aim to determine the Cas9 engineering efficiency of the pCRISPomyces-2 plasmids. Hence, no sgRNA guide sequences were inserted into the pCRISpomyces-2 plasmids. Primarily, the aim was to establish an intergeneric conjugation to determine if the introduction of pCRISPomyces-2 plasmids in *S. albulus* BCRC11814 was possible. The foundational insights obtained from this work could subsequently enable future metabolic engineering in *S. albulus* BCRC11814 with pCRISPomyces-2 plasmids.

3.2 Methods

3.2.1 Culturing of *Streptomyces* spores and mycelia

Streptomyces albulus BCRC11814 and *S. coelicolor* A3(2) strains donated, by Professor Yoshimitsu Hamano, were each spread-plated from glycerol stocks on soya flour mannitol (SFM; 20 g Mannitol; 20 g Soya Flour; 20 g agar, 1L dH_20, pH 7.0) agar and incubated at 30°C for 7-8 days until sporulation occurred. Subsequently, spore suspensions were prepared from adequately sporulated lawns of *S. albulus* BCRC11814 and *S. coelicolor* A3(2). The plates were washed with 3-5 ml 0.1% Tween80 and the spores were gently dislodged by scraping the lawn's surface with a sterile spreader. The spore suspensions were transferred into a 50 ml centrifuge tube with a sterile pipette and vortexed thoroughly for 1 min to

disaggregate spore chains. Thereafter, the spore suspensions were filtered through a Whatman® 8 µm filter disk and collected in a 50 ml centrifuge tube for centrifugation at 7000 x g for 10 min, at 4°C. The supernatants were immediately discarded, and the pellet was resuspended in 2 ml 20% glycerol. The spore suspensions were enumerated with a haemocytometer and diluted with 20% glycerol to obtain a final concentration of 10^6 cfu/ml. The final spore suspensions were aliquoted in 400 µl volumes and stored at -20°C until required.

The mycelia stocks of *S. albulus* BCRC11814 and *S. coelicolor* A3(2) were also cultured and prepared similarly to the spores; however, the incubation time was reduced to 3-4 days until early mycelia appeared and the 400 µl aliquots of mycelia were suspended in 30% glycerol and stored at -80°C for subsequent use.

Gram Stains were performed on *S. albulus* BCRC11814 and *S. coelicolor* A3(2) to verify the bacteria according to the method of Hans Christian Gram (Coico, 2005). Smears of *S. albulus* BCRC11814 and *S. coelicolor* A3(2) were each heat-fixed to microscope slides. The slides were flooded with crystal violet staining reagent and stained for 1 min before being washed with water. Iodine was used to flood the slides and fix the stain for 1 min before washing them again. A decolorising agent (95% ethanol vol/vol) was then used to flood the slides for 15 sec before flooding them with the counterstain safranin for 1 min. The slides were washed for a final time and dried before viewing them under a microscope.

3.2.2 Intergeneric conjugation

Intergeneric conjugation was performed with *Streptomyces* strains and transformed *E. coli* WM6026/pCRISPomyces-2, as described by Du et al.,(2012), Flett et al.,(1997) and Kieser et al.,(2000) with minor modification.

a. Preparation of Streptomyces recipient cells

Streptomyces albulus BCRC11814 and *S. coelicolor* A3(2) spores and mycelia were prepared in parallel as recipient cells for intergeneric conjugation. The spore and mycelial suspensions (400 µl) were thawed on ice and pelleted at 7000 x g for 10 min, at 4°C, and washed twice with an equal volume of 2X yeast extract tryptone media (YT; 16 g tryptone, 10 g yeast extract, 5 g NaCl, 1 L dH_2O, pH 7.0). The resultant pellets were resuspended in 400 µl 2X YT to prepare recipient cells. Thereafter, the spore recipient cells were either heat-shocked and pre-germinated at 50°C for 10 min then incubated at 30° for 5-6 h, or used directly without pre-germination. Pre-germination was omitted for mycelial recipient cells.

b. Preparation of transformed E. coli WM6026 *donor cells*

The donor cells for intergeneric conjugation were prepared by inoculating 50 µl *E. coli* WM6026/pCRISPomyces-2 inoculum into 5 ml LB, supplemented with 19 µg/ml DAP and 50 µg/ml Apr. The inoculum was incubated overnight at 30°C with shaking at 250 rpm. Due to the lagged growth of the transformed *E. coli* WM6026, the overnight culture was used directly with minor adjustments to obtain OD_{600} of 0.4-0.6 (~10^8 cfu/ml). The OD_{600} readings of the inoculum were measured with a S-200 VIS spectrophotometer (BOECO, Hamburg, Germany). The overnight culture was pelleted at 4000 x g for 10 min, at 4°C then washed twice in LB to remove residual antibiotics before resuspending in an equal volume LB. The donor cells were incubated on ice until required.

c. Conjugal Mating

Parallel conjugal transfer experiments were performed for recipient spores of *S. albulus* BCRC11814 and *S. coelicolor* A3(2). The conjugation experiments were conducted stepwise under different conditions (conjugation media type, pre-germination, and donor: recipient ratio) to obtain the highest conjugation frequency

(CF). The donor and spore recipient cells were mixed in a 1:1 (10^7: 10^7) or 1:3 (10^2: 10^7) ratio in a total volume of 400 µl. The conjugation mixture was briefly vortexed to homogenise cells and serially diluted 10^{-1}-10^{-3} in a volume of 200 µl. Thereafter, 100 µl of undiluted and diluted conjugation mixtures were spread-plated on non-selective conjugation media: International *Streptomyces* Project-2 Medium (ISP2; 4 g Yeast extract, 4 g dextrose, 10 g malt extract, 10 g agar,1 L dH_2O, pH 7.0), SFM and Modified R2 agar (refer to **Appendix A** for preparation). The SFM and ISP2 plates were supplemented with 19 µg/ml DAP to support the growth of *E. coli* WM6026 donor cells as well as 10 mM $MgCl_2$ to increase conjugation efficiency (Choi et al.,2004). The conjugation plates were incubated at 30°C for 16 h before being evenly overlayed with 1.5 ml sterile dH_2O containing 500 µg nalidixic acid (Nal) and 1.25 mg Apr to halt conjugal transfer and select for exconjugants. The plates were dried under sterile conditions and then further incubated for 3-5 days until exconjugants appeared and were counted. The conjugation experiment for mycelial recipient cells was performed similarly in a 1:1 (200 µl:200 µl) ratio with donor cells. All conjugation experiments included control experiments which lacked the addition of donor cells or the antibiotic overlay. Average CFs were calculated from three independent conjugation experiments conducted with each different condition as a ratio of the number of exconjugants on a conjugation plate to the number of recipient cells. The average CF for mycelial conjugations were calculated as exconjugants to donor cells as it was impossible to count individual mycelial fragments.

3.2.3 Screening of exconjugants by colony PCR

Post-conjugation, the *S. albulus* BCRC11814 and *S. coelicolor* A3(2) exconjugants were screened by colony PCR to verify the transfer of pCRISPomyces-2 plasmid as described in **Chapter 2, section 2.3.3** with minor modification. Three exconjugants each were randomly isolated from spore and mycelial conjugation plates and streaked on selective ISP2 agar, supplemented with 50 µg/ml Nal and 150 µg/ml or 250 µg/ml Apr for *S. albulus* BCRC11814 and *S. coelicolor* A3(2) respectively, to purify the colonies. After two purification cycles on selective ISP2

plates, single exconjugant colonies were isolated and subjected to colony PCR using M13/pUC primers. The exconjugant colonies were initially lysed, as described by Packeiser et al.,(2013), by extraction of a single colony with a sterile inoculating needle and resuspension in 20 µl of filter-sterilised 0.2% sodium dodecyl sulphate (SDS). The suspension was vortexed for 10s and incubated at 98°C for 5 min to obtain lysates. The lysates were micro-centrifuged at 10 000 x g and the supernatant was collected and diluted to a 1:5 ratio with nuclease-free water and subsequently subjected to colony PCR. The colony PCR reaction mix in a total volume of 50 µl contained 3 µl diluted DNA template,5 µl x10 ThermoFisher DreamTaq buffer, 1 µl 10 mM DNTP mix, 0.5 µl ThermoFisher DreamTaq Polymerase (5U/µL), 2.5 µl each of 10 mM M13/pUC forward and reverse primer, and 35.5 µl nuclease-free water. The reaction mixes were amplified with the same cycling conditions and viewed by gel electrophoresis as per **Chapter 2, section 2.2.3**. A negative and positive control contained unconjugated *Streptomyces* strains and "pure" extracted pCRISPomyces pDNA, respectively were included in the colony PCR

3.2.4 Electro-transformation of *S. albulus* BCRC11814 spores

Electro-transformation of *S. albulus* BCRC11814 spores was performed without pre-preparation of protoplasts as described by Hamano et al.,(2006) and Pigac & Schrempf (1995), with minor modification.

Fresh *S. albulus* BCRC11814 spores were grown and obtained similarly as per **section 3.2.1**; however, spore pellets were resuspended in 10 ml CRM (10 g glucose, 103 g sucrose, 10.12 g $MgCl_2$ hexahydrate, 15 g Oxoid tryptic soy broth and 5 g yeast extract, 1 L dH_2O, pH 7.0) and germinated at 30°C for 5h. The germinated spores were centrifuged and washed twice with 5 ml chilled dH_2O and resuspended in 2 ml 10% glycerol to obtain a spore concentration of 10^7 cfu/ml. The spore suspensions were aliquoted in 100 µl volumes into sterile microcentrifuge tubes and mixed with 4 µl pCRISPomyces pDNA (10 ng/µl) obtained from transformed *E. coli* WM6026 and *E. coli* DH5-alpha. The mixtures

were transferred into chilled sterile 0.2 cm gap electroporation cuvettes and electroporated with a Bio-Rad Micropulser™ (Johannesburg, South Africa) at 3 kV voltage and 2.4 msec time constant. Post-electroporation, the transformed mixtures were immediately resuspended in 500 µl CRM and incubated at 30°C with shaking at 150 rpm for 16 h without any antibiotic selection. Subsequently, 10x dilutions in 100 µl of transformed *Streptomyces* spores were spread-plated on prewarmed SFM, supplemented with 150 µg/ml Apr, and then incubated at 30°C for 3 days until electro-transformants appeared and were counted. The experiments were performed with a negative control lacking the addition of pCRISPomyces-2 pDNA. An average transformation efficiency (cfu/µg) was calculated from three independent electro-transformation experiments.

3.2.5 Screening of electro-transformants by colony PCR

Electro-transformed *S. albulus* BCRC11814 were screened post-incubation to confirm uptake of pCRISPOmyces-2 plasmids. Four randomly picked electro-transformants were isolated and screened as described in **section 3.2.3**.

3.2.6 Statistical analysis

Statistical significance of conjugation experiments was performed with unpaired t-tests (α=0.05) on triplicate data where necessary.

3.3 Results and Discussion

3.3.1 Gram staining of *S. coelicolor* A3(2) and *S. albulus* BCRC11814

Gram stains were performed to verify the bacteria and ensure there was no contamination of gram-negative species. The stains performed for *S. coelicolor* A3(2) and *S. albulus* BCRC11814 were viewed under a microscope at 100x magnification. Gram-positive stain results were observed for the mycelia and spores of both bacteria as expected (Bentley

et al., 2002). The magnification of the slides for both bacteria displayed the mycelia with a purple stain where the spores were stained blue (**Figure 14**).

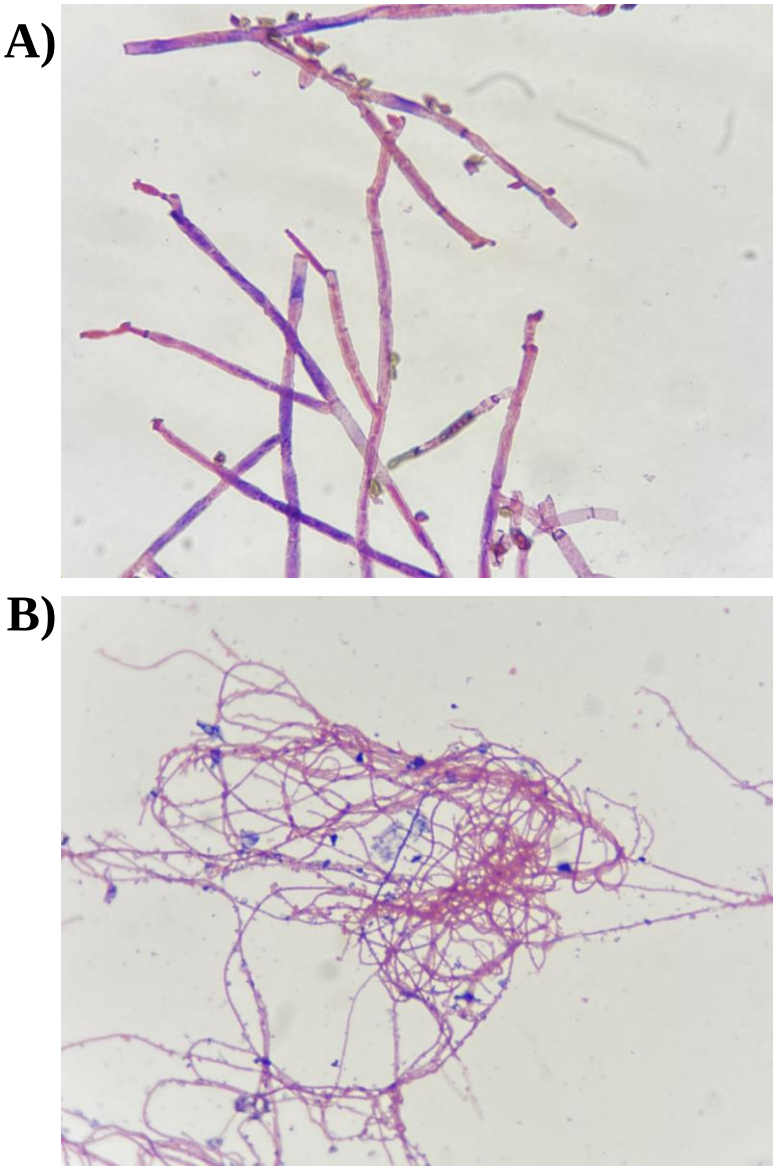

Figure 14: Gram-positive stains of *S. coelicolor* A3(2) and *S. albulus* BCRC11814. Stains performed for A) *S. coelicolor* A3(2). B) *S. albulus* BCRC11814 were visualised at 100x to reveal gram-positive stains.

3.3.2 Intergeneric conjugation

Intergeneric conjugation was attempted with *S. coelicolor* A3(2) and *S. albulus* BCRC11814 to introduce pCRISPomyces-2 plasmids as achieved with other *Streptomyces* strains reported in the principal study by Cobb et al.,(2015). This was achieved by conjugally mating *hsd*- methylation deficient *E. coli* WM6026 harbouring the pCRISPomyces-2 plasmids with the respective *Streptomyces* strains in this study. The development of the conjugal mating systems was conducted stepwise by changing different factors to achieve the best average baseline CF for the respective *Streptomyces* strains.

Table 2: Summary of conjugational frequencies obtained from stepwise optimisation for *S coelicolor* A3(2) and *S. albulus* BCRC11814.

	CONJUGATIONAL FREQUENCY	
	***S. albulus* BCRC11814**	***S coelicolor* A3(2)**
Media Type		
ISP2	-	6.51×10^{-5}
SFM	-	1.86×10^{-4}
Modified R2	-	-
Spore Heat-shock and Pre-germination		
Untreated	-	1.81×10^{-4}
Treated	-	1.88×10^{-4}
Donor: Recipient Ratio		
10^7: 10^7	-	1.96×10^{-4}
10^2: 10^7	-	3.15×10^{-5}

3.2.2.1 Conjugation media type

Initial conjugation experiments were performed with three magnesium (Mg^{2+}) ion-supplemented conjugation media, SFM and ISP-2 and modified R2, to determine which was better suited for *S coelicolor* A3(2) and *S. albulus* BCRC11814. Conjugation was only observed with *Streptomyces coelicolor* A3(2) on two media, SFM and ISP-2. A 10-fold difference in CF was observed between the two media

with SFM performing significantly better with a CF of 1.86×10^{-4} compared to ISP-2 which only achieved a CF of 6.51×10^{-5} (**Table 2**). The lack of CFs obtained for *S. albulus* BCRC11814 with different media types was suspected to be caused by other influencing factors. This was based on the control experiments which lacked the addition of the antibiotic overlay which displayed positive growth on SFM and ISP-2. No CFs were obtained for either *Streptomyces* strain on modified R2 suggesting that modified R2 was not nutritionally suited for either strain.

The response of different *Streptomyces* strains to different conjugation media has been reported to be highly variable (Xu et al., 2015). This may be attributed to a *Streptomyces* strain's specific metabolic requirements and the nutritional makeup of the conjugation media. In addition, CF is positively impacted by the presence of magnesium (Mg^{2+}) ions; however, excessive amounts can inhibit *Streptomyces* spore formation (Wang & Jin, 2014; Zhang et al., 2018). The exact mechanism through which Mg^{2+} ions improve CF is unknown but has been hypothesised to stabilise a mating pair (Johnson & Grossman, 2016). This study used 15 mM $MgCl_2$ which is a standard concentration for most conjugal mating studies(Choi et al., 2004; Zhang et al., 2018).

In consideration of the average CFs observed for *S coelicolor* A3(2) for the different conjugation media types, SFM was chosen for subsequent conjugation experiments.

3.3.2.2 Spore pre-germination

Different *Streptomyces* strains respond differently to spore heat-shock and pre-germination. Some strains display no difference in CF whereas other strains display increased CF (Liu et al., 2016; Zhang et al., 2018). The rationale of heat-shock and pre-germinating the *Streptomyces* spores before conjugation was to advance the slow growth of *Streptomyces* such that it reaches the mycelial substrate phase faster. It is during this phase, which typically takes approximately 18h, that conjugal mating occurs (Thoma et al., 2019). Accelerating the *Streptomyces* growth to reach

the mycelial substrate phase faster would benefit the growth synchronisation between donor and recipient cells; such that it would reduce donor cell overpopulation and maintain the effectiveness of antibiotic overlay (Zhang et al., 2018). This would accelerate the overall conjugation process which takes an average of three days (Liu et al., 2016). Additionally, heat-shock of the *Streptomyces* strain has been suggested to temporarily inactivate *Streptomyces* methyl-specific-restriction systems, thus improving CF, but it may be at the expense of spore viability (Engel, 1987; Sun et al., 2014). The results observed in this study indicated no significant impact on CF by spore heat-shock and pre-germination. The CF obtained for untreated and treated *S. coelicolor* A3(2) spores was 1.81×10^{-4} and 1.88×10^{-4} respectively (**Table 2**). Furthermore, no improvement in the lack of CF for *S. albulus* BCRC11814 was achieved. Due to the insignificant change in heat-treated and pre-germinated spores, subsequent conjugation experiments were performed with untreated spores.

3.3.2.3 Donor-Recipient ratio

Previous studies have reported variable CF impacted by donor-recipient ratios. Therefore, to determine any effect on CF, conjugal mating was performed at ratios of 1:1 and 1:3 of donor to recipient cells. As with the prior conditions, no CF was obtained for S. *albulus* BCRC11814. Alternatively, a significantly higher CF was obtained at a ratio of 1:1 compared to 1:3 for *S. coelicolor* A3(2), represented in **Table 2**. This was in line with previous studies which reported that the highest CFs for different *Streptomyces* strains were achieved with a 1:1 ratio (Lampkowska et al., 2008; Wan et al., 2011). The 1:1 ratio enabled the establishment of an equilibrium point between donor and recipient population growth such that satisfactory interaction of cells for conjugal mating occurs within the conjugation timeframe.

Contrastingly, the decreased CF obtained with a 1:3 ratio likely occurred because the ratio did not establish an equilibrium point between donor and recipient population growth within the conjugation time frame, thus satisfactory cell

interaction did not occur. The excessive recipient cell concentration could have reached its substrate mycelia phase and overpopulated the conjugation media while the donor cells had only established a small population. Therefore, the interaction for conjugal mating was decreased as not enough donor cells were present to donate the pCRISPomyces-2 plasmids to the larger recipient cell population.

Alternatively, this study did not test a higher donor cell concentration compared to the recipient cell concentrations as the resulting population dynamics would have negatively influenced conjugal mating. Population dynamics states the growth rate of a population is limited by its carrying capacity such that its environment has limited nutrition (Wan et al., 2011). Given that the *E. coli* WM6026 donor cells have a faster doubling time compared to that of *Streptomyces* recipient cells, a higher or excessive concentration of donor cells would consequently lead to donor cell overpopulation. Thus, accelerating nutrient depletion of the conjugation media before recipient cells had established an adequate substrate mycelium for conjugal mating. Therefore, a ratio with a higher concentration of donor cells ultimately would have caused severely low CF or lack of conjugation, as with a high recipient cell concentration.

The cumulative CF achieved for *S. coelicolor* A3(2) from the stepwise development of the conjugal mating was 1.96×10^{-4}. This CF was similar to those CFs obtained for conjugation experiments with non-Cas9 plasmid uptake or genome integration (Peng et al., 2018; Zhou et al., 2012). Despite attempts under different conditions on ratios no CF was achieved for S. *albulus* BCRC11814.

3.3.2.4 Spores vs Mycelia

Streptomyces mycelia presents as a promising alternative to spores in metabolic engineering, particularly for non-spore forming *Streptomyces*. Moreover, high CF has been reported with *Streptomyces* mycelial conjugation exceeding CF achieved by spores (Du et al., 2012; Paranthaman & Dharmalingam, 2003; Zhang et al., 2018). In an attempt to optimise the CF achieved by *S. coelicolor* A3(2) and obtain

conjugation for S. *albulus* BCRC11814, mycelial conjugation was performed. Successful mycelial conjugation was achieved with *S. coelicolor* A3(2), but no conjugation was observed with S. *albulus* BCRC11814 as per prior conjugation attempts. A CF of 2.80×10^{-4} was obtained for *S. coelicolor* A3(2) mycelial conjugation. This CF was significantly higher (p-value: $2.27\times 10^{-4} < \alpha$) than the cumulative CF obtained for *S. coelicolor* A3(2) spore conjugation at 1.96×10^{-4} (**Figure 15**). The obtained CF in this study was in line with previous studies mentioned (Du et al., 2012; Paranthaman & Dharmalingam, 2003; Zhang et al., 2018). No further optimisation was performed for conjugal mating with *S. coelicolor* A3(2) mycelia.

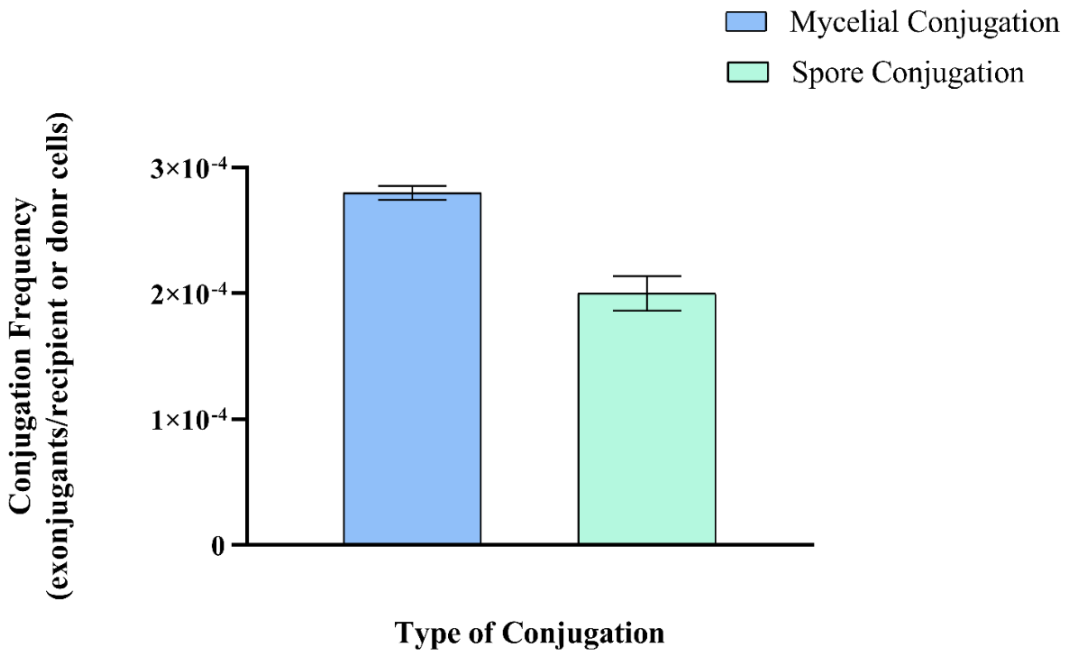

Figure 15: Conjugation frequency comparison between spore and mycelial conjugation observed in *S. coelicolor* A3(2). Mycelial conjugation was performed with a 1:1 ratio of donor and recipient cells. A significantly higher CF at 2.80×10^{-4} was obtained compared to that of spore conjugation CF at 1.96×10^{-4} (p-value of $2.27\times 10^{-4} < \alpha$).

Concerning the absence of S. *albulus* BCRC11814 mycelial and spore conjugation, two possibilities were considered. Firstly, as previously mentioned *Streptomyces* strains are known for their methyl-specific restriction systems which cleave exogenous methylated DNA (MacNeil, 1988). *Streptomyces albulus* strains also possess these restriction systems such that they cleave methylated pCRISPomyces-2 pDNA upon entry into the cell (Sun et al., 2014; Zhou et al., 2012). The *E. coli* WM6026 donor cells possessed a mutated *hsd* gene to disrupt methylation by this methyltransferase; however, methyltransferases encoded by *dam* and *dcm* genes were still functional (Blodgett et al., 2007). Therefore, it is suspected that the inability to perform conjugation with S. *albulus* BCRC11814 was due to *dam* or *dcm* methylated pCRISPomyces-2 pDNA obtained from the donor cells which recipient cells would strongly restrict.

Secondly, *Streptomyces* strains have varying growth times, thus their conjugation timeframes may also differ (Shepherd et al., 2010). All conjugation experiments performed were halted at 16h as it was observed from initial conjugation attempts that longer conjugation timeframes resulted in decreased effectivity of the antibiotic overlay against donor cells as an inoculum effect occurred (Brook, 1989). This was also reported in a previous study by Zhang et al.,(2018), who observed failed conjugation experiments when the conjugation timeframe surpassed 18 h. However, it was considered that S. *albulus* BCRC11814 required a longer conjugation timeframe to interact with donor cells before conjugal mating was halted by the addition of the antibiotic overlay.

3.3.3 Screening of exconjugants by colony PCR

Positive *S. coelicolor* exconjugants from spore and mycelial conjugations were screened for successful uptake of pCRISPomyces-2 plasmids. Three random exconjugants each for spore and mycelial conjugations were verified by colony PCR. The subsequent colony PCR products were observed by gel electrophoresis. Positive bands confirming the successful uptake of pCRISPomyces-2 plasmids were observed for all colony PCR products **(Figure 16)**. Positive bands with an

amplicon size of 181 bp for spore conjugation were displayed in lanes 3-5; whereas positive bands for mycelial conjugation were displayed in lanes 6-8.

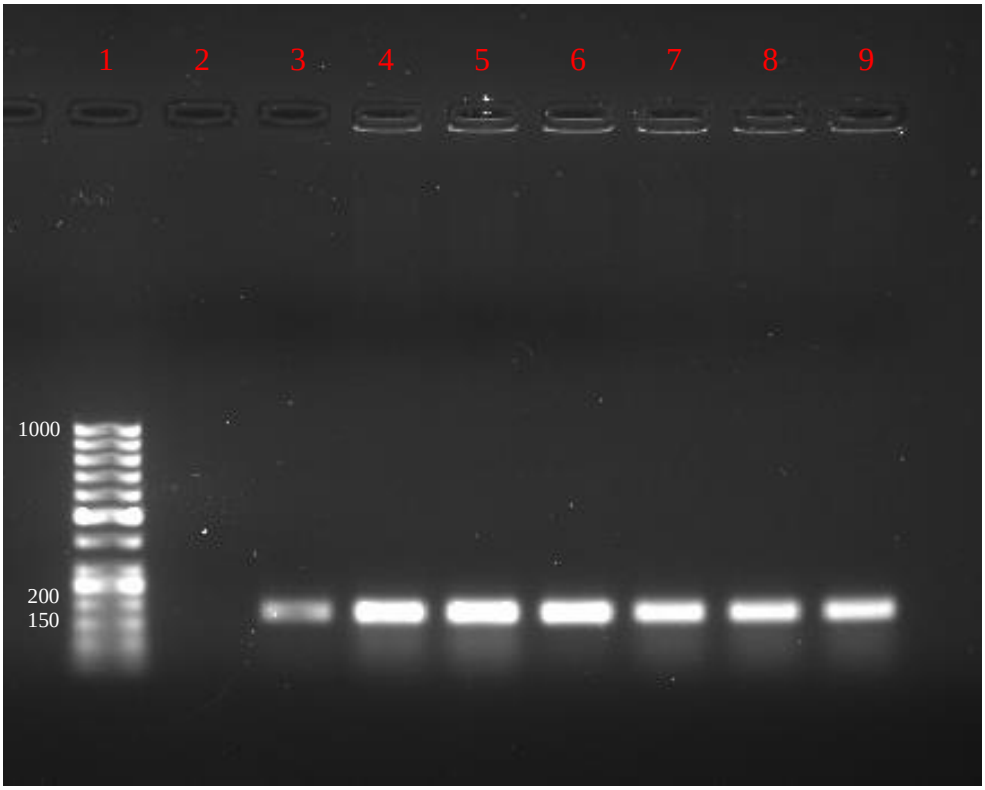

Figure 16: Colony PCR screening of *S. coelicolor* A3(2) exconjugants obtained from spore and mycelial conjugations. Obtained *S. coelicolor A3(2)* exconjugants were screened for uptake of pCRISPomyces-2 plasmids. Successful conjugation was observed as lanes 3-5 and 6-8 displayed positive bands for spore and mycelial experiments, respectively. Negative and positive controls were displayed in lanes 2 and 3, respectively.

3.3.4 Electro-transformation of *Streptomyces* spores with pCRISPomyces-2 *dam* and *dcm* non-methylated pDNA

To determine why conjugation was absent in S. *albulus* BCRC11814, verification of exogenous DNA restriction from the *E. coli* WM6026 donor cells was required. The verification had to be achieved without intergeneric conjugation given that the maintenance strain *E. coli* DH5-alpha could not be used as a donor strain.

Additionally, it was hypothesised that the pCRISPomyces-2 pDNA was being methylated by the *dam* and *dcm* encoding methyltransferases functional in the *E. coli* WM6026 conjugative donor strain. This was based on the observation that several *dam* and *dcm* restriction sites are contained in pCRISPomyces-2 plasmid and the reported presence of strong methyl-specific restrictions systems in other *S. albulus* strains (Cobb et al., 2015; Gu et al., 2016; Hamano et al., 2005).

Thus, verification was achieved by electro-transformation of *S. albulus* BCRC11814 spores with *dam* and *dcm* non-methylated pDNA. Electro-transformation of *S. albulus* BCRC11814 spores using pCRISPomyces-2 pDNA extracted from *E. coli* WM6026 was unsuccessful. However, electro-transformation experiments performed with pCRISPOmyces-2 pDNA extracted methylation defective *E. coli* DH5-alpha were successful. The *E. coli* DH5-alpha cells, in which the pCRISPomyces-2 plasmids were originally cloned and maintained, contained *dam* and *dcm* defective genes such that cloned pDNA was not methylated by the respective encoded methyltransferases (Cobb et al., 2015). Therefore, the hypothesised presence of a methyl-specific R-M system in *S. albulus* BCRC11814 with specificity to methylated DNA, by *dam* and *dcm* encoding methyltransferases, was verified.

With respect to methylation occurring by the *hsd* gene encoded methyltransferase, it was observed that very few *hsd* restriction sites are contained in the pCRISPomyces-2 plasmid sequence. However, both donor and maintenance *E. coli* strains contained a defective *hsd* gene which prevents cleavage at this restriction site (Blodgett et al., 2007; Cobb et al., 2015). Therefore, it could not be determined if *S. albulus* BCRC11814 restricts methylated DNA from this specific methyltransferase. Further experimentation by introducing *hsd* methylated DNA into *S. albulus* BCRC11814 is required to confirm *hsd* specificity of the methyl-specific R-M system.

The number of transformants observed in this study was too few (<20 colonies) to determine an accurate TE. The poor TE was likely attributed to several factors such

as pDNA concentration, field strength, pulse length and resistance (Fan et al., 2013; Pigac & Schrempf, 1995). Previous studies on the electro-transformation of *Streptomyces* have reported significant improvement in TE when these factors were optimised (Gong et al., 2004; Hamano, Maruyama, et al., 2006). No optimisation was performed to improve TE as this study was performed to verify *S. albulus* BCRC11814 methyl-specific-restriction systems. The pCRISPomyces-2 pDNA concentration for initial experiments was set to 10 ng/µl to reduce metabolic burden effects of Cas9 protein expression as observed in previous experiments.

Successful *S. albulus* BCRC11814 electro-transformants were screened for uptake of pCRISPOmyces-2 plasmids extracted from *E. coli* DH5-alpha cells. This was achieved by colony PCR and was analysed by gel electrophoresis. Results displayed positive bands for colony PCR products in lanes 4-6 with an amplicon size of 181 bp **(Figure 17)**. Therefore, successful electro-transformation was verified for *S. albulus* BCRC11814 which confirmed the hypothesis of this study. However, intergeneric conjugation with *S. albulus* BCRC11814 and a methylation defective donor strain such as *E. coli* ET12567 (dam-13::Tn9 dcm-6 hsdM Cmr) must also be carried out (Flett et al., 1997; MacNeil, 1988).

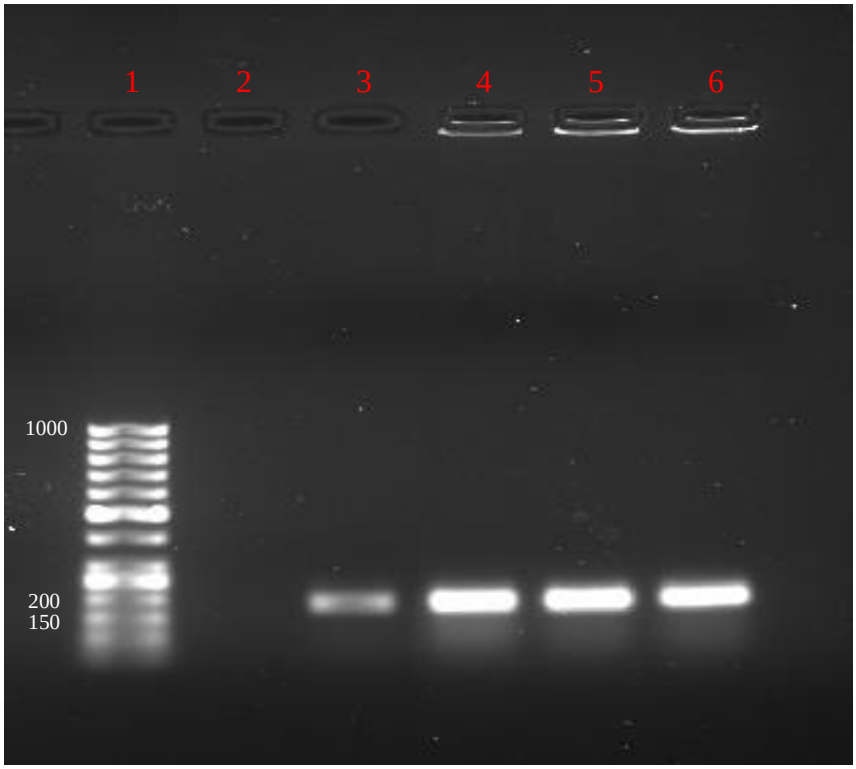

Figure 17: Colony PCR screening of *S. albulus* BCRC11814 electro-transformants. The uptake of pCRISPOmyces-2 pDNA (10 ng/ µl) by *S. albulus* BCRC11814 electro-transformants was verified by M13/pUC primers. Positive bands with an amplicon size of 181 bp were displayed in lanes 4 to 6. Lanes 2 and 3 displayed negative and positive controls, respectively

3.4 Conclusion

Intergeneric conjugation with recipient S. *coelicolor A3(2)* and donor *E. coli* WM6026 donor cells for the uptake of pCRISPomyces-2 plasmids was achieved through spore and mycelial conjugation. An average CF of 1.96×10^{-4} was obtained from spore conjugations through optimisation of CF influencing factors, such as donor-recipient ratios and type of conjugation media. Furthermore, an improved CF of 2.80×10^{-4} was achieved for mycelial conjugation. Successful conjugation and uptake of pCRISPomyces-2 plasmids were verified by colony PCR. Contrastingly, all attempts of spore and mycelial intergeneric conjugation with *S. albulus* BCRC11814 despite optimisation were unsuccessful. This was

attributed to *S. albulus* BCRC11814 expected restriction of methylated pCRISPomyces-2 pDNA, by *dam* and *dcm* encoded methyltransferases, obtained from *E. coli* WM6026 or ill-synchronisation of the permitted conjugation timeframe. The restriction of methylated pCRISPomyces-2 pDNA by *S. albulus* BCRC11814 was verified by electro-transforming *S. albulus* BCRC11814 with *dam* and *dcm* methylated pDNA from *E. coli* WM6026 and *dam* and *dcm* non-methylated pDNA from *E. coli* DH5-alpha. Positive electro-transformants, verified by colony PCR, were obtained with *dam* and *dcm* non-methylated pDNA, whereas no electro-transformants were obtained from *dam* and *dcm* methylated pDNA. Therefore, the hypothesis was confirmed. However, an accurate average TE from positive electro-transformants could not be determined as too few colonies were observed. Optimisation of electro-transformation influencing factors such as pDNA concentration, field strength, pulse length and resistance could improve TE.

Chapter 4: Minimum inhibitory concentration assay confirming *Streptomyces* antibiotic resistance

4.1 Introduction

Antibiotic resistance (AR) in *Streptomyces* has been well reported as the majority of antibiotics are produced by the genus itself. There are a few modes in which AR occurs in *Streptomyces* (Ogawara, 2016; Pacios-Michelena et al., 2021). Firstly, self-resistance is inherent in antibiotic-producing strains of *Streptomyces,* which occurs through a variety of mechanisms that simultaneously ensure the strain is entirely unaffected by the antibiotics (Hotta & Okami, 1996; Schmutz et al., 2003). Secondly, AR may occur by environmental adaptation through horizontal gene transfer or expression of the same AR genes in non-producing *Streptomyces* strains because of their closely related antibiotic-encoding BCGs (Wiener et al., 1998). Lastly, AR acquired from the aforementioned modes can be inherited through vertical gene transfer.

Resistance to aminoglycoside antibiotics (AGAs) occurs by self-resistance mechanisms as several *Streptomyces* strains share the same AR genes, thus there is lack of antibiotic resistance specificity within the genus (Benveniste & Davies, 1973; Hotta & Okami, 1996). The two mechanisms of AGA resistance are aminoglycoside inactivating enzymes and ribosome-dependent resistance caused by mutations of the 16S rRNA (Galimand et al., 2003; Hamano et al., 2004). Antibiotic resistance by aminoglycoside inactivating enzymes is more common of the two mechanisms and occurs through enzymes of the classes: aminoglycoside nucleotidyltransferases, aminoglycoside phosphotransferases and aminoglycoside acetyltransferases (Cundliffe, 1989; Yamamoto et al., 1982).

Although resistance to the acetyltransferase (3)-IV class of aminoglycoside inactivating enzymes is rare, it has still reported (Juhas et al., 2019). This specific class of aminoglycoside-modifying enzymes has been implicated in the majority of AGA resistance by *S. coelicolor* A3(2) and *S. albulus* strains. *Streptomyces*

coelicolor A3(2) has also been reported to be resistant to AGAs such as streptomycin, gentamycin and kanamycin (Ogawara, 2019; Wang et al., 2008; Westhoff et al., 2017). Similarly, AGA resistance has also been reported in *S. albulus* strains to kanamycin A and streptomycin through the aminoglycoside acetyltransferase (3)-IV class (Hamano et al., 2004; Liu et al., 2019). The shared AR to AGAs, previously mentioned to result from self-resistance mechanisms, could be observed by phylogenetic tree analysis of *Streptomyces* strains that displayed shared lineage-specific genes. These lineage-specific genes confer benefits and adaptations such as AR between strains (Wang et al., 2015; Zhou et al., 2012). Therefore, this chapter aimed to confirm the presence of spontaneous resistance by *S. coelicolor* A3(2) and *S. albulus* BCRC11814 to Apr.

Apramycin, an AGA produced by *S. tenebrarius*, is a common selectable marker for many genetic engineering experiments with *Streptomyces* strains (O'Connor et al., 1976; Tong et al., 2015; Yao et al., 2018). This is due to the unique bicyclic eight-carbon dialdose moiety in its structure that makes Apr immune to common AGA resistance mechanisms through most aminoglycoside-modifying enzyme classes or ribosomal methyltransferases (Dorman et al., 1976; Quirke et al., 2020; Westhoff et al., 2017) . In this work, Apr was used as the selectable marker for pCRISPomyces-2 plasmids to confirm the uptake of plasmids during intergeneric conjugation or electro-transformation studies. Based on the control experiments for these studies it was hypothesised that the *Streptomyces* strains possessed spontaneous resistance to Apr.

4.2 Methods

Minimum inhibitory concentration experiments were conducted with *S. albulus* BCRC11814 and *S. coelicolor* A3(2) to determine spontaneous resistance to Apr by the agar dilution method, according to Hamid, (2011) and Clinical and Laboratory Standards Institute (CLSI) guidelines (Woods et al., 2019). *Streptomyces albulus* BCRC11814 and *S. coelicolor* A3(2) spores were freshly isolated from a pure culture as per **Chapter 3, section 3.2.1** and resuspended in 2-

3 ml dH_2O. The spores were enumerated with a haemocytometer and diluted accordingly to obtain spore suspensions of ~10^6-10^8 cfu/ml. Subsequently, 20 µl of the spore suspension was inoculated onto 20 ml tryptic soy agar (TSA; Difco) supplemented with Apr in diluted concentrations of 30, 50, 70, 90, 100, 150, 200, 250 and 300 µg/ml prepared from 10 mg/ml stock solution. The inoculated plates were dried under sterile conditions and incubated at 30°C for 72 h. Control experiments were conducted with TSA plates lacking the addition of Apr as well as plates testing susceptibility to kanamycin. The control experiment with kanamycin were prepared at the following concentrations 50, 55, 70, 75, 90, 95, 100, 150, 300 µg/ml and 1mg/ml.

The MIC assays were performed in triplicate independently. The obtained MICs were assessed post-incubation and concentrations were scored as resistant(R) or susceptible(S) corresponding to the growth of *Streptomyces* strains.

4.3 Results and Discussion

Apramycin was the resistance marker for the pCRISPOmyces-2 plasmids used for intergeneric conjugation or electro-transformation of *Streptomyces* strains. Previous work involving intergeneric conjugation with *S. albulus* BCRC11814 and *S. coelicolor* A3(2) suspected spontaneous resistance of Apr. This was confirmed by performing a MIC assay by agar dilution that indicated spontaneous resistance for both *Streptomyces* strains (Table 3). In comparison to the CLSI MIC control reference of 2-8 µg/ml, the MIC results observed for both *Streptomyces* strains were significantly higher. The MIC for *S. albulus* BCRC11814 and *S. coelicolor* A3(2) was observed at 150 µg/ml and 250 µg/ml, respectively. The obtained MICs indicating spontaneous resistance for both strains were not unexpected as the concentration reference for Apr at which several *Streptomyces* strains begin to show susceptibility was 50 µg/ml (Fouces et al., 2000; Kieser et al., 2000). However, the resistance was observed at higher concentrations than the reported susceptibility concentrations. The increased resistance was likely to have been acquired through

environmental horizontal gene transfer or shared *Streptomyces* lineage-specific genes.

Table 3: MIC Assay for *S. albulus* BCRC11814 and *S. coelicolor* A3(2) susceptibility to apramycin

Test strain	Apramycin concentration (µg/ml)								
	<50	50	70	90	100	150	200	250	300
S. albulus BCRC 11814	R	R	R	R	R	S	S	S	S
S. coelicolor A3(2)	R	R	R	R	R	R	R	S	S

The only reported AGA mechanism for Apr resistance has been attributed to aminoglycoside acetyltransferase (3)-IV class enzymes (Quirke et al., 2020). The Apr resistance mechanism, likely by the aminoglycoside acetyltransferase (3)-IV class enzymes, observed for the *Streptomyces* strains in this study must be verified for further applications or possible AGA resistance reversal. This could be achieved through the sequence analysis of isolated aminoglycoside acetyltransferase (3)-IV genes and enzymatic assays of aminoglycoside acetyltransferases would be required.

The control experiments also confirmed resistance to kanamycin by *S. albulus* BCRC11814 as growth was observed at concentrations ≥64 µg/ml (MIC control range reference 8-64 µg/ml) **(Figure 18)**. This observation has not been reported by other studies on *S. albulus* BCRC11814 but also correlates with a study on *S. albulus* IF01414 that reported resistance to kanamycin A as well as other AGAs through the same rare aminoglycoside acetyltransferase (3)-IV class which confers

Apr resistance (Hamano et al., 2004). Therefore, verification of the resistance mechanism by aminoglycoside acetyltransferase (3)-IV enzymes in *S. albulus* BCRC11814 and *S. coelicolor* A3(2) could provide insights of the strains' resistance mechanism to other AGAs. This would be important when engineering *S. albulus* BCRC11814 as many *Streptomyces* strains acquire AR post-engineering (Wang et al., 2008).

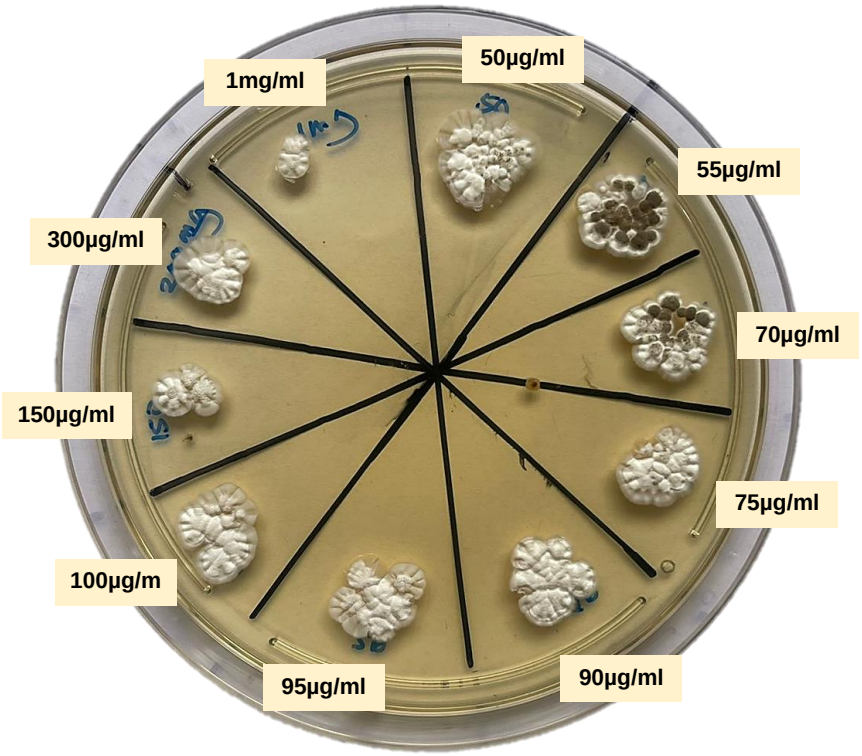

Figure 18: *Streptomyces albulus* BCRC11814 displayed resistance to kanamycin A. Minimum inhibitory concentration assay verified *S. albulus* BCRC11814 resistance to aminoglycoside antibiotic kanamycin A as growth was observed at concentrations ≥ 64 µg/ml which was the MIC resistant control reference.

4.4 Conclusion

Apramycin, known for its immunity against most AGA resistance mechanisms, is the typical selectable marker for several vectors used in the metabolic engineering of *Streptomyces* strains. However, spontaneous resistance in *S. albulus* BCRC11814 and *S. coelicolor* A3(2) to Apr was suspected because of previous genetic recombination work. Minimum inhibitory concentration assays by agar dilution, over an Apr concentration range from 30 to 300 µg/ml, confirmed the spontaneous resistance as MICs for *S. albulus* BCRC11814 and *S. coelicolor* A3(2) were observed at 150 and 250 µg/ml, respectively. The MICs for both strains were significantly above the standard MIC control reference (8-64 µg/ml) for Apr. This observation was not unexpected as the reference concentration for which the majority of *Streptomyces* strains display susceptibility has been reported at 50 µg/ml. However, MICs for both strains were observed above the relative reference as well. The resistance mechanism to Apr must be verified for future applications. Furthermore, resistance to kanamycin A by *S. albulus* BCRC11814 was also confirmed.

Chapter 5: Conclusion

5.1 Summary

The search for sustainable and efficient sources of new and existing antibiotics as well as other industrially relevant compounds has been a continuous global effort. The microbial world has played a significant role as source and tool to obtain such compounds. One particular bacterial family that has demonstrated the foremost impact is gram-positive *Streptomyces*. The *Streptomyces* genus is characterised by its BCGs that are responsible for producing a plethora of versatile and industrially relevant metabolites. However, the inability to extract these metabolites, due to inherent epigenetic BCG inactivation, or harness *Streptomyces* as an engineering host has resulted in a significant setback. In light of this, several research avenues have been conducted to explore existing metabolic engineering tools to resolve this. Recent engineering efforts with a CRISPR/Cas9 -based plasmid, coined the pCRISPomyces expression system, to engineer gram-positive bacteria such as *Streptomyces* have demonstrated promise.

The pCRISPomyces expression system, in particular pCRISPomyces-2, has been studied and displayed high engineering efficiency with a number of *Streptomyces* strains. This study aimed to ultimately introduce the pCRISPomyce-2 expression system into a fairly novel strain, *S. albulus* BCRC11814, for future metabolic engineering applications to harness the industrial potential of its SMs, such as ε-poly-L-lysine. Initial work aimed to verify the pCRISPomyces-2 plasmid structural and functional integrity to avoid potential bottlenecks caused by detrimental mutations in the pCRISPomyces-2 plasmid sequence. This was achieved through pDNA extraction followed by Sanger sequencing and ClustalW alignment with the reference *Streptomyces* expression codon optimised Cas9 nucleotide sequence. The obtained results confirmed a sequence identity greater than 98%.

Subsequently, the verified pCRISPomyces-2 plasmids were transformed into the conjugative donor *E. coli* WM6026 by electroporation and heat-shock. A relatively

low TE after optimisation was obtained for both transformation methods at 1.06×10^5 cfu/µg and 1.49×10^5 cfu/µg for electroporation and heat-shock, respectively. This suggested that the pCRISPomyces-2 plasmid size and pDNA concentration's relation to inherent toxicity of the Cas9 protein expression were the influencing factors that resulted in the low TE. However, considering the dynamics of bacterial fitness and its modulation of donor cell ability, the low TE and associated reduced fitness presented by a slower doubling time (T_d=1.49 h) for the transformed donor *E. coli* WM6026 cells were observed as a favourable indicator for downstream intergeneric conjugation.

Intergeneric conjugation was conducted for the introduction of pCRISPomyces-2 plasmids in the *Streptomyces* strains, *S. coelicolor* A3(2) and *S. albulus* BCRC11814, with donor transformed donor *E. coli* WM6026 cells. Successful intergeneric conjugation was only observed with the model strain *S. coelicolor* A3(2). Conjugational frequencies for optimised spore conjugation at 1.96×10^{-4} and mycelial conjugation at 2.80×10^{-4} were obtained. The absence of spore and mycelial intergeneric conjugation observed for *S. albulus* BCRC11814 was hypothesised to be caused by the restriction of methylated pCRISPomyces-2 pDNA by *dam* and *dcm* encoded methyltransferases functional in *E. coli* WM6026. This hypothesis was confirmed by comparative electro-transformation of *S. albulus* BCRC11814 spores with methylated and non-methylated pCRISPomyces-2 pDNA by *dam* and *dcm* encoded methyltransferases.

Furthermore, this study also confirmed spontaneous resistance of *S. albulus* BCRC11814 and S. *coelicolor* A3(2) to Apr. Spontaneous resistance was suspected from control experiments performed for intergeneric conjugation as Apr functioned as the selectable marker. This was verified by MIC assays which indicated the MICs for both *Streptomyces* strains were higher than the standard MIC control reference of 2-8 µg/ml. In addition, the MICs for both *Streptomyces* strains were also observed higher than the reported reference concentration of 50 µg/ml at which most *Streptomyces* strains exhibit susceptibility. In addition, resistance to

kanamycin A by *S. albulus* BCRC11814 was also confirmed as observed in other *S. albulus* strains.

In conclusion, the work completed in this study accomplished its objectives except that a successful intergeneric conjugation system to introduce pCRISPomyces-2 plasmids in *S. albulus* BCRC11814 could not be established. This was likely due to the presence of *dam* or *dcm* methylated pDNA occurring from the *E. coli* WM6026 donor strain. However, this study displayed the introduction of pCRISPomyces-2 plasmids in *S. albulus* BCRC11814 by electro-transformation at a low level. Thus, the introduction of pCRISPomyces-2 was possible, and the overall hypothesis of this work was achieved. However, optimisation is required to improve workflow and efficiency. Furthermore, this dissertation serves as a foundational research base for future applications of prospective multiplex genome editing and metabolic engineering in *S. albulus* BCRC11814.

5.2 Future perspectives

Intergeneric conjugation to introduce pCRISPomyces-2 plasmids into *S. albulus* BCRC11814 should be re-attempted with a *dam* and *dcm* methylation defective donor strain such as *E. coli* ET12567. Furthermore, electro-transformation as a secondary approach for the introduction of pCRISPomyces-2 into *S. albulus* BCRC11814 should be optimised. Both these techniques will enable CRISPR/Cas9 editing work in *S. albulus* BCRC11814 and successive metabolic engineering studies. In addition, further studies on Apr and other AGA resistance profiles, by sequence analysis and enzymatic assays, in *S. albulus* BCRC11814 and *S. coelicolor* A3(2) will be beneficial for reversal of AGA resistance and use of Apr as a selectable marker in genetic engineering.

Appendix A
Buffer and Media Recipes

TB-Transformation buffer

15 mM $CaCl_2$, 250 mM KCl, 55 mM $MnCl_2$,10 mM PIPES, and 1L dH_20.

All reagents excluding $MnCl_2$ were mixed then pH was adjusted with KOH to 6.7. Finally, $MnCl_2$ was added and dissolved then the solution was filter sterilized with a 0.45 μm filter unit and stored at 4°C.

Modified R2 media

0.1 g casamino acids, 10 g glucose, 0.25 g K_2SO_4, 10.12 g $MgCl_2.6H_2O$, and 800 ml dH_2O

The above solution was prepared then divided by pouring 80 ml volumes into 250 ml flasks. To each flask 2.2 g agar was added and autoclaved. When required, the medium was remelted and the following pre-autoclaved solutions were added in the order listed:

8 ml CaCl2.2H2O (3.68%), 1 ml KH_2PO4 (0.5%), 1.5 ml L-proline (20%), 10 ml TES buffer (5.73%, measured to pH7.2), 0.2 ml trace element solution (see below), 0.5 ml NaOH (1N) and growth factors for auxotrophs.

Trace element solution: 10 mg $CuCl_2$. $2H_2O$, 200 mg $FeCl_3$. $6H_2O$, 10 mg $MnCl_2$. $4H_2O$, 10 mg $(NH_4)_6Mo_7O_{24}$. $4H_2O$, 10 mg $Na_2B_4O_7$. $10H_2O$ and 40 mg $ZnCl_2$.

Appendix B

Growth Curve Calculations

Exponential curve fitting: $y = Ae^{Bx}$ (Masters & Ela, 2008)

a)

$$x = \ln\left(\frac{y}{A}\right)/B$$

$$T_d = x2 - x1 = \frac{(ln2 - lnA) - (ln2 - lnA)}{B}$$

$$T_d = \ ln2/B = 0.693/B$$

b) Derived from **Figure B1** for untransformed *E. coli* WM6026 cells:

$$y = 0.036e^{0.6104x}$$

$$T_d = \ ln2/B = 0.693/0.6104 \ = 1.14\ h$$

Derived from **Figure B1** for transformed *E. coli* WM6026/pCRISPomyces-2 cells:

$$y = 0.0223e^{0.4637x}$$

$$T_d = \ ln2/B = 0.693/0.4637 = 1.49\ h$$

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